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

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4 Hailee N. Nerber and Joseph A. Sorg*

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6 Department of Biology, Texas A&M University, College Station, TX 77845

7

8 *corresponding author

9

10 PH: 979-845-6299

11 Email: jsorg@bio.tamu.edu

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12 **Abstract**

13 *Clostridioides difficile* is a nosocomial pathogen which causes severe diarrhea and colonic
14 inflammation. *C. difficile* causes disease in susceptible patients when endospores germinate
15 into the toxin-producing vegetative form. The action of these toxins results in diarrhea and the
16 spread of spores into the hospital and healthcare environments. Thus, the destruction of spores
17 is imperative to prevent disease transmission between patients. However, spores are resilient
18 and survive extreme temperatures, chemical exposure, and UV treatment. This makes their
19 elimination from the environment difficult and perpetuates their spread between patients. In the
20 model spore-forming organism, *Bacillus subtilis,* the small acid-soluble proteins (SASPs)
21 contribute to these resistances. The SASPs are a family of small proteins found in all
22 endospore-forming organisms, *C. difficile* included. Although these proteins have high sequence
23 similarity between organisms, the role(s) of the proteins differ. Here, we investigated the role of
24 the main α/β SASPs, SspA and SspB, and two annotated SASPs, CDR20291_1130 and
25 CDR20291_3080, in protecting *C. difficile* spores from environmental insults. We found that
26 SspA is necessary for conferring spore UV resistance, SspB minorly contributes, and the
27 annotated SASPs do not contribute to UV resistance. In addition, none of these SASPs
28 contribute to the resistance of tested chemicals. Surprisingly, the combined deletion of *sspA* and
29 *sspB* prevented spore formation. Overall, our data indicate that UV resistance of *C. difficile*
30 spores is dependent on SspA and that SspA and SspB regulate / serve as a checkpoint for
31 spore formation, a previously unreported function of SASPs.

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36 **Importance**

37 *C. difficile* infections remain problematic and elimination of spores within an environment is
38 essential to limit person-to-person spread. A deeper understanding of how spores resist
39 cleaning efforts could lead to better strategies to eradicate the spores in a contaminated
40 environment. The small acid-soluble proteins (SASPs), found in all endospore-forming
41 organisms, are one mechanism that allows for spore resilience. Here, we find that *C. difficile*
42 SspA and SspB protect against UV light. Unexpectedly, these SASPs also regulate spore
43 formation, a role not described for any SASP to date.

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57 **Introduction**

58 *Clostridioides difficile* is the leading cause of antibiotic associated diarrhea with
59 ~224,000 annual infections in the United States [1-3]. Prior antibiotic treatment is the greatest
60 risk factor for *C. difficile* infection due to their broad-spectrum activity that can lead to a dysbiotic
61 colonic microbial community [4, 5]. Upon inoculation into a susceptible host, *C. difficile* spores
62 germinate to the toxin-producing vegetative form [4]. These toxins lead to the disruption of the
63 colonic epithelium and the common symptoms of disease (*e.g.*, diarrhea and colitis) [4].
64 Vancomycin and fidaxomicin are the recommended treatments for *C. difficile* infection [4, 6].
65 While these antibiotics treat the infection by targeting the actively-growing vegetative cells, the
66 spore form is resistant to antibiotics [5, 7]. In contrast to the anaerobic nature of the *C. difficile*
67 vegetative cell, spores can survive the oxygen-rich environment outside of a host, and are
68 considered the transmissible form [5, 8-10].

69 Endospores are dormant forms of bacteria that can withstand extreme environmental
70 conditions and chemical exposures [11]. In all endospore-forming bacteria, sporulation is
71 controlled by the master transcriptional regulator, Spo0A [10]. Activation of Spo0A by
72 phosphorylation results in the global activation of gene expression with the end goal of
73 optimizing / surviving post-exponential phase growth [12-14]. Upon the initiation of sporulation,
74 the vegetative cell divides asymmetrically, resulting in a large mother cell compartment and a
75 smaller forespore compartment [13]. Subsequently, a cascade of sigma factor activation occurs
76 and leads to the development of a dormant endospore [13]. In *Bacillus subtilis*, a model
77 organism for studying sporulation and germination, the activation of σ^F in the forespore, leads to
78 σ^E activation in the mother cell. The activation of σ^E in the mother cell, in turn, leads to activation
79 of σ^G in the forespore and then results in the activation of σ^K in the mother cell. The result of this
80 crisscross sigma factor activation cascade is the engulfment of the forespore by the mother cell,
81 maturation of the forespore, and the eventual release of the spore by lysis of the mother cell; the

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82 same sigma factors drive *C. difficile* sporulation but the crisscross activation across
83 compartments does not occur [12, 13, 15-17]. The resulting spores are extremely resistant to
84 environmental conditions and common cleaning methods [11, 18]. Thus, with a deeper
85 understanding of the resistance properties of spores, and the mechanisms that confer this
86 resistance, novel interventions could be developed to clean contaminated environments.

87 The small acid-soluble proteins (SASPs) confer resistance to spores [11, 19]. The
88 SASPs are a family of proteins that are less than 100 amino acids in length and are conserved
89 among all endospore forming organisms [19]. They are produced late in sporulation under the
90 forespore-specific sigma factor, σ^G, and account for approximately 20% of the total spore
91 protein content [11, 16, 19]. Most spore-forming bacteria encode the two major α/β-type SASPs
92 (SspA and SspB), however there are other minor SASPs that vary in number depending on the
93 organism [19-23]. *B. subtilis* also encodes a γ-type SASP that is hypothesized to serve as an
94 amino acid reservoir for use upon outgrowth of the vegetative cell from the germinated spore
95 [24]. Clostridial species, to date, have not been found to contain γ-type SASPs [20, 21]. In *C.*
96 *difficile,* the R20291 strain encodes *sspA* and *sspB* orthologues and two genes annotated as
97 putative SASPs, *CDR20291_1130* and *CDR20291_3080*. SASPs are well-conserved across
98 genera with 70% similarity between *C. difficile* and *B. subtilis* SspA and SspB [25]. However, the
99 annotated SASPs have less similarity to *C. difficile* or *B. subtilis* SspA and SspB, ranging from
100 40-60%. Interestingly, CDR20291_3080 shares 80% similarity to *C. perfringens* Ssp4, a novel
101 SASP, which protects the spores from nitrous acid and extreme heat associated with food
102 processing [23, 26].

103 In *B. subtilis*, the α/β-type SASPs contribute to spore resistance against several
104 chemicals, such as nitrous acid, formaldehyde, glutaraldehyde, iodine, or hydrogen peroxide
105 [18, 26-34]. Moreover, *B. subtilis* Δ*sspA* mutants completely lose viability after 3 minutes of
106 exposure to 254 nm UV light, and *B. subtilis* Δ*sspB* mutants have 10% survival after 7 minutes

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107 of exposure [35]. Interestingly, a double mutant was even more sensitive to UV exposure than
108 were vegetative cells, highlighting the importance of these proteins in spore survival [35]. These
109 UV-sensitive phenotypes could be complemented by expressing, *in trans*, either *sspA* or *sspB*
110 [36]. Moreover, a SASP from *B. megaterium* complemented the phenotype, suggesting that they
111 could play interchangeable roles in UV resistance [31, 36].

112 In other spore-forming bacteria, the role of the SASPs vary among organisms. In *C.*
113 *botulinum,* SASPs were found to be necessary for protection against nitrous acid, similar to
114 what is observed in *B. subtilis* [28]. However, they are not necessary for protection against
115 hydrogen peroxide or formaldehyde, contrary to what is observed in *B. subtilis* [28]. In *C.*
116 *perfringens* isolates that cause food poisoning, Ssp4 was necessary for spores surviving food
117 processing events (*e.g.*, high heat and use of nitrites) [23, 26]. Other *C. perfringens* SASPs
118 were found to protect spores against UV light, hydrogen peroxide, nitrous acid, formaldehyde,
119 and hydrochloric acid [29, 37, 38].

120 The α/β-type SASPs of *B. subtilis, C. perfringens,* and *C. acetobutylicum* all bind to DNA
121 *in vitro* [37, 39-41]. In *B. subtilis*, the binding of these proteins changes the confirmation of the
122 DNA to one between an A and a B form [39, 41-43]. In this unique conformation, an alternative
123 form of UV damage is induced, a thymidyl-thymidine adduct, called the spore photoproduct. The
124 spore photoproduct is repaired by the spore photoproduct lyase, SPL, during germination and
125 outgrowth [25, 44-48].

126 Here, we investigate the functions of *C. difficile sspA*, *sspB*, and the annotated SASPs,
127 *CDR20291_1130* and *CDR20291_3080.* We found that *C. difficile* SspA is the major contributor
128 to UV resistance of the spores and that SspB is minorly involved in UV resistance.
129 CDR20291_1130 and CDR20291_3080 do not contribute to UV resistance. Additionally, none
130 of the SASPs contribute to spore chemical resistance in the concentrations and exposure times
131 tested. Surprisingly, we found that the deletion of both *sspA* and *sspB* prevented spore

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132 formation. Our results indicate that, in addition to providing UV resistance to spores, the major
133 *C. difficile* SASPs are involved in spore formation.

134

135 **Results**

136 *Conservation of SASPs in C. difficile*

137 The main α/β type SASPs are conserved in spore-forming bacteria [19]. Within *C.*
138 *difficile*, SspA and SspB are 85% identical. The putative SASPs are approximately 40% identical
139 to the main α/β type SASPs. All 4 contain the conserved "EIA" sequence that is cleaved by the
140 germination protease (GPR) upon germination (Figure 1) [49-51]. Even with strong sequence
141 similarity / identity, SASPs have varying roles between spore forming organisms. To investigate
142 the roles of *C. difficile sspA*, *sspB*, *CDR20291_1130*, and *CDR20291_3080* in UV resistance,
143 outgrowth, and chemical resistance, single deletions and pairwise deletions were generated
144 using CRISPR-Cas9 genome editing [52]. Total amounts of spores produced and the
145 sporulation rates by the single deletion strains, *C. difficile* Δ*sspA, C. difficile* Δ*CDR20291_1130,*
146 and *C. difficile* Δ*CDR20291_3080* were indistinguishable from the wildtype *C. difficile* R20291
147 parental strain (Figure S1). Surprisingly, the *C. difficile* Δ*sspB* strain did not make spores and
148 will be discussed later. These results indicate that the single deletions of *C. difficile sspA, C.*
149 *difficile CDR20291_1130* and *C. difficile CDR20291_3080* do not impact *C. difficile* sporulation.

150

151 *sspA is required for spore UV resistance*

152 Because of the strong phenotype of *B. subtilis* SASPs in UV resistance, we
153 hypothesized that *C. difficile* SspA, and / or the two annotated SASPs (CDR20291_1130 and
154 CDR20291_3080) may function similarly. The viability of SASP mutants after a 10 minute

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155 exposure to 302 nm UV light was tested. Viable spores were quantified by colony formation on
156 media supplemented with taurocholate (a *C. difficile* spore germinant) and then compared to
157 spores derived from the wildtype parental strain [53]. Exposure of the *C. difficile* Δ*sspA* strain to
158 UV light resulted in a ~1,000x decrease in spore survival. Spores derived from the *C. difficile*
159 Δ*CDR20291_1130* and *C. difficile* Δ*CDR20291_3080* mutants had a statistically significant
160 difference in UV resistance compared to spores derived from the wildtype strain, but this
161 difference is likely not biologically relevant (Figure 2A). These results indicate that *C. difficile*
162 SspA is the most important SASP for UV resistance while the genes annotated as putative
163 SASPs are not involved in UV resistance. In *B. subtilis*, SASP binding to DNA helps to protect
164 from UV by encouraging the formation of spore photoproducts (SP) instead of cyclobutane
165 thymine dimers [18, 54]. The spore photoproduct lyase, SPL, repairs the SP [44-46, 55]. To
166 understand the impact of SPL on UV resistance in *C. difficile,* we engineered a deletion in the *C.*
167 *difficile spl* gene. Spores derived from *C. difficile* Δ*spl* strain have approximately 10x reduced
168 survival compared to spores derived from the wildtype *C. difficile* R20291 strain (Figure 2A).
169 Unsurprisingly, the *C. difficile* Δ*sspA* Δ*spl* double mutant remains at the level of *sspA* mutant
170 alone (0.1% of wildtype), suggesting that SPL functions in a capacity where without SspA, SPL
171 is not necessary.

172 To determine if the putative SASPs have redundant roles in UV resistance, we
173 generated deletions of all pairwise combinations of *C. difficile CDR20291_1130, C. difficile*
174 *CDR20291_3080,* and *C. difficile sspA*. After 10 minutes of UV exposure, spores derived from
175 the *C. difficile* Δ*CDR20291_1130* Δ*CDR20291_3080* were as resistant as wildtype spores,
176 indicating that these annotated SASPs are not compensating for each other during UV exposure
177 (Figure 2B). Spores derived from the *C. difficile* Δ*sspA* Δ*CDR20291_1130* mutant had no further
178 reduction in survival compared to the *C. difficile* Δ*sspA* mutant alone. However, spores derived
179 from the *C. difficile* Δ*sspA* Δ*CDR20291_3080* double mutant and the Δ*sspA* Δ*CDR20291_1130*

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180 Δ*CDR20291_3080* triple mutant were not as sensitive to UV light as the *C. difficile* Δ*sspA* or the
181 *C. difficile* Δ*sspA* Δ*CDR20291_1130* strains (Figure 2B).
182
183 *The sspA promoter is necessary for complementation of C. difficile ΔsspA UV resistance*
184 To understand the extent of protection that *C. difficile* SspA provides against UV
185 damage, we quantified the viability of spores exposed to UV over time. Spores derived from the
186 *C. difficile* Δ*sspA* strain showed a 10% loss of viability after 2.5 minutes of UV exposure. This
187 loss increased to approximately 3 $\log_{10}$ after 15 minutes of exposure. Expression of *sspA* from a
188 plasmid under the control of its native promoter restored viability to the *C. difficile* Δ*sspA* strain
189 (Figure 3A).
190 To further understand the role of *C. difficile* SspA in UV resistance, we tested the impact
191 of different *sspA* expression constructs on spore survival. The *sspA* complement consisting of
192 the *sspA* gene under its native promoter region resulted in restoration of spore viability. A 6x-
193 histidine tag inserted on the C-terminus of *sspA* also resulted in restoration of spore viability
194 upon UV exposure (Figure 3B). In *B. subtilis,* SASP genes can cross-complement a SASP
195 mutant [36]. Therefore, plasmids were constructed that consisted of *C. difficile sspB,*
196 *CDR20291_1130,* or the *CDR20291_3080* genes, driven by their native promoter regions,
197 introduced into the *C. difficile* Δ*sspA* mutant strain. After 10 minutes of exposure to UV light,
198 spores derived with these plasmid constructs revealed that *C. difficile sspB, CDR20291_1130,*
199 or *CDR20291_3080* were unable to restore the UV resistance to spores derived from the Δ*sspA*
200 mutant strain (Figure 3B). To determine if this is an issue with differences in expression, the
201 promoter regions were changed and these genes were again tested for their ability to
202 complement the *C. difficile* Δ*sspA* strain. When the *sspA* gene was placed under control of the
203 *sspB* promoter region, complementation no longer occurred. This further supported our

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204 hypothesis of the SASPs containing differences in expression levels. Swapping the promoter
205 regions of *C. difficile sspB, CDR20291_1130*, or *CDR20291_3080* complementation plasmids
206 for the *C. difficile sspA* promoter region resulted in a restoration to approximately 5% of wildtype
207 levels (Figure 3C). However, as negative controls, the *sspA* promoter region alone, or the *sspA*
208 promoter driving the gene encoding mCherry, could not complement the UV phenotype. These
209 results suggest that despite what is observed for cross-complementation in other organisms, *C.*
210 *difficile sspB*, *CDR20291_1130*, or *CDR20291_3080* cannot fully complement the *C. difficile*
211 Δ*sspA* strain phenotype, and to provide any complementation, they must be expressed from the
212 *sspA* promoter.

213

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

215 *C. difficile CDR20291_1130* and *CDR20291_3080* do not contribute to spore UV
216 resistance, but it is possible that their main role is to protect against other harsh environmental
217 conditions or to serve as amino acid reservoirs (like γ-type SASPs of *B. subtilis*) during
218 outgrowth of a vegetative cell from the germinated spore [24]. To determine if these annotated
219 SASPs contribute to outgrowth, the OD_{600} of spores derived from wildtype and mutant strains
220 were analyzed over 12 hours in complex medium supplemented with germinants [53, 56]. No
221 difference in the outgrowth of vegetative cells was observed for the mutant strains, compared to
222 the wildtype parental strain.

223 It is possible that the complex medium masked the hypothesized phenotype due to the
224 sheer abundance of nutrients in the medium eliminating the need for the amino acids derived
225 from the SASPs. Instead, we tested if outgrowth of a spore in minimal medium would be
226 influenced in these mutant strains. Unfortunately, using a minimal medium resulted in an
227 extreme delay in outgrowth that was not possible to practically measure. Therefore, spore

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228 outgrowth was analyzed in half-strength complex medium. In addition, to eliminate the
229 possibility of extra resources being packaged into the spore when grown on a complex medium,
230 and thus reducing the need for SASPs during outgrowth, spores were generated on minimal
231 medium. Again, despite spore production on minimal medium and using half-strength complex
232 medium during the assay, there were no differences between the outgrowth of wildtype spores
233 or spores derived from the *C. difficile* Δ*sspA* or the *C. difficile* Δ*CDR20291_1130*
234 Δ*CDR20291_3080* double mutant strain (Figure 4A).

235 Due to the possibility that the SASPs can compensate for the deletion of one, a triple
236 mutant was generated of *sspA, CDR20291_1130,* and *CDR20291_3080.* These spores were
237 also generated on minimal medium and outgrowth analyzed in half-strength complex media.
238 Spores derived from the *C. difficile* Δ*sspA* Δ*CDR20291_1130* Δ*CDR20291_3080* triple mutant
239 had an approximate 2-hour delay in outgrowth compared to the wildtype strain (Figure 4B). To
240 eliminate the possibility that the difference in outgrowth is due to a germination defect in this
241 triple mutant, we monitored germination by OD_{600} in buffer supplemented with germinants [53,
242 56]. During the very early events of endospore germination, the dormant, phase bright, spore
243 transitions to a phase dark, germinated spore. Mutant spores germinated similarly to wildtype,
244 suggesting no defect in germination (Figure 4C). These results suggest that *C. difficile* SspA,
245 CDR20291_1130, and CDR20291_3080 could be used as a nutrient / amino acid source during
246 outgrowth of a germinated spore.

247

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

249 To further characterize the role of these proteins in the spore, spores were exposed to
250 various chemicals. Spores derived from the single deletion of *C. difficile sspA*,
251 *CDR20291_1130*, and *CDR20291_3080* and their complements, when necessary, were

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252 exposed to chemicals for 1 minute, 5 minutes, 10 minutes, and 30 minutes and spore viability
253 was assessed by plating onto rich medium supplemented with germinant. Colony forming units
254 were compared to T_0 and then this ratio compared to the ratio of wildtype survival. Spores
255 exposed to 3% H_20_2, 75% EtOH, 0.25% glutaraldehyde, 1 M HCl, 0.05% hypochlorite, and 2.5%
256 formaldehyde did not exhibit reduced viability in comparison to spores derived from the wildtype
257 strain (Figure S2A-F). The mutant strains did have a slight reduction in viability after 5 or 10
258 minutes of exposure to 250 mM nitrous acid (Figure S2G).

259

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

261 When generating the *C. difficile* Δ*sspB* mutant strain, we found that spores derived from
262 this strain were phase gray and were not released from the mother cell, compared to the
263 wildtype strain (Figure 5A and 5B). This phenotype could be complemented by expression of
264 the *sspB* gene under the control of the *sspB* promoter region (Figure 5C) or the *sspA* gene
265 under control of the *sspA* promoter region (Figure 5D). However, only a σ^G-controlled promoter
266 was able to complement the phenotype, other σ-factor controlled promoters could not
267 complement (Figures 5E - H). The rate of sporulation of the *C. difficile* Δ*sspB* mutant was also 5
268 $\log_{10}$ less than the wildtype strain and was complemented by expression of *sspB* from a plasmid
269 (Figure S3). Surprisingly, whole genome resequencing of the *C. difficile* Δ*sspB* strain revealed
270 an additional, single nucleotide mutation in *sspA*. This mutation resulted in an *sspA*$_{G52V}$ allele, in
271 addition to the *sspB* deletion (referred to as *sspB*$_{SNP}$).

272 Due to the phenotype of this strain, normal spore purification processes were
273 unsuccessful. To encourage release of the immature spores from the mother cells, cultures from
274 sporulating cells were incubated with lysozyme before resuming normal spore purification steps.
275 This encouraged the spores to release from the mother cells and the recovery of a limited

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276 number of phase gray spores. The phase bright phenotype of wildtype spores is partially
277 attributed to the dipicolinic acid (DPA) content of the spore core. Approximately 2.5 x 10^5 spores
278 of the *C. difficile* wildtype, Δ$sspB_{SNP}$, and Δ$sspB_{SNP}$ p*sspB* strains were boiled and the DPA
279 levels determined by Tb^{3+} fluorescence [57]. The DPA content of the *C. difficile* Δ$sspB_{SNP}$ strain
280 was significantly lower than wildtype content and this was able to be complemented by
281 expression of the *sspB* gene from its native promoter region (Figure 5I).

282 Because germination assays rely heavily on the release of DPA during germination
283 (phase bright to phase dark transition), we were unable to use these assays to determine if this
284 deletion and SNP combination altered germination capabilities [57, 58]. Instead, the spores
285 were exposed to germinants and the processing of spore proteins during germination were
286 analyzed by immunoblot. In response to germinants, spores derived from the *C. difficile*
287 Δ$sspB_{SNP}$ strain processed proSleC to its active form, indicating that they are capable of
288 receiving the germinant signals (Figure 5J) [59-61].

289 Finally, we tested the ability of spores derived from the *C. difficile* Δ$sspB_{SNP}$ p*sspB* strain
290 to resist UV damage. The *C. difficile* Δ$sspB_{SNP}$ alone was not assessed due to the difficulty in
291 producing and purifying spores. In the assay, the *C. difficile* Δ$sspB_{SNP}$ p*sspB* strain was
292 complemented to wildtype levels, revealing that the $sspA_{G52V}$ allele is not impaired in its ability to
293 protect against UV damage but SspA may regulate *C. difficile* sporulation with SspB (Figure
294 5K).

295

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

297 Due to the G52V mutation in *sspA*, we created a clean *C. difficile* Δ*sspA* Δ*sspB* strain to
298 determine if the double mutant had the same phenotype as the *C. difficile* Δ$sspB_{SNP}$ strain.
299 Similar to above, the *C. difficile* Δ*sspA* Δ*sspB* strain produced phase gray, immature spores

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300 (Figure 6A). The rate of sporulation was also 1,000x less than wildtype and could be
301 complemented with expression of *sspA* and *sspB* from a plasmid (Figure S3). Moreover,
302 analysis of the DPA content also showed little DPA in the double mutant strain, in comparison to
303 wildtype (Figure 6B). Finally, to evaluate whether these spores can germinate, we monitored
304 SleC activation. These double mutant spores also processed proSleC to the active SleC form,
305 showing that they are still able to germinate (Figure 6C) [59-61]. Again, due to the difficulty in
306 purifying this strain and despite density gradient purification, spore preparations still contained
307 debris making it difficult to quantify the spores by microscopy. Therefore, we were forced to use
308 approximate spore counts. The Coommassie stained SDS PAGE gel shows that more protein is
309 present in the double mutant sample than in wildtype or the complemented strains, even though
310 the SleC bands detected by western indicate similar concentrations (Figure S4). This is possibly
311 due to the presence of contaminating vegetative cells in the double mutant preparation because
312 of the difficulty in purifying or the spore counts could have been underestimated.

313

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

316 After discovering the SNP in the *C. difficile* Δ*sspB* strain, we generated a clean deletion
317 that produces phase bright, released spores. The *C. difficile* Δ*sspB* strain had a sporulation rate
318 identical to wildtype (Figure S3).

319 Since a clean deletion produces spores, the UV resistance could be determined. Spores
320 derived from the wildtype *C. difficile* R20291, *C. difficile* Δ*sspB* mutant, and *C. difficile* Δ*sspB*
321 p*sspB* strains were exposed to UV light for 10 minutes before plating on media with germinants
322 to determine CFUs. Spores derived from the *C. difficile* Δ*sspB* strain had approximately 10% of
323 the resistance observed for the wildtype strain. This phenotype was complemented by

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324 expression of *sspB* from its native promoter *in trans* (Figure 7A). Therefore, *C. difficile* SspB
325 plays a minor role in UV resistance.

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

331 Spores from *C. difficile* Δ*sspB* were also tested for chemical resistance in the same
332 manner as previously discussed. In comparison to *C. difficile* R20291 wildtype spores, *C.*
333 *difficile* Δ*sspB* spores exposed to 3% H_20_2, 75% EtOH, 0.25% glutaraldehyde, 1 M HCl, 0.05%
334 hypochlorite, 2.5% formaldehyde, and 250 mM nitrous acid did not have reduced viability
335 (Figure S2A-G).

336

337 **Discussion**

338 *C. difficile* infections occur in individuals with a disrupted microbiota, commonly due to
339 the use of broad-spectrum antibiotics [1, 4, 5]. Vegetative *C. difficile* colonizes the lower GI tract
340 and produces toxins that disrupt the epithelial lining integrity and cause colitis and diarrhea [4].
341 While the toxin producing vegetative cells are eliminated with antibiotic treatment, the spores
342 can persist in the gut and become vegetative cells again, or they can shed into the environment
343 [4, 5]. This shedding allows for the transmission of disease by passing spores into an
344 environment where they can then transfer to other individuals [4, 9]. Spores can withstand
345 environmental insults, such as UV light, and many chemicals, and persist long term in most
346 environments [11, 18, 25].

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347 UV-B light (between 315 – 280 nm) is the UV wavelength range most responsible for
348 DNA breaks and DNA lesions (*e.g.,* cyclobutane pyrimidine dimers) [62]. DNA does not absorb
349 UV-A (400 – 315 nm) but UV-C (less than 280 nm) has high energy and is known to cause
350 some DNA mutations, but fewer cyclobutane pyrimidine dimers than UV-B [62]. Though *C.*
351 *difficile* spores are more likely to encounter UV-B from environmental UV radiation, UV-C is
352 absorbed by ozone making it less likely that spores encounter this spectrum in nature [62].
353 Several studies have shown that treatment with 254 nm UV light reduced viable *C. difficile* spore
354 load in the hospital environment [63, 64]. However, this method is not ideal due to the potential
355 exposure of dangerous UV-C light to individuals implementing this cleaning technique and the
356 long exposure time of approximately 1 hour. An interesting study evaluated the effects of a
357 hospital biocide, sodium dichloroisocyanurate (NaDCC), on *C. difficile* spores. In this study, a
358 10x less lethal concentration of biocide was tested [65]. At this concentration, spores are not
359 killed as effectively as the lethal concentration and the hydrophobicity of the spores was greatly
360 reduced (to approximately 20%) [65]. The reduction in hydrophobicity is of concern because it is
361 possible that spores would spread easier due to reduced adherence to surfaces. This further
362 perpetuates the problem of trying to clean a *C. difficile* contaminated environment.

363 The small acid-soluble proteins (SASPs) are well established to protect the spores from
364 environmental insults (*e.g.*, UV light or chemicals) [11, 18, 19, 25]. Here we investigated the
365 functions of *C. difficile* SASPs in response to UV light and chemicals. We found that *C. difficile*
366 SspA and SspB have a role in UV resistance but none of the SASPs significantly contribute to
367 chemical resistances. Surprisingly, we discovered that the deletion of *C. difficile* Δ*sspA* Δ*sspB*
368 resulted in phase gray, unreleased spores and we hypothesize that SspA and SspB are working
369 together to influence sporulation, a SASP function not previously reported.

370 *C. difficile* SASPs are involved in protecting spores from UV exposure. This was not
371 surprising since the SASPs in all other organisms have had a large role in UV resistance [29,

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372 35, 38, 66, 67]. While *B. subtilis* Δ*sspB* had almost 100% survival after 3 minutes of exposure to
373 254 nm UV, *B. subtilis* Δ*sspA* spores were sensitive to UV light with only 0.1% survival [35]. In
374 *C. difficile*, SspA is the major contributor to UV resistance. *C. difficile* Δ*sspA* spores exposed to
375 UV 302 nm light for 10 minutes had 0.1% survival in comparison to *C. difficile* Δ*sspB* which had
376 10% survival; *C. difficile* Δ*CDR20291_1130* and *C. difficile* Δ*CDR20291_3080* did not lose
377 viability. UV protection is contributed to the binding of the SASPs to the DNA, which changes its
378 conformation and encourages spore photoproduct mutations over cyclobutane pyrimidine
379 dimers [19, 25, 39, 41, 44, 46, 54, 68]. The loss of the spore photoproduct repair system, SPL,
380 interestingly only resulted in a 10x loss of viability. This highlights the importance of SspA in
381 protecting the genome from lethal UV irradiation. However, in a *C. difficile* Δ*sspA* Δ*spl* double
382 mutant, the viability remained the same as for a *C. difficile* Δ*sspA* single deletion, suggesting
383 SspA is the major contributor to UV resistance. This also suggests that SspA primarily
384 influences the change in DNA structure that encourages spore photoproduct formation upon UV
385 exposure.

386 In *B. subtilis,* other SASPs than SspA and SspB are considered minor SASPs with many
387 redundant roles [19, 25, 36]. To evaluate if the *C. difficile* annotated SASPs can compensate for
388 each other or only minorly contribute to UV resistance, pairwise deletions were generated. The
389 annotated SASPs do not compensate for each other, as a *C. difficile* Δ*CDR20291_1130*
390 Δ*CDR20291_3080* double deletion does not have a UV defect. A *C. difficile* Δ*sspA*
391 Δ*CDR20291_1130* deletion does not further the UV defect from the *C. difficile* Δ*sspA* strain
392 alone. Unexpectedly, whenever the double deletion *C. difficile* Δ*sspA* Δ*CDR20291_3080* or the
393 triple deletion *C. difficile* Δ*sspA* Δ*CDR20291_1130* Δ*CDR20291_3080* were tested, they
394 resulted in 10% viability. We hypothesized that these deletions would result in either the same
395 defect of 0.1% viability seen with *C. difficile* Δ*sspA* alone or that UV sensitivity would be
396 increased upon more deletions. This increase in survival would suggest that another factor may

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397 be compensating for the loss of these SASPs. Another possibility is that the loss of certain
398 SASPs influences the DNA structure so that UV light affects the strains differently than when
399 those SASPs are present. However, further work is necessary to determine the cause of the
400 partial UV resistance with *C. difficile* Δ*sspA* and Δ*CDR20291_3080* co-deletions. We also found
401 that the other 3 *C. difficile* SASPs, *sspB, CDR20291_1130,* and *CDR20291_3080* are unable to
402 complement a *C. difficile* Δ*sspA* deletion. We hypothesized that this could possibly be due to
403 differences in expression levels. Indeed, when the *sspA* promoter region was used to drive
404 expression of the other SASPs, complementation occurred to approximately 5% of wildtype
405 level. This suggests that the *sspA* promoter region was able to increase expression levels of
406 these SASPs which in turn led to some protection from UV light in comparison to *C. difficile*
407 Δ*sspA* alone. We hypothesize that these other SASPs can bind DNA but need higher
408 expression to do so.

409 Other analyses revealed that the SASPs SspA, CDR20291_1130, and CDR20291_3080
410 only minorly contribute to outgrowth. A triple mutation was needed before a ~2 hour defect was
411 observed. We predict that deletion of all three SASPs depletes the amino acid pool and results
412 in a delay in protein production and outgrowth of the vegetative cell.

413 In other organisms, SASPs play large roles in protection from chemicals. Interestingly,
414 *C. difficile* SASPs were not important for survival of the spores upon chemical exposure. Nitrous
415 acid was the only chemical where the SASPs may play a minor role in protection. At 5 minutes
416 of exposure, *C. difficile* Δ*sspA* has approximately 3% of wild type survival and this is
417 complemented to wildtype levels. At 10 minutes of exposure, *C. difficile* Δ*sspA* has 8% of wild
418 type survival that, again, complements to wildtype levels. Also at 10 minutes, *C. difficile*
419 Δ*CDR20291_1130* survival is 10% of wildtype and *C. difficile* Δ*CDR20291_3080* is 16%; both
420 could be complemented to wildtype levels. Of note, there were some reductions in spore
421 survival of some mutants compared to wildtype across the other chemicals tested. However, in

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422 these cases complementation plasmids did not restore viability, leading to the conclusion that
423 some other factor, besides the SASP deletion, was causing the reduced viability. For evaluating
424 the effect of hypochlorite and glutaraldehyde, reproducibility was an issue. Every trial varied in
425 response to these chemicals, making it difficult to draw a confident conclusion from these data.
426 We conclude that *C. difficile* SASPs do not contribute to chemical resistance. This could be due
427 to protection being evolutionarily unnecessary because *C. difficile* would not typically encounter
428 many of these chemicals in nature or that the spore coat proteins are better suited to resist
429 these conditions. Indeed, the *C. difficile* spore coat is established to contain proteins involved in
430 some resistances [69, 70].

431 In *B. subtilis*, the Δ*sspA* Δ*sspB* strain is commonly used to characterize the SASPs. It
432 was very surprising that the *C. difficile* Δ*sspB* $sspA_{G52V}$ allele ($sspB_{SNP}$) and the *C. difficile*
433 Δ*sspA* Δ*sspB* double mutant were unable to form phase bright spores. Based on the phase-
434 gray cells, we hypothesized that the forespore did not have DPA packaged. Indeed, DPA
435 content is significantly reduced in these SASP-mutant strains. Because DPA contributes to heat
436 resistance and heat resistance is a classically tested feature of endospores and used in
437 sporulation assays, this could have impacted the results of the sporulation assay by killing fully
438 formed spores that do not package DPA and thus exacerbating the sporulation rate phenotype
439 [11, 58, 71-73].

440 In the *C. difficile* $\Delta sspB_{SNP}$ strain, complementation of the spore phenotype only
441 occurred when *sspB* was driven by a σ^G promoter, indicating the importance of expression at the
442 correct time and in the correct compartment during sporulation. The use of the *bcla2* promoter
443 region for σ^E was based off Fimlaid *et al.* However, Saujet *et al.* classified *bcla2* as σ^K
444 dependent [16, 17]. Either way, mother cell specific promoters do not complement the
445 phenotype, further driving the conclusion that expression must occur in the forespore. This

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446 strain could also be complemented by expression of *C. difficile sspA*, suggesting that SspA and
447 SspB may work together.

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

458 Furthermore, evaluation of UV viability on the *C. difficile* Δ$sspB_{SNP}$ strain complemented
459 with *sspB* shows that these spores have approximately 60% of wildtype viability, a large
460 difference between 0.1% viability in a *C. difficile* Δ*sspA* or the 10% viability of the *C. difficile*
461 Δ*sspB*. Thus, the $sspA_{G52V}$ allele does not impair the ability of SspA to protect against UV
462 damage. Due to the observed phenotypes, we hypothesize that SspA and SspB together
463 regulate spore formation. Interestingly, the *C. difficile* Δ*sspA* Δ*sspB* phenotype is similar to the
464 phenotype of a *C. difficile* Δ*spoVT* mutant [17, 74]. In *B. subtilis*, SpoVT is involved in a feed
465 forward loop to regulate sporulation. While it's expression is through σ^G , it works to enhance
466 some, and repress other, σ^G controlled genes [75]. SpoVT regulates the SASPs, SspA and
467 SspB, with a mutant having a 30% reduction in these proteins [75]. This mutant strain is still
468 able to form phase bright spores but they have a reduced sporulation rate [75]. In *C. difficile*, the
469 *spoVT* mutant has a different phenotype than that observed in *B. subtilis*. This protein is under
470 σ^G regulation and possibly also σ^F [16, 17, 74]. The *C. difficile* Δ*spoVT* strain forms immature

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471 spores that are phase dark [17]. Similarly to *B. subtilis*, SASP expression was reduced in *C.*
472 *difficile*; *C. difficile sspA* had a 70 fold reduction and *sspB* a 12 fold reduction [17]. The authors
473 suggested that this SASP reduction was probably not sufficient to explain the phenotype [17].
474 However, our data suggests that the *C. difficile* Δ*spoVT* phenotype may be due to reduced *sspA*
475 and *sspB* levels. This further highlights the differences in sporulation and SASP function in *C.*
476 *difficile* compared to the model *B. subtilis* and also furthers our hypothesis of SspA and SspB
477 regulating spore formation. Furthermore, in the *B. subtilis* ΔsspA ΔsspB double mutant,
478 expression levels of some genes were changed [76]. However, the expression levels suggest
479 that the SASPs work to negatively regulate multiple forespore genes and even one mother cell
480 gene [76]. They do note that the changes in transcription may not be due to regulation but to
481 actual binding of the SASPs to the genome, which represses transcription. The novel finding of
482 immature spore formation in *C. difficile* Δ*sspA* Δ*sspB* double mutant suggests that *C. difficile*
483 SASPs perform a previously unreported function in the sporulation process. This insight will
484 open new doors for understanding the regulation of sporulation of *C. difficile*.

485

486

487

488

489

490 **Materials and Methods**

491 ***Bacterial growth conditions:*** *C. difficile* strains were grown in a Coy anaerobic chamber at
492 >4% H_2, 5% CO_2, and balanced N_2 at 37 °C in either brain heart infusion supplemented with 5 g
493 / L yeast extract (BHIS) and 0.1% L-cysteine or tryptone yeast medium with 0.1% thioglycolate

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494 [77]. When necessary, media was supplemented with thiamphenicol (10 µg / mL), taurocholate
495 (TA) (0.1%), anhydrous tetracycline (100 ng / mL), kanamycin (50 µg / mL), or xylose (1%). *E.*
496 *coli* strains were grown on LB at 37 °C and supplemented with chloramphenicol (20 µg / mL) for
497 plasmid maintenance. *B. subtilis* BS49 was grown on LB agar or in BHIS broth at 37 °C and
498 supplemented with 2.5 µg / mL chloramphenicol for plasmid maintenance and 5 µg / mL
499 tetracycline during conjugations.

500

501 ***Plasmid construction:*** The *C. difficile sspA* targeted CRISPR plasmid was constructed by
502 amplifying 500 bp upstream and downstream from the *sspA* gene using primer pairs
503 5'sspA_MTL, 3'sspA_up and 5'sspA_down, 3'sspA_downMTL, respectively. The fragments
504 were cloned into the *Not*I and *Xho*I site of pKM126 by Gibson assembly [78]. The gRNA was
505 retargeted to *sspA* by inserting gBlock CRISPR_sspA_165 into the *Kpn*I and *Mlu*I sites by
506 Gibson assembly, generating the pHN05 plasmid. The *spl, sspB, CDR20291_1130* and
507 *CDR20291_3080* targeting plasmids were similarly constructed. For *spl* plasmid construction, 5'
508 spl_UP and 3' spl_UP amplified the upstream homology arms and the downstream homology by
509 5' spl_DN and 3' spl_DN. These were cloned into pKM197 at the *Not*I and *Xho*I sites. The gRNA
510 was retargeted by cloning into the *Kpn*I and *Mlu*I sites gBlock CRISPR_spl_647 resulting in
511 plasmid pHN61. Primers 5' sspB UP and 3' sspB UP amplified the upstream region, while
512 primers 5' sspB DN and 3' sspB DN amplified the downstream portion. These fragments were
513 also cloned into pKM126 at the same sites as previously used for homology. The gRNA was
514 switched with CRISPR_sspB_144 at *Kpn*I and *Mlu*I sites, generating pGC05. For
515 *CDR20291_1130,* primer pairs 5'CDR20291_1130_UP, 3' CDR20291_1130_UP and
516 5'CDR20291_1130_DN, 3'CDR2021_1130_DN were used, respectively, to amplify upstream
517 and downstream portions of *CDR20291_1130* homology. For *CDR20291_3080,* primer pairs
518 5'CDR20291_3080_UP, 3' CDR20291_3080_UP and 5'CDR20291_3080_DN,

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519 3'CDR2021_3080_DN were used, respectively, to amplify upstream and downstream portions
520 of *CDR20291_3080* homology. These homology arms were cloned by Gibson assembly into
521 pKM126 [52] at sites *Not*I and *Xho*I [78]. gRNAs CRISPR_CDR20291_1130_114 and
522 CRISPR_CDR20291_3080_184 were cloned into the plasmids at the *Kpn*I and *Mlu*I sites. Next,
523 because of issues with the *tet* promoter (in pKM126) causing leaky expression of *cas9*, and
524 causing potential problems during conjugation, *tetR* was replaced with *xylR* from pIA33 [79].
525 The *xylR* region was amplified with primers 5' sspB.xylR and 3'cas9_Pxyl2 for sspB plasmid,
526 5'CD1130_HR_xylR and 3'cas9_Pxyl2 for the CDR20291_1130 plasmid and the primers
527 5'CD3080_HR_xylR and 3'cas9_Pxyl2 for the CDR20291_3080 plasmid to create pHN101,
528 targeting *sspB*, pHN32, targeting *CDR20291_1130,* and pHN34, targeting *CDR20291_3080.*

529 For *C. difficile* Δ*sspA* complementation plasmids, the genes and promoter regions were
530 inserted by Gibson assembly into the *Not*I and *Xho*I sites of pJS116 [53] by Gibson assembly
531 [78]**.** The *sspA* gene plus 500 bp upstream was amplified using primer pair 5'sspA_MTL and 3'
532 sspA.pJS116 to create plasmid pHN11. The vector, pHN14, consisting of the *sspB* gene and
533 500 bp upstream was generated using primers 5' sspB UP and 3'sspBpJS116. The *sspA*
534 portion of the *sspA* and *sspB* double mutant complementation vector, pHN30, was amplified
535 with 5'sspApJS116 and 3' sspAsspB. The *sspB* portion of the double mutant complement was
536 amplified with 5' sspAsspB and 3' sspBpJS116. For pHN84, the *sspA* complement with a 6x
537 histidine tag on the C-terminus, the primers 5'sspA_MTL and 3' sspA.His_pJS116 were used.
538 The primers 5' sspB UP with 3' PsspB_sspA and 5' PsspB_sspA with 3' sspA.pJS116 were
539 used to generate a plasmid with 500 bp upstream of the *sspB* gene driving expression of the
540 *sspA* gene for pHN91. The 500 bp upstream of *sspA* was amplified with 5'sspA_MTL and
541 3'sspB_sspA and used to drive expression of the *sspB* gene, amplified with 5'sspB_sspA and
542 3'sspBpJS116 for the pHN83 complementation vector. To generate the pHN56 vector of the
543 *CDR20291_1130* gene and 500 bp upstream, the primer pair 5' 1130comp and 3' 1130comp

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544 were used. For amplification of the *CDR20291_3080* gene and 500 bp upstream, pHN57, the
545 primers 5' 3080comp and 3' 3080comp were used. Generation of 500 bp upstream of *sspA* was
546 amplified with primers 5'sspA_MTL and 3' sspA_CD1130 and used to drive expression of the
547 *CDR20291_1130* gene amplified with primers 5' CD1130_sspA and 3' 1130comp for plasmid
548 pHN96. The plasmid pHN97 was generated with 500 bp upstream of *sspA*, with primers
549 5'sspA_MTL and 3' sspA_CD3080 and the *CDR20291_3080* gene amplified with 5'
550 CD3080_sspA and 3' 3080comp. For the negative control, pHN109, of *mCherry* driven by *sspA*
551 promoter region, the *sspA* promoter region was amplified with primers 5'sspA_MTL and 3'
552 PsspA.mCherry, while the *mCherry* gene was amplified from pRAN473 [80] with primers 5'
553 PsspA.mCherry and 3' mCherry.PsspA. Another negative control of just the sspA promoter
554 region, pHN102, was generated by using 5'sspA_MTL and 3' PsspA.pJS116 to amplify the *sspA*
555 promoter region. To generate the σ^E_sspB plasmid, the promoter region of *bclA*2 was amplified
556 with 5' sigE.bclA2_pJS116 and 3' sigE.bclA2_sspB and the *sspB* gene with 5' sigE.bclA2_sspB
557 and 3' sspB_pJS116, creating pHN80. For the σ^K_sspB complement vector, the promoter region
558 of *sleC* was amplified with 5' pJS116_sigK and 3' sigK_sspB and the *sspB* gene with 5'
559 sigK_sspB and 3'sspB_pJS116, generating pHN49. The promoter region of *gpr* was amplified
560 with 5' pJS116_sigF and 3' sigF_sspB and the sspB gene with 5' sigF_sspB and 3'
561 sspB_pJS116 to generate the σ^F_sspB plasmid, pHN47. All plasmid sequences were confirmed
562 by DNA sequencing. Cloning was done in *E. coli* DH5α. A complete list of oligonucelotides used
563 and the strains and plasmids generated can be found in Tables S1 and S2, respectively.

564

565 ***Conjugations:*** The resulting plasmids, pGC05, pHN05, pHN61, pHN101, pHN32, and pHN34
566 were conjugated separately into *C. difficile* R20291 using *B. subtilis* BS49 as a conjugal donor,
567 as described previously [52]. Briefly, 0.25 mL of *C. difficile* R20291 overnight culture was back
568 diluted into 4.75 mL fresh BHIS and incubated for 4 hours. Meanwhile, 5 mL of BHIS

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569 supplemented with chloramphenicol and tetracycline was inoculated with 1 colony of *B. subtilis*
570 BS49 containing the plasmid and was incubated at 37 °C for 3 hours. After incubation, the *B.*
571 *subtilis* culture was passed into the anaerobic chamber and 100 µL culture was plated on TY
572 agar medium, along with 100 µL of *C. difficile* R20291. This was incubated for approximately 24
573 hours. Growth was then suspended in 1.5 mL of BHIS and plated onto BHIS agar supplemented
574 with thiamphenicol and kanamycin for selection. Resulting transconjugant colonies were
575 streaked, twice, onto BHIS supplemented with thiamphenicol and kanamycin (TK) or BHIS
576 supplemented with tetracycline (to screen for the conjugal transfer of the *Tn*916 transposon).
577 Thiamphenicol resistant and tetracycline sensitive colonies were tested by PCR for plasmid
578 components.
579

580 ***CRISPR induction:*** Overnight cultures of 5 mL TY medium supplemented with thiamphenicol
581 were inoculated with one colony of a *C. difficile* R20291 strain containing a CRISPR
582 mutagenesis plasmid with *tet*-driven *cas9* (pGC05 and pHN05). After approximately 16 hours of
583 growth, 0.25 mL of overnight culture was back diluted with 4.75 mL TY broth and supplemented
584 with thiamphenicol and anhydrous tetracycline and incubated for 6 hours [52]. A loopful
585 (approximately 10 µL) of culture was plated onto BHIS and the individual colonies were tested
586 by PCR for the mutation. Once PCR confirmed, the plasmid was cured by passaging onto BHIS
587 plates. For induction of *xyl*-driven *cas9* plasmids (pHN61, pHN101, pHN32 and pHN34),
588 colonies were passaged 3 times on TY agar supplemented with 1% xylose and thiamphenicol.
589 Mutants were detected by PCR and the plasmid was cured by passaging in BHIS broth
590 supplemented with 0.5% xylose. Pairwise mutants were generated by conjugating the
591 appropriate plasmid into the necessary mutant strain and inducing as described above.
592

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593 ***Spore Purification:*** Cultures were plated onto 70:30 media or CDMM minimal media where
594 indicated and incubated 5 days. Spores were purified as previously described [53, 57, 58, 81].
595 Briefly, plates were scraped and contents suspended in dH_2O overnight at 4 °C. The pellets
596 were resuspended then centrifuged at max speed. The upper, fluffy-white, layer was removed
597 and resuspended again in dH_2O. This process was repeated approximately 5 times. The spores
598 were then separated by density gradient in 50% sucrose solution at 3,500 xg for 20 minutes.
599 The spore pellet was washed approximately 5 times in dH_2O. The spores were stored at 4 °C
600 until use.

601 To purify the Δ$sspB_{SNP}$ and the Δ*sspA* Δ*sspB* double mutant spores, the pellets were scraped
602 into dH_2O and left overnight at 4 °C. The pellets were then resuspended with 1 µg of lysozyme
603 and incubated for 4 hours at 37 °C. The suspension was centrifuged at max speed for 1 minute
604 and the upper phase removed, and the pellet resuspended with dH_2O. This process was
605 repeated approximately 5 times before 5 mL of spores were layered onto a HistoDenz gradient
606 of 10 mL of 50% and 10 mL of 25%. This was centrifuged 30 minutes at 18,900 xg at 4 °C. The
607 pellet was then washed approximately 5 times in dH_2O and stored at 4°C until use.

608

609 ***Germination assay and DPA content*:** Germination was monitored using a Spectramax M3
610 plate reader (Molecular Devices, Sunnyvale, CA). 5 µL of OD_{600} = 100 spores were added to 95
611 µl germination buffer consisting of a final concentration 1x HEPES, 30 mM glycine, 10 mM TA
612 and the OD_{600} was monitored for 1 hour at 37 °C. To assay total DPA content, 1 x 10^6 spores in
613 20 µL, were boiled at 95 °C for 20 minutes. 5 µL (an equivalent of 2.5 x 10^5 spores) of the
614 solution was added to 95 µL of 1X HEPES buffer with 250 µM $TbCl_3$ and analyzed by excitation
615 at 275 nm and emission at 545 nm with a 420 nm cutoff [56, 57, 61, 72, 82].

616

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617 ***Western blotting:*** For the Δ*sspB*$_{SNP}$ strain, approximately 1 x 10^7 spores were incubated for 1
618 hour in 50 µL of BHIS with or without 10 mM TA at 37 °C. The solutions were boiled for 20
619 minutes in 2x NuPAGE buffer at 95 °C. 10 µL (equivalent to approximately 1 x 10^6 spores) of
620 each solution was separated on a 15% SDS PAGE gel. For the double mutant strain, Δ*sspA*
621 Δ*sspB*, approximately 4-8 x 10^4 total spores were separated. The protein was transferred to
622 polyvinylidene difluoride (PVDF) membranes at 0.75 amps for 1.5 hours for the Δ*sspB*$_{SNP}$ strain
623 and at 1 amp for 30 minutes for the Δ*sspA* Δ*sspB* strain. The membranes were blocked
624 overnight in 5% milk in TBST. The following day, the membranes were washed 3 times, 15
625 minutes each, at room temperature. SleC anti-sera was added to 5% milk in TBST at a 1:20,000
626 dilution for 1 hour. The membranes were washed again, as above. The anti-rabbit secondary
627 antibody was diluted to 1:20,000 in 5% milk in TBST and incubated for 1 hour. The membranes
628 were again washed, as above. The membranes were incubated with Pierce ECL Western
629 Blotting Substrate (ThermoScientific) for 1 minute and then x-ray film was exposed and
630 developed.

631

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

637

638 ***Sporulation:*** Sporulation assays were completed as previously described [73]. Briefly, three
639 70:30 plates were inoculated with fresh colonies of the indicated strains. These were grown for
640 48 hours before half of the plate was harvested into 600 µL of pre-reduced PBS solution and the

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641 pellets resuspended. 300 µL of suspension was removed from the chamber and heated for 30
642 minutes at 65 °C and mixed every 10 minutes by inversion. After heating, these samples were
643 passed into the anaerobic chamber for enumeration. Both the heat-treated and the remaining
644 300 µL of untreated suspension was serially diluted and plated in technical triplicates of 4.5 µL
645 spots on rich, BHIS, medium supplemented with 0.1% TA. After 22 hours of growth, the CFUs
646 were enumerated. The ratios of treated to untreated were calculated and then the efficiency of
647 sporulation determined in comparison to the wildtype strain. The experiment was performed in
648 biological triplicate.

649

650 ***UV exposure:*** The 302 nm UV lamp (Fisher Scientific) was allowed to warm-up for 20 minutes
651 before UV exposure. Spores were diluted in PBS to 1 x 10^7 spores / mL. A sample was
652 collected at T_0 for initial, untreated, spore calculations. 1 mL of a spore solution was added to a
653 miniature glass (UV penetrable) petri dish. These dishes were placed under the UV lamp and
654 exposed with constant agitation for the indicated time. After exposure, the untreated and treated
655 samples were serially diluted then introduced into the anaerobic chamber where they were
656 plated onto BHIS medium supplemented with taurocholic acid and thiamphenicol, for plasmid
657 maintenance. After 48 hours of incubation, the CFUs were enumerated. Treated spore counts
658 were normalized to untreated and then this ratio was normalized to the ratio for wildtype spores.

659

660 ***Outgrowth:*** Outgrowth, post-germination, was performed in half-strength BHIS supplemented
661 with 30 mM glycine and 10 mM taurocholic acid. Thiamphenicol was supplemented where
662 necessary to maintain plasmids. Spores were added to an OD_{600} of 0.05 and the OD_{600} was
663 recorded over time.

664

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665 ***Chemical resistance:*** 1 x 10^7 spores were suspended in PBS with the indicated chemical
666 concentration for various exposure times. The exposed spores were immediately serially diluted
667 into PBS tubes and plated onto rich media supplemented with taurocholate. For generation of
668 nitrous acid, a one to one volume of 1 M sodium acetate, pH 4.5 and 1 M sodium nitrite were
669 combined and incubated for at least 30 minutes before spore exposure. For formaldehyde
670 exposure, the exposed solution was serially diluted into PBS with 400 mM glycine and
671 incubated for at least 20 minutes to quench the formaldehyde before plating.

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683 Figure Legends

684 **Figure 1. SASP homology.** Alignment of *B. subtilis* SspA with *C. difficile* SspA, SspB,
685 CDR20291_1130 and CDR20291_3080. The site of GPR cleavage is indicated. Also illustrated
686 is the site of insertion in Clostridial Ssp proteins.

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687

688

689 **Figure 2. *C. difficile sspA* is necessary for UV protection.** Spores were exposed to 302 nm
690 UV light for 10 minutes. After exposure, they were serially diluted and plated onto rich medium
691 supplemented with germinants and CFUs were enumerated. All data was normalized to T_0 then
692 the ratio of the mutants was normalized to the ratio of wildtype. A.) Spores derived from wild
693 type R20291 with the indicated deletion strains. B.) spores derived from pairwise deletions of *C.*
694 *difficile* Δ*sspA*, Δ*CDR20291_1130*, and Δ*CDR20291_3080.* pEV indicates an empty vector.

695 All data points represent the average from three independent experiments. Statistical analysis
696 was performed by one way ANOVA with Dunnett's multiple comparisons test in comparison to
697 wildtype. * $P< 0.01$, ** $P< 0.0001$

698

699 **Figure 3. Complementation of the *C. difficile* Δ*sspA* strain.** A) Spores derived from wildtype
700 *C. difficile* R20291 pEV ●, the *C. difficile* Δ*sspA* pEV deletion strain □, and the *C. difficile* Δ*sspA*
701 p*sspA* strain ■ were exposed to UV for varying times and the viability assessed by normalizing
702 to T_0. B) Spores from the *C. difficile* Δ*sspA* mutant strain containing genes under control of the
703 *sspA* promoter were exposed to 302 nm UV light for 10 minutes. After exposure, the spores
704 were serially diluted and plated onto rich medium supplemented with germinants. The CFUs
705 were enumerated and then normalized to T_0 CFUs. The ratio of the complemented *C. difficile*
706 Δ*sspA* mutant strains were normalized to the ratio of the wildtype strain. C) Spores from the *C.*
707 *difficile* Δ*sspA* mutant strain containing genes under the control of the *sspA* or *sspB* promoter
708 regions were exposed to UV for 10 minutes. pEV indicates an empty vector.

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709 All data represents the average of three independent experiments. Statistical analysis was
710 performed by ANOVA with Dunnett's multiple comparison. A) two way, in comparison to
711 wildtype, B) and C) one way, in comparison to *C. difficile* Δ*sspA* pEV. * $P<0.05$, ** $P<0.0001$.

712

713 **Figure 4. *C. difficile* Δ*sspA* Δ*CDR20291_1130* Δ*CDR20291_3080* affects spore outgrowth.**
714 Outgrowth was determined by monitoring the OD_{600} of spores inoculated into half-strength
715 complex medium supplemented with germinants (10 mM taurocholic acid and 30 mM glycine).
716 All spores used for outgrowth were generated on minimal medium. A) Outgrowth of of *C. difficile*
717 R20291 pEV ●, *C. difficile* Δ*sspA* pEV □ and *C. difficile* Δ*CDR20291_1130* Δ*CDR20291_3080*
718 pEV Δ. B) Outgrowth of wildtype R20291 pEV ● and triple mutant spores of *C. difficile* Δ*sspA*
719 Δ*CDR20291_1130* Δ*CDR20291_3080* pEV ▼. C) Spore germination was monitored at OD_{600}
720 upon exposure of spores to germinants taurocholic acid and glycine in a buffered solution. The
721 OD_{600} was normalized to T_0. R20291 pEV ● and triple mutant spores of *C. difficile* Δ*sspA*
722 Δ*CDR20291_1130* Δ*CDR20291_3080* pEV ▼. pEV indicates an empty vector.

723 All data points represent the average from three independent experiments. Statistical analysis
724 by two way ANOVA with Dunnett's multiple comparisons test. A) *C. difficile* Δ*sspA,* $P<0.001$ at
725 11 minutes.

726

727 **Figure 5. *C. difficile* Δ*sspB*$_{SNP}$ results in phase gray engulfed spores.** A-H) Day 6 cultures
728 of the indicated strains were fixed in 4% formaldehyde and 2% glutaraldehyde before imaging
729 on a Leice DM6B microscope. I) DPA content was determined by boiling spore solutions and
730 analyzing approximately 2.5×10^5 spores by terbium florescence. J) Western blot of spores
731 exposed to 10 mM taurocholic acid in a rich medium for 1 hour and blotted against SleC. ←
732 indicates processed SleC. K) Spores were exposed to 302 nm UV light for 10 minutes before

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733 serial dilution and plating on rich medium supplemented with germination. All values were
734 normalized to T_0 and then those ratios were normalized to the ratio of wildtype survival. pEV
735 indicates an empty vector.

736 All data points represent the average from three independent experiments. I) Statistical analysis
737 by one way ANOVA with Dunnetts multiple comparisons test. K) Statistical analysis by unpaired
738 t test. * P<0.05, ** P<0.01, *** P<0.001.

739

740 **Figure 6. Phenotypic characterization of *C. difficile* Δ*sspA* Δ*sspB* double mutant.** A) Day
741 6 cultures were fixed in 4% formaldehyde and 2% glutaraldehyde before imaging on a Leica
742 DM6B microscope. B) DPA content was determined by boiling spore solutions and analyzing
743 approximately 2.5 x 10^5 spores by terbium florescence. C) Western blot of spores exposed to 10
744 mM taurocholic acid in a rich medium for 1 hour and blotted against SleC. ← indicates the
745 cleaved SleC band. pEV indicates an empty vector.

746 Data represents the average of three independent experiments. Statistical analysis was
747 performed by one way ANOVA with Dunnett's multiple comparison test. * P<0.01.

748

749 **Figure 7. *C. difficile* Δ*sspB* has a minor UV defect and no role in outgrowth.** A) Spores
750 derived from *C. difficile* R20291 pEV, *C. difficile* Δ*sspB* with pEV, and *C. difficile* Δ*sspB* p*sspB*
751 were exposed to UV light for 10 minutes. The spores were serially diluted and plated onto
752 medium containing germinants. Strains were normalized to CFUs at T_0. The ratios were then
753 normalized to the ratio of wildtype survival. B) Outgrowth of spores from *C. difficile* R20291 pEV
754 ● and *C. difficile* Δ*sspB* pEV ◊ was monitored at OD_{600} over 12 hours. Starting OD was 0.05 into
755 half-strengthed complex medium supplemented with germinants. pEV indicates an empty
756 vector.

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757 All data represents the average of three independent experiments. A) Statistical analysis by one
758 way ANOVA with Dunnett's multiple comparison test. * P<0.001. B) Statistical analysis by two
759 way ANOVA with Dunnett's multiple comparison test, P<0.0001 at 10 hours.

760

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1048

Figure 01

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Figure 02

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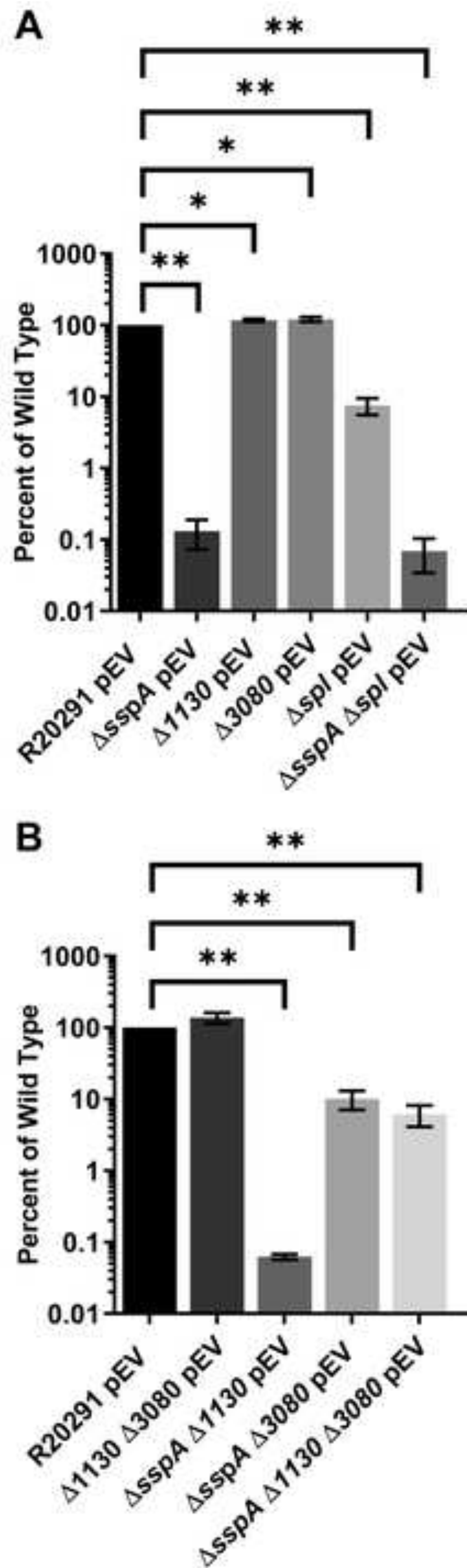

Figure 03

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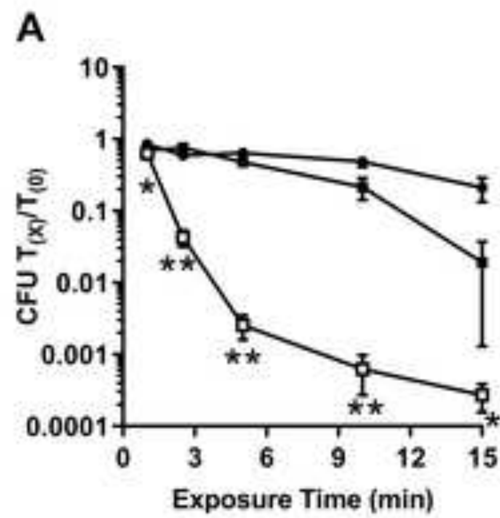

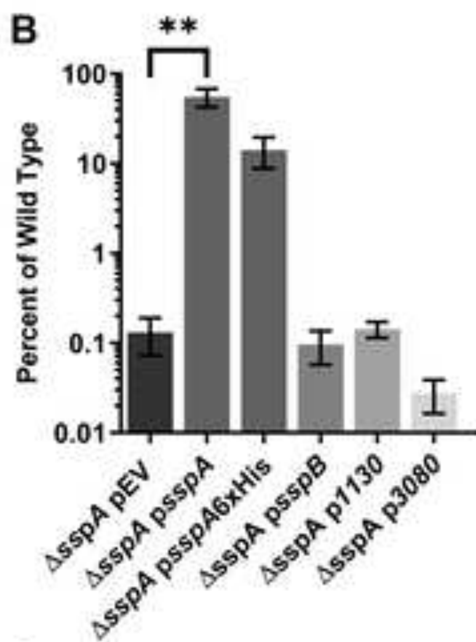

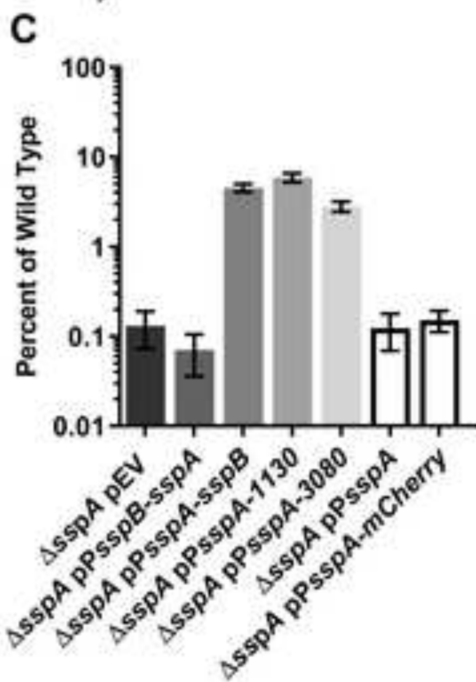

Figure 04

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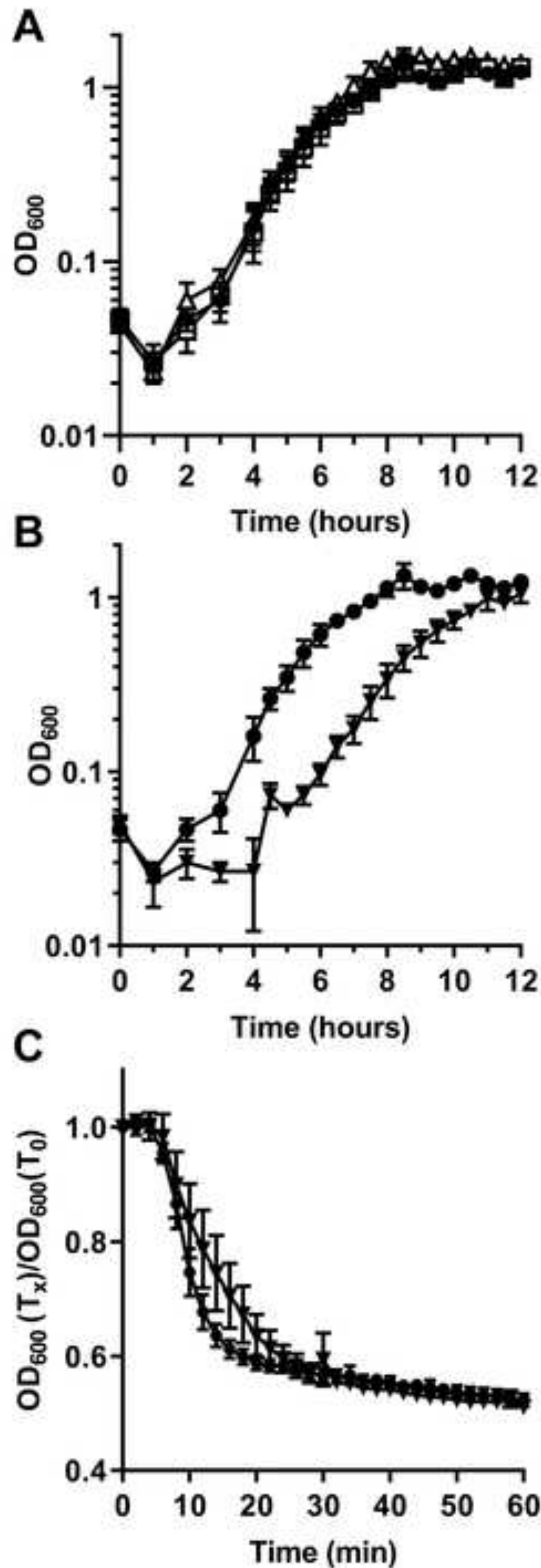

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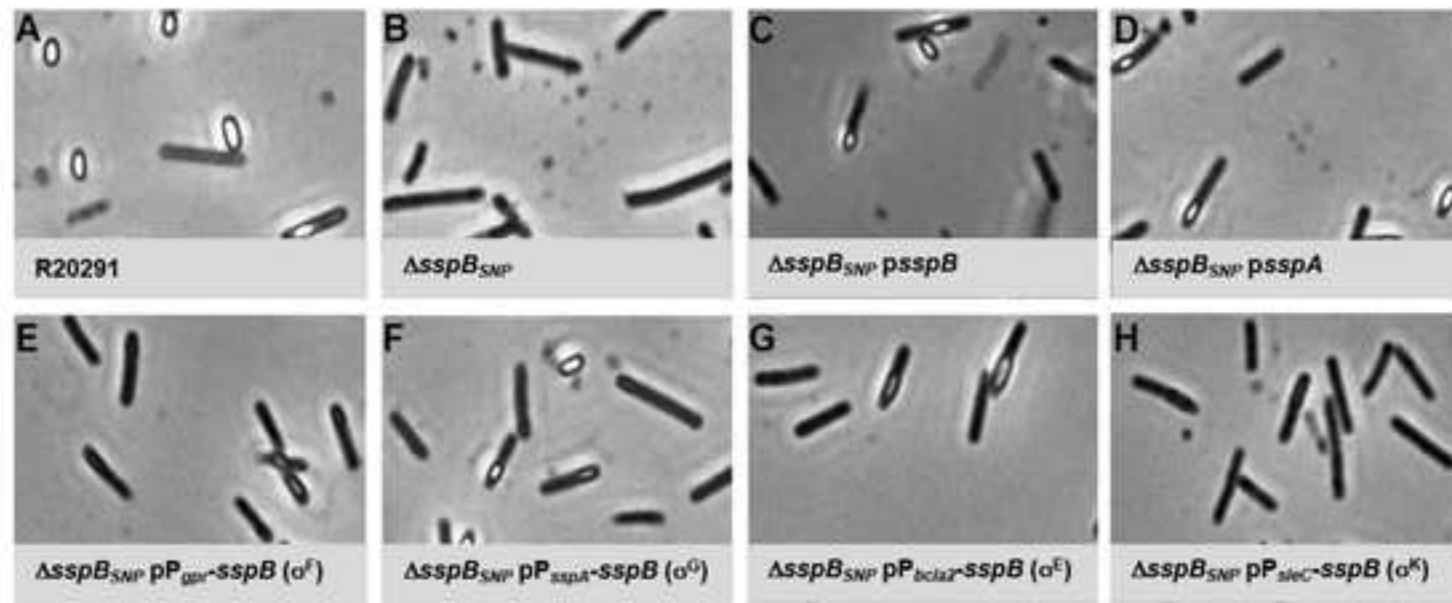

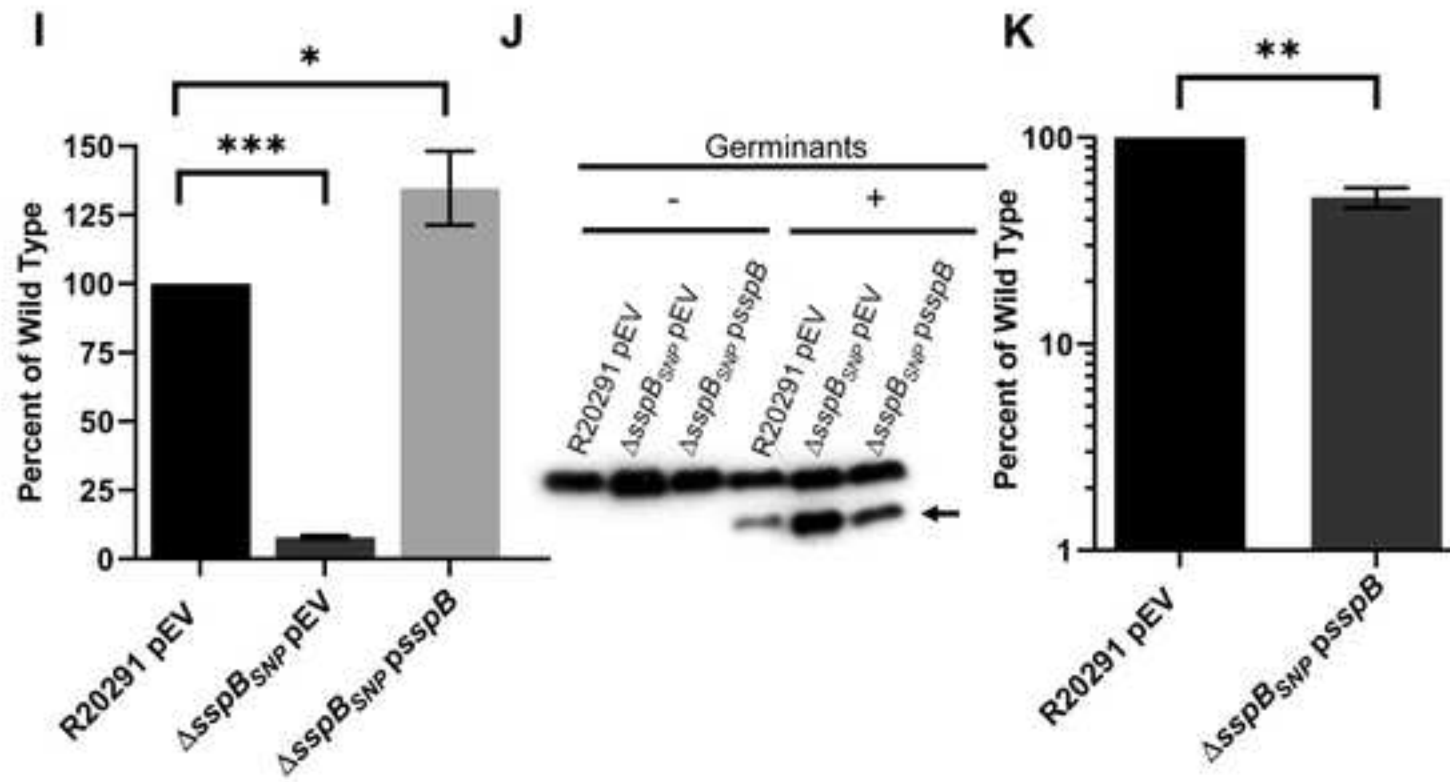

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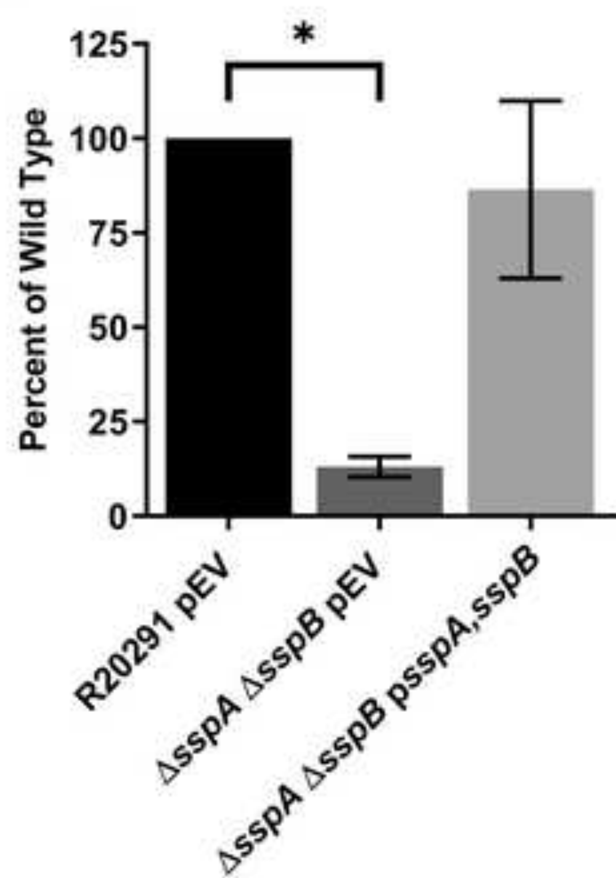

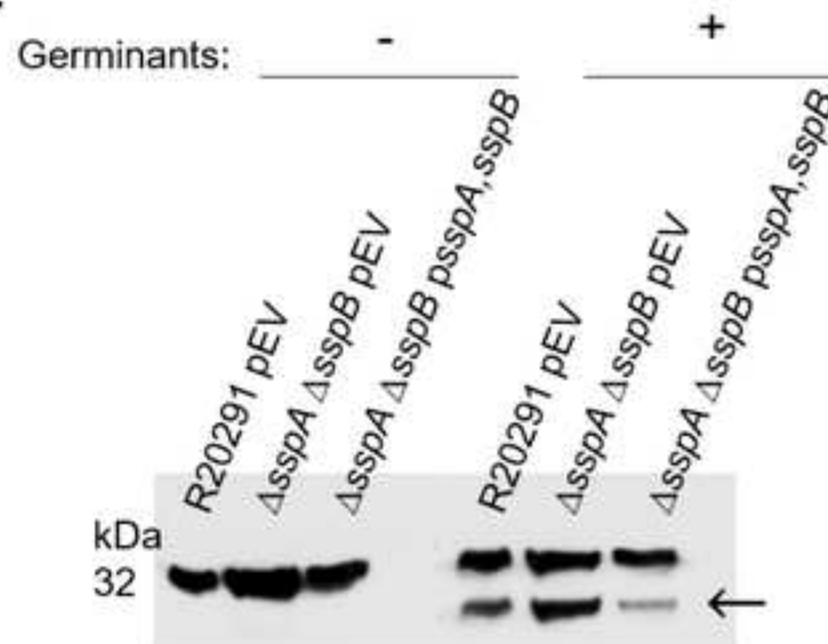

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Figure 07

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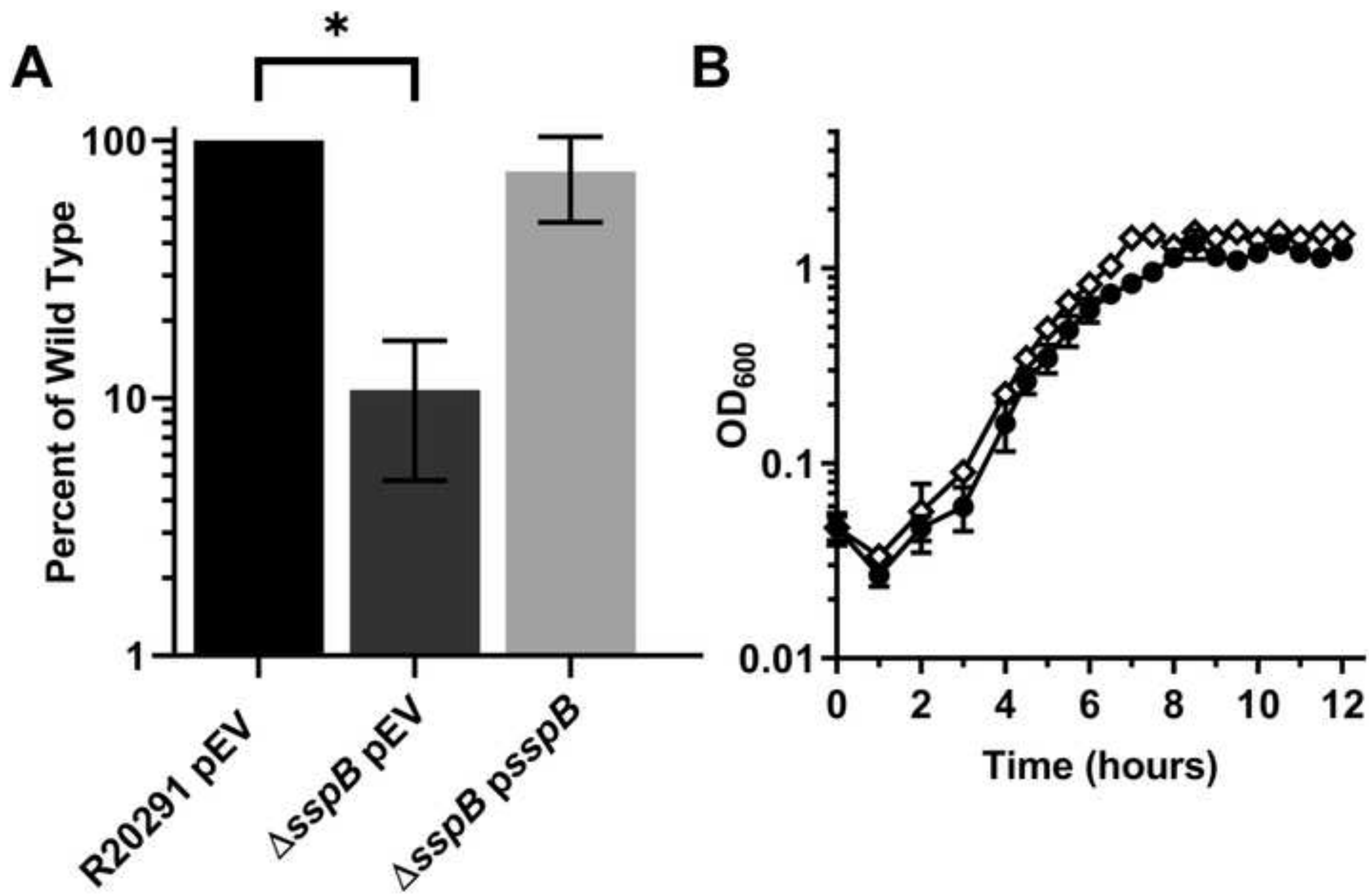

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Supplement Figure 1. Sporulation efficiency is not affected by deletion of *sspA, CDR20291_1130* or *CDR20291_3080* alone. Strains were grown on sporulation medium for two days. Sporulating cultures were heat treated at 65 °C. Sporulation rate was 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.

Supplement Figure 2. Chemical resistance is not impacted by loss of individual SASPs. 1 x 10^7 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% H202 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* Δ*sspA* pEV and *C. difficile* Δ*sspA* p*sspA* P<0.0001 at 30 minutes. C) P<0.001 for *C. difficile* Δ*sspB* p*sspB* 5 minutes and *C. difficile* Δ*sspA* p*sspA* at 10 minutes. P<0.05 for *C. difficile* Δ*CDR20291_3080* pEV at 10 minutes. D) P<0.05 for *C. difficile* Δ*sspA* p*sspA* at 5 minutes and *C. difficile* Δ*sspB* p*sspB* at 30 minutes. P<0.01 for *C. difficile* Δ*sspA* p*sspA* at 10 minutes. P<0.001 for *C. difficile* Δ*sspA* pEV and *C. difficile* Δ*sspA* p*sspA* at 30 minutes. E) P<0.01 for *C. difficile* Δ*sspB* p*sspB* at 1 minute. F) P<0.05 for *C. difficile* Δ*CDR20291_3080* at 10 minutes. G) P<0.05 for *C. difficile* Δ*CDR20291_1130* at 5 minutes, *C. difficile* Δ*sspA* pEV and *C. difficile* Δ*CDR20291_1130* pEV

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at 10 minutes. P<0.01 for *C. difficile* Δ*CDR20291_3080* at 5 minutes. P<0.001 for *C. difficile* Δ*sspA* pEV at 5 minutes.

Supplement Figure 3. Sporulation efficiency of mutant strains. 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.

Supplement Figure 4. Coomassie-stained gel of Δ*sspA* Δ*sspB* samples. The same sample volumes used for the Δ*sspA* Δ*sspB* SleC cleavage assay were separated by a 15% SDS PAGE and stained with Coomassie. pEV indicates an empty vector.

Supplement Table 1. Primers used in this study.

Primer Name	Sequence
5'sspA_MTL	ttatcaggaaacagctatgaccgcggccgcttagatgaggaaaaactggataa

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3'sspA_up	ttatttataactatctgttgctttttccaggttgattaccttccttctgttta
5'sspA_down	aataaattaaacagaaggaaggtaatcaacctggaaaaagcaacagatagt
3'sspA_downMTL	ctgcgatcgcgcatgtctgcaggcctcgagctattgaacttggaaatgagag
CRISPR_sspA_165	gtgtgctataattaaactgtaaaacgcgtGACTAAAAAATTAGTTGAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTaagctctgcaactatttttagat
5' spl_UP	ttatcaggaaacagctatgaccgcggccgctattatagaaagttcatggg
3' spl_UP	atatagcaaatctttaggtaagatttgccatttatttcacctctacttaa
5' spl_DN	taaaaactttaagtagaggtgaaataaatggcaaatcttacctaaagatt
3' spl_DN	caggcttcttatttttatgctagctcgaggatttgctctatatttttctg
CRISPR_spl_647	gtgtgctataattaaactgtaaaacgcgtgtcagctgtaaaacttgctaGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTaagctctgcaactatttttagat
5' sspB UP	attttttatcaggaaacagctatgaccgcggccgcttttaaaatatcatccatattat
3' sspB UP	tgtcaaaatttactatttattttccagccacctcaaataattagtttatgatg
5' sspB DN	tgttagacatcataaactaattatttgaggtggctggaaaataaatagta
3' sspB DN	tctgcgatcgcgcatgtctgcaggcctcgagatacttgtctatttttttcagtaaca
CRISPR_sspB_144	aattaaactgtaaaggtaccagagaaaatggatatgttggGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTaagctctgcaacta
5' sspB.xylR	gtgttactgaaaaaatagacaagtatctcgagctagcataaaaataagaagcct
5'CDR20291_1130_UP	attttttatcaggaaacagctatgaccgcggccgcgcatttatcagaagatggt
3'CDR20291_1130_UP	tttctcacatttaactttttttattccataagaatcctcctatcagtaaaatttat
5'CDR20291_1130_DN	tactgataggaggattcttatggaataaaaaaagttaaatgtgagaaaataag
3'CDR20291_1130_DN	tctgcgatcgcgcatgtctgcaggcctcgagatcaacaaatccttcaaca

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CRISPR_CDR20291_1130_114	gtgtgctataattaaactgtaaaacgcgttctaataaaggtgttttagaGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTaagctctgcaactatttttagat
5'CDR20291_3080_UP	attttttatcaggaaacagctatgaccgcggccgcattttgccacatatacgctatat
3'CDR20291_3080_UP	ttaattgacaaaaatacaaataagaagggggtgattttgcgtttgtatatg
5'CDR20291_3080_DN	cataagttcatcatatacaaacgcaaaatcacccccttcttatttgtatttttgtc
3'CDR20291_3080_DN	tctgcgatcgcgcatgtctgcaggcctcgagcagaagtgtatgagaaaatgaag
CRISPR_CDR20291_3080_184	gtgtgctataattaaactgtaaaacgcgtTGCTTACATCACGCAAAAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTATGGAGAAATCTAGATCAGCATGATGTCTGACTAGACGCGTaagctctgcaactatttttagat
3'cas9_Pxyl2	taatcctatactatatttttttatccatttaattaactctcctctttaccctcctt
5'CD1130_HR_xylR	agctggaagtgttgaaggatttgttgatctcgagctagcataaaaataagaagcct
5'CD3080_HR_xylR	ctctaagcttcattttctcatacacttctgctcgagctagcataaaaataagaagcct
3' sspA.pJS116	tgccaagcttgcatgtctgcaggcctcgagctatctgttgctttttccag
3'sspBpJS116	cagtgccaagcttgcatgtctgcaggcctcgagttattttccagccatttgtc
3' PsspB_sspA	gttctgttgttgttattgttacttgccatataattagtttatgatgtcta
5' PsspB_sspA	agtgttgttagacatcataaactaattatatggcaagtaacaataacaac
3'sspB_sspA	ttgaatttgacatagtaaatttcctcctcaagttgattaccttccttctg
5'sspB_sspA	ataaattaaacagaaggaaggtaatcaacttgaggaggaaatttactatg
5' 1130comp	ttatcaggaaacagctatgaccgcggccgcaaagcatttatcagaagatg
3' 1130comp	gccaagcttgcatgtctgcaggcctcgagttattctaaatgcctagatatacc
5' 3080comp	ttatcaggaaacagctatgaccgcggccgccccagaagtgtatgagaaaa
3' 3080comp	caagcttgcatgtctgcaggcctcgagctatattgactcatcctttttattc
3' sspA_CD1130	tagcattttgtcttgaaacatcatccatgttgattaccttccttctgttta
5' CD1130_sspA	ataaattaaacagaaggaaggtaatcaacatggatgatgtttcaagacaaaa
3' sspA_CD3080	tttctctttgtactttttctttcaaaaaaatcacgttgattaccttccttctgtt

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5’ CD3080_sspA	aataaattaaacagaaggaaggtaatcaacgtgattttttgaaagaaaaagtac
3' PsspA.mCherry	gccatattatcttcttctcctttagataccatgttgattaccttccttctg
5' PsspA.mCherry	taaattaaacagaaggaaggtaatcaacatggtatctaaaggagaagaag
3' mCherry.PsspA	gtgccaagcttgcatgtctgcaggcctcgagttaaaacttataggatccgg
3’ PsspA.pJS116	agtgccaagcttgcatgtctgcaggcctcgaggttgattaccttccttc
5’ sigE.bclA2_pJS116	tttatcaggaaacagctatgaccgcggccgcttagtgccagattattgtgg
3’ sigE.bclA2_sspB	tttgacatagtaaatttcctcctcaaataattaatcctcctttttaaag
5’ sigE.bclA2_sspB	ctctaactttaaaaaaggaggattaattatttgaggaggaaatttactatg
3’ sspB_pJS116	tgccaagcttgcatgtctgcaggcctcgagttattttccagccatttgtc
5’ pJS116_sigK	ttttatcaggaaacagctatgaccgcggccgctcagagcttttacaccttct
3’ sigK_sspB	aatttgacatagtaaatttcctcctcaacaaatcaccctttctttaaatg
5’ sigK_sspB	ctaaattcatttaaagaaagggtgatttgttgaggaggaaatttactatg
5’ pJS116_sigF	ttatcaggaaacagctatgaccgcggccgcaagaaatgaagtgcgttaa
3’ sigF_sspB	atttgacatagtaaatttcctcctcaaataaacctccagtataaattaaatg
5’ sigF_sspB	attagcatttaatttatactggaggtttatttgaggaggaaatttactatg
5’sspApJS116	cgaattcgagctcggtacccggggatcctctagattagatgaggaaaaactggataaaag
3’ sspAsspB	ggaactgataatatggatgatattttaaaactatctgttgctttttccagccatttg
5’ sspAsspB	caaatggctggaaaaagcaacagatagttttaaaatatcatccatattat
3’ sspA.His_pJS116	tgcaggcctcgagctagtggtggtggtggtgtctgttgctttttccagc

Supplement Table 2. Strains and plasmids used in this study.

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Strain	**Description**	**Reference**
E. coli DH5a	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG□□80d*lacZ*ΔM15 Δ(*lacZ*YA-*argF*)U169, hsdR17(r□$^-$m□$^+$),□-	[83]
E. coli MB3436	*recA*$^+$ *E. coli* strain	Gift from Dr. Michael Benedik
B. subtilis BS49	*Tn*916 donor strain, Tet^R	[84]
C. difficile R20291	Wild type, ribotype 027	[85]
C. difficile KNM10	*spo0A* CRISPR-*cas9* mutant	[86]
C. difficile HNN03	*sspA* CRISPR-*cas9* mutant	This study
C. difficile HNN04	*sspB* CRISPR-*cas9* mutant with an $sspA_{G52V}$ allele	This study
C. difficile HNN05	*sspA* and *sspB* CRISPR-*cas9* double mutant	This study
C. difficile HNN06	*CDR20291_1130* CRISPR-*cas9* mutant	This study
C. difficile HNN07	*CDR20291_3080* CRISPR-*cas9* mutant	This study
C. difficile HNN10	*spl* CRISPR-*cas9* mutant	This study
C. difficile HNN11	*sspA* and *spl* CRISPR-*cas9* double mutant	This study
C. difficile HNN12	*CDR20291_1130* and *CDR20291_3080* CRISPR-*cas9* double mutant	This study
C. difficile HNN14	*sspA* and *CDR20291_3080* CRISPR-*cas9* double mutant	This study
C. difficile HNN15	*sspA* and *CDR20291_1130* CRISPR-*cas9* double mutant	This study
C. difficile HNN16	*sspA* and *CDR20291_1130* and *CDR20291_3080* CRISPR-*cas9* triple mutant	This study
C. difficile HNN17	*sspB* CRISPR-*cas9* mutant	This study
Plasmid	**Description**	**Reference**
pJS116	*B. subtilis* – *C. difficile* shuttle vector pCD6 ColE1 *Tn916 oriT* Cm^R	[53]
pKM126	CRISPR plasmid with *tetR* promoter driving *cas9*	[52]
pKM197	CRISPR plasmid with *xylR* promoter driving *cas9*	[87]
pIA33	*xylR* containing plasmid	[79]
pRAN473	*mCherry* containing plasmid	[80]
pGC05	*sspB* targeting CRISPR plasmid	This study

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pHN05	*sspA* targeting CRISPR plasmid	This study
pHN11	*sspA* promoter region and gene	This study
pHN14	*sspB* promoter region and gene	This study
pHN30	*sspA* and *sspB* complement	This study
pHN32	*1130* targeting CRISPR plasmid	This study
pHN34	*3080* targeting CRISPR plasmid	This study
pHN47	*gpr* promoter region and *sspB* gene	This study
pHN49	*sleC* promoter region and *sspB* gene	This study
pHN56	*1130* promoter region and gene	This study
pHN57	*3080* promoter region and gene	This study
pHN61	*spl* targeting CRISPR plasmid	This study
pHN80	*bclA2* promoter region and *sspB* gene	This study
pHN83	*sspA* promoter region and *sspB* gene	This study
pHN84	*sspA* promoter region and gene with 6x His tag on the C-terminus	This study
pHN91	*sspB* promoter and *sspA* gene	This study
pHN96	*sspA* promoter region and *1130* gene	This study
pHN97	*sspA* promoter region and *3080* gene	This study
pHN101	*sspB* targeting CRISPR plasmid, *xylR* promoter	This study
pHN102	*sspA* promoter region	This study
pHN109	*sspA* promoter region and *mCherry* gene	This study

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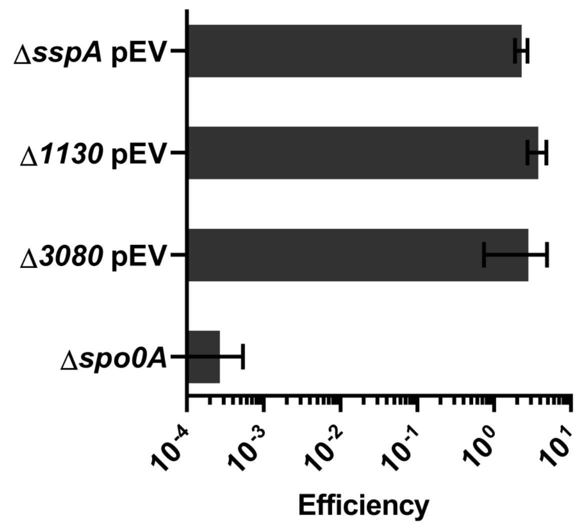

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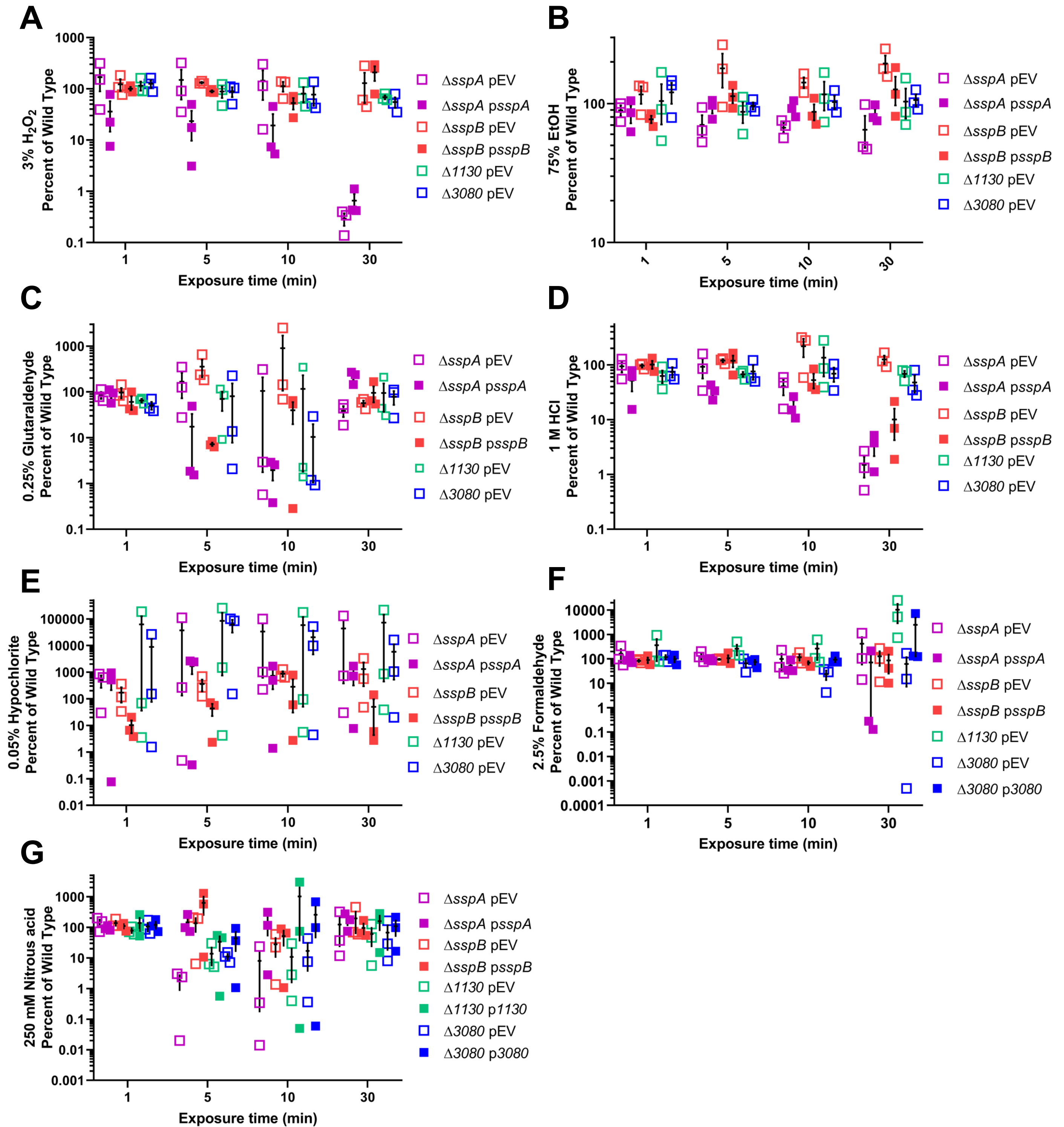

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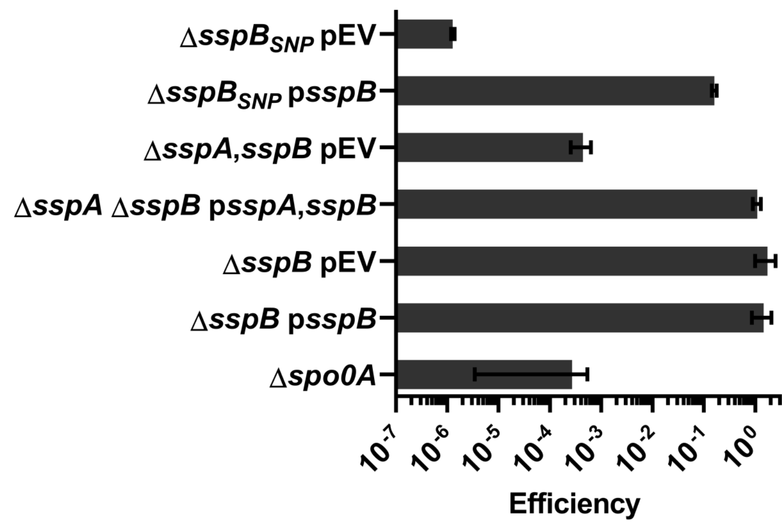

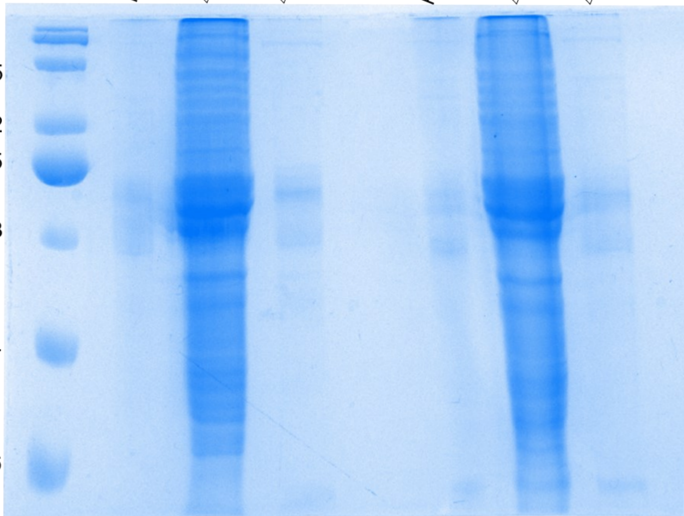
Germinants:
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+
R20291 pEV
$\Delta sspB_{SNP}$ pEV
$\Delta sspB_{SNP}$ psspB
R20291 pEV
$\Delta sspB_{SNP}$ pEV
$\Delta sspB_{SNP}$ psspB
kDa
95
72
55
43
34
26