1 **Research Article**

2 **Characterization of a hypersporulating strain derivative *of Clostridioides***

3 ***difficile* R20291**

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Abstract

Clostridioides difficile is a Gram-positive and obligate anaerobe pathogen capable of forming spores, which are essential for the transmission and recurrence of *C. difficile* infections. A major challenge in sporulation studies in *C. difficile* is the low yield and asynchronous nature of this process. In this work, a hypersporulating strain, derivative of R20291, with an early sporulation onset and enhanced sporulation efficiency was isolated by serendipity. It showed up to a 1000-fold higher sporulation efficiency than the parental R20291 strain in sessile culture conditions. Electron micrographs reveal that spores of both strains have similar hair-like projections, electron-dense outer exosporium layer features. Whole genome sequencing and genomic analyses revealed that the hypersporulating strain had a deletion of 2356 bp spanning three ORF, including a non-essential *proC1* involved in proline metabolism, and a missense mutation in *rsbV*, an anti-anti-SigB factor of RsbW. These observations suggest that RsbV-variant might contribute to constitutive repression of the SigB-dependent general stress response, and therefore, derepressing sporulation.

42 **Introduction**

43

44 *Clostridioides difficile* is an anaerobic, Gram-positive, spore-forming pathogen and the
45 principal etiologic agent of *C. difficile* infection (CDI), as well as, currently being the most
46 frequent healthcare-associated cause of infectious diarrhea in industrialized countries (1, 2).
47 CDI typically occurs after antibiotic-mediated disruption of the normal gut microbiota,
48 enabling endogenous or ingested *C. difficile* spores to germinate and colonize (3, 4). Clinical
49 manifestations range from mild diarrhea to pseudomembranous colitis, toxic megacolon and,
50 in severe cases, death (3, 4). Moreover, recurrent CDI (R-CDI) remains a major public-health
51 challenge, which affects 20-30% of CDI patients and may increase to nearly 60% after
52 multiple episodes (5, 6).

53

54 The infection cycle of *C. difficile* is primarily driven by its ability to produce metabolically
55 dormant and environmentally resilient spores (7). After ingestion, spores survive passage
56 through the stomach and germinate in the gastrointestinal tract in response to bile acids and
57 other cues (7, 8), leading to colonization, toxin production, and disease development (9). A
58 subset of cells initiate sporulation, generating new spores that can persist in the host and are
59 shed into the environment (10).

60

61 Although the core regulatory stages of sporulation have been characterized in *C. difficile*,
62 sporulation-related studies are challenging. Several studies have shown that *C. difficile*
63 sporulates at relatively low frequencies under laboratory conditions (11-14), and that cultures
64 undergo sporulation in an inherently asynchronous manner (12, 15). Unlike other genetically
65 trackable organisms such as *B. subtilis* and *C. perfringens*, this heterogeneity complicates

66 detailed investigations of sporulation-specific phenotypes, limits the ability to obtain stage-
67 enriched populations, and reduces yields of purified spores for biochemical, structural, or
68 high-throughput assays. Attempts to create high sporulating strains have met partial success,
69 such as overexpression of Spo0A (11), however, tunning sporulation commitment requires
70 consideration of additional regulators such as the phosphatase Spo0E and RtsA, both shown
71 to act as repressors sequestering Spo0A (16, 17).
72
73 In the context of unrelated culturing of *C. difficile*, we serendipitously identified a derivative
74 of the epidemic lineage strain R20291 that exhibits a distinguishable hypersporulation
75 phenotype. In this study we characterize this hypersporulating strain ($R20291_{CM196}$) and
76 assess its potential as a laboratory tool for generating high-yield and more synchronous spore
77 preparations. We observed that $R20291_{CM196}$ presented a 30 to 1,000-fold increase in ethanol-
78 resistant CFU, depending on the sporulation media composition, and reached a total of ~ 60
79 % sporulating cells after 24 h of incubation in 70:30 agar plates. Moreover, electron
80 micrographs show that hyper sporulating spores are identical to the parental strain. Whole
81 genome sequence revealed the presence of a ~2000 bp deletion including a pyrroline-
82 carboxylate reductase, *proC1*, involved in proline biosynthesis and a single nucleotide
83 polymorphism in *rsbV*, an antianti-SigB factor, involved in the general stress response and
84 inhibition of sporulation by Spo0E activation. Because this sporulation phenotype occurs
85 under *in vitro* conditions, the strain offers a practical platform for detailed studies of *C.*
86 *difficile* sporulation and spore physiology.

87 **Materials and Methods**

88

89 **Bacterial strains and growth conditions**

90 *C. difficile* strains were routinely grown at 37 °C under anaerobic conditions in a Coy Vinyl
91 Anaerobic chamber (COY laboratory products, USA) in BHIS medium: 3.7% weight vol^{-1}
92 brain heart infusion (BD, USA) supplemented with 0.5% weight vol^{-1} yeast extract (BD,
93 USA) and 0.1% weight vol^{-1} L-cysteine (Thermo Fisher Scientific, USA); TY medium: 3%
94 weight vol^{-1} trypticase (BD, USA) with 0.5% weight vol^{-1} yeast extract (BD, USA) or 70:30
95 medium: 6.3% weight vol^{-1} peptone (BD, USA), 0.35% weight vol^{-1} protease peptone (BD,
96 USA), 0.07% weight vol^{-1} ammonium sulfate $(NH_4)_2SO_4$ (Merck USA), 0.106%
97 weight vol^{-1} Tris base (Omnipur, Germany), 1.11% weight vol^{-1} brain heart infusion (BD,
98 USA) and 0.15% weight vol^{-1} yeast extract (BD, USA). To grow strains in plates, 1.5%
99 weight vol^{-1} agar (VWR, USA) was added to medium. Strains used in this work are
100 summarized in Table S1.

101

102 **Growth assays**

103 *C. difficile* strains were cultured anaerobically on pre-reduced BHIS broth supplemented with
104 0.1% sodium taurocholate (NaTC, Sigma, USA) and 0.5% glucose (BHISG + NaTC) for 16
105 h at 37 ºC to prevent sporulation. Overnight culture was diluted 1:3 with pre-warmed BHISG
106 + NaTC broth and incubated for 2 h at 37 ºC to allow cells to enter exponential phase.
107 Subsequently, culture was diluted 1:100 in fresh BHIS and 100 µL were added to wells in a
108 96-well plate. Optical density (OD_{600}) was measured every 3 minutes for 22 hours at 37 ºC
109 using Cerillo's Alto™ microplate reader. Three independent biological replicates with four
110 technical replicates each, were performed per sample.

111

112 **Biofilm formation**

113 Biofilm formation was measured as previously described (18, 19). Briefly, *C. difficile*
114 inoculated BHISG broth was diluted 1:50, 1 mL aliquots were added to 24-well plate and
115 incubated at 37 °C in anaerobic conditions for 24 h. Biofilm biomass was stained with 0.1%
116 crystal violet (CV) for 1h, gently washed with 1X PBS and extracted with 100% ethanol for
117 2 h at room temperature. Solubilized CV was diluted 1:10 and absorbance was measured at
118 OD_{570} with plate reader (BioTek, Synergy H1).

119

120 **Spore purification**

121 As previously described (19, 20), 250 µL of a *C. difficile* inoculated BHISF (0.2% Fructose)
122 + NaTC broth was plated on pre-reduced 70:30 agar plates (35 mL) and incubated for 5 days
123 at 37 °C in a COY anaerobic chamber. Sporulating culture was harvested and cleaned with
124 ice-cold sterile Milli-Q water. Nycodenz (Axell USA) 45% solution was used to purified
125 spores by centrifugation at 18,400 × g for 20 min, cellular debris was removed, spore pellet
126 separated and washed with sterile Milli-Q water and stored at −80 °C until use.

127

128 **Whole genome sequence**

129 *C. difficile* strains were whole genome sequenced (SeqCenter, USA) and Illumina data was
130 analyzed using Geneious Prime® 2025.1.3. Reads were mapped to *C. difficile* R20291
131 reference genome (GenBank: CP029423) using Bowtie2 v7.2.2, with default parameters.
132 Whole genome variant calling was performed by Geneious variant finder with a minimum
133 coverage of five reads and a minimum variant frequency of 0.95 and long INDEL (insertion
134 or deletion) regions were predicted using Find Low/High Coverage tool.

135

136 **Sporulation assay by ethanol resistance and phase-contrast microscopy**

137 *C. difficile* sporulation efficiency was assayed as previously described (19, 21). Briefly,
138 plating was performed as previously described in spore purification methods in BHIS, TY
139 and 70:30 agar plates. Cells were harvested after 24 h in 1X PBS, OD_{600} was measured and
140 absorbance of sporulating culture was adjusted to 1. An aliquot of 250 µL was treated with
141 100% ethanol for 15 minutes at room temperature, serially diluted and spot plated in triplicate
142 onto BHIS + NaTC plates under anaerobic conditions. Sporulation frequency was calculated
143 as the proportion of spores that germinated after ethanol treatment, divided by the total
144 number of CFU in the untreated sample. The ratio of vegetative cells vs spores/sporulating
145 cells was also determined using phase contrast microscopy (Leica DMRX; Germany).

146

147 For sporulation timing, cultures were plated in 70:30 plates and incubated at 37 ºC under
148 anaerobic conditions. Samples were harvested after 8, 10, 12, 14, 16, 18, 20 and 24 h into 1
149 mL 1X PBS. Cells were centrifuged at 18,400 × g for 5 min and fixed with 4%
150 paraformaldehyde. Vegetative and spores/sporulating cells count was determined using
151 phase contrast microscopy (Leica DMRX; Germany).

152

153 **Transmission electron microscopy (TEM)**

154 As previously described with some modifications (10, 22), spores were fixed overnight with
155 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer. Secondary
156 fixation was performed with 1% osmium tetroxide for 2 h, followed by 30 min staining with
157 1% tannic acid. Samples were serially dehydrated 30 min each with acetone 30% (with 2%
158 uranyl acetate), 50%, 70%, 90%, and twice with 100% acetone, after which samples were

159 embedded in Spurr resin (22, 23). Thin sections (90 nm) were obtained using a microtome,
160 placed on glow-discharge carbon-coated grids, and double-stained with 2% uranyl acetate
161 followed by lead citrate. Spores were analyzed using a Philips Tecnai 12 Bio Twin
162 microscope at the Unidad de Microscopía Avanzada, Pontificia Universidad Católica de
163 Chile.
164
165 **Statistical analyses**
166 Statistical significance was determined by Welch's t-test in GraphPad Prism v9.0. <0.05
167 (*), <0.01 (**), <0.001 (***), <0.0001 (****), ns: no significance. All experiments were
168 performed in triplicate with at least three technical replicates.

169 **Results and discussions**

170

171 **Hyper-sporulating R20291 derivative.**

172 A canonical feature of *C. difficile* spore formation is its asynchronous nature and low
173 efficiency yield, therefore, a major challenge in *C. difficile* spore-related research, is the
174 production of enough mass of said spores. By serendipity, it was observed in a routine
175 experiment that an isolate of *C. difficile* R20291 (from here on address as $R20291_{CM196}$)
176 presented a marked increase in the number of spores after 24 h incubation in 70:30
177 sporulation media in comparison with the usual levels obtained in lab with this strain (wild-
178 type strain from here on address as $R20291_{CM210}$) (**Fig. 1A**).

179

180 To confirm and evaluate if the increased amounts of spores observed in $R20291_{CM196}$ strain
181 were confined to specific nutritional conditions, a sporulation efficiency assay by ethanol
182 resistance of cells grown in three different media widely used in *C. difficile* research; BHIS,
183 TY and 70:30, was performed. There was no significant difference in the amount of ethanol-
184 resistant CFU obtained by the two strains when they were grown in any broth media ($p > 0.5$)
185 (**Fig. 1B,1D and S1**). However, when cells were plated in 70:30 and BHIS, but no in TY,
186 agar plates, a significant difference ($p < 0.01$ and $p < 0.05$, respectively) was observed in
187 ethanol-resistant CFU of $R20291_{CM196}$ reaching ~92% and 1% in comparison with a 3% and
188 0% of $R20291_{CM210}$, respectively (**Fig. 1E, 1G and S2**), reaching sporulation levels more
189 than 30-times higher with 70:30 media.

190

191 This phenotype was confirmed by phase contrast microscopy counts of vegetative cells and
192 spores/sporulating cells of cultures grown in broth and agar plates. It was observed that

193 R20291$_{CM196}$ strain grown in 70:30 broth had a total of 4.9% cells that were phase bright
194 spores but reached 65% when grown in 70:30 agar plates. As for R20291$_{CM210}$ strain, the
195 percentage of spores/sporulating cells ranged from 0.21% in broth and 4.5% cells in agar
196 plate (**Fig. 1C and 1F**). The difference between ethanol-resistance CFU and micrograph
197 counts could be attributed to R20291$_{CM196}$ vegetative cells in the sporulating culture being
198 prone to lyse, during being in ethanol resistance assay by spot plating and counting of cells
199 by phase contrast microscopy (92% vs 65%, respectively), could be due to loss of viability
200 of vegetative cells.
201
202 **R20291$_{CM196}$ exhibits an early onset of sporulation**
203 To determine if the sporulation process of R20291$_{CM196}$ and R20291$_{CM210}$ initiated at the
204 same time or deferred, a sporulation timing assay was conducted. Strains were grown in
205 70:30 agar plates and harvested at 8 and 24 h and analyzed by phase contrast microscopy.
206 Free spores were observed first at 12 h incubation time in R20291$_{CM196}$ strain, at least 2 h
207 before R20291$_{CM210}$ and with ~7 times more spores (6.5% vs 0.9%, respectively) (**Fig. 2A**
208 **and 2B**). At 14 h, the amount of R20291$_{CM196}$ spores reached 31% compared to 1% in
209 R20291$_{CM210}$, accounting for an increase of 4.8% vs a 1.1%, respectively. By 24 h,
210 R20291$_{CM196}$ sporulating culture had more than half (57.4%) of phase bright spores, 10-times
211 higher than observed in R20291$_{CM210}$ (5.3%) sporulating cultures (**Fig. 2A and 2B**). This
212 validated that the onset of sporulation of R20291$_{CM196}$ was not only earlier but that more cells
213 committed to this process in waves than in the case of R20291$_{CM210}$ strain. Unfortunately, the
214 speed of sporulation cannot be measured, so this attribute remains unknown.
215
216 **Ultrastructural analysis of R20291$_{CM196}$ spores.**

217 Furthermore, to determine if the organization of the spore ultrastructure was affected,
218 transmission electron microscopy images were analyzed. R20291$_{CM196}$ spores remained
219 seemingly unchanged, with the presence of hair-like projections on top of the electron-dense
220 outer coat (ED-OC) layer and lamellae structure within the coat (Ct) of thin and thick spores
221 morphotypes (**Fig. 2C**). Therefore, the hypersporulation trait of R20291$_{CM196}$ appears to only
222 affect the onset and quantity of sporulating cells but not the structure of the spores.
223

224 **R20291$_{CM196}$ exhibit differences in growth and biofilm formation**

225 To gain more insight into the physiology of R20291$_{CM196}$ strain, planktonic growth and
226 biofilm formation phenotypes were assayed. Growth of R20291$_{CM196}$ reached a lower optical
227 density, with a maximum OD_{600} of 0.1082 in comparison with R20291$_{CM210}$ that had a
228 maximum OD_{600} of 0.2259. Additionally, the decline phase in R20291$_{CM196}$ appears to begin
229 approximately at 10 h after inoculation, meanwhile R20291$_{CM210}$ is at 18 h, 8 hours after (**Fig**
230 **3A**). Biofilm formation was also significantly decreased in R20291$_{CM196}$, with a value of
231 OD_{570} 0.31 in comparison with R20291$_{CM210}$ of OD_{570} 0.59 ($p < 0.0001$) (**Fig. 3B**).This data
232 suggests that the differences in commitment to sporulate observed in R20291$_{CM196}$, might be
233 impacting planktonic growth and the ability to form sessile communities.
234

235 **Whole genome sequencing of R20291$_{CM196}$ reveals unique polymorphism.**

236 To evaluate if the aforementioned differences in sporulation commitment in R20291$_{CM196}$
237 was attributed to genetic differences, R20291$_{CM196}$ and R20291$_{CM210}$ were whole genome
238 sequenced, and performed comparative genomics against *C. difficile* reference genome
239 CP029423. Five mutations are shared between these two R20291 isolates. Two missense
240 mutations, one in *rsbW* (G82V, CDIF27147_00009), a serine protein kinase that acts as the

241 anti-sigma factor of SigB and the second one in *vncR* (D202G, CDIF27147_02036), a two-
242 component system response regulator (Fig. 4A). As well as two nonsense mutations by single
243 nucleotide deletions in ribokinase, *rbsK* (CDIF27147_00429) and a two-component sensor
244 histidine kinase (CDIF27147_02733) (Fig. 4A); additionally, a single nucleotide insertion in
245 a non-coding region between the end of two ORF; i) 336 bp from CDIF27147_00700 and ii)
246 144 bp from CDIF27147_00701 were observed (**Fig. 4A**).
247
248 Notably, R20291$_{CM196}$ possess two additional polymorphisms. The first one being a missense
249 mutation (L20F) in *rsbV*, that encodes for an anti anti-SigB, that plays a crucial role in the
250 general stress response against pH, antimicrobial peptides and reactive oxygen species (ROS)
251 (24, 25). In *B subtilis*, its function is to sequester RsbW, detaching it from the complex
252 RsbW-SigB, allowing for the activation of the SigB regulon (26). The induction of SigB has
253 been shown to reduce the sporulation process in *B. subtilis* (27), by the positive regulation of
254 Spo0E, an aspartyl phosphatase that dephosphorylates the master of sporulation Spo0A (27,
255 28). In *C. difficile* it has been shown that *spo0E* mutants sporulated twice wild-type levels,
256 indicating that sporulation is being repressed by Spo0E as seen in *B. subtilis* (16). Sporulation
257 is a high energy process, utilized as a last resource to survive an unfavorable environment;
258 endosporeformers it will try to survive by first by activating the general stress response before
259 committing to sporulation (29). The mutation in RsbV, could therefore hinder binding to
260 RsbW, in which case SigB will remain sequestered by its anti-sigma factor, inhibiting the
261 activation of the SigB regulon and its impact in sporulation repression.
262
263 The second mutation present in R20291$_{CM196}$ is a 2,356 bp deletion affecting a transcriptional
264 regulator (CDIF27147_01521), a pyrroline-5-carboxylate reductase (*proC1*,

265 CDIF27147_01522) involved in the last step of L-proline biosynthesis obtained from
266 glutamate, and the last 284 bp of an uncharacterized protein (CDIF27147_01523) (**Fig. 4A**
267 **and 4B**). In *B. subtilis*, that only the absence of the three genes that encode for pyrroline-5-
268 carboxylate reductase (*proG*, *proH*, and *proI*) render a proline auxotroph (30), but
269 individually they are not essential genes. *C. difficile* R20291 genome encodes two pyrroline-
270 5-carboxylate reductase *proC1* and *proC2* (CDIF27147_03465) that share an identity
271 between 29 to 33% with the *B. subtilis proH/proI*, but only a 23% identity to *proG*. This
272 suggests that the single deletion of *proC1* may not affect $R20291_{CM196}$ ability to produce
273 proline, since *proC2* is still intact. Moreover, while this deletion also included
274 CDIF27147_01521, the putative response regulator, and CDIF27147_01523, their roles
275 remains unclear and are matter of current work in the lab.
276
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286 **Figure legends**

287

288 **Fig. 1.** Hypersporulating *C. difficile* R20291 derivative strain. (A) Phase contrast microscopy
289 of a 24 h sporulating culture. Sporulation efficiency evaluated by ethanol resistant sporulation
290 assay of 24 h sporulating cultures grown in BHIS, TY or 70:30 media (B) broth or (E) agar.
291 Sporulation efficiency evaluated by phase contrast microscopy images of 24 h sporulating
292 cultures grown in BHIS, TY or 70:30 media (C) broth or (F) agar. At least 800 spores/cells
293 were counted from each sample. Representative images of serial spot dilution of ethanol
294 resistant sporulation assay in (D) broth and (G) agar plate. Cells were spotted at 10-fold serial
295 dilutions on BHIS agar plates supplemented with 0.1% sodium taurocholate. ND: no data.
296 Welch's t-test. < 0.05 (*), < 0.01 (**), ns: no significance.

297

298 **Fig. 2. Early sporulation onset and spore ultrastructure analysis of R20291$_{CM196}$.** (**A**)
299 Sporulation timing in 70:30 agar plates. Sporulating culture was monitored at 8, 10, 12, 14,
300 16, 18, 20 and 24 h. (**B**) Representative phase contrast microscopy images of sporulation
301 timing assay. Numbers on the lower right corner corresponds to the percentage of
302 spores/sporulating cells in the sample at that time. (**C**) Transmission electron microscopy
303 (TEM) of R20291$_{CM196}$ and R20291$_{CM210}$ spores. ED-OC: Electron-dense outer coat. Ct:
304 Coat.

305

306 **Fig. 3. R20291$_{CM196}$ physiology traits.** (**A**) Growth curve of R20291$_{CM196}$ (teal) and
307 R20291$_{CM210}$ (yellow) in BHIS media over 22 h. (**B**) Biofilm formation was evaluated at 24
308 h by crystal violet staining and measured at OD_{570}. Welch's t-test. < 0.0001 (****)

309

310 **Fig. 4. Mutations in hypersporulating *C. difficile* R20291$_{CM196}$ strain.** (**A**) Description of
311 single nucleotide variants and INDELs present in R20291$_{CM196}$ and R20291$_{CM210}$ strains in
312 comparison with *C. difficile* R20291 reference genome (CP029423). (**B**) Genetic deletion in
313 R20291$_{CM196}$ vs wildtype region in R20291$_{CM210}$.
314
315 **Fig. S1. Ethanol-resistant cells grow in broth media.** Representative images of serial spot
316 dilution of ethanol resistant sporulation assay of R20291$_{CM196}$ and R20291$_{CM210}$. Cells were
317 spotted at 10-fold serial dilutions on BHIS agar plates supplemented with 0.1% sodium
318 taurocholate. U: untreated cells, T: ethanol-treated cells.
319
320 **Fig. S2. Ethanol-resistant cells grow in agar media.** Representative images of serial spot
321 dilution of ethanol resistant sporulation assay of R20291$_{CM196}$ and R20291$_{CM210}$. Cells were
322 spotted at 10-fold serial dilutions on BHIS agar plates supplemented with 0.1% sodium
323 taurocholate. U: untreated cells, T: ethanol-treated cells.

324 **Reference**

325 1. Rupnik M, Wilcox MH, Gerding DN. 2009. Clostridium difficile infection: new
326 developments in epidemiology and pathogenesis. Nat Rev Microbiol 7:526-36.
327 2. Paredes-Sabja D, Cid-Rojas F, Pizarro-Guajardo M. 2022. Assembly of the
328 exosporium layer in Clostridioides difficile spores. Curr Opin Microbiol 67:102137.
329 3. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM,
330 Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago
331 BM, Fridkin SK, Gerding DN, McDonald LC. 2015. Burden of Clostridium difficile
332 infection in the United States. N Engl J Med 372:825-34.
333 4. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. 2016. Clostridium difficile
334 infection. Nat Rev Dis Primers 2:16020.
335 5. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke
336 ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox
337 MH. 2018. Clinical Practice Guidelines for Clostridium difficile Infection in Adults
338 and Children: 2017 Update by the Infectious Diseases Society of America (IDSA)
339 and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis
340 66:987-994.
341 6. Kelly CP. 2012. Can we identify patients at high risk of recurrent Clostridium difficile
342 infection? Clin Microbiol Infect 18 Suppl 6:21-7.
343 7. Paredes-Sabja D, Shen A, Sorg JA. 2014. *Clostridium difficile* spore biology:
344 sporulation, germination, and spore structural proteins. Trends Microbiol 22:406-16.
345 8. Sorg JA, Sonenshein AL. 2008. Bile salts and glycine as cogerminants for
346 *Clostridium difficile* spores. J Bacteriol 190:2505-12.

347 9. Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW,
348 Fairweather NF, Dougan G, Lawley TD. 2012. The *Clostridium difficile spo0A* gene
349 is a persistence and transmission factor. Infect Immun 80:2704-11.
350 10. Castro-Córdova P, Mora-Uribe P, Reyes-Ramírez R, Cofré-Araneda G, Orozco-
351 Aguilar J, Brito-Silva C, Mendoza-León MJ, Kuehne SA, Minton NP, Pizarro-
352 Guajardo M, Paredes-Sabja D. 2021. Entry of spores into intestinal epithelial cells
353 contributes to recurrence of Clostridioides difficile infection. Nat Commun 12:1140.
354 11. Dembek M, Willing SE, Hong HA, Hosseini S, Salgado PS, Cutting SM. 2017.
355 Inducible Expression of *spo0A* as a Universal Tool for Studying Sporulation in
356 *Clostridium difficile*. Front Microbiol 8:1793.
357 12. Fimlaid KA, Bond JP, Schutz KC, Putnam EE, Leung JM, Lawley TD, Shen A. 2013.
358 Global analysis of the sporulation pathway of *Clostridium difficile*. PLoS Genet
359 9:e1003660.
360 13. Saujet L, Pereira FC, Serrano M, Soutourina O, Monot M, Shelyakin PV, Gelfand
361 MS, Dupuy B, Henriques AO, Martin-Verstraete I. 2013. Genome-wide analysis of
362 cell type-specific gene transcription during spore formation in *Clostridium difficile*.
363 PLoS Genet 9:e1003756.
364 14. Underwood S, Guan S, Vijayasubhash V, Baines SD, Graham L, Lewis RJ, Wilcox
365 MH, Stephenson K. 2009. Characterization of the sporulation initiation pathway of
366 *Clostridium difficile* and its role in toxin production. J Bacteriol 191:7296-305.
367 15. Pereira FC, Saujet L, Tome AR, Serrano M, Monot M, Couture-Tosi E, Martin-
368 Verstraete I, Dupuy B, Henriques AO. 2013. The spore differentiation pathway in the
369 enteric pathogen *Clostridium difficile*. PLoS Genet 9:e1003782.

370 16. DiCandia MA, Edwards AN, Alcaraz YB, Monteiro MP, Lee CD, Vargas Cuebas G,
371 Bagchi P, McBride SM. 2024. A conserved switch controls virulence, sporulation,
372 and motility in C. difficile. PLoS Pathog 20:e1012224.
373 17. Edwards AN, Anjuwon-Foster BR, McBride SM. 2019. RstA Is a Major Regulator
374 of Clostridioides difficile Toxin Production and Motility. mBio 10.
375 18. Reyes Ruiz LM, King KA, Agosto-Burgos C, Gamez IS, Gadda NC, Garrett EM,
376 Tamayo R. 2022. Coordinated modulation of multiple processes through phase
377 variation of a c-di-GMP phosphodiesterase in Clostridioides difficile. PLoS Pathog
378 18:e1010677.
379 19. Cid-Rojas F, Guerrero-Araya E, Brito-Silva C, Pizarro-Guajardo M, Rodríguez-
380 Sanchéz C, Paredes-Sabja D. 2025. Phylogenomic analysis of the collagen-like BclA
381 proteins in Clostridioides difficile. bioRxiv:2025.06.24.661383.
382 20. Edwards AN, McBride SM. 2016. Isolating and Purifying Clostridium difficile
383 Spores. Methods Mol Biol 1476:117-28.
384 21. Edwards AN, McBride SM. 2017. Determination of the in vitro Sporulation
385 Frequency of Clostridium difficile. Bio Protoc 7.
386 22. Pizarro-Guajardo M, Calderón P, Romero-Rodriguez A, Paredes-Sabja D. 2020.
387 Characterization of exosporium layer variability of *Clostridioides difficile* spores in
388 the epidemically relevant strain R20291. Front Microbiol 11:1345.
389 23. Paredes-Sabja D, Cofre-Araneda G, Brito-Silva C, Pizarro-Guajardo M, Sarker MR.
390 2012. *Clostridium difficile* spore-macrophage interactions: spore survival. PLoS One
391 7:e43635.
392 24. Hecker M, Pané-Farré J, Völker U. 2007. SigB-dependent general stress response in
393 Bacillus subtilis and related gram-positive bacteria. Annu Rev Microbiol 61:215-36.

394 25. Kint N, Janoir C, Monot M, Hoys S, Soutourina O, Dupuy B, Martin-Verstraete I.
395 2017. The alternative sigma factor σ(B) plays a crucial role in adaptive strategies of
396 Clostridium difficile during gut infection. Environ Microbiol 19:1933-1958.
397 26. Brigulla M, Hoffmann T, Krisp A, Völker A, Bremer E, Völker U. 2003. Chill
398 induction of the SigB-dependent general stress response in Bacillus subtilis and its
399 contribution to low-temperature adaptation. J Bacteriol 185:4305-14.
400 27. Reder A, Albrecht D, Gerth U, Hecker M. 2012. Cross-talk between the general stress
401 response and sporulation initiation in Bacillus subtilis - the σ(B) promoter of spo0E
402 represents an AND-gate. Environ Microbiol 14:2741-56.
403 28. Reder A, Gerth U, Hecker M. 2012. Integration of σB activity into the decision-
404 making process of sporulation initiation in Bacillus subtilis. J Bacteriol 194:1065-74.
405 29. Kint N, Morvan C, Martin-Verstraete I. 2022. Oxygen response and tolerance
406 mechanisms in Clostridioides difficile. Curr Opin Microbiol 65:175-182.
407 30. Belitsky BR, Brill J, Bremer E, Sonenshein AL. 2001. Multiple genes for the last step
408 of proline biosynthesis in Bacillus subtilis. J Bacteriol 183:4389-92.
409

Figure 1

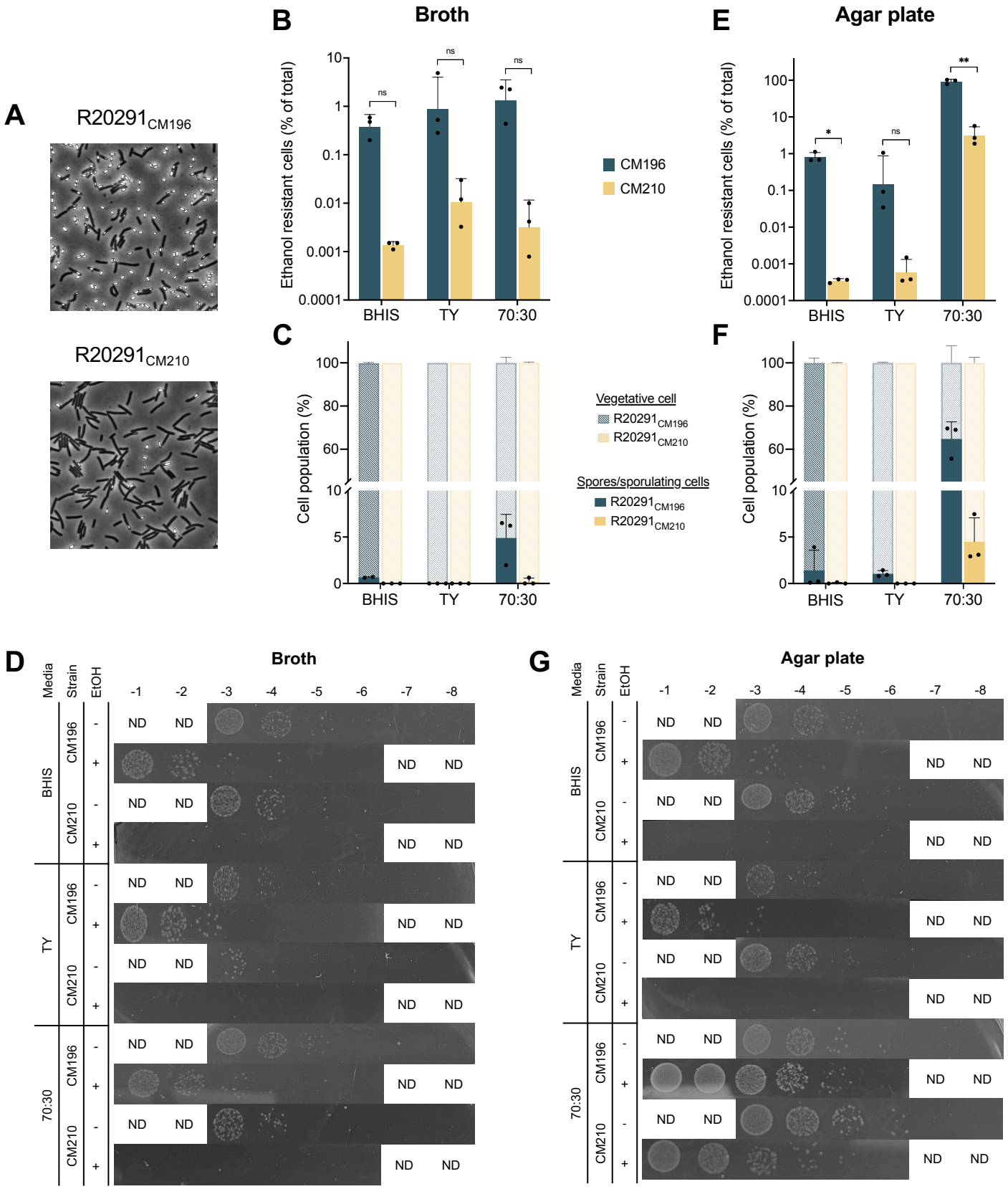

A
R20291CM196
R20291CM210
B
Broth
Ethanol resistant cells (% of total)
10
1
0.1
0.01
0.001
0.0001
ns
ns
ns
BHIS
TY
70:30
CM196
CM210
C
Cell population (%)
100
80
60
10
5
0
BHIS
TY
70:30
Vegetative cell
R20291CM196
R20291CM210
Spores/sporulating cells
R20291CM196
R20291CM210
E
Agar plate
Ethanol resistant cells (% of total)
100
10
1
0.1
0.01
0.001
0.0001
*
ns
**
BHIS
TY
70:30
F
Cell population (%)
100
80
60
10
5
0
BHIS
TY
70:30
D
Broth
Media
Strain
EtOH
-1
-2
-3
-4
-5
-6
-7
-8
BHIS
CM196
CM210
TY
CM196
CM210
70:30
CM196
CM210
-
+
ND
G
Agar plate
Media
Strain
EtOH
-1
-2
-3
-4
-5
-6
-7
-8
BHIS
CM196
CM210
TY
CM196
CM210
70:30
CM196
CM210
-
+
ND

Figure 2

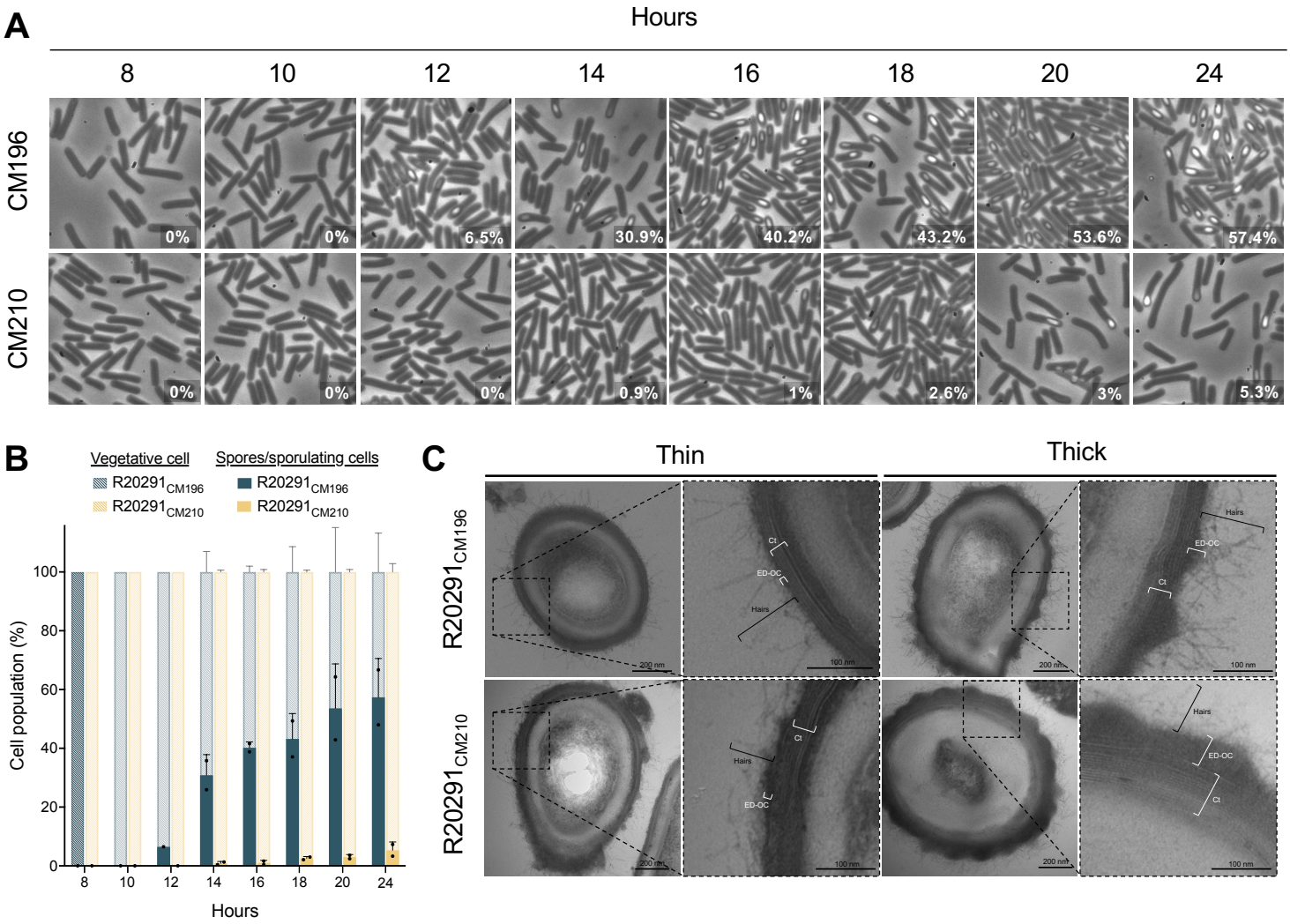

A
Hours
8
10
12
14
16
18
20
24
CM196
CM210
0%
0%
6.5%
30.9%
40.2%
43.2%
53.6%
57.4%
0%
0%
0%
0.9%
1%
2.6%
3%
5.3%
B
Vegetative cell
Spores/sporulating cells
R20291CM196
R20291CM210
R20291CM196
R20291CM210
Cell population (%)
Hours
C
Thin
Thick
R20291CM196
R20291CM210
Hairs
ED-OC
CI
200 nm
100 nm

Figure 3

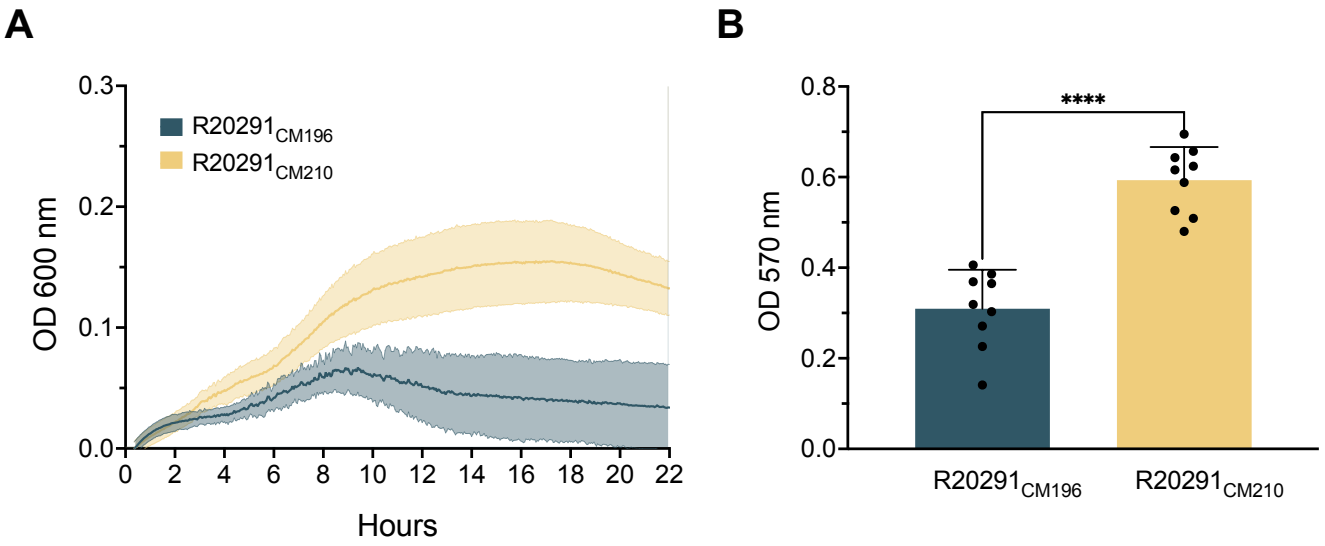
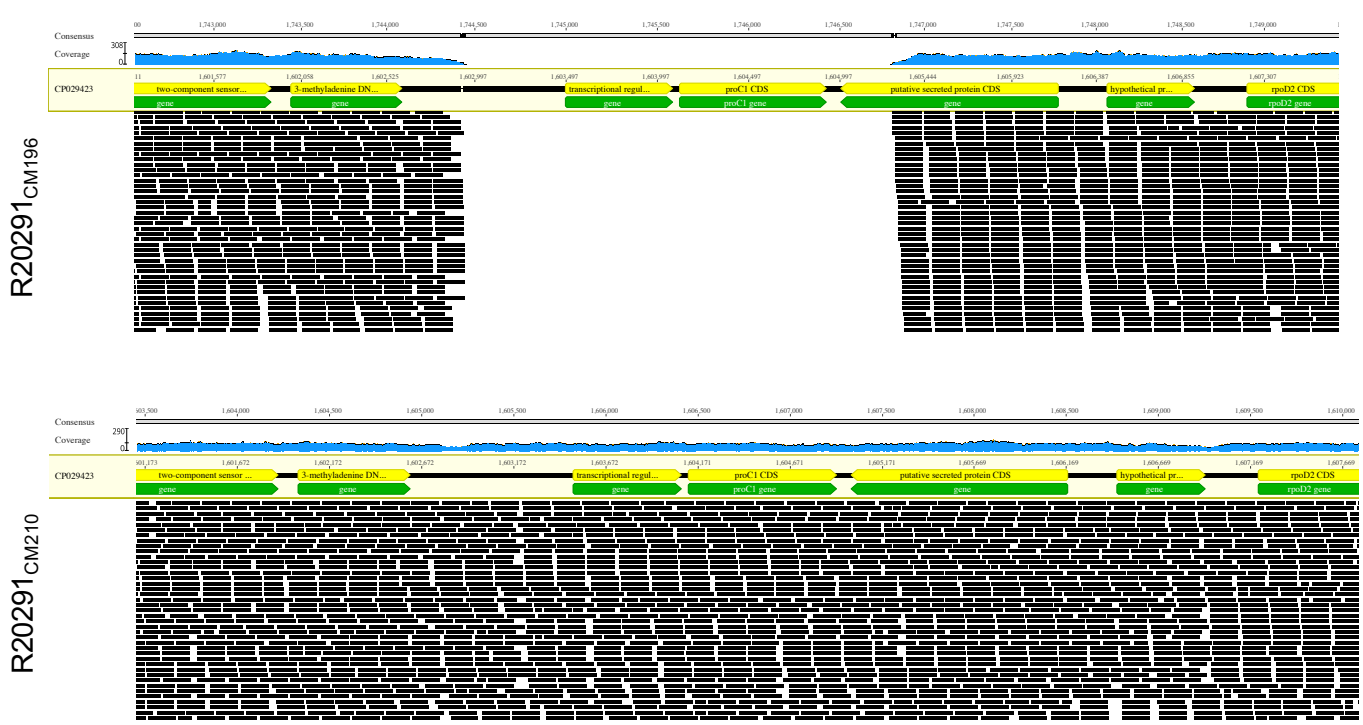

A
B
R20291CM196
R20291CM210
OD 600 nm
0.0
0.1
0.2
0.3
0
2
4
6
8
10
12
14
16
18
20
22
Hours
OD 570 nm
0.0
0.2
0.4
0.6
0.8
****
R20291CM196
R20291CM210

Figure 4

A

locus_tag	Gene name	Description	Genome position (pb)	Polymorphism type	Mutation	nucleotide change	Amino acid change	Coverage	Variant frequency (%)	Genomes	
										CM196	CM210
Coding region polymorphisms											
CDIF27147_00009	*rsbV*	anti-sigma-B factor antagonist	9,186	SNV	Missense	C -> T	L20F	213	99.5	X	-
CDIF27147_00010	*rsbW*	serine-proteine kinase	9,694	SNV	Missense	G -> T	G82V	189	99.7	X	X
CDIF27147_00429	*rbsK*	ribokinase	371,626	Deletion	Nonsense	(A)5 -> (A)4	V55=fs*2	165	95.3	X	X
CDIF27147_02036	*vncR*	two-component system response regulator	2,134,234	SNV	Missense	A -> G	D202G	173	100	X	X
CDIF27147_02733	-	two-component sensor histidine kinase	2,895,029	Deletion	Nonsense	(T)7 -> (T)6	K436=fs*12	162	96.2	X	X
Non-coding region polymorphisms											
-	-	-	685626	Insertion	-	(A)5 -> (A)6	-	177	96.9	X	X
-	-	-	2090870	Deletion	-	- C	-	281	97.9	-	X
INDEL											
CDIF27147_01521	-	Transcriptional regulator	1,602,931 - 1,605,287	Deletion	-	-	-	-	100	X	-
CDIF27147_01522	*proC1*	pyrroline-5-carboxylate reductase									
CDIF27147_01523	-	Uncharacterized protein									

fs: frame shift

: Stop codon. Number after "" denotes new stop codon position after indel event.

B