1 A regulatory protein that represses sporulation in

2 *Clostridioides difficile*

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17 **Running title:** Inhibition of sporulation initiation in *C. difficile*

18 **Key words:** Sporulation; *Clostridioides difficile*; Anaerobe; Spo0A

19 Abstract

20 Bacteria that reside in the gastrointestinal tract of healthy humans are essential for
 21 our health, sustenance and well-being. About 50 to 60% of those bacteria have the
 22 ability to produce resilient spores, important for the life cycle in the gut and for host-
 23 to-host transmission. A genomic signature for sporulation in the human intestine
 24 was recently described, which spans both commensals and pathogens such as
 25 *Clostridioides difficile*, and contains several genes of unknown function. We report
 26 on the characterization of a signature gene, *csiA*, which, as we show, is involved in
 27 the control of sporulation initiation in *C. difficile*. Spo0A is the main regulatory
 28 protein controlling entry into sporulation and we show that an in-frame deletion of
 29 *csiA* results in increased sporulation, and increased expression of *spo0A* per cell.
 30 Spo0A also drives transcription of the *spollA* and *spollG* operons, coding for the
 31 first forespore- (σ^F) and mother cell-specific (σ^E) RNA polymerase sigma factors.
 32 Strikingly, deletion of *csiA* increases expression of the *spollG* operon, but not that
 33 of the *spollA* operon. Increased expression of *spollG* results in increased
 34 production and proteolytic activation of pro- σ^E , suggesting that normally, the levels
 35 of active σ^E are limiting for sporulation. While other regulatory proteins affect both
 36 sporulation and several processes during the transition phase of growth, including
 37 toxin production or motility, deletion of the *csiA* gene does not alter the expression
 38 of the genes coding for the TcdA and TcdB cytotoxins or the genes involved in
 39 motility. Thus, our results establish that CsiA acts to modulate sporulation by
 40 reducing expression of the *spo0A* gene.

41

42 Introduction

43 Many members of the Firmicutes phylum produce endospores (hereafter
44 spores for simplicity), resilient and metabolically dormant structures that can
45 remain viable for long periods of time. A large proportion of recently identified
46 members of the human gut microbiota are spore formers and many of which are
47 anaerobic organisms (Almeida *et al.*, 2019, Browne *et al.*, 2016). For anaerobic
48 bacteria, pathogens or commensals, the production of oxygen-resistant spores is
49 an important mechanism for transmission between hosts and persistence inside
50 and outside the host (Browne *et al.*, 2016). Gut spore forming bacteria are
51 important members of the human commensal microbiota that only in rare cases
52 cause opportunistic infections (Atarashi *et al.*, 2011, Browne *et al.*, 2016). Other
53 spore formers, however, have evolved to become dedicated pathogens that can
54 cause a striking variety of diseases. Despite variations in disease presentation, the
55 infectious agent is often the spore, with toxins produced by the actively growing
56 cells that result from spore germination playing a central role in the
57 pathophysiology of infection (Zhu *et al.*, 2018). One example is *Clostridioides*
58 *difficile*, a spore-former obligate anaerobe. *C. difficile* is a major nosocomial
59 pathogen and the main causative agent of a range of intestinal diseases
60 associated with antibiotic therapy in adults (Carroll & Bartlett, 2011).

61 Soon after the initiation of sporulation, the sporulating cell undergoes an
62 asymmetric division originating two compartments of unequal sizes, the large
63 mother cell and the small forespore (Errington, 2003, Higgins & Dworkin, 2012,
64 Piggot & Hilbert, 2004)(Fig. 1A). The forespore and the mother cell will follow
65 different lines of gene expression (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet
66 *et al.*, 2013). Entry into sporulation involves phosphorylation of the Spo0A

67 response regulator, a transcription factor conserved among spore formers and
68 essential for sporulation initiation in all organisms in which its function was
69 experimentally assessed (Abecasis *et al.*, 2013, Galperin *et al.*, 2012, Traag *et al.*,
70 2013). The role of Spo0A in sporulation has been studied in detail in *Bacillus*
71 *subtilis* (reviewed in (Deakin *et al.*, 2012, Fujita & Losick, 2005, Molle *et al.*, 2003,
72 Pettit *et al.*, 2014, Sonenshein, 2000)). In pre-divisional cells, the Spo0A-encoding
73 gene, *spo0A*, is transcribed under the control of σ^A and σ^H (Pettit *et al.*, 2014).
74 Spo0A is post-translationally activated *via* phosphorylation by a phosphorelay
75 consisting of several sensor kinases and phosphotransfer proteins, with multiple
76 points of control of the flow of phosphate to Spo0A. The kinases respond to
77 external signals by auto-phosphorylating and subsequently transferring a
78 phosphate residue *via* the intermediary proteins Spo0F and Spo0B to Spo0A
79 (Jiang *et al.*, 2000). In contrast, no Spo0F or Spo0B homologs are found in *C.*
80 *difficile*, and therefore presumably no phosphorelay is present; rather, Spo0A is
81 believed to be phosphorylated directly by orphan histidine kinases that respond to
82 as yet unknown signals (Steiner *et al.*, 2011, Underwood *et al.*, 2009, Worner *et al.*,
83 2006). The *C. difficile* genome codes for five orphan kinases, three of which,
84 CD1492, CD1579 and CD2492, share a high degree of sequence similarity with
85 the sensor histidine kinases involved in the *B. subtilis* phosphorelay (Underwood *et*
86 *al.*, 2009). These three kinases were initially proposed to be responsible for Spo0A
87 phosphorylation. CD1579, however, was the only kinase shown to directly
88 phosphorylate Spo0A. CD2492 was proposed to positively regulate sporulation
89 (Underwood *et al.*, 2009), however no complementation was shown for the mutant.
90 In contrast, CD1492 is now known to inhibit sporulation initiation and to affect toxin
91 and motility through the regulatory proteins RstA and SigD (Childress *et al.*, 2016).

92 In *C. difficile*, Spo0A controls approximately 300 genes (Fimlaid *et al.*, 2013,
93 Pettit *et al.*, 2014), which have been linked to biofilm formation, swimming motility,
94 toxin production and sporulation (Dawson *et al.*, 2012, Fujita & Losick, 2005, Pettit
95 *et al.*, 2014, Underwood *et al.*, 2009). Among the latter, are the genes coding for
96 the first cell type-specific regulators of sporulation, (σ^F and σ^E), as well as genes
97 required for the asymmetric division of the cell at the onset of sporulation
98 (Errington, 2003, Higgins & Dworkin, 2012, Pettit *et al.*, 2014, Piggot & Hilbert,
99 2004, Rosenbusch *et al.*, 2012).

100 Following asymmetric division, the mother cell initiates engulfment of the
101 forespore, a process with similarities to phagocytosis (Fig. 1A). At this stage, the
102 forespore is surrounded by an inner and outer membranes, that derive from the
103 polar division septum. Following engulfment completion, two types of
104 peptidoglycan are layered between the inner and outer membranes surrounding
105 the forespore. The surface of the inner spore membrane is the site of assembly of
106 a thin layer of peptidoglycan called primordial cell wall, similar in composition to the
107 vegetative cell wall. A second, thicker layer of chemically distinct peptidoglycan,
108 called the spore cortex, is assembled across the forespore outer membrane, which
109 faces the mother cell cytoplasm. The spore cortex is essential for the attainment
110 and maintenance of the dehydrated state of the spore core, and hence for heat
111 resistance and dormancy (Henriques & Moran, 2007). Finally, in *C. difficile*, the
112 ordered assembly of proteins produced in the mother cell, form the coat and the
113 exosporium (Henriques & Moran, 2007, Paredes-Sabja *et al.*, 2014, Stewart,
114 2015). These proteinaceous layers protect the cortex against the action of
115 peptidoglycan-breaking enzymes, confer protection against small noxious
116 molecules and UV light, and influence the interaction of spores with molecules able

117 to trigger germination (Driks & Eichenberger, 2016, Henriques & Moran, 2007,
118 McKenney *et al.*, 2013). In addition, the exosporium plays an important role not
119 only in the adhesion of spores to abiotic surfaces but also to host cells (Stewart,
120 2015). At the end of the sporulation process the mother-cell lyses, releasing the
121 spore into the environment.

122 We previously established a genomic signature for sporulation, defined as
123 the genes found in 90% of the endospore forming bacteria, and present in no more
124 than 10% of the non-spore-formers ((Abecasis *et al.*, 2013); Fig. 1B). This
125 signature not only allows prediction of spore formation by organisms for which
126 sporulation has not been shown in the laboratory, but it also identified new genes
127 involved in sporulation (Abecasis *et al.*, 2013). More recently, a genomic signature
128 for endosporulation in the human gastro-intestinal tract was established ((Browne
129 *et al.*, 2016); Fig. 1B). This signature includes 65 genes and is dominated by
130 genes with a known function in sporulation. Approximately 30% of the signature
131 genes, however, have no known function in sporulation and/or code for products
132 with no similarity to known proteins; nevertheless, their presence in a genomic
133 signature for sporulation suggests a role in spore development, at least in the gut.
134 One of these genes is *CD25890* (Fig. 1B) and here we show that deletion of
135 *CD25890* increases the expression of *spo0A*, and also the expression of the
136 Spo0A- and σ^A -dependent *spoIIG* operon, coding for the first mother cell specific
137 RNA polymerase sigma factor, σ^E . In contrast, deletion of *CD25890* does not
138 increases the expression of the Spo0A- and σ^H -dependent *spoIIA* operon, coding
139 for the first forespore-specific sigma factor, σ^F . Expression of the *spoIIG* operon
140 results in the production of an inactive form of σ^E , pro- σ^E . Cleavage of pro- σ^E
141 requires a forespore signalling protein, which in *C. difficile* is mostly but not

142 completely dependent on σ^F (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Saujet *et*
 143 *al.*, 2013). Strikingly, increased expression of *spoIIIG* in the *CD25890* mutant
 144 results in increased production and proteolytic activation of pro- σ^E , suggesting that
 145 the levels of active σ^E , and not forespore signalling, are a limiting factor for
 146 sporulation. Deletion of the *CD25890* gene does not alter the expression of the
 147 gene coding for the TcdA cytotoxin, or the genes involved in motility, or biofilm
 148 formation. Based on these phenotypes we renamed the *CD25890* locus *csiA*, to
 149 reflect its role in the control of sporulation initiation.

Results

Functional analysis of a *csiA* mutant

Phylogenetically, the *csiA* gene and its genomic context are highly conserved within the Firmicutes (Fig. S1). In each genome we examined, CsiA is encoded immediately upstream of the *gmk* gene encoding a guanylate kinase or of the *remA* gene encoding a biofilm regulatory protein (Winkelman *et al.*, 2009, Winkelman *et al.*, 2013). These genes are always upstream of the *rpoZ* gene, encoding for the RNA polymerase omega subunit. Although its genomic context is conserved, there is no evidence for the possible function of *csiA*. Nevertheless, its inclusion in a genomic signature for sporulation suggested an important role in this developmental process. We started by the phenotypic characterization of a mutant in the *csiA* gene. An in-frame deletion mutant of *csiA*, lacking codons 5 – 291 of the 294-codons-long open reading frame was generated by allelic-coupled exchange (ACE) in the background of the widely used 630 Δ *erm* strain (Fig. 1C; Fig. S2; Table S1; (Ng *et al.*, 2013)). The mutant was then complemented in single copy upon restoration of the *pyrE* gene (Fig. S2).

We then examined cultures of the *csiA* mutant after 14h of incubation on sporulation liquid medium (SM). SM has been shown to efficiently induce sporulation in strain 630 Δ *erm* and is well suited for kinetics analyses and fluorescence microscopy (Pereira *et al.*, 2013, Wilson *et al.*, 1982). The cells were stained with the lipophilic membrane dye FM4-64, which allows identification of the stages of sporulation up to engulfment completion, prior to imaging by phase contrast and fluorescence microscopy (Pereira *et al.*, 2013). As shown in Fig. S3A, the *csiA* mutant presents more cells with signs of sporulation than the wild-type

174 strain as determined by fluorescence and phase-contrast microscopy (630 Δ *erm*,
175 29%; Δ *csiA*, 69%).

176 To rule out that the increased sporulation phenotype came from differences
177 in vegetative growth, the wild-type and the *csiA* mutant strains were grown in SM
178 and the optical density of the cultures was measured at 600 nm (OD₆₀₀) at two-
179 hour intervals for 20 hours after inoculation. Both strains showed similar growth
180 patterns and, after about 10 hours, both entered in stationary phase (Fig. S3B).
181 Therefore, the *csiA* mutation does not affect growth of *C. difficile* under the
182 conditions tested.

183 To determine whether the kinetics of sporulation was altered in the *csiA*
184 mutant, we analysed the formation of heat resistant spores along time. Strains
185 were grown in SM broth, and at 12, 24, 48 and 72 hours the titer of heat resistant
186 spores/ml of culture was determined as described in the materials and methods
187 section (Fig. 2A and Table 1). In line with earlier results (Pereira *et al.*, 2013), the
188 titer of spores for the wild-type strain increased from 7.5x10³ spores/ml at hour 12,
189 to 1.0 x10⁴ spores/ml at hour 24, 1.3 x10⁵ spores/ml at hour 48 and 1.7x10⁵
190 spores/ml at hour 72 (Fig. 2A and Table 1). As expected from the previous results,
191 the *csiA* mutation did not affect cell viability (Table 1). In contrast, the titer of heat
192 resistant spores in the mutant strain was higher than the wild-type for all time
193 points: 3.1x10⁴, 8.5x10⁴ spores/mL, 1.5x10⁶ and 2.2x10⁶ spores/mL at hour 12, 24,
194 48 and 72, respectively (Fig. 2A and Table 1). To determine whether the *csiA*
195 deletion was responsible for the mutant phenotype, sporulation was assessed in
196 the complemented strain (Table 1). As shown in Fig. 2A and Table 1,
197 complementation restored the wild-type kinetics of spore formation (1.8x10⁴,
198 1.5x10⁴, 7.8x10⁴ and 6.0x10⁴ spores/ml at hour 12, 24, 48 and 72, respectively).

199 Thus, the deletion of *csiA* is responsible for the increased sporulation frequency of
200 the mutant.

201 A second frequently used method to induce sporulation is growth on 70:30
202 agar plates, in which the rate of sporulation is higher (Putnam *et al.*, 2013). We
203 also tested the sporulation efficiency of the *csiA* mutant in this medium. When
204 sporulation was induced by this method, the *csiA* mutant shows a percentage of
205 sporulation similar to the wild-type strain (Fig. S4A). Thus, the culture medium
206 influences the *csiA* mutant phenotype (see also below).

207

208 ***csiA* negatively impacts sporulation**

209 We next followed progress through the morphological stages of sporulation
210 in SM, by phase contrast and fluorescence microscopy (Pereira *et al.*, 2013). The
211 wild-type and the mutant strain were grown in SM and samples were collected 8,
212 10, 12, 14, 16, 18 and 20 hours after inoculation. The cells were stained with the
213 lipophilic membrane dye FM4-64 prior to microscopic examination. Cells
214 representative of several distinctive morphological classes are shown on Fig. 2B.
215 The results shown in Fig. 2C demonstrate that 8 hours after inoculation a
216 significant fraction of the *csiA* cells had undergone polar division (class *a*). In
217 contrast, class *a* cells were detected for the wild-type strain 2 hours later than in
218 the mutant. Moreover, phase bright spores (class *e*) were observed at 14 hours for
219 the mutant and at 18 hours for the wild-type strain.

220 To test whether spore morphogenesis proceeded faster in the *csiA* mutant,
221 we determined the time at which at least 10% of the cell population had undergone
222 polar division (class *a*), initiated engulfment (class *b*), showed complete engulfment
223 of the forespore by the mother cell (class *c*), or presented phase-gray or phase-

224 bright spores (classes *d* and *f*). The results in Fig. 2D show that progress through
225 these stages of sporulation occurred at the same pace for the two strains. We
226 conclude that the *csiA* mutant initiated sporulation earlier than the parental wild-
227 type or that the fraction of cells entering sporulation at the end of growth is larger
228 for the mutant. In any event, CsiA negatively affects the entry into sporulation.

229

230 **CsiA accumulates during growth independently of Spo0A**

231 We further investigated accumulation of CsiA during growth and sporulation
232 in SM by immunoblot analysis using an anti-CsiA antibody (Fig. 3A). CsiA starts to
233 accumulate early during growth and is present also during entry into stationary
234 phase when the cells start to sporulate. No signal is detected in the *csiA* mutant,
235 while its accumulation is restored in the complementation strain, confirming the
236 specificity of the antibody. CsiA accumulation is independent of Spo0A, the master
237 regulator of sporulation (Fig. 3B). These results show that CsiA starts to
238 accumulate during vegetative growth, independently of the main sporulation
239 transcription regulators.

240

241 **Deletion of *csiA* increases transcription of *spo0A***

242 Since Spo0A is the master regulatory protein governing entry into
243 sporulation (Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012, Underwood *et al.*, 2009),
244 we wanted to test whether deletion of *csiA* increased the expression of the *spo0A*
245 gene in individual cells. We constructed a fusion of the *spo0A* promotor region to
246 the *SNAP* reporter and we introduced the P_{*spo0A*}-*SNAP* fusion in the *csiA* mutant
247 and in the parental wild-type strain (Table S1). To monitor production of SNAP,

248 samples of cultures expressing the transcriptional fusion were collected 10 hours
249 after inoculation in SM and the cells were labelled with the TMR-Star SNAP
250 substrate (Fig. 4A). Expression of P_{spo0A} -SNAP was detected in 100% of the cells
251 for both the wild-type and the *csiA* mutant (Fig. 4A). Quantification of the
252 fluorescence signal per cell (in arbitrary units, or AU; Fig. 4B), however, shows that
253 transcription from the *spo0A* promoter occurred at a lower intensity in the wild-type
254 (average signal, 137.6 AU) as compared to the *csiA* mutant (average signal, 277.3
255 AU) (Fig. 4B).

256 To investigate whether the increased transcription from the *spo0A* promoter
257 could also lead to increased accumulation of Spo0A, we compared the levels of
258 Spo0A throughout sporulation by immunoblot analysis using a previously described
259 anti-Spo0A antibody ((Serra *et al.*, 2014); Fig. 4C). We found that Spo0A is
260 detected as early as 6 hours following inoculation in SM for all strains, but that in
261 the *csiA* mutant Spo0A accumulates at greater levels than in the wild-type or the
262 complementation strain (Fig. 4C). Together, these results suggest that more cells
263 of the mutant reach a threshold level of Spo0A that triggers sporulation, and that
264 CsiA antagonizes expression of *spo0A* and/or the activity of Spo0A, since *spo0A* is
265 subject to positive auto-regulation (Rosenbusch *et al.*, 2012).

266 Since we did not observe a phenotype for the *csiA* mutant when sporulation
267 was induced on 70:30 medium (see above), we compared the expression of *spo0A*
268 in SM with that in 70:30 medium using fluorescence microscopy and single cell
269 analysis (Fig. S3B). Quantification of the fluorescence signal per cell (Fig. S4B)
270 shows that transcription from the *spo0A* promoter is higher in 70:30 medium in
271 both the wild-type (average signal, 267.1 AU) and *csiA* mutant (average signal,
272 332.8 AU), reaching levels similar to those observed in the mutant when

sporulation is induced in SM (average signal, 372 AU) (Fig. S4B). This result suggests that in 70:30 medium, the wild-type strain reaches the threshold levels of *spo0A* required for sporulation in a higher number of cells than in SM, similar to what was observed for the *csiA* mutant in both media. Hence, CsiA acts as a repressor only under certain culturing conditions that induce sporulation, with the effect of the *csiA* mutation being masked in 70:30. medium

Phosphorylated Spo0A accumulates at higher levels in the *csiA* mutant

Spo0A is activated by phosphorylation (Fujita & Sadaie, 1998, Lewis *et al.*, 1999). The activated Spo0A protein, Spo0A~P, binds to DNA promoter regions containing a Spo0A-binding motif and then regulates the expression of Spo0A-dependent genes. Since *spo0A* is auto-regulatory, and higher expression per cell is seen in the *csiA* mutant in SM, we asked whether Spo0A~P would accumulate at higher levels in the mutant. For this, we used Phos-tag SDS-PAGE, where migration of Spo0A~P is delayed, and the two forms (phosphorylated and unphosphorylated) are then detected by western blot using anti-Spo0A antibodies. The wild-type and the mutant strain were grown in SM and samples were collected 6, 8, 10 and 12 hours after inoculation. Whole cell extracts were prepared and the proteins resolved by Phos-tag SDS-PAGE (Fig. 5A). Two bands were detected in this manner. The upper band disappeared when the samples were boiled prior to electrophoretic resolution, which indicates that the upper band corresponds to Spo0A~P and the lower band to the unphosphorylated form (Yasugi *et al.*, 2016). We found that in the *csiA* mutant, both forms of Spo0A accumulate earlier and at higher levels than in the wild-type. We then quantified band intensities in order to estimate the ratio of the phosphorylated to unphosphorylated form of Spo0A

298 (Spo0A~P/Spo0A) using Image-J software. The Spo0A~P/Spo0A ratio was higher
299 at hour 8 and 10 in the mutant as compared to the wild-type strain (Fig. 5B).
300 However, at hour 12 the ratio between the two forms was similar between the two
301 strains.

302 This result suggests that the level of Spo0A phosphorylation is directly
303 proportional to the level of the protein. Together with the observation that *spo0A* is
304 expressed in most if not all of the pre-divisional cells (Fig. 4A), our findings suggest
305 that phosphorylation of Spo0A is not a limiting factor for the initiation of sporulation.
306 Possibly then, the level of *spo0A* per cell could limit entry into sporulation.

307

308 **CsiA acts at the level of *spo0A* transcription**

309 Since we found increased transcription of P_{spo0A} -SNAP^{Cd} in the *csiA* mutant,
310 we reasoned that the increased sporulation in the mutant could be due to
311 increased transcription or to increased activity of Spo0A. To uncouple transcription
312 from activation of Spo0A by phosphorylation, we placed the *spo0A* gene under the
313 control of the anhydrotetracycline (ATc) responsive P_{tet} promoter (Dembek *et al.*,
314 2017). In this way Spo0A could accumulate to similar levels in the wild-type and in
315 the *csiA* mutant strains, allowing us to test whether increased levels of Spo0A~P
316 accumulated in the mutant. The strains were grown in SM supplemented with 50
317 nM of ATc, a concentration at which Spo0A accumulates to levels similar to the
318 wild-type strain (Dembek *et al.*, 2017), and proteins in whole cells extracts were
319 resolved by Phos-tag SDS-PAGE (Fig. 5C). In both strains Spo0A accumulates at
320 similar levels and the Spo0A~P/Spo0A ratio was similar (Fig. 5C and D). In
321 agreement with this observation, heat test revealed no difference in the number of
322 heat resistant spores produced by both strains (Table 2).

323 Together these results suggest that CsiA acts mainly by decreasing the
324 levels of *spo0A* transcription per cell.

325

326 **The *csiA* mutant shows increased expression of Spo0A-dependent genes**

327 The increased expression of *spo0A* and the increased sporulation of the
328 *csiA* mutant suggested that a larger fraction of the population entered the
329 sporulation pathway. To independently test this idea, we analysed the expression
330 of the genes coding for the early sporulation sigma factors *sigE* and *sigF*, whose
331 transcription is under Spo0A control. To monitor transcription of *sigE* and *sigF*, we
332 used transcriptional fusions of the *spoIIA* (driving σ^F production) and *spoIIG* (driving
333 σ^E production) promoters to the SNAP^{Cd} reporter (Pereira *et al.*, 2013). Samples of
334 cultures expressing each of the promoter- SNAP^{Cd} fusions were collected 14 hours
335 after inoculation in SM and the cells labelled with TMR-Star and the membrane dye
336 MTG, to allow identification of the different stages of sporulation (Fig. 6A).

337 Expression of both *sigF* and *sigE* was first detected in pre-divisional cells and cells
338 that had undergone polar division, as reported previously (Pereira *et al.*, 2013).

339 Quantification of the fluorescence signal shows that although for *sigF* the
340 percentage of cells with a fluorescence signal is higher in the *csiA* mutant (52%)
341 than in the wild-type strain (35%), the average intensity of the signal did not differ
342 much between the two strains (1187.05 AU for the wild type and 1110.50 AU for
343 the mutant) (Fig. 6B). Transcription of *sigE*, however, was lower in the wild-type,
344 with 76% of the cells showing a signal with an average intensity of 857.96 AU, as
345 compared to the mutant, in which 87% of cells showed a fluorescence signal with a
346 significantly higher average intensity of 1295.78 AU (Fig. 6B).

347 We next examined the effect of the *csiA* mutant on the activity of σ^F and σ^E
 348 by monitoring expression of transcriptional fusions of the *gpr* (controlled by σ^F) and
 349 *spoIIIA* (controlled by σ^E) promoters to the SNAP^{Cd} reporter (Pereira *et al.*, 2013).
 350 Expression of *gpr* was detected in 83% of the sporulating cells of the wild-type and
 351 in 94% of the *csiA* sporangia (Fig. 6A), while expression of *spoIIIA* was detected in
 352 77% of the sporulating cells in the wild-type and in 91% of the *csiA* sporangia (Fig.
 353 6A). A quantitative analysis revealed no effect of the *csiA* mutation on *gpr*
 354 expression (average intensity of 4412.30 AU for the wild type and 4551.54 AU for
 355 the mutant; Fig. 6B). In contrast, the average intensity of the fluorescence signal
 356 from P_{*spoIIIA*}-SNAP^{Cd} was higher in cells of the mutant (1277.84 AU for the mutant)
 357 as compared to the wild-type (827.90 AU) (Fig. 6B).

358 This increase likely results from the augmented expression of *sigE* in the
 359 *csiA* mutant (see above).

360

361 **Earlier accumulation and processing of pro- σ^E in the *csiA* mutant.**

362 The increased activity of σ^E , but not of σ^F , suggested to us that σ^E could
 363 accumulate to higher levels in cells of the *csiA* mutant as compared to the wild-type
 364 and that in contrast, the levels of σ^F would be similar in the mutant and the wild-
 365 type. To test this prediction, we monitored the accumulation of σ^F and σ^E by
 366 immunoblotting during entry into sporulation. The mature, active form of σ^E was
 367 first detected 8 hours after inoculation in the mutant and only at 10 hours in the
 368 wild-type (Fig. 6C). Importantly, we found that the accumulation of the mature form
 369 of σ^E is also higher for the *csiA* mutant as compared to the wild-type, with the
 370 protein accumulating at levels 8 times higher in the mutant 10 hours after

371 inoculation (Fig. 6C). On the other hand, there was no significant difference on the
372 accumulation of σ^F between the wild-type and the mutant. Thus, the lack of CsiA
373 leads to premature accumulation and activation of pro- σ^E , but does not affect the
374 accumulation of σ^F .

375

376 **Expression of *sigE* establishes a threshold for sporulation.**

377 Higher levels of Spo0A~P accumulate in the *csiA* mutant, leading to
378 increased expression of the Spo0A-dependent genes. Consequently, the higher
379 levels of Spo0A~P increase the number of cells showing asymmetric division (Fig.
380 2C), and on transcription from the *spoIIG* promoter, but not from the *spoIIA*
381 promoter (Fig. 6B). These results strongly suggest that asymmetric division and the
382 level of active σ^E are a limiting factor for sporulation. To test this idea we inserted
383 the P_{sigF} and P_{sigE} -SNAP^{Cd} fusions in the strain in which *spo0A* is under the control
384 of P_{tet} promoter (Dembek *et al.*, 2017). The strains were grown in SM to an OD₆₀₀
385 of approximately 0.2, supplemented with a range of ATc concentrations, from 0–
386 250 ng/ml, and 2 hours after addition the cells were labelled with TMR. In the
387 absence of the inducer, no fluorescence was detected in either strain. In contrast, a
388 clear dose-dependent response in the percentage of cells showing fluorescence
389 from the P_{sigE} -SNAP fusion was observed at concentrations of inducer from 0 to 50
390 ng/ml (Fig. 6D), reaching a plateau at higher aTc concentrations (approximately
391 70% of the cells). Interestingly, this is also the concentration at which wild type
392 levels of heat resistance spores are reached in the strain carrying *spo0A* under P_{tet}
393 control (Dembek *et al.*, 2017). In contrast, low levels of *spo0A* (10 ng/ml) are
394 required to detect expression of *sigF* in nearly all of the cells in the population
395 (approximately 95%; Fig. 6D). Since the formation the polar septum is also

governed by Spo0A~P (Ben-Yehuda & Losick, 2002, Levin & Losick, 1996), we also measured the number of cells showing asymmetric septa in the $P_{ter} spo0A$ strain at the same range of ATc concentrations, following staining of the cells with the membrane dye FM4-64 prior to microscopic examination (Fig. 6D). At 10 ng/ml of aTc only approximately 35% of the cells showed asymmetric septa or were at intermediates stages during engulfment; this percentage increased to more than 90% at 25 ng/ml of aTc.

Thus, our results suggest that there are different Spo0A~P thresholds for the formation of the asymmetric septum, and for the activation of *spolIA* and *spolIG* transcription. At least under some conditions, a fraction of the cells that activate *spolIA* expression may not reach a point that allows *spolIG* expression or asymmetric division.

Deletion of *csiA* mainly affects sporulation

Spo0A regulates directly or indirectly a number of virulence-associated factors such as motility, biofilm formation and toxin production (Pettit *et al.*, 2014, Rosenbusch *et al.*, 2012, Underwood *et al.*, 2009). Therefore, we wanted to determine whether the *csiA* mutation had a more global effect on gene expression, for that we decided to compare the transcriptome of the mutant with the wild type after 10 hours of growth in SM. We used two biological replicates and genes were considered differentially expressed if the fold change was > 2 and the adjusted p value <0.01. We found that 165 genes were overexpressed in the mutant, while only 21 genes were under-expressed (Table 3). From these genes, 69% are genes regulated by Spo0A or by sporulation-specific cell type-specific sigma factors (Fig.

420 7A and Table 3). No toxin, biofilm or motility genes are among these genes (see
421 also Fig. S5).

422

423 Discussion

424 A genomic signature of sporulation in the human intestinal microbiome
425 (Browne *et al.*, 2016) includes not only genes with an established function in
426 sporulation (Fig. 1) but also a group of 20 genes of unknown function. We
427 hypothesized that these uncharacterized genes are important for sporulation. To
428 test this hypothesis, we characterized a mutant in the *csiA* (*CD25890*) gene. We
429 show that the CsiA protein accumulates during growth in a medium in which
430 sporulation is induced, and also during sporulation (Fig. 3). Attempts to localise
431 *csiA* expression during sporulation using a SNAP transcriptional fusion to the 331
432 bp region upstream of *csiA* failed, even if this same region is sufficient to allow the
433 expression of *csiA* at the *pyrE* locus at levels that permit complementation of the
434 *csiA* deletion mutation.

435 We found that under certain culturing conditions, *csiA* deletion has an
436 impact on sporulation. The *csiA* mutant reaches a higher titer of spores than the
437 wild type strain when grown in sporulation medium (SM). We show here that in the
438 *csiA* mutant spore differentiation is triggered in a larger fraction of the population
439 (Fig. 2). This mutant maintains high levels of *spo0A* expression and shows higher
440 expression of genes required for sporulation, such as *spoIIIG* (Fig. 4 and 6). Since
441 Spo0A is auto-regulatory, the transcriptional fusion reports both transcription of
442 *spo0A* as well as the activity of Spo0A which requires phosphorylation of the
443 protein (Rosenbusch *et al.*, 2012). We show that higher levels of Spo0A~P

444 accumulate in the *csiA* mutant earlier during growth, leading to increased
445 expression of Spo0A-dependent genes. Uncoupling of transcription from activation
446 by using an inducible promoter to control *spo0A* expression showed that CsiA does
447 not act on the phosphorylation of Spo0A but rather at the level of *spo0A*
448 transcription (Fig. 5). In cells growing in SM, Spo0A~P is directly proportional to the
449 total level of protein, indicating that under these conditions phosphorylation is not a
450 limiting step for the initiation of sporulation.

451 Our finding that higher accumulation of Spo0A correlates with enhanced
452 sporulation contrasts with studies performed in *B. subtilis*, where accelerated
453 accumulation of Spo0A independent of the phosphorelay was detrimental to
454 sporulation (Fujita & Losick, 2005, Vishnoi *et al.*, 2013). Rather, Spo0A needs to
455 accumulate in a gradual manner to trigger sporulation and this requires its
456 phosphorylation through the phosphorelay (Vishnoi *et al.*, 2013). This suggests that
457 a gradual build-up of Spo0A may not be important for the sporulation process in *C.*
458 *difficile*. Previous studies have already shown that *spo0A* expression can be
459 increased from an inducible promoter in a dose-dependent manner, and that the
460 level of expression correlates with sporulation efficiency, which reaches values
461 higher than those observed in a congenic wild-type strain (Dembek *et al.*, 2017).

462 Intriguingly, in the *csiA* mutant, higher Spo0A~P levels impact the formation
463 of the asymmetric septum, possibly through *spoIIIE*, which is also under the control
464 of Spo0A and is required for proper septum formation (Ben-Yehuda & Losick,
465 2002, Levin & Losick, 1996) . The mutation also increased *spoIIIG* transcription,
466 while not affecting *spoIIA* transcription (Fig. 2 and 6). These events are under the
467 control of Spo0A (Ben-Yehuda & Losick, 2002, Levin & Losick, 1996, Pettit *et al.*,
468 2014, Rosenbusch *et al.*, 2012, Saujet *et al.*, 2011). The *spoIIA* operon (*spoIIAA*-

469 *spolIAB-sigF*) is transcribed under the joint control of the σ^H sigma factor and
 470 Spo0A, which binds to Spo0A boxes in the *spolIA* promoter region (Pettit *et al.*,
 471 2014, Rosenbusch *et al.*, 2012, Saujet *et al.*, 2011). Our results, however,
 472 suggests that a factor or factors other than Spo0A contributes to expression of
 473 *sigF*, as also shown in *C. acetobutylicum* (Alsaker *et al.*, 2004). *spolIE* is involved
 474 in the Spo0A-dependent switch in the septum localization from mid-cell to the pole,
 475 and its expression is also σ^H - and Spo0A-dependent (Ben-Yehuda & Losick, 2002,
 476 Khvorova *et al.*, 1998, Levin & Losick, 1996, Saujet *et al.*, 2011). In contrast, the
 477 *spolIG* operon (*spolIGA-sigE*) is under the control of σ^A and is also positively
 478 regulated by Spo0A. Therefore, differences on the role of CsiA in the expression
 479 from different promoters are unlikely to be related to the regulation of the σ factor.
 480 Rather, CsiA may affect the complexes made by σ^H and Spo0A and by σ^A and
 481 Spo0A at different promoters.

482 In line with the *SNAP^{Cd}*-based single cell transcriptional analysis, we also
 483 found that accumulation of the active, processed form of σ^E is higher in the *csiA*
 484 mutant as compared to the wild-type. In contrast, there was no significant
 485 differences on the accumulation of σ^F . This is in agreement with previous results
 486 showing that the expression of the σ^E regulon in the mother cell was not strictly
 487 under the control of σ^F (Saujet *et al.*, 2013).

488 Previous studies have shown that the DNA-binding domain of Spo0A
 489 (Spo0A-DBD) is able to bind to the promoter regions of *sigF*, *spolIE* and *sigE*,
 490 (Rosenbusch *et al.*, 2012). Spo0A-DBD binds at low concentration to *spolIA* and
 491 *spolIE*, and a higher concentration is needed to detect binding to the *sigE*
 492 promoter. Here, we used a strain in which *spo0A* expression is under the control of
 493 an inducible promoter (Dembek *et al.*, 2017) to test for the requirement of different

amounts of Spo0A to trigger *sigF* and *sigE* expression, as well as formation of asymmetric septa (an event that is also under Spo0A control). Under the conditions tested, phosphorylation was not a limiting step for sporulation (Fig. 5). Therefore, we infer that the amount of *spo0A* expression is proportional to the level of Spo0A~P. We found that expression from the *spoIIA* promoter increases dramatically at low levels of *spo0A* expression (10 ng/ml of the inducer aTc), while higher levels of *spo0A* expression (250 ng/ml of aTc) are required for *sigE* to be expressed in the majority of the cells. Asymmetric division is achieved in almost 100% of the cells when *spo0A* was induced with 25 ng/ml of aTc (Fig. 6). We were not able to detect *spoIIIE* expression using a *SNAP* transcriptional fusion. These results indicate that as in *B. subtilis* many of the *spo0A*-dependent genes respond to the transcriptional factor in a dose-dependent manner (Fujita *et al.*, 2005). In *B. subtilis* the threshold of Spo0A activity required for σ^F activation is below that for the activation of σ^E (Narula *et al.*, 2012). In *B. subtilis*, cells that form an asymmetrical septum but do not activate σ^F or σ^E are able to resume vegetative growth (Dworkin & Losick, 2005); commitment to sporulation occurs only after the activation of both of the first cell type-specific σ factors, σ^F and σ^E . Since spore formation is an energy consuming process and irreversible after specific points, in *C. difficile* commitment to sporulation may be postponed until σ^E accumulates to sufficient levels. This may be achieved by controlling *spo0A* expression, and *csiA* may be involved in this process (Fig. 7D).

The impact of *csiA* on sporulation and on expression of sporulation-specific genes is significantly influenced by the culture medium. The two sporulation conditions used here differ in medium composition, and this may indicate that the signalling to enter sporulation may be different, dependent on the environment.

519 The specific external signals that activate sporulation in *C. difficile* are not known,
520 but it is anticipated that, like in *B. subtilis*, the sporulation-initiating and -inhibiting
521 signals for *C. difficile* act on Spo0A (Childress *et al.*, 2016, Underwood *et al.*,
522 2009).

523 Many factors that influence the sporulation pathway have been identified.
524 Two global regulators, CcpA and CodY, affect the initiation of sporulation by
525 repressing the expression of sporulation-related genes in high-nutrient conditions
526 (Antunes *et al.*, 2012, Dineen *et al.*, 2007, Girinathan *et al.*, 2018, Nawrocki *et al.*,
527 2016). Both of these transcription repressors also repress the expression of the
528 toxin encoding genes (Antunes *et al.*, 2012). Another negative regulator of
529 sporulation initiation is the histidine kinase CD1492. The *CD1492* mutant shows
530 increased expression of *spo0A*-dependent genes, but also exhibits decreased toxin
531 production and motility. RstA, acts as a positive regulator of sporulation, although
532 the exact nature of this effect remains unclear (Edwards *et al.*, 2016). The DNA
533 binding domain of RstA was shown to directly bind to toxin and motility genes,
534 repressing their expression, but this domain is not required for the positive effect
535 exerted on sporulation (Edwards *et al.*, 2019). The genome of *C. difficile* also
536 encodes for two oligopeptide permeases (Opp and App) that in *B. subtilis* are
537 involved in the control of sporulation initiation in a cell density-dependent manner
538 (Koide & Hoch, 1994, Perego *et al.*, 1991). Given the ecological niche of *C. difficile*,
539 cell density may not be a signal that triggers sporulation, and in contrast to *B.*
540 *subtilis*, the *opp* and *app* mutants show increased sporulation-specific gene
541 expression, and exhibit a hyper-sporulation phenotype with no impact on toxin
542 production or motility (Edwards *et al.*, 2014). It was suggested that *opp* and *app*
543 may be involved in sporulation as a response to nutrient availability. Like *csiA*,

544 these mutants also show an increase on the expression of the *sinRR'* operon
545 (Table 3), previously shown to be involved in sporulation, toxin production and
546 motility (Girinathan *et al.*, 2018). It was shown that overexpression of *sinR*
547 increased sporulation efficiency, while overexpression of *sinR'* reduced sporulation.
548 However, to our knowledge the overexpression of the entire operon, as occurs in
549 the *csiA* and *opp*, *app* mutants, was not tested. With the exception of the *opp* and
550 *app* peptide transporter mutants, all the mutants in genes involved in the control of
551 initiation of sporulation also have an impact on toxin production and/or motility.
552 Since deletion of *csiA* has no impact on toxin production and motility, the *csiA*
553 function is most likely independent from SigD, RstA, CD1492, and CD2492. In
554 addition, *csiA* must not be impacting activity of CodY or CcpA, both of which also
555 affect toxin production.

556 *B. subtilis csiA* orthologue (*yloC*) is in an operon with the *remA* gene, which
557 was also previously identified as part of a sporulation genomic signature
558 ((Abecasis *et al.*, 2013, Traag *et al.*, 2013), Fig. S1, orange gene). RemA is
559 required for activating expression of the extracellular matrix biosynthetic operons
560 during biofilm development by undomesticated strains of *B. subtilis* (Winkelman *et al.*,
561 2009, Winkelman *et al.*, 2013). While *remA* is not present in *C. difficile* and
562 neither YloC nor CsiA show sequence similarity to proteins of known function, the
563 genomic context suggested a role in the regulation of genes at the onset of the
564 stationary growth, which may have different outcomes in the two bacteria (biofilm
565 or sporulation) due to differences on the specific signals that lead to different
566 responses.

567 Our study has identified a new factor that affects sporulation initiation, CsiA,
568 which negative impacts sporulation by affecting the expression of *spo0A*. The

569 medium dependence of CsiA function may reflect differences in nutrient-signalling
 570 of *spo0A* expression and this observation may be explored to identify some of
 571 those signals. In all, our analysis shows that the genomic signature of sporulation
 572 within the human intestinal microbiome can be used to identify new genes
 573 important during the sporulation process. Further studies are needed to
 574 established the function of the other signature genes. This may lead to the
 575 identification of new genetic determinants for spore formation whose products
 576 could act as important targets for the design of drugs effective against all spore-
 577 formers.

578

579

580 Materials and Methods

581 **Growth conditions and general methods.** Bacterial strains and their relevant
582 properties are listed in Table S1. The *Escherichia coli* strain DH5 α (Bethesda
583 Research laboratories) was used for molecular cloning, while the strain HB101
584 (RP4) was used as the donor in *C. difficile* conjugation experiments (Hussain *et al.*,
585 2005). Luria-Bertani medium was routinely used for growth and maintenance of *E.*
586 *coli*. When appropriate, ampicillin (100 μ g/ml) or chloramphenicol (15 μ g/ml) was
587 added to the culture medium. The *C. difficile* strains used in this study are isogenic
588 derivatives of the wild-type strain 630 Δ *erm* (Hussain *et al.*, 2005) and were
589 routinely grown anaerobically (5% H₂, 15% CO₂, 80% N₂) at 37°C in brain heart
590 infusion (BHI) medium (Difco) (Wilson *et al.*, 1982). Assays for toxin production
591 were done in tryptone yeast extract (30g/L tryptone; 20g/L yeast extract) (TY).
592 When necessary, cefoxitin (25 μ g/ml) and thiamphenicol (15 μ g /ml) were added to
593 *C. difficile* cultures. A defined minimal media (CDMM) (Karasawa *et al.*, 1995) with
594 1% agar was used as uracil-free medium when performing genetic selections.

595 Sporulation was tested on liquid Sporulation Medium (SM; for 1L: Bacto
596 tryptone 90 g, Bacto peptone 5 g, (NH₄)₂SO₄ 1 g, Tris base 1.5 g, pH 7; (Pereira *et*
597 *al.*, 2013)) and 70:30 agar medium (for 1L: Bacto peptone 63 g, proteose peptone
598 3.5 g, (NH₄)₂SO₄ 0.7 g, Tris base 1.06 g, brain heart infusion extract 11.1 g, yeast
599 extract 1.5 g, cysteine 0.3 g and agar 15 g, (Putnam *et al.*, 2013)). Sporulation was
600 regularly induced by inoculating in SM liquid. To determine the total number of
601 cells, the cells were serially diluted and plated on BHI with 0.1 % taurocholate
602 (Sigma-Aldrich) to ensure efficient spore germination. To determine the number of

603 spores, the cells were heat killed by incubation for 30 min at 70°C prior to plating
604 on BHI with 0.1 % taurocholate.

605 **Construction of the *csiA* mutant.** A *csiA* in-frame deletion mutant was generated
606 using allele-couple exchange (ACE) in *C. difficile* 630 Δ *erm* Δ *pyrE* as described (Ng
607 *et al.*, 2013). The homology regions upstream and downstream of the desired
608 junction point within *csiA* were PCR-amplified and cloned into pMTL-YN3 as
609 follows (Ng *et al.*, 2013). The upstream fragment (742 bp) was amplified using the
610 primers *csiA*_Ascl_Fwd and *csiA*_LHA_Rev and the downstream fragment (735
611 bp) was amplified using the primers *csiA*_RHA_Fwd and *csiA*_Sbfl_Rev. The
612 fragments were then joined by overlapping PCR, the resulting fragment cleaved
613 with Ascl and Sbfl and cloned between the same sites of pMTL-YN3, yielding
614 pAM37. This plasmid was introduced into *E. coli* HB101 (RP4) and then transferred
615 to strain 630 Δ *erm* Δ *pyrE* by conjugation (Heap *et al.*, 2007). Following two
616 passages on BHI agar supplemented with 25 μ g/mL cefoxitin and 15 μ g/mL
617 thiamphenicol, colonies that were noticeably larger (indicative of plasmid
618 integration) were screened by colony PCR to identify single-crossover mutants
619 using primers flanking the upstream and downstream homology regions in
620 conjunction with a plasmid-specific primer (P3 with P2 and P4 with P1) to amplify
621 across the integration junction (Fig. S2). Clones positives for single crossover
622 mutants were streaked onto *C. difficile* minimal medium (CDMM) supplemented
623 with 5 μ g/mL uracil and 2 mg/mL 5-fluoroorotic acid (FOA) to select for plasmid
624 excision. The isolated FOA-resistant colonies were screened by PCR using
625 primers P3 and P4. Double-crossover mutants, in which the mutant allele was
626 successfully integrated yielded products smaller than those seen in WT revertants
627 (1630 bp instead of 2472 bp). In order to restore the *pyrE*⁺ phenotype, plasmid

628 pMTL-YN1 carrying the WT *pyrE* allele was conjugated into the isolated double-
629 crossover mutants. The resulting colonies were restreaked onto non-supplemented
630 CDMM agar to select for uracil prototrophy indicating successful allele exchange.
631 Successful restoration of the WT *pyrE* allele was confirmed by colony PCR using
632 primers flanking the *pyrE* locus (P5 and P6, Fig. S2).

633 ***In trans* complementation of the Δ *csiA* mutation.** To complement the *csiA*
634 mutation the coding sequence and its expected promoter region were amplified by
635 PCR using primers *csiA*_Fwd_BamHI and *csiA*_Rev_XhoI yielding a 1515 bp
636 fragment. The fragment was then digested with BamHI and XhoI and inserted
637 between the same sites of pMTL-YN1 (Ng *et al.*, 2013), yielding pAM38. This
638 plasmid was introduced into *E. coli* HB101 (RP4) and then transferred to strain
639 630 Δ *erm* Δ *pyrE* Δ *csiA* by conjugation (Heap *et al.*, 2007). Following two passages
640 on BHI agar supplemented with 25 μ g/mL cefoxitin and 15 μ g/mL thiamphenicol,
641 colonies that were noticeably larger (indicative of plasmid integration) were
642 streaked onto onto non-supplemented CDMM to select for uracil prototrophy
643 indicating successful allele exchange. Successful restoration of the WT *pyrE* allele
644 was confirmed by colony PCR using primers flanking the *pyrE* locus (P5 and P6,
645 Fig. S2).

646 ***SNAP*^{Cd} transcriptional fusions.** To construct transcriptional *SNAP*^{Cd} fusions to
647 the *spo0A* promoter, a 568 bp DNA fragment containing the Spo0A promoter
648 region was PCR-amplified using genomic DNA from strain 630 Δ *erm* and primer
649 pairs CDspo0A_598_Fw/CDspo0A_SNAP_Rev. These fragments were cloned into
650 pFT47 (Pereira *et al.*, 2013) to create pMS463 (Table S3). Plasmid pMS463 was
651 transferred to 630 Δ *erm*, and congenic Δ *csiA* mutants by conjugation from
652 derivatives of *E. coli* HB101 (RP4) (Table S1).

SNAP^{Cd} labelling, fluorescence microscopy and image analysis. Samples of 1 ml were withdrawn from SM cultures at the desired times following inoculation, and the cells collected by centrifugation (10 min, 4000xg, at 4°C). The cells were washed with 1ml of phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4), and resuspended in 0.1 ml of PBS supplemented with the lipophilic styryl membrane dye *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)-hexatrienyl pyridinium dibromide (FM4-64, Molecular Probes, Invitrogen; 10 µg.ml⁻¹) (Pogliano *et al.*, 1999). For SNAP labelling, TMR-Star was added to cells in culture samples inside an anaerobic chamber to a final concentration of 250 nM (New England Biolabs) and the mixture incubated for 30 minutes in the dark. Following labelling, the cells were collected by centrifugation (4000xg for 5 min), washed four times with 1 ml of PBS, and finally suspended in 10-20µl of PBS. For phase contrast and fluorescence microscopy, cells were mounted on 1.7% agarose coated glass slides and observed on a Leica DM6000B microscope equipped with a phase contrast Uplan F1 100x objective and captured with a CCD Andor Ixon camera (Andor Technologies). Images were acquired and analysed using the Metamorph software suite (version 5.8; Universal Imaging) and adjusted and cropped using Photoshop S4. Statistical analysis was carried out using GraphPad Prism (Version 7.0; GraphPad Software Inc.). The non-parametric Kolmogorov-Smirnov test (KS-test) was applied to compare distributions obtained from quantifications of the SNAP-TMR signal. The P-value is indicated for all comparisons whose differences were found to be statistically significant. Although the results presented are from a single experiment, all experiments involving quantification of a fluorescence signal were performed independently three times and only results that were considered statistically significant by a KS-test in all three experiments were considered to be statistically relevant.

679 Preparation of *C. difficile* whole cell extracts and immunoblotting. Whole cell
680 extracts were obtained by withdrawing 20 ml samples from SM or TY cultures of *C.*
681 *difficile* 6, 8, 10 and 12 hours of after inoculation. The cells were collected by
682 centrifugation (4000xg, for 5 min at 4°C), the cell sediment was washed with
683 phosphate-buffered saline (PBS) and suspended in 1 ml French press buffer (10
684 mM Tris pH 8.0, 10 mM MgCl₂, 0.5 mM EDTA, 0.2 mM NaCl, 10% Glycerol, 1 mM
685 PMSF). The cells were lysed using a French pressure cell (18000 lb/in²). Proteins
686 in the extracts were resolved on 12% SDS-PAGE gels. The anti- σ^F , anti- σ^E
687 antibodies (Saujet *et al.*, 2013), anti-Spo0A (Serra *et al.*, 2014) and anti-CsiA were
688 used at a 1:1000 dilution, and an anti-rabbit secondary antibody conjugated to
689 horseradish peroxidase (Sigma) was used at a 1:10000 dilution. The monoclonal
690 anti-TcdA primary antibody (Santa Cruz Biotechnology) were used at a 1:1000
691 dilution, and an anti-mouse secondary antibody conjugated to horseradish
692 peroxidase (Sigma) was used at a 1:2000 dilution. The immunoblots were
693 developed with enhanced chemiluminescence reagents (Amersham Pharmacia
694 Biotech). Images were adjusted and cropped and quantified using ImageJ
695 (<http://rsbweb.nih.gov/ij/>).

696 Detection of Spo0A phosphorylation using Phos-tag acrylamide gels. Phos-
697 tag acrylamide gels were prepared according to the instructions provided (Wako);
698 10% acrylamide gels were copolymerized with 25 nM Phos-tag acrylamide and 10
699 nM MnCl₂. 20-ml of bacterial cultures were centrifuged at 5,000 g at 4°C for 10
700 min, and the pellets suspended in 1 ml 10 mM Tris pH 8.0. The cells were lysed
701 using a French pressure cell (18000 lb/in²). Samples were stored on ice prior to
702 loading onto Phos-tag acrylamide gels and run at 4°C. Gels were fixed for 10 min
703 in transfer buffer with 10 mM EDTA and then washed for 10 min in transfer buffer

704 without EDTA twice. After transfer to a nitrocellulose membrane, the samples were
705 probed with rabbit polyclonal anti-Spo0A or anti-FliC antibodies at a 1:1000 dilution
706 and an anti-rabbit secondary antibody conjugated to horseradish peroxidase
707 (Sigma) was used at dilution 1:10000. Images were adjusted and cropped and
708 quantified using ImageJ (<http://rsbweb.nih.gov/ij/>). In order to dephosphorylate
709 Spo0A~P, samples were incubated at 100°C for 5 min.

710 **RNA extraction and RNA-sequencing.** RNA for RNA-Seq was extracted from two
711 independent biological replicates of WT and *csiA* mutant *C. difficile* strains, after 10
712 hours of growth in SM, using a RNeasy Kit (Qiagen). Contaminating genomic DNA
713 was depleted by two DNase treatments (Promega), according to manufacturer's
714 recommendations. DNase-treated RNA (5 µg) was mRNA enriched using a Ribo-
715 Zero Magnetic Kit (Epicentre). After Illumina sequencing the reads were mapped to
716 *C. difficile* 630 genome using Hisat. Statistical analyses were performed with
717 DESeq2. A gene was considered differentially expressed when the fold change
718 was > 2 and the adjusted p value was < 0.01.

719 **His₆-CsiA overproduction purification and polyclonal antibody production.** A
720 DNA fragment encoding the *csiA* gene was generated by PCR from *C. difficile*
721 630Δ*erm* genomic DNA using primers *csiA*_BamHI_Fw/ *csiA*_NotI_Rev. The
722 resulting DNA fragment was cut with BamHI and NotI and cloned between the same
723 sites of pETDuet-1 (Novagen) to produce pDM35. Plasmid pDM35 was introduced
724 into BL21 (DE3) cells and the *E. coli* strain was grown in autoinduction medium. The
725 cells were then harvested by centrifugation (4000 x *g*, for 10 min, at 4°C) and the
726 sediment resuspended in lysis buffer (20mM phosphate pH7.4, 1 mM PMSF, 10 mM
727 Imidazole). The suspension was lysed using a French pressure cell (at 18000 lb/in²)
728 and the lysate cleared by centrifugation (15000 x *g*, 30 min at 4°C), and the

729 supernatant was loaded onto a 1 ml Histrap column (Amersham Pharmacia
730 Biotech). The bound protein was eluted with a discontinuous imidazole gradient and
731 the fractions containing His₆-CsiA were identified by SDS-PAGE. The antibody was
732 produced by Eurogentec (Seraing, Belgium).

733

734

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Figure Legends

Figure 1. Genomic signature of sporulation. A: main stages of sporulation starting with asymmetric division of the rod-shaped cell and the production of the larger mother cell and the smaller forespore (the developing spore) through the development of full spore refractivity, until the release of the spore into the environment. The onset of sporulation is controlled by phosphorylation of Spo0A. After asymmetric division, the forespore and the mother cell compartments follow different lines of gene expression. **B:** The inner circle contains the signature defined as those genes present in 90% of sporulating bacteria and in no more than 10% of the remaining bacterial species (Abecasis *et al.*, 2013). The outer circle has the remaining genes identified in a genomic signature of sporulation within the human intestinal microbiome (Browne *et al.*, 2016). Both signatures are enriched with known sporulation-associated genes involved in spore morphogenesis and germination. Genes not specifically associated with a sporulation stage or uncharacterized genes are shown inside the circles. The positions of the genes are shown in degrees in the *C. difficile* 630 chromosome. The *csiA* gene (CD25890) is a signature gene not previously characterized (blue arrow). **C:** schematic representation of the *csiA* (CD25890) region of the *C. difficile* 630 Δ *erm* chromosome. The Δ *csiA* in-frame deletion removed codons 5 through 291 of the 293-codons-long *csiA* gene.

Figure 2. Sporulation dynamics of the Δ *csiA* mutant. A: Cells were grown in liquid SM and the titer of heat resistant spores (filled symbols) and total viable cells (open symbols) measured 12, 24, 48 and 72 hours following inoculation. WT, triangles; Δ *csiA* mutant, circles; *csiA*^C (complementation strain), squares. The date

956 in the graph represent the mean of three independent experiments (see also Table
957 1). Asterisks indicate statistical significance determined with a two-way ANOVA
958 (ns, no significant; **** $p < 0.0001$). **B:** Samples of an SM liquid culture of the WT
959 strain were collected at 8, 10, 12, 14, 16, 18 and 20 hours after inoculation, stained
960 with the membrane dye FM4-64 and examined by phase contrast or fluorescent
961 microscopy. The panels illustrate the sequence of the stages in sporulation. **C:**
962 Quantification of the percentage of cells in the morphological classes represented
963 in B (a to f), relative to the total viable cell population, for the WT (red) and $\Delta csfA$
964 mutant (blue) at the indicated times following inoculation in liquid SM. The data
965 represent the mean $\pm$ SD of three independent experiments. The total number of
966 cells scored (n) is indicated in each panel. **D:** Time, in hours, when at least 10% of
967 WT or $\Delta csfA$ population reached the indicated stages of sporulation. The top
968 columns shows phase-bright, phase-grey spores or free spores (classes d, e and
969 f), the middle columns engulfment completion (c), and the bottom columns
970 asymmetric septation (a).

971 **Figure 3. Accumulation of CsfA during growth and sporulation.** The wild type
972 strain (WT), the $\Delta csfA$ mutant, the complementation strain ($csfA^C$) (**A**) and the
973 $spo0A$ mutant (**B**) were grown in SM and samples were collected at 6, 8, 10 and 12
974 hours after inoculation for Western blot analysis using anti-CsfA (* cross-reactive
975 species). The position of molecular weight markers (in kDa) is indicated on the left
976 side of the panels.

977 **Figure 4. Increased expression of $spo0A$ in the $\Delta csfA$ mutant.** **A:** Microscopy
978 analysis of *C. difficile* cells carrying a P_{spo0A} -SNAP^{Cd} transcriptional fusion in the
979 WT and congenic $\Delta csfA$ mutant. The cells were collected after 10 h of growth in
980 SM, stained with TMR-Star, and examined by fluorescence microscopy to monitor

981 SNAP production. The panels are representative of the expression patterns
 982 observed. The numbers refer to the percentage of cells showing SNAP
 983 fluorescence. Data shown are from one experiment, representative of at least three
 984 independent experiments. Scale bar, 1 μ m. **B:** Quantitative analysis of the
 985 fluorescence intensity (FI.) for single cells with no signs of sporulation (n = 100
 986 cells) of the two strains in A. Data shown are from one experiment, representative
 987 of at least three independent experiments. The numbers in the legend represent
 988 the mean and the SD of fluorescence intensity. ***, p<0.001. **C:** Samples were
 989 collected from the wild type strain (WT), the Δ *csiA* mutant and the
 990 complementation strain (*csiA*^C) grown in liquid SM, at the indicated times after
 991 inoculation. Extracts were prepared and proteins (15 μ g) resolved by SDS-PAGE
 992 and subjected to immunoblotting using an anti-Spo0A antibody. The position of
 993 molecular weight markers (in kDa) is indicated on the left side of the panels.

994 **Figure 5. Phosphorylation of Spo0A in the Δ *csiA* mutant.** **A:** Samples were
 995 collected from the WT and the Δ *csiA* mutant grown in liquid SM, at the indicated
 996 times after inoculation. Extracts were prepared and proteins (15 μ g) resolved by
 997 Phos-tag SDS-PAGE and subjected to immunoblotting using an anti-Spo0A
 998 antibody. In the bottom panel the same extracts were loaded in a SDS-PAGE and
 999 subjected to immunoblotting using an anti- FliC antibody, as a loading control. The
 1000 position of molecular weight markers (in kDa) is indicated on the left side of the
 1001 panels. **B and D:** The ratio of Spo0A~P to Spo0A was assessed with Image J
 1002 software and represented for the WT and the Δ *csiA* mutant at the indicated times
 1003 (in hours) after inoculation in liquid SM. All data represent the means $\pm$ SD from
 1004 three independent experiments. Asterisks indicate statistical significance
 1005 determined by two-tailed Student t-test (ns, no significant; **** p<0.0001, *** p <

0.001). **C:** Extracts were prepared from liquid SM cultures of the $P_{tet}\text{-spo0A}$ and $\Delta\text{csiA } P_{tet}\text{-spo0A}$ strains grown in the presence (50 nM) or in the absence of anhydrotetracycline, 8 hours after inoculation. Proteins (15 μg) were resolved by Phos-tag SDS-PAGE and subject to immunoblotting using an anti-Spo0A antibody. In **A** and **C:** The faster migrating bands (black arrows) show the unphosphorylated form of Spo0A (Spo0A), and the slower migrating bands indicate the phosphorylated form of Spo0A (Spo0A~P; red arrows). The samples heated at 100°C for 5 min were loaded as a control for the position of unphosphorylated Spo0A.

Figure 6. Increased sporulation-specific gene expression in the ΔcsiA mutant. **A:** Microscopy analysis of *C. difficile* cells carrying transcriptional fusions of the *sigF*, *sigE*, *gpr* and *spoIIIA* promoters to SNAP in the WT and in the ΔcsiA mutant. The cells were collected after 12 h of growth in liquid SM, stained with TMR-Star and the membrane dye MTG, and examined by fluorescence microscopy to monitor SNAP production. The merged image shows the overlap between the TMR-Star (red) and MTG (green) channels. The images are representative of the expression patterns observed. The numbers refer to the percentage of cells showing SNAP fluorescence. Data shown are from one experiment, representative of at least three independent experiments. Scale bar, 1 μm . **B:** Quantitative analysis of the fluorescence intensity (in arbitrary units, AU) of the reporter strains for *sigF*, *sigE*, *gpr* and *spoIIIA* transcription, as indicated. The numbers in the legend represent the average and the standard deviation of fluorescence intensity for the cells considered (n = 50 cells). ***, p<0.0001. **C:** Extracts were prepared from liquid SM cultures of the WT, ΔcsiA mutant and the complementation strain (*csiA^C*), grown, at the indicated times after inoculation.

1031 Proteins (15 µg) were resolved by SDS-PAGE (12%) and subject to
 1032 immunoblotting with anti- σ^E and anti- σ^F antibodies. The position of molecular
 1033 weight markers (in kDa) is indicated on the left side of the panels. **D:** measurement
 1034 of the increase in Spo0A activity as a function of aTc using a P_{sigF} -SNAP (light
 1035 grey) or P_{sigE} -SNAP (dark grey) reporter and the formation of asymmetric septa.
 1036 Asterisks indicate statistical significance determined by two-way ANOVA (ns, no
 1037 significant, *** $p < 0.001$, * $p < 0.01$).

1038 **Figure 7. Model for the function of the *csiA* gene.** **A:** Functional classes of the
 1039 genes that are up-regulated in the $\Delta csiA$ mutant based on RNA seq data (left; the
 1040 number represent the percentage of each functional class) and the up-regulated
 1041 sporulation genes in the indicated cell type-specific regulons (right; the numbers
 1042 represent the percentage of each of the regulons). See also Table 3 **B.** Model for
 1043 the function of the *csiA* gene in controlling sporulation initiation in *C. difficile*.

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1047 **Table 1 – Sporulation efficiency of the $\Delta csiA$ mutant.** The total viable cell count and the spore titer was determined 12, 24, 48 and
1048 72 hours following inoculation into sporulation medium. The heat resistant spore count by determining the CFU/ml before and after

	12h		24h		48h		72h	
	Total viable cells	Spore titer	Total viable cells	Spore titer	Total viable cells	Spore titer	Total viable cells	Spore titer
WT	$1.6 \times 10^8 \pm 7.4 \times 10^7$	$7.5 \times 10^3 \pm 9.8 \times 10^2$	$1.7 \times 10^8 \pm 8.7 \times 10^7$	$1.0 \times 10^4 \pm 1.2 \times 10^3$	$8.5 \times 10^7 \pm 1.1 \times 10^7$	$1.3 \times 10^5 \pm 5.1 \times 10^4$	$1.0 \times 10^7 \pm 1.3 \times 10^6$	$1.7 \times 10^5 \pm 5.8 \times 10^4$
$\Delta csiA$	$1.6 \times 10^8 \pm 2.8 \times 10^7$	$3.1 \times 10^4 \pm 5.3 \times 10^3$	$1.3 \times 10^8 \pm 6.5 \times 10^7$	$8.5 \times 10^4 \pm 6.2 \times 10^4$	$3.0 \times 10^7 \pm 2.1 \times 10^7$	$1.5 \times 10^6 \pm 1.0 \times 10^5$	$3.0 \times 10^7 \pm 9.8 \times 10^6$	$2.2 \times 10^6 \pm 1.2 \times 10^6$
$csiA^C$	$1.6 \times 10^8 \pm 7.1 \times 10^6$	$1.8 \times 10^4 \pm 7.8 \times 10^3$	$2.3 \times 10^8 \pm 1.4 \times 10^8$	$1.5 \times 10^4 \pm 6.8 \times 10^3$	$3.0 \times 10^7 \pm 1.7 \times 10^7$	$7.8 \times 10^4 \pm 2.0 \times 10^4$	$1.0 \times 10^7 \pm 5.3 \times 10^6$	$6.0 \times 10^4 \pm 2.0 \times 10^4$

1049 incubation at 70°C for 10 min. The results shown are averages and standard deviations for three biological replicates.

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Table 2 – Sporulation efficiency of the *P_{tet}-spo0A* alleles. The total viable cell count and the spore titer was determined 24 hours following inoculation into sporulation medium supplemented with 50 nM of anhydrotetracycline. The heat resistant spore count by determining the cfu/mL obtained after treatment at 70°C. The results shown are averages and standard deviations for three biological replicates.

	24h	
	Total viable cells	Spore titer
WT <i>P_{tet}-spo0A</i>	$2.1 \times 10^7 \pm 1.3 \times 10^6$	$6.8 \times 10^6 \pm 1.3 \times 10^5$
$\Delta csiA$ <i>P_{tet}-spo0A</i>	$2.2 \times 10^7 \pm 1.6 \times 10^6$	$7.6 \times 10^6 \pm 1.7 \times 10^5$

Martins *et al.* – Figure 2

Martins *et al.* – Figure 5