Spo0A suppresses *sin* locus expression in *Clostridioides difficile*

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Short title: *C. difficile sin* locus regulation

Key Words: *Clostridioides difficile*, *C. difficile*, SinR, Spo0A, gene regulation

Abstract

Clostridioides difficile is the leading cause of nosocomial infection and is the causative agent of antibiotic-associated diarrhea. The severity of the disease is directly associated with the production of toxins, and spores are responsible for the transmission and persistence of the organism. Previously we characterized *sin* locus regulators SinR and SinR', where SinR is the regulator of toxin production and sporulation, while the SinR' acting as its antagonist. In *Bacillus subtilis*, Spo0A, the master regulator of sporulation, regulates SinR, by regulating the expression of its antagonist *sinI*. However, the role of Spo0A in the expression of *sinR* and *sinR'* in *C. difficile* is not yet reported. In this study, we tested *spo0A* mutants in three different *C. difficile* strains R20291, UK1, and JIR8094, to understand the role of Spo0A in *sin* locus expression. Western blot analysis revealed that *spo0A* mutants had increased SinR levels. The qRT-PCR analysis for its expression further supported this data. By carrying out genetic and biochemical assays, we have shown that Spo0A can bind to the upstream region of this locus to regulates its expression. This study provides vital information that Spo0A regulates *sin* locus, which controls critical pathogenic traits such as sporulation, toxin production, and motility in *C. difficile*.

47 **IMPORTANCE**

48 *Clostridioides difficile* is the leading cause of antibiotic-associated diarrheal disease in the
49 United States. During infection, *C. difficile spores* germinate, and the vegetative bacterial
50 cells produce toxins that damage host tissue. In *C. difficile*, *sin* locus is known to regulate
51 both sporulation and toxin production. In this study, we have shown that Spo0A, the
52 master regulator of sporulation to control the *sin* locus expression. We performed various
53 genetic and biochemical experiments to show that Spo0A directly regulates the
54 expression of this locus by binding to its upstream DNA region. This observation adds
55 new detail to the gene regulatory network that connects sporulation and toxin production
56 in this pathogen.

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70 **INTRODUCTION**

71 *Clostridioides difficile* is a Gram-positive, anaerobic bacillus and is the principal causative
72 agent of antibiotic-associated diarrhea and pseudomembranous colitis (1–3). Antibiotic
73 use is the primary risk factor for the development of *C. difficile* associated disease
74 because it disrupts normal protective gut flora and provides a favorable environment for
75 *C. difficile* to colonize the colon. Two major pathogenic traits of *C. difficile* are toxin (toxin
76 A and B) and spores (3–5). Deaths related to *C. difficile* increased by 400% between 2000
77 and 2007, in part because of the emergence of more aggressive *C. difficile* strains (6, 7).
78 Robust sporulation and toxin production were suspected of contributing to the widespread
79 of the *C. difficile* infections associated with these highly virulent strains (8–14). How *C.*
80 *difficile* triggers toxin production and sporulation in the intestinal environment is only
81 beginning to be understood.

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83 Recently, we reported the identification and characterization of master regulator SinR in
84 *C. difficile* that was found to regulate sporulation, toxin production, and motility (15). SinR
85 in the Gram-positive model organism *B. subtilis* is well characterized and is known to
86 regulate multiple pathways, including sporulation, competence, motility, and biofilm
87 formation (16–18). In *B. subtilis*, SinR is encoded by the downstream gene of the two
88 gene operon called *sin* (sporulation inhibition) locus and its transcription is driven from
89 two promoters. The second gene in the operon, *sinR,* is transcribed by an internal
90 promoter and is constitutively expressed. SinR represses the first committed (stage II)
91 genes in the sporulation pathway (17).The promoter upstream of the operon is activated
92 by phosphorylated Spo0A, leading to the expression of *sinI*, along with *sinR*. The SinI

93 protein binds and inhibits the DNA binding activity of SinR (19–21). The combined effect
94 of positive regulation by Spo0A~P and the inactivation of the negative regulator SinR
95 activates the sporulation pathway. In *C. difficile* also the *sin* locus is a two-gene operon
96 and encodes for SinR and SinR'. In our initial characterization of the *C. difficile sin* locus,
97 we have shown that disruption of *sin* locus (absence of both SinR and SinR') resulted in
98 asporogenic, less toxic, and less motile phenotype (15). Another study which reports that
99 *C. difficile sin* locus suppresses biofilm formation corroborates our finding (22). Further
100 investigation showed that among the two regulators, SinR positively influences
101 sporulation, toxin production, and motility, while SinR' acts as an antagonist to SinR and
102 control its activity. Since *sin* locus has a role in regulating various pathogenic traits in *C.*
103 *difficile*, understanding the regulation of its expression is important. Earlier, we have
104 shown that disrupting the first gene *sinR* in the operon to affects both the *sinR* and the
105 downstream *sinR'* transcription. This led to the assumption that unlike *B. subtilis*, the *C.*
106 *difficile sin* locus is transcribed from a single upstream promoter. Real-time RT PCR
107 analysis of the cells grown *in vitro*, showed *sin* locus expression at 10-12 hour time point,
108 indicating its tight regulation (15). From various gene expression data, we can observe
109 that mutations in *sigH* and *spo0A* positively influence the expression of *sinRR'* (23–26)
110 and mutations in *tcdR* to downregulate their expression (27). We have also demonstrated
111 that CodY can directly bind to *sin* locus upstream DNA to transcriptionally repress its
112 expression (15). In the same line of investigation, in this study, we discovered that Spo0A,
113 the sporulation master regulator, represses *sinR* expression. The effect was directly
114 caused by the specific binding of Spo0A to the promoter region upstream of the locus.
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116 # MATERIAL AND METHODS

117 **Bacterial strains and growth conditions**

118 *C. difficile* strains (Table S1) were grown in TY (Tryptose and Yeast extract) agar or broth
119 culture in an anaerobic chamber which is maintained at 10% H_2 , 10% CO_2 and 80% N_2
120 as described previously (27–30). Lincomycin (Linc 20ug/ml) and thiamphenicol (Thio; 15
121 ug/ml) were added to the culture medium when required. S17-1, an *E.coli* strain used for
122 conjugation (31), was cultured aerobically in LB (Luria-Bertani) broth or agar and was
123 supplemented with ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml) when necessary.

124

125 **General DNA techniques**

126 Chromosomal DNA was extracted from *C. difficile* cultures using DNeasy Blood and
127 Tissue Kit (Qiagen). PCR reactions were carried out using gene specific primers (Table
128 S2). PCR products were extracted from the gel using Geneclean Kit (mpbio). Plasmid
129 DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen). Standard procedures were
130 used to perform routine cloning.

131

132 **Construction and complementation of *C. difficile spo0A* mutant strains**

133 The *spo0A* mutants in JIR8094 and UK1 strains were created using ClosTron gene
134 knockout system as described previously (24, 25, 32, 33). Briefly, for *spo0A* disruption,
135 the group II intron insertion site between nucleotides 178 and 179 in *spo0A* gene in the
136 antisense orientation was selected using a web-based design tool called the Perutka
137 algorithm. The designed retargeted intron was cloned into pMTL007-CE5 as described

138 previously (34). The resulting plasmid pMTL007-CE5::*spo0A*-178-179a was transferred
139 into *C. difficile* UK1 and JIR8094 cells by conjugation. The potential LI.ltrB insertions
140 within the target genes in the *C. difficile* chromosome was conferred by the selection of
141 lincomycin resistant transconjugants in 20 µg/ ml lincomycin plates. PCR using gene-
142 specific primers (Table S2) in combination with the EBS-U universal was performed to
143 identify putative *C. difficile* mutants. *C. difficile spo0A* mutants were complemented by
144 introducing pRG312 which contains the *spo0A* gene with the 300 bp upstream region,
145 through conjugation. Complementation was confirmed by PCR and western blot
146 analysis.
147
148 **Western blot analysis**
149 *C. difficile* cultures for western blot analysis were harvested and washed in 1xPBS
150 solution. The pellets were resuspended in sample buffer (Tris 80 mM; SDS 2%; Glycerol
151 10%) and lysed by sonication. The whole cell extracts were then centrifuged at 17,000 g
152 at 4°C for 1 min. The lysate was heated at 100°C for 7 min and the proteins were
153 separated by SDS-PAGE and electro-blotted onto PVDF membrane. The blots were then
154 probed with specific primary and the secondary antibodies at a dilution of 1:10,000.
155 Immuno-detection of proteins was done using ECL kit (Millipore) following the
156 manufacturer's recommendations and were developed using G-Box iChemi XR scanner.
157 Blot images were overlapped with the original images of the membrane to visualize pre-
158 stained marker.
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161 **Construction of reporter plasmids and beta-glucuronidase assay**

162 The *sin* locus upstream DNA regions of various lengths were amplified by PCR using
163 specific primers with *KpnI* and *SacI* (Table S2) recognition sequences. R20291 strain
164 chromosomal DNA was used as a template for this amplification. Plasmid pRPF185
165 carries a *gusA* gene for beta-glucuronidase under tetracycline-inducible (*tet*) promoter.
166 The *tet* promoter was removed using *KpnI* and *SacI* digestion and was replaced with the
167 *sin* locus upstream regions of various lengths to create plasmids pBA009, pBA029,
168 pBA037, pBA038 and pBA039 (Table S1). The control plasmid pBA040 with promoter
169 less *gusA* was created by digesting with *KpnI* and *SacI* to remove *tet* promoter and then
170 self-ligated after creating blunt ends. Plasmids were introduced into R20291 and
171 R20291::*spo0A* strains through conjugation as described previously (15, 27). The
172 transconjugants were grown in TY medium in the presence of thiamphenicol (15 ug/ml)
173 overnight. Overnight cultures were used as an inoculum at a 1:100 dilution to start a new
174 culture. Bacterial cultures were harvested at 10 hr of growth and the amount of for beta-
175 glucuronidase activity was assessed as described elsewhere (35, 36). Briefly, the cells
176 were washed and resuspended in 1 ml of Z buffer (60 mM $Na_2HPO_4.7H_20$ pH 7.0, 40 mM
177 $NaH_2PO_4.H_2O$, 10 mM KCl, 1mM $MgSO_4.7H_2O$ and 50mM 2ME) and lysed by
178 homogenization. The lysate was mixed with 160 ul of 6mM p-nitrophenyl β-D-glucuronide
179 (Sigma) and incubated at 37°C. The reaction was stopped by the addition of 0.4 ml of 1.0
180 M $NaCO_3$. β-Glucuronidase activity was calculated as descried earlier (35, 36).

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184 **Mutagenesis of *sin* locus promoter region**

185 Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used
186 to carry out site directed mutagenesis whereby G and C residues of the potential Spo0A
187 binding '0A' boxes were substituted with A residues. The mutagenic oligonucleotide
188 primers used are listed in Table S2.

189

190 **DNA binding**

191 DNA binding was carried out as described elsewhere (37). Briefly, the promoter region of
192 interest was biotin labelled and was coupled to immobilized Monomeric Avidin Resin (G
193 Biosciences) in B/W Buffer (37). The DNA and the beads were incubated at room
194 temperature for 30 min in a rotor. The bead-DNA complex was washed with TE Buffer to
195 remove any unbound DNA. To prepare cell lysates, *C. difficile* R20291 strain was grown
196 to late exponential phase (16 hour) in 500 ml TY medium, pH 7.4. After washing with
197 1XPBS, the cells were resuspended in BS/THES buffer (37) and lysed using French
198 press. The whole lysate was centrifuged at 20,000 g for 30 min at 4°C and the supernatant
199 was incubated with the bead-DNA complex and allowed to rotate at 4°C overnight. The
200 bead-DNA-protein complex was washed with BS/THES Buffer (5 times). Elution was
201 carried out with 50mM, 100 mM, and 200 mM NaCl in Tris-HCl pH 7.4. The eluates were
202 analyzed by SDS-PAGE and Western Blotting using Spo0A specific antibody.

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207 **RESULTS**

208 **Elevated level of SinR is present in *spo0A* mutant**

209 In *C. difficile,* we have previously shown that *sin locus* mutant is asporogenic and this
210 phenotype is associated with downregulation of *spo0A* expression. Interestingly,
211 disruption of *sinR',* the second gene in the locus resulted in elevated levels of sporulation.
212 This result suggested that SinR as a positive regulator of the sporulation. Gene
213 expression data of *spo0A* mutants from different studies have shown an elevated levels
214 of *sin locus* expression when compared to their respective parents (25, 32, 38). These
215 observations taken together suggest that these two master regulators, Spo0A and SinR
216 regulate each other's transcription. To understand the possible regulatory relationship
217 between SinR and Spo0A in *C. difficile*, we created *spo0A* mutant in two different *C.*
218 *difficile* strains, JIR8094 and UK1 strain using the Clostron mutagenesis technique.
219 Mutation in *spo0A* was confirmed by PCR (Fig. S1ABC) and western blot analysis using
220 Spo0A specific antibodies (Fig. S1D). The *spo0A* mutant in R20291 obtained from Dena
221 Lyras Lab (39) was also included in the analysis. As previously reported, mutation in
222 *spo0A* resulted in the asporogenic phenotype (24, 25, 33, 39). For complementation,
223 plasmid pRG312 carrying *spo0A* under its own promoter was into the mutants.
224 Introduction of the *spo0A* expressing plasmid was successful in the JIR8094::*spo0A* and
225 in R20291::*spo0A* mutants, but unsuccessful in the UK1::*spo0A* mutant. Heat resistant
226 spores were observed in the complemented strains, however the levels were significantly
227 lower than wild-type (Fig. S2). To test whether Spo0A influences expression of the *sin*
228 locus genes, we performed quantitative reverse transcription-PCR (qRT-PCR) analysis
229 of the *sinR* and *sinR'* transcripts in *spo0A* mutants and their respective parent strains. As

230 previously reported, the level of *sinR* and *sinR'* transcripts were increased several folds,
231 (Fig. 1A) in all three *spo0A* mutants compared to their parent strains. To further confirm
232 this result, we performed western blot analysis using SinR specific antibodies. We grew
233 the mutants and the respective parent strains in TY medium for 10 hours and observed
234 the levels of SinR in their cytosol. We found that *spo0A* mutants in all three strains
235 produced higher amounts of SinR compared to their respective parents (Fig. 1B,
236 Fig.S1D). However, in our complementation of R20291::*spo0A* and JIR8094::*spo0A*, we
237 did not see lower levels of SinR (Fig. 1B).
238
239 **Spo0A represses the expression of *sinR***
240 Spo0A is a transcriptional regulator and is a DNA binding protein. Spo0A binds to specific
241 DNA sequences in the promoter region of its target gene to regulate their expression. To
242 determine if the elevated levels of SinR observed in *spo0A* mutants is due to the repressor
243 activity of Spo0A, we performed reporter fusion assays. We fused 600 bps of *sin* locus
244 upstream DNA with the *gusA* reporter gene coding for beta-glucuronidase and the
245 construct was introduced into the R2091::*spo0A* mutant and its parent strain. The plasmid
246 carrying a promoter less *gusA* was used as a negative control. We also cloned the
247 promoter region of *spoIIAB* known to be regulated by Spo0A, with the *gusA* and used this
248 construct as a positive control. The *spoIIAB* promoter is positively regulated by Spo0A
249 and was found to be active only in the parent strain and not in the *spo0A* mutant (Fig.
250 2A). We observed significantly higher beta glucuronidase activity when it was expressed
251 from the *sin* locus promoter in the R20291::*spo0A* mutant strain compared to the parent
252 strain, where very minimal reporter activity was recorded. This observation is consistent

253 with our western blot results, where we detected elevated levels of SinR in *spo0A* mutant
254 strains. Taken together these results suggest that Spo0A represses the transcription of
255 *sinR* either directly or indirectly. To narrow down the Spo0A controlled region in the *sin*
256 locus promoter, we cloned the 475 and 340 bps of the upstream DNA with the *gusA* gene
257 and performed the reporter fusion assays. The reporter gene activity was similar in the
258 cultures carrying the 600 bp as well as 340 bp upstream fusions (Fig. 2B). This indicated
259 that both the *sin* locus promoter and the Spo0A regulated regions are present within this
260 340 bp region.
261
262 **Spo0A binds to the promoter region of *sinR***
263 The results in Figure 2AB show that expression of P*sin-gusA* was less in the R20291
264 background while the expression of the reporter gene was at higher levels in
265 R20291::*spo0A* background. To determine whether the repression of *sinR* by Spo0A is
266 due to Spo0A binding specifically to the promoter region of *sinR*, we carried out a DNA
267 binding experiment. Considering that Spo0A needs to be phosphorylated to bind to the
268 target DNA and inability to purify Spo0A-P, we did not attempt the *in vitro* electrophoretic
269 gel shift assay. Instead, we used a biotin labelled DNA pulldown assay to determine the
270 DNA binding ability of Spo0A under native conditions. The DNA segment representing
271 the promoter region of *sinR* was biotinylated and was coupled to Immobilized Monomeric
272 Avidin Resin. This bead-DNA complex was incubated with the cell lysate from the parent
273 R20291 strain. The bound proteins were eluted and were run in SDS page and
274 immunoblotted with Spo0A antibody. We first standardized the binding experiment by
275 using *spo0IIAB* promoter region as a positive control. Spo0A protein could be detected in

276 the elutes when the *spo0IIAB* upstream DNA was used the bait. The biotinylated *gluD*
277 upstream DNA also processed similarly and served as a negative control. We applied the
278 same protocol using the biotinylated 340 bp *sin* upstream DNA as bait. Results showed
279 that it could pull down Spo0A, suggesting that Spo0A binds specifically to the promoter
280 region of the *sin* locus (Fig. 3AB). Next, to narrow down the Spo0A binding site within that
281 340 bps we created three biotin-labelled fragments covering the first 118 bps (340-222
282 bps upstream), the last 140 bps and overlapping 135 bps mid-region (237 to 102 bps
283 upstream) (Fig. 3A) and used them as bait in the pull-down experiment. More Spo0A was
284 detected when the 140 bps mid-region was used as a bait compared to the first 118 bps
285 (Fig. 3C). The Spo0A protein appears to bind with greater affinity to the middle fragment,
286 when compared to the other two fragments. Since the biotin DNA pulldown assay is semi
287 quantitative in nature, this assumption needs further validation. Spo0A could not be
288 recovered from the elute from the binding of *gluD* upstream region, suggesting the
289 specificity of the Spo0A binding with the *sin* locus promoter.
290

291 **Mutational analysis of the *sinR* upstream region**

292 In *B. subtilis* Spo0A-P is known to bind to 7 bp DNA element 5'-TGNCGAA-3', commonly
293 known as Spo0A box (40). However, there are certain exceptions where Spo0A binds to
294 degenerated Spo0A boxes with mismatches in the upstream of some targets (41, 42).
295 The DNA binding domain of *C. difficile* Spo0A is highly homologous to *B. subtilis* Spo0A
296 and the key residues of Spo0A known to mediate the interaction with the bases of the 0A
297 box are highly conserved in *Bacillus* and *Clostridium* species (24, 43). In *C. difficile*,
298 Spo0A is known to bind to *spo0A* upstream and *sigH* upstream. Both of these genes have

299 the TGTCGAA consensus Spo0A box sequence (23, 44). *C. difficile* Spo0A also bind to
300 the upstream of *spoIIAA-spoIIE-spoIIGA* operon with low affinity, where the binding
301 sequence is a degenerated Spo0A box with TACGACA sequence (23). We scanned the
302 upstream region of *sinR* for potential Spo0A binding consensus sequence. From the DNA
303 binding pull-down experiment we could predict that Spo0A binds to sequences within the
304 237-102 bps upstream of *sin* locus. Classical Spo0A binding boxes couldn't be identified
305 in this region. However, two repeats with TATTGTAG sequences could be seen in this
306 region. We mutated the TATTGTAG into TATTATAA, created reporter fusions with
307 mutation in these sequences and analyzed their effect on the reported expression. None
308 of the mutations affected the expression of the reporter gene. Mutations were introduced
309 in the repeat sequence TAGTCTAT that occurred within the first 88 bps of *sin* locus
310 upstream (data not shown). These changes also didn't affect the *sin* locus expression.
311 Even though, we could not identify the specific Spo0A binding boxes in the *sin* locus
312 upstream, we have mapped the region to which it binds.
313

314 **DISCUSSION**

315 Sporulation in a cell is an intense response to stress and is particularly expensive, in
316 terms of both time and materials (45). The exact conditions and timing for sporulation are
317 likely to be under strong selective pressure as both premature and belated spore
318 production can have disastrous effects on cell growth and survival. In *B. subtilis*,
319 the *sin* (*s*porulation *in*hibition) operon is central to the timing and early dynamics of this
320 network (46–48), and its regulation is controlled by the sporulation master regulator
321 Spo0A itself . In this study, we have demonstrated that similar to *B. subtilis*, the *sin* locus

322 in *C. difficile* is also regulated by Spo0A. We created *spo0A* mutant in two different *C.*
323 *difficile* strains JIR8094 and UK1, and included the previously created R20291::*spo0A*
324 mutant in the analysis. As previously noted, we found both the JIR8094::*spo0A* and the
325 UK1::*spo0A* strain to be asporogenic in nature. Complementation of sporulation
326 phenotype was not complete in JIR8094::*spo0A* and we couldn't complement the
327 UK1::*spo0A* mutant strain even after multiple attempts for reasons unknown. Western
328 blot analysis detected higher amount of SinR in all three different *spo0A* mutant strains.
329 However, similar to the sporulation phenotype complementation of R20291::*spo0A* and
330 JIR8094::*spo0A* did not lower the level of SinR. Unlike *B. subtilis* SinR, *C. difficile* SinR
331 acts as a positive regulator of sporulation (15). How SinR controls sporulation is yet to be
332 characterized. As we noted previously, *sin* locus is expressed at a low level only at a short
333 window of time at 10-12 h of growth (15), suggesting that the presence of these regulators
334 in the right amount at a specific time point could be important to regulate sporulation
335 initiation and progression. In *spo0A* mutant however, *sinR* is expressed at a very high
336 level which can affect the temporal nature of the sporulation pathway. It's worth noting
337 that asporogenic phenotype of *sinRR'* mutant could not be complemented either (15).
338 Failures to complement a *spo0A* mutation have been previously observed in *C. difficile*.
339 Two independent studies showed incomplete restoration of sporulation phenotype in
340 R20291::*spo0A* (32, 39)**.** However, when Deakin *et al.* (2012) tested the *spo0A* mutants
341 in 630Δ*erm* and R20291 strains and they found *in vitro* levels of sporulation to be restored
342 to wild type levels in their complemented derivative (33). When they tested the R20291
343 strains for toxin production, however, the complemented strain still produced increased
344 levels of toxin compared to wild type (33). These observations suggest that this method

345 of introducing *spo0A* using a multicopy plasmid may not be the suitable method for
346 complementation considering the complex nature of Spo0A regulatory networks.
347
348 Like in many Gram-positive bacteria, Spo0A is the master regulator of sporulation in *C.*
349 *difficile* (24, 33, 39). When the post-exponential phase begins, Spo0A activates the
350 expression of the genes involved in the sporulation initiation process and positively
351 regulates the sigma factor cascade required for sporulation (38). In many other
352 pathogenic spore-forming bacteria the gene regulatory networks that influence
353 sporulation and virulence are closely linked with each other (49–53). In *C. difficile*, the
354 mutation in *spo0A* affected many pathogenic traits, including toxin production, flagella
355 expression and biofilm formation (5, 32, 33, 39). Mackin *et. al* observed a clear increase
356 in the production of toxin A and B upon disruption of *spo0A* in the ribotype 027 isolates
357 R20291 and M7404 (39). In a similar study, Deakin *et al.* found that a
358 R20291 *spo0A* mutant caused more severe disease in a murine model than the wild type
359 strain, and associated this increase in severity with an increase in the amount of toxin A
360 and toxin B produced by the mutant *in vitro* (33). Dawson et. al. showed that Spo0A in
361 630Δerm strain promotes sporulation cascade and biofilm formation in addition to
362 negatively regulating virulence factor expression (toxins and flagella) (32). We found
363 UK1::*spo0A* strain to produce higher toxins compared to its parent strain, while no
364 significant difference was observed between the JIR8094 parent and JIR8094::*spo0A*
365 mutant (Fig S3A). This observation was consistent with the previous report, where
366 mutation in *spo0A* influenced the toxin production only in the 027 ribotype, which includes
367 UK1 and R20291 strains, but not in the 630Δerm strain that belongs to 012 ribotype as

368 the JIR8094. We measured the cytosolic toxins in all the three *spo0A* mutants and
369 observed increased toxin production only in the R20291 and UK1 background, but not in
370 the JIR8094 strain (Fig. S3A). Reduced biofilm formation was also found only in
371 R20291::*spo0A*, UK1::*spo0A,* but not in JIR8094::*spo0A* strains (Fig. S3BC). The
372 mechanism of Spo0A regulation over these pathways remains to be answered. In the *C.*
373 *difficile* genome >100 open reading frames have potential 0A boxes within 500 bp of their
374 start codons, indicating direct regulation by Spo0A (24). However, *tcdA* and *tcdB*,
375 encoding toxin A and toxin B, respectively, are not among them, indicating the indirect
376 influence of Spo0A on toxin production (24). Motility and biofilm formation could also be
377 indirectly controlled by Spo0A, since many candidate regulators are encoded by the
378 genes putatively under the direct control of Spo0A in *C. difficile* (24, 33). Our current
379 finding of Spo0A mediated *sin* locus regulation can partly explain many of the phenotypes
380 displayed by *spo0A* mutants, especially in the ribotype 027 strains (32, 39). In our initial
381 characterization of the *sin* locus, we have shown decreased toxin production and motility
382 in the absence of SinR and SinR' (15). Expression of *sinR* alone was sufficient to
383 complement these phenotypes and suggested SinR as a positive regulator of these
384 pathways (15). We have further shown that SinR controls toxin production by regulating
385 *sigD*, a sigma factor that positively regulates *tcdR*, which is needed for the transcription
386 of toxin genes (15, 54, 55). SigD is also needed for the transcription of flagellar operon in
387 *C. difficile* (54, 55). In this study, we have shown an increased SinR production in the
388 absence of Spo0A (Fig. 2B). Quantitative RT-PCR results showed increased expression
389 of *sigD*, *tcdR*, and *tcdB* in the R20291::*spo0A* and in UK1::*spo0A*, compared to their
390 respective parent strains (Fig. S4). Increased *sigD* expression can lead to increased

391 flagellar, toxin production and a reduced biofilm formation in the *spo0A* mutant (22, 32)
392 (Fig. S3BC).
393
394 In this study, we have shown that Spo0A binds to the promoter of the *C. difficile sin* locus
395 and suppresses the expression of both *sinR* and *sinR'*. Previously, we have shown that
396 disrupting *sinR* by insertion mutagenesis affects both *sinR* and *sinR'* transcription (15),
397 suggesting that *sinRR'* is transcribe as a bicistronic message. Our QRT-PCR analysis
398 detected lower levels of *sinR'* transcripts than the *sinR* transcripts. Since the reduction is
399 observed both in the parent and the *spo0A* mutants, we can conclude that this effect is
400 independent of Spo0A. The reason for the reduced level of SinR' over SinR is not clear.
401 In *B. subtilis* the polycistronic *sinRI* transcripts are produced from two upstream
402 promoters. The monocistronic *sinR* transcripts are driven from a promoter located within
403 the coding region of *sinI*. In *B. subtilis*, Spo0A activates the expression of *sinI* by binding
404 to the upstream promoter of the operon and indirectly regulating SinR activity (19, 20) .
405 Similar to *B. subtilis*, in *C. difficile* also there is a possibility that *sinR'* may have an
406 independent promoter within the *sinR* coding sequence and could be controlled by an
407 unknown regulator in *C. difficile*.
408
409 In summary, we have demonstrated that Spo0A, the master regulator of sporulation,
410 regulates the expression of *sin* locus. We have further shown that Spo0A can bind to the
411 upstream region of *sin* locus have successfully mapped the region to which it binds.
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585 ## FIGURE LEGENDS

586

587 **Figure 1. In the absence of Spo0A, *C. difficile* produces elevated levels of SinR.**
588 **A.** QRT-PCR results of *sin* locus transcripts in R20291; R20291::*spo0A* ; JIR8094 and
589 JIR8094::*spo0A* strains collected at 10 hr time point. The representative results from
590 three independent experiments are shown. The asterisks (***) indicate statistical
591 difference at a *P* value of <0.005. **B.** Western blot analysis parents (R20291 and
592 JIR8094), and their respective *spo0A* mutants using SinR and Spo0A specific
593 antibodies demonstrating upregulated SinR in the absence of Spo0A. The SinR
594 negative R20291::*sinRR'* mutant served as a negative control. GDH detection using
595 anti-GDH antibodies was used as loading control.

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598 **Figure 2**. **Spo0A represses *sin* locus expression. A.** Beta-glucuronidase activity of the
599 P*sin-gusA* fusions in the parent R20291 and R20291::*spo0A* mutant. Plasmid (pBA038)
600 with *gusA* as the reporter gene fused to 600 bp of *sin* locus upstream. Plasmids carrying
601 P*spoIIAB-gusA* (pBA029) and a promoter less *gusA* (pBA040) were used as positive and
602 negative controls, respectively. **B.** Expression of beta-glucuronidase in parent R20291
603 and *spo0A* mutant carrying plasmids pBA037 (475 bps P*sin-gusA*), pBA038 (600 bp P*sin-*
604 *gusA*) and pBA009 (340 bp P*sin-gusA*). The error bars in panels **A** and **B** correspond to
605 standard errors of the means of results from 3 biological replicates, where ** and ***
606 indicates P<0.05 and P<0.005, respectively (by two-tailed Student *t* test). At least three
607 independent experiments were performed.

608

609 **Figure 3. A. Spo0A binds to *sin* locus upstream DNA.** Schematics of the 340 bp
610 upstream *sin* locus denoting the general location of the TATTGTAG repeats (not to
611 scale) with respect to the translation start (+1) of *sinR*. The lower lines indicate the
612 location and the sizes of the DNA fragments used for the biotinylated DNA-pulldown
613 assay. **B.** Western blot analysis using Spo0A specific antibody to detect endogenous
614 Spo0A in input and elute fractions. For the biotin-DNA pulldown assay, the promoter
615 regions of *spoIIAB* and *gluD* were used as positive and negative controls, respectively.
616 **C.** Three DNA fragments (118 bp; 135 bp; 140 bp) spanning different regions of the 340
617 bp *sin* locus upstream were used independently to carry out the binding and Spo0A was
618 detected as in panel B. **D.** Expression of beta glucuronidase in parent R20291
619 and R20291::*spo0A* mutant strains carrying plasmids with *gusA* as the reporter gene
620 fused to the promoter of *sinR*. The predicted Spo0A box was mutagenized either on M1
621 or M2 site or both M1, M2. Strain carrying a promoter less *gusA* plasmid (pBA040) was
622 used as control. Data represent the means ± standard errors of the means (SEM) (n =
623 3). The asterisks (***) in panel A indicate statistical difference at a P value of <0.005.
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632 ## Supporting Information

633 **S1 Fig. Construction and confirmation of the *spo0A* mutant in *C. diffcile* JIR8094**
634 **and UK1 strain. (A)** Schematic representation of ClostTron (group II intron) mediated
635 insertional inactivation of *spo0A* gene in *C. difficile*. **(B)** PCR verification of the intron
636 insertion and complementation of *spo0A* in JIR8094 with intron-specific primer EBS
637 universal [EBS(U)] with *spo0A* specific primers ORG 551 and ORG 552. **(C)** PCR
638 verification of the intron insertion in UK1 strain with EBS(U), ORG 551 and ORG 552. **(D)**
639 Western blot analysis of Spo0A and SinR production in UK1 and UK1::*spo0A*. GDH was
640 used as loading control.

641

642 **S2 Fig. Sporulation in *spo0A* mutants**. Percentage sporulation (the CFU/ml from
643 ethanol resistant spores) of the parent and *spo0A* mutants. The representative results
644 from three independent experiments are shown.

645

646 **S3 Fig**. **Toxin gene transcription and biofilm formation in R20291::*spo0A* mutant**
647 **strain. (A)** Toxin production measured by ELISA. Statistical analysis was performed
648 using student t-test. (**; <0.05 *P* value). **(B)** Crystal violet stained biofilm in the 12 wells
649 tissue culture plate, showing poor biofilm formation in R20291::*spo0A* and in UK1::*spo0A*
650 mutants.(**C**) Quantification of crystal violet dye attached to the cells forming biofilms.

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653 **S4 Fig. qRT-PCR analysis.** Relative expression of the transcripts of *tcdR, sigD, and*
654 *sinR* genes from *C. difficile* parent and *spo0A* mutant strains in JIR8094, R20291 *and*
655 UK1 background. RNA was collected at 10 hr time point.

Figure 1

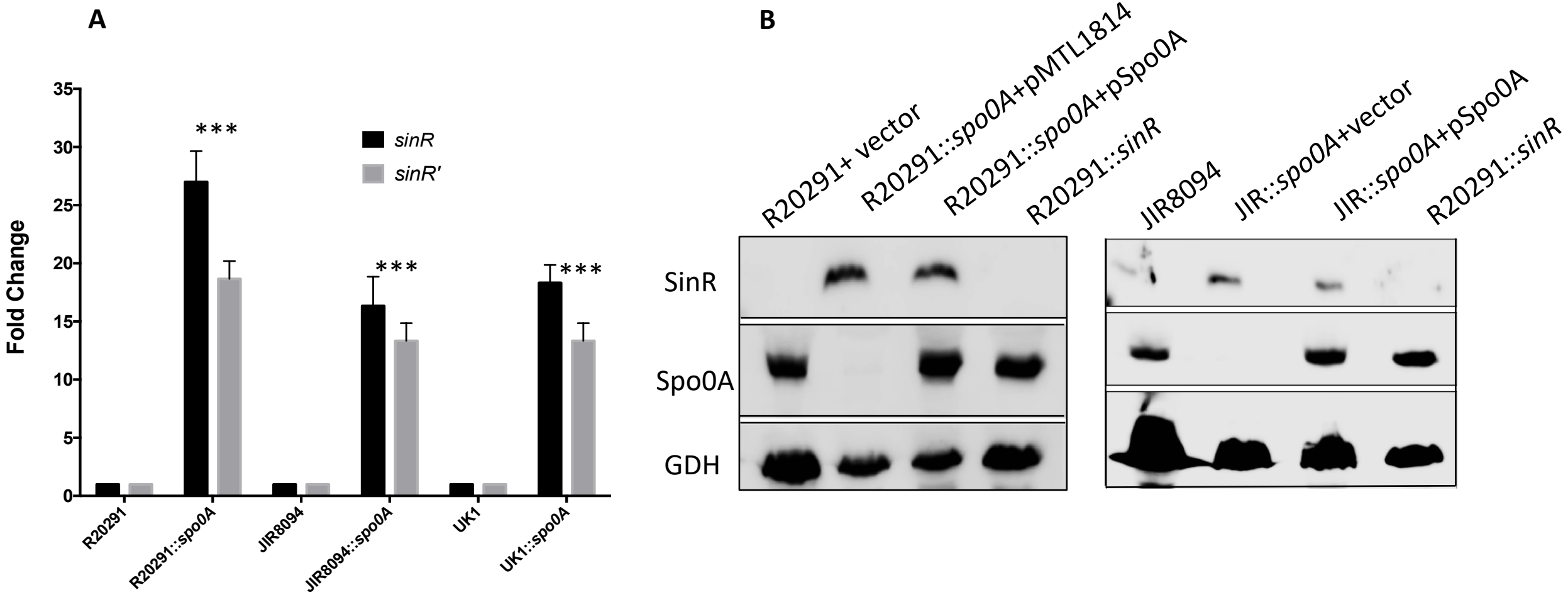
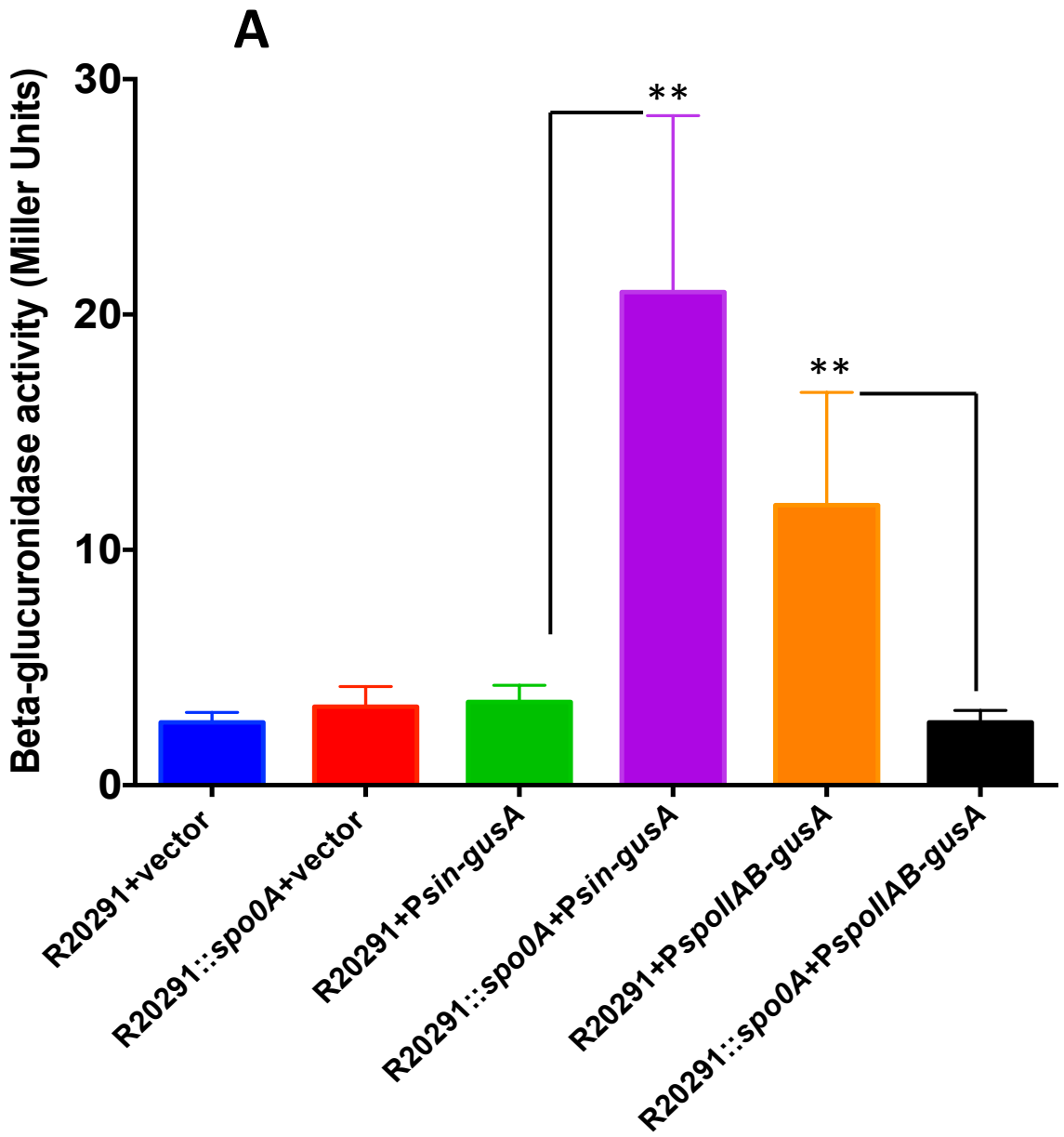

A
Fold Change
35
30
25
20
15
10
5
0
***
***
***
sinR
sinR'
R20291
R20291::spo0A
JIR8094
JIR8094::spo0A
UK1
UK1::spo0A
B
R20291+ vector
R20291::spo0A+pMTL1814
R20291::spo0A+pSpo0A
R20291::sinR
JIR8094
JIR::spo0A+vector
JIR::spo0A+pSpo0A
R20291::sinR
SinR
Spo0A
GDH

Figure 2


A
Beta-glucuronidase activity (Miller Units)
30
20
10
0
**
**
R20291+vector
R20291::spo0A+vector
R20291+Psin-gusA
R20291::spo0A+Psin-gusA
R20291+PspoIIAB-gusA
R20291::spo0A+PspoIIAB-gusA

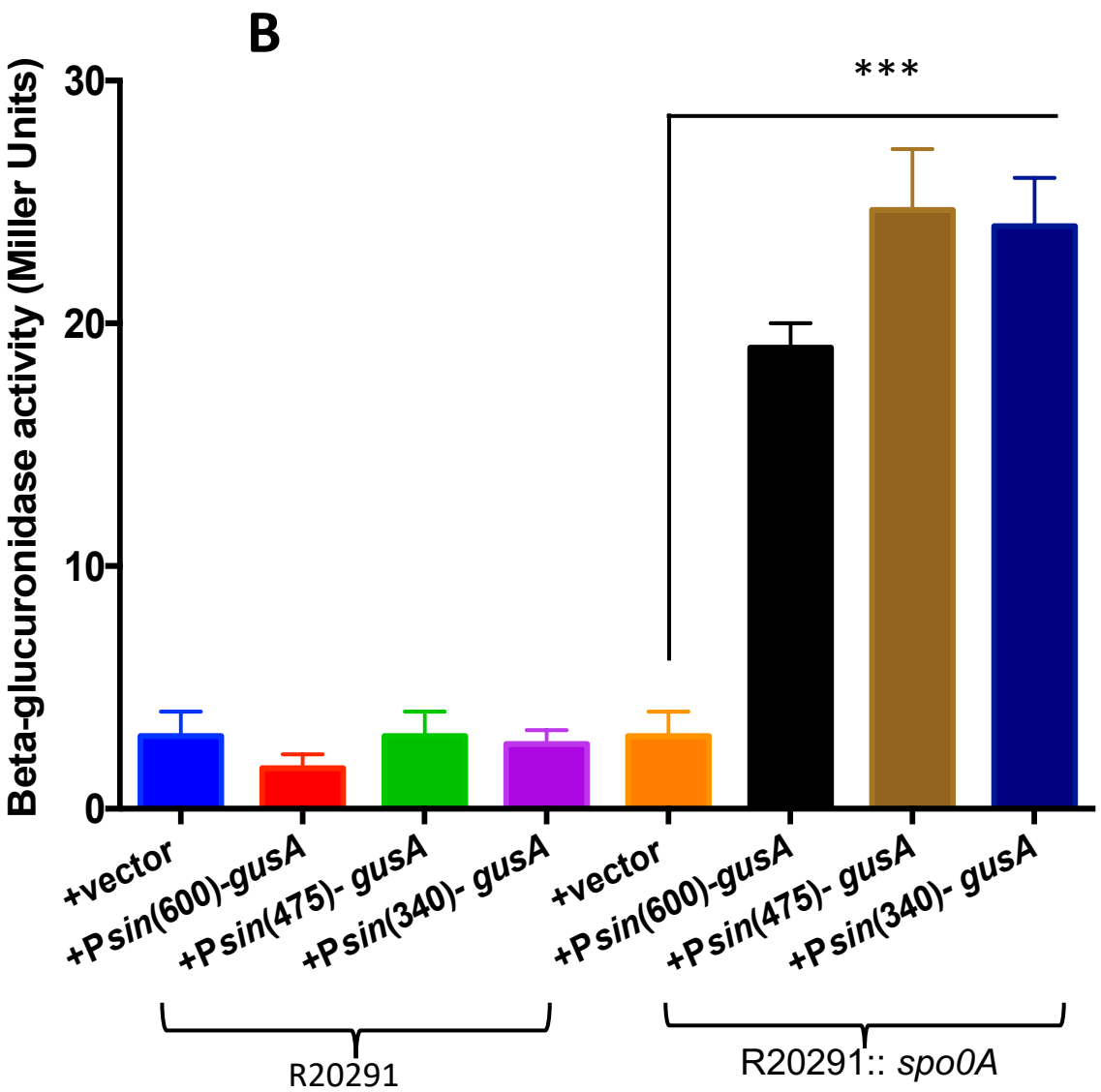
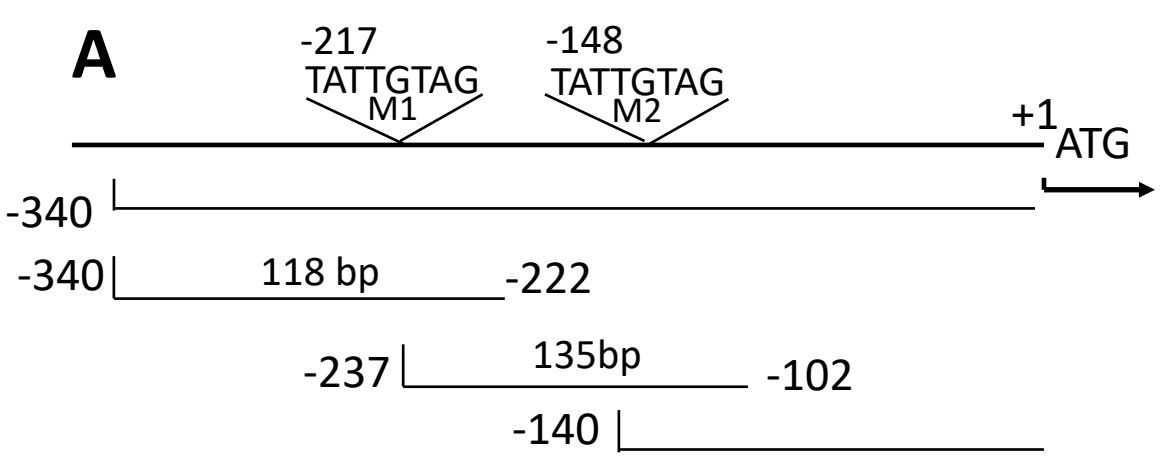
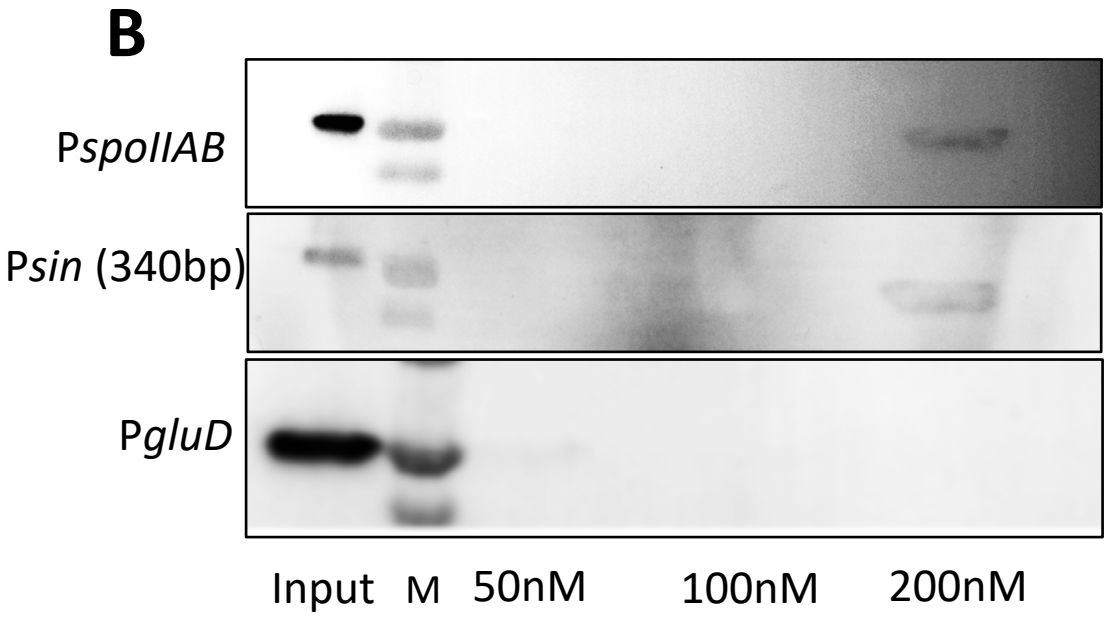
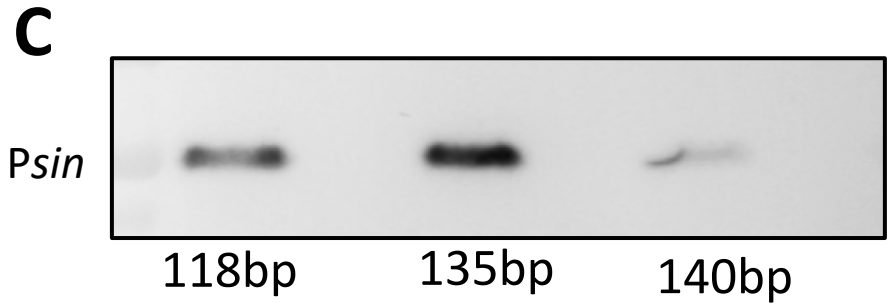
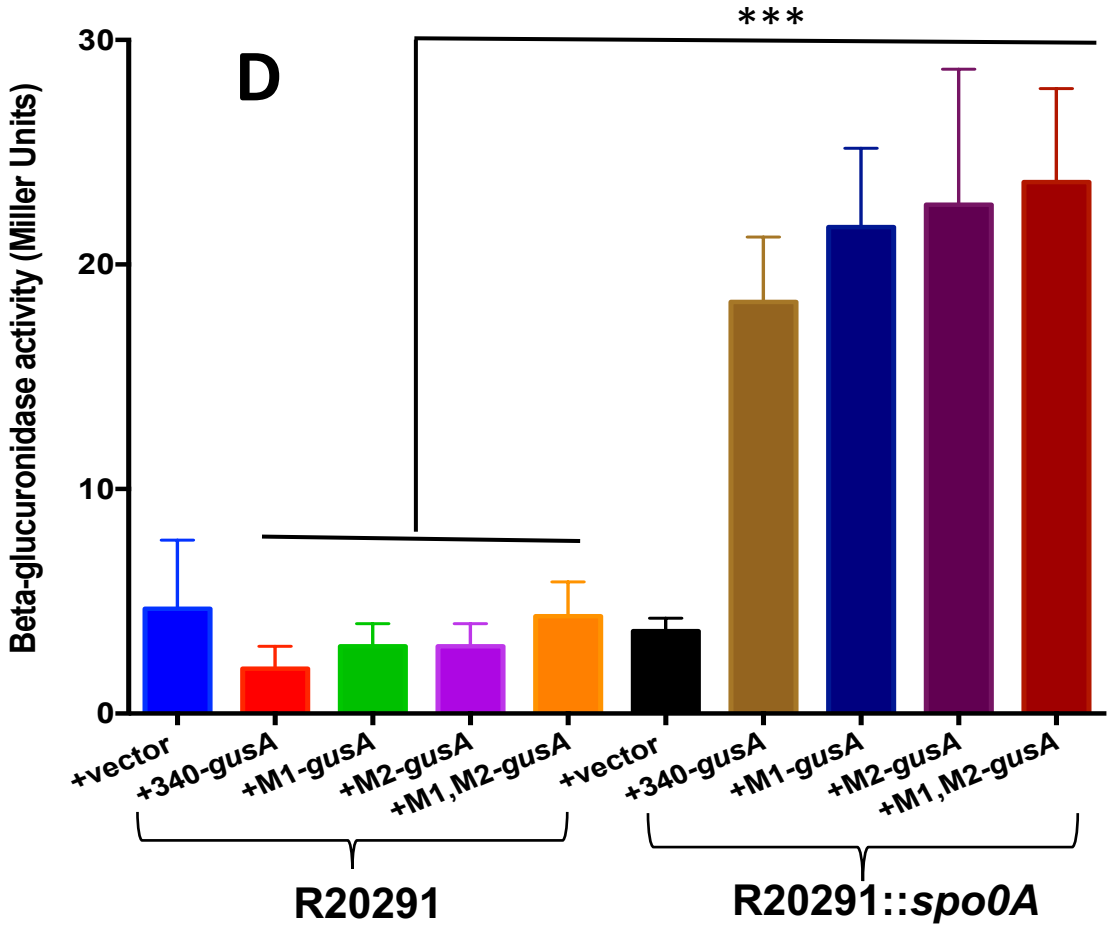

B
Beta-glucuronidase activity (Miller Units)
30
20
10
0
***
+vector
+Psin(600)-gusA
+Psin(475)- gusA
+Psin(340)- gusA
+vector
+Psin(600)-gusA
+Psin(475)- gusA
+Psin(340)- gusA
R20291
R20291:: spo0A

Figure 3


A
-217
TATTGTAG
M1
-148
TATTGTAG
M2
+1
ATG
-340
-340
118 bp
-222
-237
135bp
-102
-140


B
PspoIIAB
Psin (340bp)
PgluD
Input
M
50nM
100nM
200nM


C
Psin
118bp
135bp
140bp


D
***
Beta-glucuronidase activity (Miller Units)
30
20
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0
+vector
+340-gusA
+M1-gusA
+M2-gusA
+M1,M2-gusA
+vector
+340-gusA
+M1-gusA
+M2-gusA
+M1,M2-gusA
R20291
R20291::spo0A