Structure and assembly of a *Clostridioides difficile* spore polar appendage

Wilson Antunes^{1,2,§}, Fátima C. Pereira^{1,*,§}, Carolina Feliciano^{1,§}, Laure Saujet^{3,8,€}, Tiago dos Vultos¹, Evelyne Couture-Tosi³, Severine Péchiné⁴, Jean-François Bruxelles⁴, Claire Janoir⁴, Luís V. Melo⁵, Patrícia Brito^{6,7}, Isabelle Martin-Verstraete^{3,8}, Mónica Serrano¹, Bruno Dupuy^{3,8}, and Adriano O. Henriques^{1,*}

¹Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal, ²Laboratório de Bromatologia e Defesa Biológica do Exército, Centro de Investigação da Academia Militar, Avenida Doutor Alfredo Bensaúde, 1849-012, Lisboa, Portugal; ³Laboratoire Pathogenèse des Bactéries Anaérobies, Institut Pasteur, Paris, France, ⁴Université de Paris-Sud, Faculté de Pharmacie, Département de Microbiologie, Châtenay-Malabry, France, ⁵CEFEMA, Instituto Superior Técnico, Universidade de Lisboa, Portugal; ⁶Instituto Gulbenkian de Ciência, Portugal; ⁷CEDOC, NOVA Medical School/Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal; ⁸Université Paris 7-Denis Diderot, 75205 Paris, France.

[§]Equivalent contributions.

^{\$}Present address: Laboratoire Pathogenèse des Bactéries Anaérobies, Institut Pasteur, Paris, France.

[#]Present address: Division of Microbial Ecology, Department of Microbiology and Ecosystem Science, Research Network Chemistry Meets Microbiology, University of Vienna, Althanstrasse 14, Vienna, Austria.

[€]Present address: Eligo Bioscience, Paris, France.

*Corresponding author: *Phone*: +351 21 4469521; *Fax*: +351 21 441 1277; *Email*: aoh@itqb.unl.pt

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Abstract

Clostridioides difficile, a strict anaerobic spore-former, is the main cause of nosocomial disease associated to antibiotic therapy in adults and a growing concern in the community. Spores are the main infectious, persistence and transmission vehicle. Spore germination occurs in the intestine and the resulting vegetative cells will produce the toxins responsible for the disease symptoms, and spores. During sporulation, a wild type population bifurcates into two main spore morphotypes, with or without a thick exosporium. We show that this bifurcation extends to the formation of spores with a robust polar appendage or spores with a short appendage or that lack this structure. The cysteine-rich CdeM protein localizes to the appendage and around the entire surface of the spore, and is a major structural component of the exosporium, which we show is continuous with the appendage. In a *cdeM* mutant, when present, the polar appendage is short and disorganized. We show that wild type and *cdeM* spores with a short or no appendage germinate poorly in response to taurocholate, compared to those with an appendage. *cdeM* spores of the two types, however, germinate faster than their wild type counterparts. Thus, while the absence of CdeM may increase the permeability of spores to taurocholate, proper assembly of the appendage is also important for germination. Consistent with an overall enhancement of germination, a *cdeM* mutant shows increased virulence in a hamster model of disease. For a wild type population, spores with a short or no appendage germinate slower than the appendage-bearing spores. Differences in transmission, persistence and disease severity may result, in part, from their proportion in a spore population.

Introduction

Infection by *Clostridium difficile* (CDI), recently renamed *Clostridioides difficile* (Lawson *et al.*, 2016, Yutin & Galperin, 2013), is the cause of an intestinal disease mediated by two toxins, TcdA and TcdB (Aktories *et al.*, 2017, Chandrasekaran & Lacy, 2017). Symptoms of CDI can range from asymptomatic colonization or mild diarrhea, to more severe conditions that can lead to death (Rupnik *et al.*, 2009, Smits *et al.*, 2016). The last two decades have witnessed the emergence and spreading of epidemic strains which have caused outbreaks associated with more severe disease symptoms, morbidity, mortality and disease recurrence rates (Smits *et al.*, 2016). This has led to the recognition of *C. difficile* as a main nosocomial enteric pathogen and its recognition as an urgent threat (Kociulek & Gerding, 2016, Rupnik *et al.*, 2009, Smits *et al.*, 2016). Health care-associated CDI develops in hospitalized patients undergoing antibiotic treatment because *C. difficile* can colonize the gut if the protective effect of the intestinal microbiota is lifted (Rupnik *et al.*, 2009, Smits *et al.*, 2016). Recent changes in the epidemiology of this pathogen, together with its emergence at the community level and the risk of widespread zoonotic transmission, adds to the problem (Dubberke & Olsen, 2012, Isidro *et al.*, 2018, Jones *et al.*, 2013, Lessa *et al.*, 2015, Rupnik *et al.*, 2009, Smits *et al.*, 2016).

C. difficile is an obligate anaerobe, and has the ability to form endospores (spores for simplicity) which are extremely resilient and hard to eradicate (Setlow, 2014). The oxygen-resistant spores are the infectious and transmissible form of the organism; their persistence within the host or in environmental reservoirs is also linked to disease recurrence (Deakin *et al.*, 2012, Paredes-Sabja & Sarker, 2012). Sporulation begins with a polar division that creates a larger mother cell and a smaller forespore, or future spore (Fig. 1A). Following polar division, cell type-specific gene

89 expression is controlled by four RNA polymerase sigma subunits; σ^F and σ^E drive the
 90 early stages of development in the mother cell and in the forespore, and are replaced
 91 at later stages by σ^G and σ^K (Driks & Eichenberger, 2016, Fimlaid *et al.*, 2013, Fimlaid
 92 & Shen, 2015, Henriques & Moran, 2007, McKenney *et al.*, 2013, Pereira *et al.*, 2013,
 93 Saujet *et al.*, 2013) (Fig. 1A). The genes coding for these sigma factors are part of a
 94 genomic signature of sporulation (Abecasis *et al.*, 2013). Soon after polar division, the
 95 mother cell engulfs the forespore, and at late stages in development, it directs
 96 assembly of a thick layer of a modified form of peptidoglycan, called the cortex,
 97 essential to maintain spore dormancy, and of several multiprotein layers which encase
 98 the cortex (Fig. 1A). The components of these proteinaceous layers are produced
 99 under the direct control of σ^E and σ^K (Driks & Eichenberger, 2016, Henriques & Moran,
 100 2007, McKenney *et al.*, 2013) (Fig. 1A).

101 The structure and composition of the spore surface varies among species. In *B.*
 102 *subtilis*, the outermost spore structures are a coat and a crust (Driks & Eichenberger,
 103 2016, Henriques & Moran, 2007, McKenney *et al.*, 2013, Stewart, 2015). The coat is
 104 differentiated into an inner, lamellar layer, and a more external, electron-dense outer
 105 layer. The crust is formed by several cysteine-rich proteins that self-assemble into a
 106 hexagonal lattice, is glycosylated and stands in close apposition to the underlying
 107 outer coat (Jiang *et al.*, 2015, McKenney *et al.*, 2010, Waller *et al.*, 2004). In other
 108 sporeformers, the coat is surrounded by an exosporium, best characterized in spores
 109 of the pathogens *B. anthracis* and *B. cereus*, where it consists of a loose balloon-like
 110 structure separated from the edge of the coat by an interspace (reviewed by (Stewart,
 111 2015)). The exosporium has a basal layer, which contains cysteine-rich proteins
 112 related to the crust proteins of *B. subtilis*, and that also assemble into a porous
 113 hexagonal lattice; a hair-like protrudes from the basal layer (Jiang *et al.*, 2015, Kailas

114 *et al.*, 2011); reviewed by (Stewart, 2015)). The hairy nap is formed mainly by radial
115 projections of a long collagen-like glycosylated protein, BclA, which is anchored in the
116 basal layer (Stewart, 2015). While the exosporium contributes to the exclusion of lytic
117 enzymes and antibodies, BclA is involved in a specific interaction with receptors at the
118 surface of professional macrophages, essential for the course of infection in *B.*
119 *anthracis* (Stewart, 2015). An exosporium that fits the general structural organization
120 found in the *B. anthracis*/*B. cereus* group of organisms is present in several species of
121 *Bacillus* and *Clostridium* (Stewart, 2015).

122 The composition, assembly and structure of the surface of *C. difficile* spores is
123 of interest, firstly because infection begins with the ingestion of spores that have to
124 resist the gastric barrier and secondly, because germination, which takes place in the
125 intestine in response to bile salts (Sorg & Sonenshein, 2008) (Fig. 1B) and that may
126 occur in close contact with host cells (Panessa-Warren *et al.*, 1997), is influenced by
127 the structure and composition of the coat and exosporium (Barra-Carrasco *et al.*,
128 2013). Moreover, spores bind to colonic cells and to components of the intestinal
129 mucosa and binding is influenced by the status of the surface layers (Barra-Carrasco
130 *et al.*, 2013, Joshi *et al.*, 2012, Panessa-Warren *et al.*, 1997); binding to cells may
131 involve a specific spore receptor as it reaches saturation (Mora-Urbe *et al.*, 2016) (Fig.
132 1B). Finally, the interaction of spores with host cells, mediated by the spore surface
133 layers, is important for colonization and infection (Calderon-Romero *et al.*, 2018, Hong
134 *et al.*, 2017, Phetcharaburanin *et al.*, 2014). *C. difficile* spores have a lamellar coat
135 adjacent to the cortex, and an external, more electrondense layer surrounded by an
136 exosporium closely apposed to the underlying coat (Pereira *et al.*, 2013,
137 Phetcharaburanin *et al.*, 2014, Pizarro-Guajardo *et al.*, 2016a, Rabi *et al.*, 2017). At
138 least some strains produce, in the same culture, two main spore morphotypes, with
139 either a thick or a thin exosporium (Pizarro-Guajardo *et al.*, 2016a). How these

140 morphotypes are produced, and the significance of this differentiation at the population
141 level is unknown. Moreover, in spores produced by most clinical isolates, the
142 exosporium is thicker than in a standard laboratory strain such as 630 Δ *erm*, has a
143 convoluted or bumpy appearance, and a hairy-like nap protrudes from its surface
144 (Joshi *et al.*, 2012, Pizarro-Guajardo *et al.*, 2016a, Rabi *et al.*, 2017). *C. difficile*
145 possesses three homologues of BclA, which form the hair-like nap of the exosporium
146 (Phetcharaburanin *et al.*, 2014, Pizarro-Guajardo *et al.*, 2014).

147 At least BclA1 is required for colonization of the mouse gastro-intestinal tract,
148 suggesting that the protein and/or proper assembly of the exosporium layer is
149 important during the initial stages of infection (Phetcharaburanin *et al.*, 2014). Other
150 exosporium proteins have been identified and at least two of these proteins, CdeC and
151 CdeM, are cysteine-rich (Barra-Carrasco *et al.*, 2013, Calderon-Romero *et al.*, 2018,
152 Diaz-Gonzalez *et al.*, 2015). Importantly, a *cdeC* mutant forms spores that lack the
153 exosporium and the hairy nap, are more permeable to lysozyme and solvents and
154 show altered binding to epithelial cells and to components of the intestinal mucosa
155 (Barra-Carrasco *et al.*, 2013, Mora-Urbe *et al.*, 2016). *cdeM*, in turn, was previously
156 shown to be strongly induced *in vivo*, upon infection of axenic mice with vegetative
157 cells of *C. difficile* and a *cdeM* insertional mutant is impaired in colonization (Janoir *et*
158 *al.*, 2014). CdeM is an important determinant of exosporium assembly, and also
159 modulates the ability of spores to adhere to the mouse colonic mucosa (Calderon-
160 Romero *et al.*, 2018).

161 Here we show that CdeM is a critical determinant of the assembly of a spore
162 polar appendage that is continuous with and an integral part of the exosporium. In a
163 *cdeM* mutant, the exosporium is thin and when present, the polar appendage is short
164 and disorganized. Consistent with a role in the assembly of the exosporium and the
165 polar appendage, we show that CdeM localizes around the spore and at the

166 appendage, and contributes to the rigidity of spores. We found that in both the wild-
 167 type strain of a *cdeM* mutant, the spore population consists of spores with a well
 168 developed appendage or without an appendage or a much reduced one. *cdeM* spores
 169 with a short appendage germinate better than those without an appendage, but the two
 170 types of spores show enhanced germination compared to their wild-type counterparts,
 171 pointing to a direct role of the appendage in germination. While misassembly of the
 172 spore surface layers may explain the previously described colonization defect of a
 173 *cdeM* insertional mutant in a mouse axenic model (Janoir *et al.*, 2014), the mutant
 174 shows increased virulence in a hamster model of disease presumably because
 175 increased spore permeability results in enhanced germination. We suggest that the
 176 two types of spores correspond to distinct modes of germination. Since the
 177 representation of the two main spore types differs in an epidemic strain, their
 178 proportion in a population maybe an important trait for colonization and virulence.

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Results

Transcription of cdeM is directly controlled by σ^K

The proteins that compose the spore surface structures are produced in the mother cell, under the control of σ^E and σ^K in both *B. subtilis* (Henriques & Moran, 2007, McKenney *et al.*, 2013) and *C. difficile* (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Pishdadian *et al.*, 2015, Putnam *et al.*, 2013, Saujet *et al.*, 2013). The *cdeM* gene, was proposed to be controlled by σ^K as *cdeM* expression is severely curtailed in a *sigK* mutant (Fimlaid *et al.*, 2013, Saujet *et al.*, 2013). To more precisely define the requirements for *cdeM* expression, we mapped its promoter using 5'-RACE. We identified a single transcriptional start site, a T, located 45 nt upstream of the predicted start codon of *cdeM*. We found upstream of this nucleotide at position -10 and -35 sequences that conform to the consensus elements of promoters recognized by σ^K in *C. difficile* (Fig. 2A) (Fimlaid *et al.*, 2013, Saujet *et al.*, 2013). In addition, we used quantitative RT-PCR (qRT-PCR) to monitor expression of *cdeM* during sporulation of a wild type (WT) strain (630 Δ *erm*) or *sigF*, *sigE*, *sigG*, and *sigK* mutants. We have previously shown that S medium (SM; (Wilson *et al.*, 1982)) supports sporulation and that the activity of σ^F , σ^E , σ^G and σ^K can be detected and quantified between 14 h and 24 h of growth in this medium (Pereira *et al.*, 2013, Saujet *et al.*, 2013). We found that expression of *cdeM* was strongly reduced both in a *sigK* mutant (100-fold in transcriptome and 410-fold by qRT-PCR; Fig. 2B), unable to produce σ^K , and in a *sigE* mutant (100-fold in transcriptome and 500-fold by qRT-PCR), which is required for the production and activity of σ^K (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Pishdadian *et al.*, 2015, Serrano *et al.*, 2016). These results are in line with the presence of a promoter recognized by σ^K upstream of *cdeM*. In addition, expression of *cdeM* was not

207 affected in a mutant unable to produce σ^G , the late forespore-specific sigma factor
 208 (Fig. 2B) which, unlike in *B. subtilis*, is not required for the activation of σ^K in the mother
 209 cell in *C. difficile* (Fimlaid *et al.*, 2013, Pereira *et al.*, 2013, Pishdadian *et al.*, 2015,
 210 Saujet *et al.*, 2013, Serrano *et al.*, 2016). *cdeM* expression was also reduced in a *sigF*
 211 mutant (30-fold in transcriptome and by qRT-PCR) but to a lower extent compared to
 212 the effect of σ^E and σ^K (Fig. 2B). A control exerted by *sigF* over σ^K was also observed
 213 for other σ^K -target genes (Saujet *et al.*, 2013); it may result from either a partial control
 214 of σ^E activity by σ^F (Fimlaid *et al.*, 2013, Saujet *et al.*, 2013) or to a still
 215 uncharacterized mechanism of control of σ^K activity by σ^F . Together, these results
 216 indicate that σ^K directly transcribes the *cdeM* gene.

217 *cdeM* is expressed during the final stages in spore morphogenesis

218 To determine the time of *cdeM* expression in relation to morphogenesis and to
 219 compare its expression with that of genes encoding other coat/exosporium proteins,
 220 we examined the expression of *cotE*, *cdeC*, and *cdeM* during sporulation using strains
 221 harboring P_{cotE^-} , P_{cdeC^-} , and $P_{cdeM^-}SNAP^{Cd}$ transcriptional fusions ((Pereira *et al.*,
 222 2013); this work). $SNAP^{Cd}$ production was monitored by immunofluorescence
 223 microscopy using an anti-SNAP antibody. Under these conditions, we were only able
 224 to score sporangia following engulfment completion, in which phase dark or phase
 225 bright spores were visible (Fig. 2C). For all three genes, sporangia with weak and
 226 stronger fluorescence signals, always confined to the mother cell, were detected, and
 227 we defined two levels of fluorescence signal intensity [weak, lower than 1000 arbitrary
 228 units (AU) and high, equal or higher than 1000 AU; see also the Material and Methods
 229 section]. These two classes were scored independently as a function of the stage in
 230 morphogenesis (Fig. 2C; weak signal, blue arrows; white arrows, stronger signal).

231 CotE was detected in 60% of the sporangia of phase grey spores of which 55%
 232 showed a weak fluorescence signal and only 5% showed a strong signal (the
 233 remaining showing no labeling) (Fig. 2D and E). CotE was also detected in 95% of the
 234 sporangia of phase bright spores, and 49% of those showed a strong signal, while
 235 46% showed a weak signal (Fig. 2D and E). *cotE* is thought to be under the control of
 236 σ^K (Pishdadian *et al.*, 2015, Saujet *et al.*, 2013). Expression of $P_{cotE}\text{-SNAP}^{Cd}$, however,
 237 is detected prior to engulfment completion in some sporangia but increased following
 238 completion of engulfment when the spore turns phase grey, then phase white and
 239 finally phase bright (Fig. 2C-E). The early expression of $P_{cotE}\text{-SNAP}^{Cd}$ may result from
 240 utilization of the *cotE* promoter by σ^E ; alternatively, it is caused by low levels of σ^K
 241 activity in sporangia prior to engulfment completion (Pereira *et al.*, 2013, Saujet *et al.*,
 242 2013, Serrano *et al.*, 2016).

243 *cdeC* is also under the control of σ^K but additionally requires the ancillary
 244 transcription factor SpoIIID (Pishdadian *et al.*, 2015, Saujet *et al.*, 2013). Expression of
 245 *cdeC* was only detected following engulfment completion (Fig. S1B). Expression was
 246 detected in 44% of the sporangia of phase grey spores (40% of which showed a weak
 247 signal and 4% a stronger signal, the remaining with no labeling) and in 89% of the
 248 sporangia of phase bright spores (of which 65 % showed a weak signal, 24% a strong
 249 signal, and the remaining no labeling) (Fig. 2D and E). Thus, the onset of *cdeC*
 250 expression coincides with the appearance of phase grey spores. Finally, expression of
 251 *cdeM* was also only detected following engulfment completion (Fig. 2C-E) in
 252 agreement with the presence of a σ^K -dependent promoter (Fig. 2A). Expression of
 253 *cdeM* was detected in 7% of the sporangia of phase grey spores (all of which showed
 254 a weak signal) but increased to 87% of the sporangia of phase bright spores (of which
 255 44% showed a weak signal and 43% a strong fluorescence signal) (Fig. 2D and E).

256 Although the percentage of sporangia showing a fluorescence signal was lower, the
257 analysis of P_{cotE} -SNAP^{Cd}, and P_{cdeM} -SNAP^{Cd} and P_{cdeC} -SNAP^{Cd} expression using the
258 TMR-Star SNAP substrate confirmed the results obtained using immunofluorescence
259 (Fig. S1).

260 While the transcription of genes such as *cotE* begins prior to engulfment
261 completion, *cdeC* is expressed after engulfment completion. *cdeM* in turn, is activated
262 only at late stages in spore morphogenesis, when the spore becomes phase bright,
263 just prior to lysis of the mother cell. Furthermore, the data also suggests that at least
264 following engulfment completion, the levels of expression of *cotE*, *cdeC* and *cdeM*
265 show some heterogeneity across the cell population.

266 ***CdeM is an abundant component of the spore surface layers that undergoes*** 267 ***multimerization***

268 As a first step to characterize the role of *cdeM* we characterized spores of a
269 previously constructed insertional mutant, *cdeM::ermB*, constructed in the background
270 of strain 630Δ*erm* (Janoir *et al.*, 2014). We first examined the proteins extractable from
271 mature spores under our experimental conditions. Spores were purified to greater than
272 99% homogeneity by density centrifugation on gradients of metrizoic acid (see
273 Materials and Methods). A collection of about 30 polypeptides was extracted from wild-
274 type (WT) spores as revealed by Coomassie blue staining (Fig. 3A, lane 1). The
275 pattern obtained differs slightly from other recent reports in the number of bands and
276 their relative abundance. These differences likely reflect different culturing, spore
277 purification and extraction conditions. Nevertheless, 11 proteins were identified by
278 mass spectrometry analysis, which were previously found to be spore surface
279 components or part of the spore proteome (Abhyankar *et al.*, 2013, Barra-Carrasco *et*
280 *al.*, 2013, Calderon-Romero *et al.*, 2018, Diaz-Gonzalez *et al.*, 2015, Escobar-Cortes

281 *et al.*, 2013, Lawley *et al.*, 2009, Paredes-Sabja & Sarker, 2012, Permpoonpattana *et*
282 *al.*, 2011). These include Pfo (a pyruvate-ferredoxin oxidoreductase, coded for
283 CD2682, below and above the 250 kDa marker; predicted molecular mass of 128
284 kDa), AdhE, a bifunctional acetaldehyde CoA/alcohol dehydrogenase (95 kDa, coded
285 for by CD2966), CotE (coded for by CD1433; predicted size of 82 kDa, but detected in
286 the 40 kDa region of the gel possibly due to proteolytical separation of the
287 peroxiredoxin and chitinase domains; (Permpoonpattana *et al.*, 2013,
288 Permpoonpattana *et al.*, 2011)), CdeC (42 kDa, coded for by CD1067; detected in
289 both the 80 and 200 kDa regions of the gel), CotB (35 kDa, CD1511), CotA (34 kDa,
290 CD1613), CdeM (at 17 kDa; predicted molecular weight of 19 kDa; coded for by
291 CD1581), SleC (CD0551), a rubrerythrin (Rbr; 25 kDa/CD0825) and CotCB (21 kDa,
292 CD0598) (Fig. 3A, in the WT, red arrows). CdeM, which was also detected as an
293 abundant species in WT spores, was absent from extracts prepared from spores of the
294 *cdeM::ermB* mutant (Fig. 3A, green arrows). Importantly, complementation of
295 *cdeM::ermB* in single copy using transposon Tn916 (this strain is henceforth referred
296 to as *cdeM^C*; see Fig. S2) or a multicopy plasmid (pFT43) restored the presence of the
297 protein at the spore surface (Fig. 3A). The absence of CdeM was the main difference
298 between the protein profile obtained for the WT and *cdeM::ermB* spores (Fig. 3A).
299 However, the extractability of at least two other species, CotJC1 and SleC, increased
300 in the *cdeM::ermB* mutant, while the extractability of several others, including NifJ and
301 CotB, decreased (Fig. 3A). Reduced extractability of CotB in *cdeM* spores was also
302 reported in a recent study (Calderon-Romero *et al.*, 2018). These authors also
303 reported a significant decrease in the levels of two forms of CdeC (of 140 and 44 kDa),
304 in spores of the *cdeM* mutant (Calderon-Romero *et al.*, 2018). In our hands, CdeC was
305 detected as a species of about 52 kDa, which most likely corresponds to the 44 kDa
306 species detected by Calderón-Romero and co-authors (the predicted molecular weight

of CdeC is 44.7 kDa). The extractability remained unchanged, relative to the WT, in *cdeM::ermB* spores (Fig. 3B, bottom panel).

In addition to the 17 kDa, possibly monomeric form of CdeM, the immunoblot analysis of the WT spore extracts using a polyclonal antibody raised against the partially purified protein (see the Material and Methods), revealed additional forms of about 25 kDa, 37 kDa and 60 kDa (Fig. 3B). CdeM contains 14 cysteine residues, some of which part of repeat motifs previously noticed (Calderon-Romero *et al.*, 2018); 7 of the cysteine residues are clustered close to its C-terminal end, from residues 151 through 160 of the 161 residues-long protein (Fig. S3A and B). The Cys-rich C-terminal end of CdeM is preceded by a polymorphic region (Fig. S3B, region P4; see also the supplemental text). The partially purified His₆-CdeM protein runs at about 17 kDa in SDS-PAGE in the presence of reducing agents (Fig. S3C). In the absence of reducing agents, however, CdeM runs as a major species at about 60 kDa, as well as several other species of even higher apparent molecular weight (Fig. S3C). Therefore, the 25 kDa, 37 kDa and 60 kDa forms detected in the spore extracts by immunoblotting are likely to be disulfide-cross-linked homo-multimeric forms of CdeM. These species are at least partially resistant to boiling and the reducing SDS-PAGE conditions.

CdeM localizes asymmetrically at the spore surface

We next wanted to localize CdeM at the surface of mature spores. We first used a CdeM-SNAP^{Cd} translational fusion produced from a low copy plasmid in an otherwise WT background. Labeling of the purified spores with the TMR-Star substrate reveals the preferential localization of CdeM-SNAP^{Cd} at one or both of the spore poles (Fig. 3C, yellow arrows). Often, the signal overlapped with an appendage, visible in the phase contrast images, protruding from one of the spore poles, suggesting the

332 presence of CdeM-SNAP^{Cd} in this structure (Fig. 3C, yellow arrows; see also below).
 333 The SNAP-TMR signal is specific, since no fluorescence is detected in spores of the
 334 WT strain, which does not produce SNAP^{Cd}, labeled with TMR-Star (Fig. 3C, left
 335 panels).

336 To localize the native protein, we used immunofluorescence microscopy. The
 337 polyclonal anti-CdeM antibody was bound to WT and *cdeM::ermB* mutant spores, and
 338 the antigen-antibody complex detected with a secondary anti-rabbit fluorescent
 339 antibody. We found two main types of spores in the population, those with a visible
 340 polar appendage and those without (see also the following section for a quantitative
 341 description of this phenotype). Both types of spores were decorated by the antibody. In
 342 79% of the spores with a polar appendage, the antibody decorated the tip of the
 343 appendage, but not at the appendage body, and also the opposite spore pole (Fig. 3D,
 344 white arrows). Among the spores with an appendage, 21% showed a somewhat
 345 shorter appendage, and in those spores CdeM, was also found at the spore body (Fig.
 346 3D, yellow arrows). In 10% of the spores without a visible appendage, CdeM localized
 347 at the two spore poles, whereas in 56% of those spores CdeM showed a punctate
 348 pattern of fluorescence around the entire contour of the spore, with the remaining
 349 showing no decoration (Fig. 3D). Importantly, only residual unspecific labeling of
 350 *cdeM::ermB* spores was detected (Fig. 3D, right). The decoration of WT spores by the
 351 anti-CdeM antibody, along the periphery of the spores and at the edge of the
 352 appendage is consistent with the proposal that CdeM is at least partially exposed at
 353 the spore surface, and accessible to the antibody (Barra-Carrasco *et al.*, 2013,
 354 Calderon-Romero *et al.*, 2018). Consistent with this inference, incubation of purified
 355 WT or TMR-labelled CdeM-SNAP^{Cd} spores with trypsin reduced the amount of
 356 extracted CdeM (Fig. S4A) and the intensity of the fluorescence signal (Fig. S4B).

357 CdeM however, is tightly associated with the spore surface, as it was not significantly
358 released from spores by washing with a 1M solution of potassium chloride (Fig. S4C).
359 Also consistent with a surface localization, *cdeM::erm* spores showed reduced
360 hydrophobicity (Fig. S4D). Importantly, the comparison between labeling of CdeM-
361 SNAP^{Cd} with the small molecule TMR, and the decoration pattern obtained by
362 immunofluorescence suggests that CdeM localizes both at the edge and the body of
363 the appendage, but is not accessible or not recognized by the antibody at the body of
364 this structure. In any case, CdeM is an abundant component of the spore surface
365 (Barra-Carrasco *et al.*, 2013, Calderon-Romero *et al.*, 2018) that undergoes
366 multimerization at the spore surface, and that localizes both at the polar appendage as
367 well as around the spore body.

368 ***CdeM is a key determinant of assembly of a spore polar appendage***

369 The observation that a population of WT spores consisted of a mixture of
370 appendage-bearing and appendage-lacking spores (above), together with the polar
371 localization of CdeM suggested that the protein could have a role in the assembly of
372 this structure. We have shown before that the lipophilic dye FM4-64 stains spores of
373 *C. difficile* (Pereira *et al.*, 2013) and we used this dye in combination with phase
374 contrast microscopy to characterize populations of WT and *cdeM::ermB* mutant
375 spores. In both WT and *cdeM* spores, the polar appendage is clearly distinguishable
376 from the spore body, as it does not stain with FM4-64 (Fig. 4A). The most prevalent
377 appendage pattern seen for the WT is a relatively long, well-organized and partially
378 refractile appendage (Fig. 4A, type A: about 25% of the population) whereas only 3%
379 of the spores showed a short, disorganized appendage (type B), with the remaining
380 spores (72% of the population) showing no appendage (Fig. 4A). In contrast, type B
381 appendages were the most prevalent for *cdeM::ermB* spores (about 20%) whereas

only 7% showed a type A appendage, while the remaining spores (73% of the population) showed no appendage (Fig. 4A; see also the Supplemental text). The WT numbers were restored in the *cdeM^C* strain (Fig. 4A and B). Interestingly, the percentage of type A spores was higher for the epidemic strain R20191 (Fig. S5A; see also the supplemental text).

As a further characterization of the appendage, we measured the spore length in both the phase contrast and FM4-64 images for individual spores as well as the spore width (Fig. 4B, top). The length measurement in the phase contrast images gives the total length of the spore (spore body and appendage) whereas the FM4-64 images gives the length of the spore body (without the appendage), regardless of the appearance of the appendage (Fig. 4B, top). The spore width measurement coincides in the two images (Fig. 4B, top). The spore length, as measured in the phase contrast images did not differ much among spores of the three strains (Fig. 4B; WT: 1.45 ± 0.17 μm , *cdeM*: 1.49 ± 0.22 μm ; *cdeM^C*: 1.51 ± 0.19 μm). This is consistent with the presence of an appendage, albeit disorganized, in *cdeM* spores (Fig. 4A) and suggests that *cdeM* affects mainly the organization of this structure, and not its presence or absence. In contrast, the length and width of the spore body, measured in the FM4-64 images, is larger for *cdeM* spores than for WT or *cdeM^C* spores (Fig. 4B; L: 1.35 ± 0.13 μm /W: 0.81 ± 0.06 μm for *cdeM*; L: 1.20 ± 0.15 μm /W: 0.77 ± 0.07 μm for WT; L: 1.25 ± 0.14 μm /W: 0.77 ± 0.06 μm for *cdeM^C*). This suggests that expression of *cdeM* imposes a constraint on the dimensions of the spore and that in the absence of CdeM some expansion of the spore body occurs. We also note that spores of strain R20291, which have a thicker exosporium layer as compared to 630 Δerm spores (Pizarro-Guajardo *et al.*, 2016a, Pizarro-Guajardo *et al.*, 2014, Pizarro-Guajardo *et al.*, 2016b), also have a longer appendage (Fig. S5B; see also the supplemental text).

407 To next used transmission electron microscopy (TEM) to examine the impact of
 408 the *cdeM* insertional allele on the structure of the spore. The coat of *C. difficile* spores
 409 shows an inner and an outer layer, both lamellar, with the outer coat more
 410 electrodense; an electrodense exosporial layer surrounds the spore, closely apposed
 411 to the coat (Lawley *et al.*, 2009, Paredes-Sabja & Sarker, 2012, Pereira *et al.*, 2013,
 412 Permpoonpattana *et al.*, 2011, Rabi *et al.*, 2017). For WT spores, a lamellar coat layer
 413 in close contact with the spore cortex peptidoglycan (Fig. 4C, red arrows) and the
 414 electrodense exosporial layer attached to the coat were seen (Fig. 4C, blue arrows).
 415 The distinction between an inner and an outer coat layer, however, was not evident
 416 (Fig. 4C). In longitudinal sections of WT spores, a compact polar appendage is clearly
 417 seen (Fig. 4C, yellow arrow); a basal region at the spore/appendage junction is also
 418 discernible (green arrow). Importantly, the TEM images clearly show that the
 419 appendage is continuous with the exosporium. In *cdeM* spores the appendage
 420 appears shorter and less electrodense in *cdeM* spores and the spore/appendage
 421 junction region is poorly defined (Fig. 4C). In cross sections, the coat and the
 422 exosporium appear well defined for WT spores (Fig. 4C, bottom panels). In contrast, in
 423 *cdeM* spores, the coat layer appears thinner, and the exosporium is largely absent
 424 (Fig. 4C). These two features, a thin coat and the absence of the exosporium have
 425 also been described by Calderón-Romero and co-workers (Calderon-Romero *et al.*,
 426 2018). The outer layer of the coat, however, which these authors reported as being
 427 thicker in the *cdeM* mutant, was not evident (Fig. 4C), suggesting that under our
 428 experimental conditions (including sample preparation), this structure is either not
 429 formed or not retained by spores when most of the exosporium is absent.
 430 Nevertheless, disorganized material, most likely remnants of the outer coat layer
 431 and/or exosporium, remain loosely attached to the exposed edge of the inner coat
 432 (Fig. 4C). The structural features seen in the spore cross sections are in agreement

433 with previous descriptions of WT spores (Barra-Carrasco *et al.*, 2013, Calderon-
434 Romero *et al.*, 2018, Diaz-Gonzalez *et al.*, 2015, Escobar-Cortes *et al.*, 2013, Pereira
435 *et al.*, 2013, Pizarro-Guajardo *et al.*, 2016a, Pizarro-Guajardo *et al.*, 2016b). Polar
436 spore appendages have been described in other organisms, including *C.*
437 *bifermentans*, *C. sporogenes* and *C. sordellii* (Panessa-Warren *et al.*, 1997, Rabi *et*
438 *al.*, 2017, Samsonoff *et al.*, 1971). A polar appendage has been suggested to form as
439 the result of a reorganization of the spore surface during germination of *C. difficile*
440 spores (Panessa-Warren *et al.*, 1997), but has not been described, to our knowledge,
441 as part of mature *C. difficile* spores. This structure is also clearly seen in scanning
442 electron microscopy (SEM) images of WT and *cdeM* spores, although it is
443 disorganized in the latter case (Fig. 4D, red arrows in the two bottom panels).

444 In any event, the requirement for CdeM for proper assembly of the exosporium
445 and the polar appendage, is in line with the dual localization pattern of CdeM or CdeM-
446 SNAP^{Cd} along the spore body and at the appendage region (Fig. 3C and 3D). Because
447 CdeM is the most abundant protein extracted from WT spores that is absent from
448 *cdeM::erm* spore extracts, we infer that the protein is likely to be the main structural
449 component of the exosporium, which comprises the polar appendage.

450 *CdeM* contributes to the rigidity of spores

451 Assembly of the exosporium and appendage may afford rigidity to the spore, as
452 suggested by the increased length and width of the spore body in *cdeM* spores
453 (above). To test this possibility, we used atomic force microscopy (AFM) to image
454 purified WT and *cdeM::erm* spores. Topography (Fig. 5A, top panel), amplitude
455 (middle) and phase (bottom) images show a relatively smooth surface in WT spores
456 with a well-organized rigid polar region (Fig. 5A, white arrows; inserts show the spore
457 polar region at a higher magnification). The surface of *cdeM* spores was more

458 irregular, with a less organized and softer polar region from which material seems to
459 peel-off possibly through the action of the microscope tip (Fig. 5A, white arrows in the
460 amplitude images). The phase images also reveal a region of decreased rigidity that
461 runs along the longitudinal axis of WT spores (Fig. 5A, white arrows in the phase
462 panel). Imaging of this region at higher magnification suggests the presence of
463 individual protein complexes along the two sides of a crevice (Fig. 5A; black arrows in
464 the two lower panels). Importantly, this feature could not be defined for the *cdeM*
465 mutant, as the entire spore surface, including the polar region, is softer (Fig. 5A). In all,
466 the AFM results are in line with the view that CdeM participates in the assembly of the
467 entire surface of the spore, appendage included, and that it confers rigidity to the
468 spore.

469 ***The spore appendage is an integral part of the exosporium***

470 To test the hypothesis that the appendage is continuous with the exosporium,
471 we attempted to isolate this structure. Earlier work has shown that sonication of spores
472 in the presence of sarkosyl and proteases completely extracts the exosporium
473 proteins, leaving behind a seemingly intact coat (Escobar-Cortes *et al.*, 2013). That
474 several of the proteins assigned to the exosporium-like layer are Cys-rich, including
475 CdeC and CdeM (Barra-Carrasco *et al.*, 2013, Diaz-Gonzalez *et al.*, 2015), together
476 with the finding of a longitudinal region of lower rigidity in WT spores by AFM (above),
477 suggested to us that the surface layer of spores could be sensitive to mechanical
478 disruption in the presence of a reducing agent. Purified spores were shaken with either
479 glass beads, the reducing agent DTT, or both (Fig. 5B). SEM of WT spores before any
480 treatment revealed that most spores have a smooth surface, whereas the surface of
481 *cdeM* spores has a porous appearance (Fig. 5B, top panels). Treatment of WT spores
482 with glass beads results in the partial removal of an external layer, leaving behind an

483 intact spore (Fig. 5B, second row, yellow arrow). In contrast, for *cdeM* spores,
 484 treatment with glass beads often resulted in the release of a complete empty shell; this
 485 shell shows a wide opening that appears to coincide with the position of the region of
 486 low rigidity seen for WT spores by AFM and that ends close to the junction between
 487 the polar appendage and the spore body (above) (Fig. 5B, second row, white arrow).
 488 This suggests that *cdeM* spores are less rigid or less stable than WT spores, in line
 489 with the light microscopy, TEM and AFM data (above). While treatment with DTT alone
 490 had no major impact of the surface of either WT or *cdeM* spores, the combined action
 491 of the beads and the reducing agent caused the release of empty shells in most of the
 492 spores of the two strains (Fig. 5B; white arrows in the fourth row of panels). We infer
 493 that the structural rigidity of spores is in part conferred by the formation of disulfide
 494 cross-links involving the Cys-rich exosporium proteins, among which CdeM.
 495 Consistent with the view that CdeM is a key structural component of the exosporium,
 496 but not essential for its formation, the shells extracted from *cdeM* spores were thinner
 497 ($0.063 \pm 0.008 \mu\text{m}$) than for 630 Δerm spores; conversely, they were thicker for spores
 498 of epidemic strain R20291 (Fig. S5C and D; see also the supplemental text).
 499 During none of the treatments did we see cells outgrowing from spores.
 500 Nevertheless, to test whether formation of the empty shells resulted from spore
 501 germination, we used SEM in combination with energy dispersed X-ray (EDX)
 502 spectroscopy. Release of Ca^{2+} from the spore core when germination is induced by
 503 the bile salt taurocholate (TA) results in a strong reduction of the signal for this
 504 element in spores, while the signal for sulfur remains essentially unchanged (Fig. 5C,
 505 bottom panels). Thus, we measured the Ca^{2+} and sulfur signals for WT and *cdeM*
 506 spores before and after treatment with glass beads in the presence of DTT. The sulfur
 507 signal, which is not expected to change during germination, showed variation among
 508 the different conditions tested but remained high following the different treatment; the

509 signal for Ca^{2+} also showed some variation, but it never came down to nearly
510 undetectable levels, as when germination is induced by TA (Fig. 5C, compare the
511 three first sets of panels with the bottom panel, which shows measurements for WT
512 spores following induction of spore germination with TA). Although we cannot exclude
513 that some spores germinate under our experimental conditions, we infer that the
514 empty shells result mainly from the mechanical/chemical detachment of the
515 exosporium that surrounds the entire spore and is continuous with the polar
516 appendage.

517 ***Evidence for two populations of CdeM at the spore surface***

518 Following treatment with the glass beads in the presence of DTT, the spore
519 suspension was separated into a sediment containing spores and shells, and a
520 supernatant, which we expected to contain proteins released from the spores during
521 treatment. Several proteins were found in the supernatant, following SDS-PAGE and
522 Coomassie staining, including, for WT spores, the 17 kDa form of CdeM; this species
523 is also detected by immunoblotting and is absent from the supernatant of *cdeM* spores
524 (Fig. 5D, black arrow in the top and middle panels). This species seemed reduced in
525 the WT sediment by Coomassie staining relative to the supernatant, but reacted more
526 strongly with the anti-CdeM antibody (Fig. 5D, top and middle panels). We do not
527 presently know the reason for this apparent difference in recognition of CdeM in the
528 supernatant and in the sediment by the antibody, but one possibility is that the protein
529 is subject to a post-translational modification. That several multimeric forms of CdeM
530 (close to the 37 kDa marker, as well as below and above the 75 kDa marker) are
531 detected in the sediment from the WT (Fig. 5D, top and middle panels, red arrows)
532 suggests that the shells are enriched in cross-linked forms of the protein. These
533 multimeric forms of CdeM appear to be less extractable from whole spore

534 preparations, *i.e.*, without any prior treatment (see Fig. 3A). CdeC was not detected in
535 the supernatant of WT or *cdeM* spores, but was found as two species of about 60 kDa
536 and 44 kDa in the sediment of both WT and *cdeM* spores and at slightly higher levels
537 in the latter (Fig. 5D, black arrow in the bottom panel). The 44 kDa species likely
538 corresponds to the low mass CdeC previously detected in spore extracts (Calderón-
539 Romero et al., 2018). A high molecular weight form of CdeC, above the 150 kDa
540 marker, was also detected in the *cdeM* spore sediment (Fig. 5D, red arrow in the lower
541 panel). This form was not detected in the total spore extracts (Fig. 3A and B),
542 suggesting that the shells are enriched in cross-linked forms of CdeC. The results
543 suggest that CdeC is tightly associated with the spore and is not released into the
544 supernatant by the glass beads/DTT treatment. Moreover, the increased extractability
545 of CdeC from the shells isolated from *cdeM* spores suggests a more internal
546 localization for CdeC and that CdeM, somehow reduces the extractability of CdeC.
547 The CdeM protein, in contrast, exists as two populations; part is released to the
548 supernatant upon treatment with beads/DTT, suggesting a looser association with the
549 spore, while the spore and shell sediment is enriched in multimeric forms of the
550 protein.

551 ***Density gradient separation of different spore morphotypes***

552 To determine whether the multimeric forms of CdeM were preferentially
553 associated with spores bearing a polar appendage. We used density centrifugation to
554 separate spores without an appendage from those bearing either short or long
555 appendages. On 40%-60% step gradients of metrizoic acid, a WT population of spores
556 forms bands at 55%, 50% and 45% (Fig. 6A, top). As determined by SEM, the initial
557 material consisted of a heterogeneous population of spores while the denser fractions
558 appeared enriched for appendage-bearing spores (Fig. 6A, bottom). In agreement with

559 this, phase contrast microscopy shows that the spores collected from the band at 55%
 560 showed well developed appendages, as compared to those at 45% (Fig. S6). We
 561 measured the spore length in both the phase contrast and FM4-64 images as well as
 562 the spore width for individual spores in the initial material and in the various fractions,
 563 as described above (see also Fig. 4B). For both the WT and *cdeM* spores, the material
 564 at the 45% density layer consisted mainly of smooth spores that appeared to lack an
 565 external layer and have no appendage or a very short one, as assessed by SEM (Fig.
 566 6A, top row of the SEM panels). For both the WT and *cdeM* spores, the width was
 567 similar to that in the initial material (Fig. 6B). The spore length, however, measured in
 568 the phase contrast images was reduced for both strains as compared with the initial
 569 material but increased in the FM4-64 images for *cdeM* spores (Fig. 6B; $1.13 \pm 0.18 \mu\text{m}$
 570 vs $1.23 \pm 0.14 \mu\text{m}$). This again suggests longitudinal expansion of the spore in the
 571 absence of CdeM (see also above). The 50% and 55% density fractions were enriched
 572 in spores that were longer than those in the 45% fraction as measured in the phase
 573 contrast images (Fig. 6B; e.g., length of WT spores: $1.10 \pm 0.15 \mu\text{m}$ in the 45% fraction
 574 vs. $1.35 \pm 0.18 \mu\text{m}$ in the 55% fraction). In line with the idea that CdeM imparts rigidity
 575 to the spore, *cdeM* spores were however shorter in the 50% and 55% fractions, as
 576 measured in the FM4-64 images (Fig. 6B; $1.23 \pm 0.14 \mu\text{m}$ in the 45% fraction vs.
 577 $1.06 \pm 0.11 \mu\text{m}$ in the 50% fraction). Together, this analysis suggests that spores in the
 578 45% fraction may have a thin exosporium and lack an appendage, whereas spores in
 579 the 50% and 55% density fractions have a more developed exosporium and a polar
 580 appendage, even though disorganized in the case of the *cdeM* mutant.

581 *CdeM is enriched in appendage-bearing spores*

582 We next extracted the surface proteins from WT and *cdeM* spores collected
 583 from the 45%, 50% and 55% fractions, resolved the proteins by SDS-PAGE and

584 analyzed Coomassie stained gels, which were also subject to immunoblotting with
 585 anti-CdeM and anti-CdeC antibodies. Coomassie staining revealed a similar profile for
 586 the corresponding density fractions derived from the WT or *cdeM* samples with the
 587 exception of CdeM, absent from the mutant (Fig. 6C, top panel). Importantly, only the
 588 monomeric form of CdeM was found in the 45% fraction of WT spores; this form of the
 589 protein was clearly enriched in the 50% and 55% fractions in which multimeric forms
 590 were also detected by immunoblotting (Fig. 6C). As for CdeC, only the monomeric
 591 form of CdeC was found in the 45% fraction for both WT and *cdeM* spores (Fig. 6C;
 592 bottom panel). Importantly, multimeric forms of CdeC were found in the 50% and 55%
 593 fractions, which are enriched for appendage-bearing spores.

594 These results suggest that CdeC is preferentially associated with a more
 595 internal layer of this structure, upon which most of CdeM is assembled. Assembly of
 596 the exosporium around spores and/or formation of the polar appendage seems to
 597 involve multimerization of both CdeC and CdeM.

598 ***CdeM conceals other exosporium proteins***

599 Since treatment of purified spores with glass beads in the presence of DTT
 600 leads to the isolation of empty shells, which we posit is the exosporium, we next
 601 attempted to separate the shells from spores using density gradient centrifugation. WT
 602 and *cdeM* spore suspensions were shaken with glass beads in the presence of DTT
 603 and then applied to the top of 40-60% step gradients of metrizoic acid. For both the
 604 WT and *cdeM* samples, we found that a gradient band formed at a concentration of
 605 42% of metrizoic acid, was enriched in phase dark structures, confirmed by SEM to be
 606 mostly empty shells although some spores were also seen (Fig. 7A). A fraction at 45%
 607 was also enriched in empty shells, but contained more spores, especially for the *cdeM*
 608 sample, and a 55% fraction contained mainly appendage-bearing spores (Fig. 7A).

609 The material isolated from the 42% (referred to as shells) and 55% (referred to as
610 spores) gradient fractions was analyzed by SDS-PAGE and Coomassie staining, and
611 by immunoblotting. The profile of Coomassie-stained proteins was similar for shells
612 derived from the WT or *cdeM* samples, except for the absence of CdeM from the latter
613 (Fig. 7B, top panel, black arrow). Immunoblot analysis confirms the presence of
614 multimeric forms of both CdeM and CdeC in the 42% fraction (Fig. 7B, middle panels).
615 The BclA proteins are markers for the exosporium (Diaz-Gonzalez *et al.*, 2015,
616 Pizarro-Guajardo *et al.*, 2014). Several forms of BclA1 are detected in
617 coat/exosporium extracts with an anti-BclA antibody, including a form of 48 kDa and a
618 complex of about 110 kDa (Calderon-Romero *et al.*, 2018, Pizarro-Guajardo *et al.*,
619 2014). Under our conditions, only the 48 kDa form of BclA1 was detected and only in
620 the 42% fraction (shells) derived from *cdeM* spores, but not in the material obtained
621 from WT spores (Fig. 7D, bottom panel). Both monomeric and multimeric forms of
622 CdeC and CdeM were detected in the 55% fraction (appendage-bearing spores) but
623 BclA1 was not detected in this fraction in spite of an overall higher protein content (Fig.
624 7B, right panels). Thus, BclA1 is assembled in *cdeM* spores, is enriched in the “shells”
625 (the 42% gradient fraction), and its extractability increases in the absence of CdeM.

626 We used flow cytometry following immunostaining to test whether BclA1 could
627 be more exposed in spores of the *cdeM* mutant. CdeM is detected at the surface of
628 WT spores (Fig. 7C, top panel, blue trace), consistent with the localization studies (Fig.
629 3), the release of the protein following the bead/DTT treatment of spores (Fig. 5) and
630 the enrichment of the protein in appendage-bearing spores (Fig. 6). In line with the
631 idea that CdeM conceals BclA1, the signal for BclA increased in *cdeM* spores as
632 compared to the WT (Fig. 7C, middle panel, red trace). Moreover, the signal for CdeC
633 also increased in *cdeM* spores relative to the WT (Fig. 7C, red trace in the bottom

634 panel). Fluorescence microscopy of the same spores confirms the increased
635 decoration of *cdeM* spores by the anti-BclA1 and anti-CdeC antibodies (Fig. 7D, yellow
636 arrows). Thus, our results suggest that in the exosporium-like layer, both BclA1 and
637 CdeC are concealed by CdeM.

638 ***CdeM* is required for proper spore germination**

639 The abnormal assembly of the spore surface layers in the *cdeM* mutant raised
640 the possibility that spore germination could be affected. To test this possibility, we
641 monitored spore germination by measuring the drop in the absorbance at 580 nm of a
642 suspension of heat-activated spores, following addition of TA (Burns *et al.*, 2010, Sorg
643 & Sonenshein, 2008, Wilson *et al.*, 1982). In the presence of 5% TA, but not in its
644 absence, the OD₅₈₀ of the heat-activated WT spore suspension dropped by about 30%
645 of the initial in 25 min (Fig. 8A, close black symbols) whereas the OD₅₈₀ of the *cdeM*^C
646 spore suspension dropped 35% (close blue symbols). Germination was essentially
647 completed 30 min after TA addition and was followed by spore outgrowth. Strikingly,
648 for *cdeM* spores, a drop of 20% in the OD₅₈₀ of the initial suspension takes place even
649 in the absence of TA (Fig. 8A, open red circles) but the addition of TA caused a 50%
650 drop in the OD₅₈₀ in 15 min (close red circles). Thus, spores of the *cdeM* mutant seem
651 to germinate faster and more extensively than WT or *cdeM*^C spores. As a control
652 experiment, we used spores from a *s/eC* mutant that are impaired in TA-induced
653 germination (Adams *et al.*, 2013, Francis *et al.*, 2013) and found no germination under
654 our conditions (Fig. 8A, green dots). Outgrowth of the *cdeM* spores followed a kinetics
655 similar to that of WT spores (Fig. 8A). We should note that no difference in TA-induced
656 germination was seen between WT and *cdeM* spores in a recent study (Calderon-
657 Romero *et al.*, 2018). However, while we used 5% TA, Calderón-Romero and co-
658 authors used 10% TA; a higher concentration of TA could potentially mask differences

659 between WT and mutant spores. In any event, since *cdeM* spores lack the
660 electrondense outer layer of the exosporium, increased permeability of the spore
661 surface layers to TA may explain, at least in part, the increased germination.

662 ***The spore polar appendage has a direct role in germination***

663 The *cdeM* mutant produces spores with no exosporium but that also show a
664 short and disorganized appendage, raising the possibility that this structure contributes
665 directly to germination. To address this question, we fractionated the population of WT
666 and *cdeM* spores by density centrifugation and we monitored germination of the
667 fractions enriched in smooth spores (at 45% of metrizoic acid; see also Fig. 6A and B),
668 or in appendage-bearing spores (55% fraction). No germination was observed, for the
669 WT or *cdeM* fractions, in the absence of TA (Fig. 8C, open symbols). In the presence
670 of 5% TA, the smooth spores of the WT (45% fraction; Fig. 8C, close blue symbols)
671 germinated poorly as compared to those with appendages (55% fraction; close dark
672 blue symbols). For the *cdeM* mutant, the appendage-lacking spores (45% fraction; Fig.
673 8C, close green symbols) also germinated to a lower extent than the appendage-
674 bearing spores (55% fraction; close red symbols). The appendage-bearing *cdeM*
675 spores, however, germinated to a greater extent than the appendage-bearing WT
676 spores and the appendage-lacking *cdeM* spores germinated better than their WT,
677 appendage-lacking, counterparts (Fig. 8C). We infer that the appendage itself
678 contributes directly for proper spore germination.

679 ***The cdeM mutant shows enhanced virulence***

680 Because spores of the *cdeM* mutant have an altered surface and germination
681 properties, we next wanted to assess the infectivity of the mutant spores. We used the
682 hamster model of infection in which WT spores cause a rapid and fulminant infection
683 leading to high mortality (Goulding *et al.*, 2009, Sambol *et al.*, 2001). Five days

684 following administration of clindamycin by gavage, a group of 6 female hamsters were
685 challenged with WT spores, whereas 8 hamsters were challenged with *cdeM* spores,
686 both at an infective dose of 10^3 spores. For WT spores, the clinical end-point was
687 reached in 7 days, with the first animals dying at day 3 of the experiment (Fig. 9A). In
688 contrast, the clinical end-point was reached at day 5 for *cdeM* spores, with the first
689 animals dying at day 2 (Fig. 9A). Thus, *cdeM* spores are more infective than WT
690 spores. For both WT and *cdeM* spores, no animals survived (Fig. 9A). We were not
691 able to purify sufficient spores of the two main appendage morphotypes for either the
692 WT or *cdeM* mutant, and thus we were not able to directly assess the role of the
693 appendage in virulence. Since overall, unfractionated spores of the *cdeM* mutant
694 germinate more efficiently than WT spores in response to TA (Fig. 8A), it seems likely
695 that the increased infectivity of *cdeM* spores is related to their increased germination
696 (Fig. 8A; see also above).

697 Spores of the mutant, however, also show an altered surface which is likely to
698 influence binding to host tissues and cells (Calderon-Romero *et al.*, 2018, Mora-Urbe
699 *et al.*, 2016, Paredes-Sabja & Sarker, 2012) and previous work has shown that *cdeM*
700 spores are impaired in colonization of axenic mice (Janoir *et al.*, 2014). In an attempt
701 to determine whether the *cdeM* mutation affected the ability of spores to bind to host
702 cells, we measured adhesion of spores to hamster caeca *ex vivo*. Isolated and washed
703 caeca were incubated with 3×10^6 WT or *cdeM* spores for 1, 2, or 3 h, and after
704 washing, bound spores were enumerated by plating. Most of the WT spores bound to
705 the hamster caecum after an incubation of 1 hour and binding did not change over
706 time (Fig. 9B). In contrast, after 1 hour, binding of *cdeM* spores was 10-fold lower (Fig.
707 9B). Moreover, binding of *cdeM* spores increased over time but even after 3 hours of
708 incubation it remained lower than for the WT (Fig. 9B). Thus, binding of *cdeM* spores
709 to hamster caeca is impaired, in comparison to the WT spores. In a mouse colonic

710 loop model, binding of *cdeM* spores was also impaired (Calderon-Romero *et al.*,
711 2018).
712

713 Discussion

714 *Expression of cdeM in relation to spore morphogenesis*

715 Our data suggests that genes coding for components of the coat and
716 exosporium of *C. difficile* spores are expressed according to a temporal cascade that
717 may reflect the order of assembly of the various structures. It has been shown that the
718 genes coding for morphogenetic proteins SpoIVA and SipL are under the control of
719 σ^E , as are other genes coding for components of the coat layers (Fimlaid *et al.*, 2013,
720 Pereira *et al.*, 2013, Pishdadian *et al.*, 2015, Putnam *et al.*, 2013, Saujet *et al.*, 2013).
721 These genes are expressed mainly prior to engulfment completion, which is the main
722 period of activity of σ^E and some additionally require SpoIIID (Pishdadian *et al.*, 2015).
723 Some activity of σ^K is detected at this stage, but it increases following engulfment
724 completion, explaining the early expression of *cotE* (Pereira *et al.*, 2013, Serrano *et*
725 *al.*, 2016). Expression of *cdeC*, which is under the joint control of σ^K and SpoIIID, is
726 only detected following engulfment completion (Pishdadian *et al.*, 2015). The onset of
727 *cdeM* expression, which we now show to be under the direct control of σ^K (Fig. 2A and
728 B) is detected later in morphogenesis, when the spore becomes phase bright. It is
729 presently unknown how expression of *cdeM* is delayed relative to that of *cdeC*. The
730 joint requirement for σ^K and SpoIIID for the expression of *cdeC* (Pishdadian *et al.*,
731 2015) suggests that the accumulation of SpoIIID along time may activate the
732 expression of genes with SpoIIID-binding regions of progressively lower affinity. It is
733 not presently known, however, whether the late expression of *cdeM* requires direct
734 binding of SpoIIID to the promoter region of the gene. SpoIIID may only function as an
735 activator in *C. difficile* ((Pishdadian *et al.*, 2015); but see also (Saujet *et al.*, 2013)) but
736 in *B. subtilis* it also functions as a repressor (Eichenberger *et al.*, 2004, Halberg &
737 Kroos, 1994, Zhang & Kroos, 1997). Thus, an alternative scenario is that the action of

738 a *cdeM* repressor is relieved at a late stage in morphogenesis (Fig. 9C, “+/- X”, where
739 X may be SpoIIID).

740 Expression of *cdeM* is only detected at high levels in 43% of the sporangia of
741 phase bright spores, while high level expression of *cdeC* is detected in about 4% of
742 the phase grey spores and in 24% of the sporangia of phase bright spores (Fig. 2E).
743 Given the role of the two proteins in the assembly of the exosporium ((Calderon-
744 Romero *et al.*, 2018); this work), it seems plausible that the two main morphotypes
745 described herein and detected in a WT population of spores arise, at least in part, from
746 sporangia in which the combined expression of *cdeC* and *cdeM* occurs above or below
747 a certain threshold or does not take place. The fraction of appendage-bearing spores
748 produced by the epidemic strain R20191 is higher than for strain 630 Δ *erm* (Fig. S5)
749 suggesting that the ratio between spores with or without an appendage is an important
750 trait differentiating epidemic strains. The mechanism by which expression of *cdeM* is
751 restricted to a fraction of the sporangia is unknown; it may be related to the activity of
752 SpoIIID, as suggested above, or an as yet unknown factor (“X” in Fig. 9C). Previous
753 work has shown that *cdeM* was the most highly expressed gene following infection of
754 axenic mice with *C. difficile* cells (Janoir *et al.*, 2014). While suggesting that the
755 contribution of CdeM for the assembly of the exosporium is important in the host,
756 consistent with the colonization defect of a *cdeM* mutant (Janoir *et al.*, 2014), the
757 factors that contribute to the differential expression of *cdeM* at least in vivo are
758 presently unknown. Although it is tempting to conclude that most if not all of the spores
759 that are produced in vivo have a robust appendage, it is also possible that the two sub-
760 populations/morphotypes are maintained in vivo, because they serve different
761 functions (see also below).

762 *Function of CdeM in the assembly of the exosporium*

763 CdeM is a critical determinant of assembly of both the exosporium and a spore
764 polar appendage ((Calderon-Romero *et al.*, 2018); this work). This suggests that
765 CdeM is both a component of the exosporium that surrounds the spore and of the
766 appendage (Fig. 9D), consistent with the abundance of CdeM and the localization of
767 the protein around the spore and in the appendage as assessed both with a CdeM-
768 SNAP fusion and immunofluorescence. A difference between the two approaches is
769 that CdeM-SNAP labels the bulk of the appendage, whereas by immunofluorescence it
770 is only the edge of the appendage that is labeled (Fig. 2). Possibly, CdeM is either
771 modified in the appendage region and/or part of a tightly packed structure to which the
772 antibodies are not accessible. Several lines of evidence suggest that CdeM undergoes
773 disulfide cross-linking and this in turn may contribute to the rigidity of the spore surface
774 appendage included. Firstly, CdeM is a Cys-rich and the partially purified protein forms
775 multimers (Fig. S3); secondly, multimeric forms of CdeM are also detected in
776 coat/exosporium extracts prepared from WT spores (Fig. 3). Finally, enrichment for an
777 appendage-bearing sub-population of spores as well as enrichment for “shells” derived
778 from those spores through mechanical disruption of spores in the presence of reducing
779 agents, also enriches for multimeric forms of CdeM (and also of CdeC) (Figs. 5-7).
780 Cross-linking of CdeM, may also confer rigidity to the spore, as indicated by our
781 inability to produce a surface topology image of *cdeM* spores using AFM (Fig. 5).
782 Some of the CdeM protein is released upon treatment of the spores with
783 beads/DTT, suggesting the existence of a sub-population more loosely attached to the
784 exosporium and/or associated with the more internal coat layers. In contrast, no CdeC
785 is released during isolation of the exosporium, suggesting a higher degree of cross-
786 linking of this protein or a more internal localization within the exosporium (Fig. 5).
787 Other lines of evidence support a more internal localization of CdeC within the
788 exosporium. Firstly, in our hands, the assembly of CdeC is not affected by disruption

789 of *cdeM* (Fig. 3A); a decrease in both low (44 kDa) and high (120 kDa) forms of CdeC
 790 in spores of a *cdeM* mutant, recently reported, may be due to differences in extraction
 791 conditions (Calderon-Romero *et al.*, 2018). We note for example, that under our
 792 experimental conditions, only a form of CdeC of about 50 kDa is detected in whole
 793 coat/exosporium extracts (Fig. 3), but that in other forms of CdeC are detected in
 794 enriched exosporia/shells (Fig. 7B; see bands at ≈ 75 kDa and ≥ 150 kDa).
 795 Nevertheless, a reduction in these two forms of CdeC in *cdeM* spores relative to the
 796 WT is in agreement with the general conclusion that CdeC and CdeM somehow
 797 influence each other (Calderon-Romero *et al.*, 2018). Secondly, CdeC becomes more
 798 accessible to antibodies in the absence of CdeM and likewise, BclA1 becomes more
 799 extractable and more accessible to antibodies in the absence of CdeM (Fig. 7B-D).
 800 Finally, intact exosporia/shells are still isolated from a *cdeM* mutant, although they are
 801 thinner (Fig. S5), consistent with a model in which CdeM assembles on top of a
 802 scaffold or basal layer of which CdeC may be part of (Fig. 9D). This putative basal
 803 layer may contain additional Cys-rich proteins such as CdeA and CdeB which have
 804 also been identified as part of the exosporium and BclA may be attached to this basal
 805 layer (Diaz-Gonzalez *et al.*, 2015, Phetcharaburanin *et al.*, 2014, Pizarro-Guajardo *et*
 806 *al.*, 2014). Deposition of CdeM on top of this more internal layer forms both the
 807 electrondense, surface exposed part of the exosporium and the appendage (Fig. 9D).
 808 Although the intervening proteins are not related, this model calls to mind studies in *B.*
 809 *cereus* and *B. anthracis*. The exosporium has a basal layer formed by several
 810 proteins, including the Cys-rich proteins CotY and CotZ, that self assemble into a
 811 crystalline hexagonal lattice through cooperative disulfide bond formation and BclA is
 812 attached to this basal layer (Ball *et al.*, 2008, Kailas *et al.*, 2011).
 813 *Evidence for an escape route for outgrowing spores*

814 AFM revealed the existence of a region of lower rigidity running along the long
815 axis of WT spores. This region is most likely at the origin of the apertures seen upon
816 treatment of intact spores with glass beads and DTT and that allow the isolation of the
817 shells/exosporia, or at least the outermost layers of this structure. Apertures are also
818 formed during germination of *C. sporogenes* spores, although in this case close to one
819 of the spore pole, thought to facilitate spore emergence and cell outgrowth (Brunt *et*
820 *al.*, 2015). Apertures in the exosporium are also formed during germination of spores
821 of *B. anthracis*, which are formed at a “botllecap” structure located at one of the spore
822 poles (Steichen *et al.*, 2007). The apertures formed in the exosporium during
823 germination of spores of different species may form at regions of lower rigidity built into
824 the structure during its assembly and may result, in part, from increased turgor
825 pressure of the forespore cytoplasm (Brunt *et al.*, 2015, Steichen *et al.*, 2007). It
826 seems likely that the apertures that result from chemical/mechanical disruption of *C.*
827 *difficile* spores are also formed during spore germination.

828 *Biological significance of the two main spore morphotypes*

829 Our study suggests that the two main morphotypes with respect to the
830 assembly of the polar appendage, *i.e.*, robust appendage or disorganized or absent
831 appendage, show different germination properties (Fig. 9E). Both WT and *cdeM*
832 mutant spores with an appendage germinate better in response to taurocholate than
833 those without an appendage (Fig.8). The WT spores with an appendage, however,
834 germinate slower than their *cdeM* counterparts (Fig. 8C), possibly because in the
835 absence of CdeM the spore is more permeable to germinants (Fig. 8A). The more
836 permeable spores may germinate more promptly *in vivo* explaining why hamsters that
837 received *cdeM* spores succumbed to infection faster than animals that received WT

838 spores (Fig. 9A and D). Consistent with this interpretation, the overall population of
839 *cdeM* spores germinates faster than WT spores in response to taurocholate (Fig. 8A).
840 The presence of the appendage may be important for germination by allowing a
841 structural alteration of the exosporium that facilitates germination. For example, the
842 presence of the polar appendage may facilitate opening through the region of rigidity
843 along the spore longer axis. The appendage-bearing spores may germinate in contact
844 with cells in vivo. Cells of a *cdeM* insertional mutant shows impaired ability to colonize
845 axenic mice (Janoir *et al.*, 2014). The impaired colonization may result from the
846 misassembly of the spore surface in vivo and an impaired interaction of the spores
847 with the colonic mucosa ((Janoir *et al.*, 2014); this work). In line with this idea, spores
848 of the *cdeM* mutant show impaired binding in a colonic loop mouse model (Calderon-
849 Romero *et al.*, 2018), and also impaired binding to isolated hamster caeca (Fig. 9B).
850 This is line with published work indicating that the exosporium or specific components
851 of this structure, including CdeM, are needed for efficient spore binding to host cells
852 ((Calderon-Romero *et al.*, 2018)and references therein).
853 While the expression pattern of *cdeM* may be a key factor in the production of
854 the different spore morphotypes, as suggested above, the polymorphisms identified in
855 *cdeM* (Fig. S4 and S7; see also the supplemental text) may also influence, at least in
856 part, the structure of the exosporium which varies across strains (Pizarro-Guajardo *et al.*,
857 2016a, Pizarro-Guajardo *et al.*, 2016b). Perhaps significantly, the sequence length
858 polymorphism at region P3 most likely eliminates the last two helices of CdeM in strain
859 E10 (Fig. S3A and B; Fig. S7). It seems plausible that variations in the expression
860 level and/or sequence of *cdeM*, may be related to variations in the structure of the
861 spore surface layers and play a role in evasion of the host immune system.
862

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877 **Material and Methods**

878 **Strains and growth of *C. difficile*.** Bacterial strains and their relevant properties are
 879 listed in Table S1. The *Escherichia coli* strain DH5 α (Bethesda Research laboratories)
 880 was used for molecular cloning and BL21(DE3) (Novagen) was used for the over-
 881 production of a CdeM-His₆ fusion (see below). Luria-Bertani medium was routinely
 882 used for growth and maintenance of *E. coli*. When indicated, ampicillin (100 μ g /ml),
 883 chloramphenicol (15 μ g /ml) or kanamycin (30 μ g/ml) was added to the culture
 884 medium. The *C. difficile* strains used in this study are congenic derivatives of the
 885 standard laboratory strain 630 Δ erm ((Hussain *et al.*, 2005); a gift from Nigel Minton); or
 886 strain R20291 ((McEllistrem *et al.*, 2005, Stabler *et al.*, 2009); was a gift from Daniel
 887 Paredes-Sabja). *C. difficile* strains were routinely grown anaerobically (5% H₂, 15%
 888 CO₂, 80% N₂) at 37°C in brain heart infusion (BHI) medium (Difco) or in sporulation
 889 medium (SM), as described before (Pereira *et al.*, 2013, Saujet *et al.*, 2013). When
 890 necessary, thiamphenicol (15 μ g /ml) or erythromycin (5 μ g /ml) was added to *C.*
 891 *difficile* cultures. All plasmid constructions were carried out using standard procedures
 892 and are described in detail in the supplemental material. A list of plasmids is also
 893 included in Table S1.

894 **General methods.** The methods used for the construction of SNAP transcriptional and
 895 translation fusions, SNAP^{Cd} labelling, phase contrast, fluorescence microscopy and
 896 image analysis, RNA extraction, quantitative RT-PCR analysis and 5'-RACE, spore
 897 trypsin digestions, KCl washes and hydrophobicity assays and for the overproduction
 898 and purification of CdeM-His₆ for polyclonal antibody production, are given in detail in
 899 the Supplemental Material.

900 **Complementation analysis.** To complement the *cdeM::ermB* mutation in single copy,

a 1117 bp region comprising the entire coding sequence of *cdeM* (483 bp) and its expected promoter region (534 bp upstream of the *cdeM* gene) was amplified by PCR using primers PCdeM Fw/CdeM Rev (the sequence of all primers is given in Table S2). The generated PCR fragment was then cloned into the Sall and XhoI sites of pMTL84121, yielding pFT43 (Table S1). The same fragment was excised from pFT43 using Sall/HindIII restriction sites and cloned into the same sites of pSMB47, yielding pFT71. AHCD159 was created by the integration of pFT71 into the chromosomal Tn916 locus of *B. subtilis* strain BS49. AHCD715 is a transconjugant from the mating of *B. subtilis* strain AHCD159 and *C. difficile* CdeM mutant strain, resulting in the integration of the plasmid::Tn916 fusion of the donor strain into the *C. difficile* chromosome (see Figure S1).

Spore production, purification and fractionation. For spore production, 5 ml of BHI media was inoculated with an isolated colony of *C. difficile* and cultured overnight at 37°C under anaerobic conditions. The next day, the overnight culture was diluted 1:100 into 100 ml of fresh BHI media and the resulting culture incubated at 37°C under anaerobic conditions for 10 days. Cells were collected by centrifugation (at 4800 x g, for 20 min, 4°C) resuspended in cold water and stored overnight at 4°C. The cells were then collected by centrifugation (at 4800 x g, for 20 min, 4°C) and the sediment resuspended in 1 ml of a 20% solution of Gastrografin (Schering). Spores were purified by applying the suspension onto a 50% layer of Gastrografin and centrifugation at 10.000 x g, for 20 min at room temperature (Henriques *et al.*, 1995). The pellet was resuspended in cold distilled water, washed 10 times with distilled water, and stored at 4°C for further use. For spore fractionation, 1 ml of a spore suspension was layered on top of step gradients of Gastrografin (from 40% to 60% in steps of 5%) and centrifuged at 10000 x g for 30 min at room temperature. Fractions

926 (0.2 ml) were collected from the bottom and processed for scanning electron
927 microscopy, SDS-PAGE and immunoblot analysis (below).

928 **Extraction, fractionation and analysis of the spore exosporium.** Purified spores
929 (10 mg dry weight) were resuspended in 500 µl of distilled water in a Pathogen Lysis
930 Tube S (from Qiagen) and DTT added to 10 mM, final concentration. The suspension
931 was shaken in a vortex mixer for 10 min at 10.000 rpm, at room temperature. The
932 supernatant was then collected into a new tube and centrifuged for 10 min at 5.000 x
933 g, at room temperature. The supernatant contains the exosporium. The sediment was
934 washed 3 times in PBS containing Triton X-100 (at 0.1%). Finally, the sediment was
935 resuspended in 50 µl of extraction buffer (0.125 mM Tris-HCl pH 6.8, 5% beta-
936 mercaptoethanol, 2% SDS, 0.025% Bromophenol Blue, 0.5 mM DTT 5% glycerol) and
937 boiled for 10 min. Proteins were then resolved on 15% SDS-PAGE gels and subject to
938 immunoblot analysis with anti-CdeC, anti-CdeM or anti-BclA1 antibodies as described
939 below.

940 **Spore coat extraction and mass spectrometry.** Spores were resuspended in 50 µl
941 of extraction buffer (0.125 mM Tris-HCl pH 6.8, 5% beta-mercaptoethanol, 2% SDS,
942 0.025% Bromophenol Blue, 0.5 mM DTT 5% glycerol) to a final OD_{600nm} of 4, and
943 boiled as described previously (Costa *et al.*, 2006). Proteins present in the supernatant
944 fraction were resolved on 15% SDS-PAGE gels, and visualized by Coomassie brilliant
945 blue R-250 staining. For protein identification, protein bands were excised and
946 digested with trypsin before analysis by matrix-assisted laser desorption ionization
947 (MALDI) (http://www.itqb.unl.pt/Services/Analytical_Services/Mass_Spectrometry).

948 **Spore Germination.** Density-gradient-purified spores were resuspended in PBS to a
949 final OD_{600nm} of 1 and heat activated for 10 min at 71°C. Taurocholic acid (TA) (Sigma-

950 Aldrich) in PBS was then added to a final concentration of 5% in TA, to induce spore
951 germination. Germination was followed by measuring the decrease in the OD_{600nm} of
952 the spore suspension, in a plate reader, at 37°C with agitation, until no significant
953 changes in OD_{600nm} were detected. Spore germination was followed both in the
954 presence of oxygen or under anaerobic conditions (5% H₂, 15% CO₂, 80% N₂), in a
955 glove box.

956 **Whole cell lysates and immunoblot analysis.** Samples (10 ml) of *C. difficile* cultures
957 were collected, and the cells collected by centrifugation (at 4800 x g, for 20 min, 4°C).
958 The cell sediment was washed with phosphate-buffered saline (PBS), resuspended in
959 1 ml of French press buffer (10 mM Tris pH 8.0, 10 mM MgCl₂, 0.5 mM EDTA, 0.2 mM
960 NaCl, 10% Glycerol, 1 mM PMSF) and lysed using a French pressure cell (18000
961 lb/in²). Proteins in the total cell extracts (10 µg) or the spore coat or shell extracts
962 prepared as described in the preceding sections were resolved on 15% SDS-PAGE
963 gels and subject to immunoblot analysis with the following primary antibodies at the
964 indicated dilutions: anti-CdeM, 1:10000, anti-CdeC (Fimlaid *et al.*, 2013) and anti-
965 BclA1 (Phetcharaburanin *et al.*, 2014), both at 1:1000. A rabbit secondary antibody
966 conjugated to horseradish peroxidase (Sigma) was used at a dilution of 1:5000. The
967 immunoblots were developed with enhanced chemiluminescence reagents
968 (Amersham Pharmacia Biotech).

969 **Immunofluorescence microscopy of sporangia and spores.** Samples (500 µl) from
970 sporulating cells were fixed with HistoChoice (from Amresco) for 15 min at room
971 temperature. The cells were collected by centrifugation (6.000 x g, for 2 min at room
972 temperature) the sediment resuspended in 100 µl of GTE (50 mM glucose, Tris-HCl
973 pH 7.5, 10 mM EDTA), lysozyme added (to 2.5 mg/ml) and the suspension incubated
974 on ice for 30 min. Following incubation, the cells were washed three times with PBS

975 supplemented with 2% bovine serum albumin (Sigma). The primary antibodies were
 976 then added, either anti-CdeM (at a 1:1000 dilution) or anti-SNAP (from New England
 977 Biolabs, at a 1:500 dilution), and incubation continued for 1 hour. The cells were
 978 washed three times with PBS and then the Alexa Fluor 594 or Alexa Fluor 488 goat
 979 anti-rabbit IgG secondary antibody was added (Molecular probes, Invitrogen) (1:500).
 980 For immunofluorescence of spores, 100 µl of a spore suspension at an OD₅₈₀ of about
 981 1 was used as above, except that the lysozyme incubation step was omitted.

982 **Flow cytometry.** For flow cytometric analysis, immune-stained spores (as above)
 983 were diluted in PBS and directly applied to a S3 cell sorter (Bio-Rad) operating a solid-
 984 state laser at 488 nm. For each sample, 30.000 events were analysed. Data
 985 containing the fluorescent signals were collected using a 525/30-bp filter, and the
 986 photomultiplier voltage was set between 400 and 550 V. Data were acquired and
 987 processed using the ProSort software package (Bio-Rad).

988 **Scanning electron microscopy and EDX analysis.** Spores were prepared for
 989 scanning electron microscopy essentially as described (Braet *et al.*, 1997). Briefly,
 990 spores were fixed with a mixture of glutaraldehyde (2,5%) and formaldehyde (1%) in
 991 phosphate buffer (0.2 M at pH7,4) for 15 min at 22°C. The fixed spores were then
 992 washed twice in PBST (phosphate saline buffer with 0,1% tween 20) and collected by
 993 centrifugation (6000 x g, for 5 min at room temperature). Spores were then contrasted
 994 with 1% osmium tetroxide in water for 15 min at 22°C, washed twice in PBST and
 995 collected by centrifugation (6000 x g, 5 min). After contrasting, spores were
 996 dehydrated with a gradient of ethanol (from 50% to 100%, in 10% steps). Ethanol was
 997 then substituted by 100% acetone and 20 µl of the spore suspension was poured on a
 998 golden sputter glass slide lamella. A 50 µl drop of HDMS (hexamethyldisilazane) was
 999 applied over the spores and the lamella dried in a fume hood. Spores were then gold

sputtered with 10 nm gold in an electron sputter (Sputter Coater 108auto, from Cressington). Samples were imaged using a Hitachi TM3000 scanning electron microscope (SEM), at 15 kV. Spores for energy-dispersive X-ray (EDX) spectroscopy were prepared as described above and analysed using the x-ray spectroscope Quantax 70 (Bruker Corporation) in the TM3000 SEM from Hitachi. The analysis was performed at 15 kV, for a working distance of 8,5 mm, a magnification of 18.000x and an acquisition time of 200 seconds. The elements dosed were calcium and sulphur.

Transmission electron microscopy. Samples were collected from BHI cultures 72 hours after inoculation. Cells or density gradient-purified *C. difficile* spores (prepared as above) were processed for thin sectioning transmission electron microscopy as described before (Pereira *et al.*, 2013).

Atomic Force Microscopy. A suspension of density gradient purified spores (3 µl; as described above) were applied to a microscope slide and left to dry. A Di (Veeco/Bruker) Dimension 3100 microscope with a Nanoscope 3a controller was used for observations. Measurements were performed in air in tappingtm mode with Bruker OTESP (Olympus) tips (resonance frequency is tip dependent, but close to 280kHz). In tapping mode the tip is oscillated at high frequency touching the surface as the sample is scanned, reducing drag forces.

Virulence assays. Two groups of adult female hamsters (*Mesocricetus auratus*) weight of 90-100g, were treated with clindamycin (50 mg/kg) five days before challenge with purified *C. difficile* spores (above). At day 0, the animals were challenged with spores as follows: group 1 (6 animals), challenged with 2 x 10³ wild-type spores per animal; group 2 (8 animals), challenged with 7 x 10³ *cdeM::ermB* spores per animal. The protocols used were approved by the Animal Central

1024 Department of the Paris-Sud university.

1025

1026 ***Ex vivo* spore binding assays.** Six adult female hamsters (90-100g) were

1027 euthanized and six samples of the caeca (approximate size) removed and

1028 reversed. The caeca samples were washed 8 times in PBS. Each caeca sample

1029 was divided in two pieces and incubated with 10^6 wild-type or *cdeM::ermB* spores

1030 for 1, 2 and hours, after which the sections were washed 4 times in PBS, weighted

1031 and 10 ml of PBS added. The suspension was grinded for 1 min in an Ultra-Turrax

1032 homogenizer (IKA). Serial dilutions were then plated onto Columbia blood agar

1033 plates containing cysteine (concentration?) and taurocholic acid (concentration?),

1034 in duplicate.

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Figure legends

Figure 1 – Sporulation and infection by *C. difficile*. **A:** Schematic representation of the *C. difficile* infectious cycle with emphasis on the role of the spore. Infection starts with spore ingestion. Spores germinate in the intestine, in response to bile salts in the form of cholate, which are more abundant than inhibitory deoxycholate salts (CA<<CDCA). Spore germination seems to occur in close contact with epithelial cells. “R” is a putative spore receptor at the surface of epithelial cells. Following spore germination and outgrowth, a population of vegetative cells is established that produces the TcdA and TcdB toxins and spores. Shedding of spores from the infected organism completes the cycle. **B:** Stages in spore morphogenesis: *a*, asymmetric septation; *b*, an intermediate stage in engulfment; *c*, engulfment completion; *d*, phase grey spores; *e*, phase bright spores; *f*, free spores. The main spore layers are represented as follows: yellow, core; brown, cortex; blue, coats; red, crust or exosporium. The four cell-type specific sigma factors that control spore morphogenesis and their windows of activity with respect to the stages in morphogenesis are also indicated.

Figure 2 – *cdeM* is expressed in the mother cell during late stages of spore morphogenesis. **A:** the *cdeM* region of the chromosome of strain 630Δ*erm*, encompassing 11 kb of DNA, and the *cdeM* promoter region. The transcription start site (+1, yellow), as mapped by RNAseq, and the -10 and -35 promoter elements (green) that match the consensus for σ^K binding are indicated. The ribosome binding site (RBS, shaded in grey) and the *cdeM* start codon are also indicated. **B:** Effect of mutations on the indicated regulatory genes on the level of the *cdeM* transcript as measured by qRT-PCR and represented as the WT/mutant ratio. **C:** expression of

transcriptional P_{cotE} -SNAP^{Cd}, P_{cdeC} -SNAP^{Cd}, and P_{cdeM} -SNAP^{Cd} fusions in relation to spore morphogenesis. The cells were collected after 24h of growth in SM liquid medium and subject to immunofluorescence microscopy (IMF) with an anti-SNAP antibody. Arrows: blue, phase grey spore; red, phase bright spore; white, strong mother cell-specific signal; grey, weak mother cell signal. Scale bar, 1 μ m. **D**: the percentage of sporangia of phase grey and phase bright spores expressing P_{cotE} -SNAP^{Cd}, P_{cdeC} -SNAP^{Cd}, or P_{cdeM} -SNAP^{Cd} is shown. **E**: the intensity of the fluorescence signal (low versus high; see material and methods for a definition) is shown for the sporangia of phase grey (G) or phase bright (B) spores, for strains expressing P_{cotE} -SNAP^{Cd}, P_{cdeC} -SNAP^{Cd}, and P_{cdeM} -SNAP^{Cd} fusions, as indicated.

Figure 3 – Localization of CdeM in spores. **A**: shows the SDS-PAGE analysis of the proteins extracted from purified spores of the wild type strain 630 Δ erm (WT), the *cdeM::ermB* mutant, and the mutant complemented with either *cdeM* in a multicopy plasmid (pFT43) or integrated into the genome in single copy (Tn916::*cdeM*). The gel was stained with Coomassie. Red arrows indicate proteins, identified by mass spectrometry in the WT (see text for details); the green arrows indicate the position of forms of CdeM. **B**: Immunoblot analysis of the gel in A with anti-CdeM (top) or anti-CdeC (bottom) antibodies. The black arrows indicate CdeC; in A and B, the green arrows indicate forms of the CdeM protein and the asterisks indicate species that cross-react with the anti-CdeM antibody. The position of molecular weight (MW) markers (in kDa) is indicated. **C**: localization of CdeM-SNAP^{Cd} in spores by fluorescence microscopy. Spores of a 630 Δ erm derivative producing CdeM-SNAP were labeled (+TMR-Star) or not (no TMR-Star) with the SNAP substrate TMR-Star and examined by fluorescence microscopy. The yellow arrows point to the strong signal associated with one of the spore poles. **D**: immunofluorescence analysis

1297 of WT and *cdeM::ermB* spores using an anti-CdeM antibody and an Alexa-488-
1298 conjugated secondary antibody. The yellow arrows point to a polar fluorescence signal
1299 and the white arrows to signals located along the long axis of the spore. The numbers
1300 refer to the percentage of spores showing the pattern represented in schematic form
1301 below. Scale bar in C and D, 1 μ m.

1302 **Figure 4 – CdeM is required for proper assembly of a spore polar structure. A:**

1303 Phase contrast (PC) and fluorescence microscopy images of the two main types of
1304 spores produced by a WT strain, the *cdeM* mutant and the complementation strain
1305 (Tn916::*cdeM*, or *cdeM^C*) following staining by FM4-64. The black arrow points to a
1306 polar structure that in the WT and the complementation strain is partially refractile
1307 (type A appendage); this structure is shorter and phase dark in the *cdeM::ermB* mutant
1308 (type B appendage). Note that neither type of appendages stains with FM4-64 (yellow
1309 arrow). The numbers refer to the percentage of type A (top) and type B (bottom)
1310 appendages scored for each strain. Scale bar, 1 μ m. **B:** The top diagram shows the
1311 measures of length and width measured for spores in phase contrast (black) and FM4-
1312 64 images (red). Note that the width coincides for the images. The bottom panel is a
1313 box plot showing the median, lower and upper quartiles of the distance measured (in
1314 μ m), for each of the indicated strains, in the phase contrast or FM4-64 images. Each
1315 point is a measurement of a single spore. Pairwise differences between the strains are
1316 statistically significant for all measures ($P < 0.05$; Kruskal-Wallis rank sum test for
1317 width and length Ph. Contrast; ANOVA for length FM4-64), except for those
1318 comparisons denoted by a black bar connecting them and labeled by N.S. (not
1319 significant). **C:** transmission electron microscopy of thin sections of WT, *cdeM::ermB*
1320 and *cdeM^C* spores. The two top rows of panels show TEM images of longitudinal
1321 sections whereas the third row are cross sections. The yellow arrows point to the polar

1322 appendage region and the green arrow points to a region between the coat and the
1323 appendage seen in WT spores; the red arrows point to the lamellar coat, and the blue
1324 arrow to the more external, exosporium-like layer, that is reduced in *cdeM::ermB*
1325 spores, or to the electrondense material that remains attached to *cdeM* spores. Cr,
1326 spore core; Cx, spore cortex. Scale bar for the three top rows of panels, 0.2 μ m. The
1327 two bottom panels are SEM images of WT and *cdeM* spores. The yellow arrow points
1328 to the spore body and the red arrow to the appendage. Scale bar for the two bottom
1329 panels, 1.0 μ m. D: scanning electron microscopy of a WT and a *cdeM::erm* spore. The
1330 yellow label points to the spore body and the yellow arrow to the appendage region.
1331 Scale bar, 0.2 μ m. **Figure 5 – Surface features of WT and *cdeM::ermB* spores. A:**
1332 atomic force imaging of purified WT and *cdeM::ermB* spores. Shown are topography
1333 (top panel), amplitude (middle) and phase (bottom) images. The amplitude image is a
1334 spatial derivative of the topography image, which is very sensitive to small, sharp
1335 features. The phase image depicts the phase shift between the excitation signal and
1336 the tip oscillation response, and it is sensitive to surface hardness. The white arrows in
1337 the amplitude images point to the spore polar appendage region. The white arrows in
1338 the phase image of WT spores point to a region of decreased rigidity that runs along
1339 the longitudinal axis of the spore. The inserts show the polar region of the spore. Scale
1340 bar, 1 μ m. **B:** scanning electron microscopy analysis of purified WT and *cdeM::ermB*
1341 spores before (top row) or after treatment with beads (second row), 10 mM DTT (third
1342 row) and beads in the presence of 10 mM DTT (bottom row). The yellow arrow points
1343 to a spore with a partially disrupted shell; the white arrows point to empty shells. The
1344 two bottom panels show higher magnification images of the shells obtained following
1345 the beads/DTT treatment. Scale bar, 3 μ m. **C:** shows the EDX analysis of the samples
1346 in B after treatment with beads in the presence of DTT. The EDX signal is shown for
1347 calcium (red) and sulfur (blue). The two bottom panels show the calcium and sulfur

1348 signals (same color code) after inducing germination of WT spores with 5%
 1349 taurocholate (TA). **D**: suspensions of spores of the indicated strains were centrifuged
 1350 and proteins in the supernatant or sediment fractions were extracted and resolved by
 1351 SDS-PAGE (top panels; Coomassie-stained gels). The presence of the CdeM and
 1352 CdeC proteins was then monitored by immunoblotting (middle and bottom panels).
 1353 Black arrows show the position of CdeC or CdeM; red arrows show the position of
 1354 multimeric species of CdeC or CdeM. The blue arrow shows the position of a species
 1355 that cross-reacts with the anti-CdeM antibody and the asterisk the position of a
 1356 possible CdeC degradation product. Molecular weight markers (in kDa) are shown on
 1357 the left side of the panels.

1358 **Figure 6 – Appendage-bearing spores are enriched in multimeric forms of CdeM**
 1359 **and CdeC. A:** Purified spores of the WT and *cdeM::ermB* mutant were separated on
 1360 discontinuous gradients of metrizoic acid (from 40 to 60%) and resolved in bands at
 1361 45%, 50% and 55% (the top panel illustrates the separation for WT spores). The
 1362 bottom series of panels shows the analysis of the three fractions by scanning electron
 1363 microscopy. The white arrow point to smooth spores, lacking an exosporium-like layer;
 1364 the yellow arrows point to spores with an exosporium-like surface layer, which is
 1365 continuous with the spore polar appendage. Scale bar, 2 μ m. **B:** Box plots showing the
 1366 median, lower and upper quartiles of the distance measured (in μ m), for the initial
 1367 spore suspension, and for the spores collected in the 45%, 50% and 55% fractions of
 1368 the metrizoic acid gradients. Each point is a measurement of a single spore. Pairwise
 1369 comparisons between fractions are statistically significant for both WT and
 1370 *cdeM::ermB* spores ($P < 0.05$; Kruskal-Wallis rank sum test for length in the
 1371 Ph.contrast and length in the FM4-64 images; ANOVA for width), except for those
 1372 comparisons denoted by a black bar connecting them and labeled by N.S. The legend

1373 applies to all panels. **C:** the spores in the 45%, 50% and 55% bands, obtained as
 1374 described in panel A, were collected, proteins extracted, resolved by SDS-PAGE (top
 1375 panel; Coomassie-stained gel) and subject to immunoblot analysis with anti-CdeM and
 1376 anti-CdeC antibodies (middle and bottom panels). Black arrows show the position of
 1377 CdeC or CdeM in Coomassie; red arrows show the position of multimeric species of
 1378 CdeC or CdeM. The blue arrow shows the position of a species that cross-reacts with
 1379 the anti-CdeM antibody. The asterisk shows the position of possible CdeM or CdeC
 1380 degradation products. The position of molecular weight markers (in kDa) is shown on
 1381 the left side of the panels.

1382 **Figure 7 – CdeM and CdeC are present in isolated exosporia. A:** following
 1383 treatment with beads in the presence of 10 mM DTT, suspensions of WT and
 1384 *cdeM::ermB* spores were separated on step gradients of metrizoic acid. The material
 1385 present in bands at 42%, 45% and 50% layers was collected and analyzed by phase
 1386 contrast and SEM. Note the enrichment of empty shells in the 42% layer. The yellow
 1387 arrows indicate empty exosporium shells; the red arrows indicate the position of
 1388 appendage/exosporium-bearing spores, enriched in the 55% fraction. Higher
 1389 magnification images of the empty shells released from WT spores are shown at the
 1390 bottom. Scale bar, 1 μ m. **B:** SDS-PAGE (top) and immunoblot analysis of the 42% and
 1391 55% gradient fractions with anti-CdeM, anti-CdeC and anti-BclA1 antibodies. Black
 1392 arrows show the position of CdeC, CdeM or BclA1; red arrows show the position of
 1393 multimeric species of CdeC or CdeM. The asterisk shows the position of a possible
 1394 CdeM degradation product. Molecular weight markers (in kDa) is shown on the left
 1395 side of the panels. **C:** purified WT and *cdeM::ermB* spores were analyzed by flow
 1396 cytometry with the indicated antibodies and subject to immunofluorescence

1397 microscopy (**D**) with anti-CdeC and anti-BclA1 antibodies. The yellow arrows indicate
1398 spores with a strong fluorescence signal. AU, arbitrary units. Scale bar, 1 μ m.

1399 **Figure 8 – The polar appendage and CdeM have opposite effects on spore**

1400 **germination. A:** Germination curves for spores of the WT strain, the *cdeM::ermB*

1401 mutant, the complementation strain *cdeM::ermB/Tn916::cdeM* and a *sleC* mutant.

1402 Purified spores were heat activated in PBS and germination induced by the addition of

1403 TA, 5% (filled symbols). In control experiments, spores were heat activated but no TA

1404 was added (open symbols). Germination was followed by the decrease in the OD_{600nm}

1405 over time at 37°C under anaerobic conditions. **B:** The kinetics of germination was

1406 monitored as described in panel A, except that the WT and *cdeM::ermB* spore

1407 suspensions were fractionated on gradients of metrizoic acid and the 45% (enriched in

1408 spores with no appendage) and 55% (enriched in appendage bearing spores) fractions

1409 used.

1410 **Figure 9 – Increased virulence caused by spores of a *cdeM::ermB* mutant. A:**

1411 Kaplan-Meier survival curve representing the cumulative results from two independent

1412 experiments in which clindamycin-treated Syrian golden hamsters were inoculated

1413 with WT or *cdeM::ermB* spores. Mean times to morbidity were: WT, 3.5 hours;

1414 *cdeM::ermB*, 2.6 hours ($P < 0.01$, log rank test). **B:** *ex vivo* adhesion of WT and

1415 *cdeM::ermB* spores to hamster caecum. Caeca were collected from hamsters, washed

1416 extensively, weighted, and incubated with 10^6 WT or *cdeM::ermB* spores for the

1417 indicated times. After washing and grinding, binding spores were determined by

1418 plating onto blood agar plates containing taurocholic acid. The results are expressed

1419 as the number of spores per gram of caeca. **C:** model for the regulation of assembly of

1420 the coat and exosporium of *C. difficile* spores. The onset of expression of *cotE* (prior to

1421 engulfment completion), *cdeC* (following engulfment completion when the forespore

1422 turns phase dark, under the joint control of σ^K and SpoIIID) and *cdeM* (when the
1423 forespore becomes phase bright) is represented as a function of the stages in
1424 morphogenesis. “X” is a putative regulatory protein, either an activator or a repressor,
1425 thought to delay expression of the σ^K -controlled *cdeM* gene (see text for details). **D:**
1426 model for the arrangement of CdeM and CdeC in *C. difficile* spores. CdeC is
1427 represented as being part of basal layer upon which CdeM assembles conferring
1428 electrondensity to the outer layers of spores. CdeM, however, may also be located in
1429 more internal layers of the coat. In the absence of CdeM, several other proteins,
1430 including BclA1 and a CdeC, become more extractable. CdeM is found around the
1431 entire spore, but multimeric forms are enriched in the spore polar appendage. **E:**
1432 characteristics of the two main spore morphotypes (1, with a well developed
1433 appendage; 2, with a short or non-existent appendage). Spores of morphotype 1 may
1434 germinate in contact with the colonic mucosa; spores of morphotype 2 do not colonize
1435 efficiently, but may germinate more promptly in vivo and show enhanced virulence.