



HAL
open science

The blueprint for building a biofilm the Clostridioides difficile way

Yannick Dn Tremblay, Bruno Dupuy

► **To cite this version:**

Yannick Dn Tremblay, Bruno Dupuy. The blueprint for building a biofilm the Clostridioides difficile way. Current Opinion in Microbiology, 2022, 66, pp.39-45. 10.1016/j.mib.2021.12.002 . pasteur-03579985

HAL Id: pasteur-03579985

<https://pasteur.hal.science/pasteur-03579985>

Submitted on 8 Jan 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **The blueprint for building a biofilm the *Clostridioides difficile* way**

2

3 Yannick D.N. Tremblay^{a,b,#} and Bruno Dupuy^{a,#}

4 ^a Institut Pasteur, Université de Paris, UMR-CNRS 2001, Laboratoire Pathogenèse des Bactéries
5 Anaérobies, F-75015 Paris, France

6 ^b Department of Biochemistry, Microbiology and Immunology, University of Saskatchewan,
7 Saskatoon, Canada

8 # To whom correspondence should be addressed:

9 YDNT: 6B65, Health Sciences Building, Department of Biochemistry, Microbiology and
10 Immunology, University of Saskatchewan, 107 Wiggins Rd Saskatoon, SK, S7N 5E5 306-966-
11 6278, yannick.tremblay@usask.ca

12 BD: Laboratoire Pathogenèse des Bactéries Anaérobies, Institut Pasteur, 25 rue du Docteur
13 Roux, 75724 Paris cedex, +33140613175, bruno.dupuy@pasteur.fr

14

15 **Highlights:**

- 16 - *Clostridioides difficile* can form aggregate biofilms and surface-attached biofilms
- 17 - Transition to a biofilm state is dependent on the production of c-di-GMP
- 18 - Cyclic-di-GMP prevents flagellar motility and promotes type IV pili production
- 19 - Initiation of biofilm formation requires metabolic adaptation for long-term viability
- 20 - Metabolic reprogramming is controlled by major transition phase regulators

21

22

23 **Abstract:** *Clostridioides difficile* is an opportunistic pathogen that causes by a high rate of
24 recurrent infections. Persistence in the gastrointestinal tract is thought to be mediated by
25 sporulation and/or biofilm formation. There is an increase interest in *C. difficile* biofilm formation
26 and recent findings have provided a framework to model surface-attached biofilm formation. For
27 *in vitro* biofilm formation, *C. difficile* must undergo a metabolic reprogramming as it enters
28 stationary phase. This helps maintain long-term viability and increases responsiveness to signals
29 leading to biofilm formation. Metabolic reprogramming and biofilm formation requires several
30 regulatory factors and these overlap with the sporulation cascade. Despite recent advances,
31 further research is needed to answer outstanding questions in the field.

32

33 Introduction

34 *Clostridioides difficile* is an opportunistic pathogen of rising public health concern due to
35 increased resistance to the antibiotics used to treat the infections [1]. *C. difficile* infections (CDI)
36 are a leading cause of hospital-acquired diarrhea and are typically associated with antibiotic-
37 induced dysbiosis [1]. Disruption of the microbiota changes the nutritional landscape of the gut
38 and alters the concentration of microbial metabolites that have anti-*C. difficile* properties [2]. For
39 example, secondary bile acids such as deoxycholate (DOCA) and short chain fatty acids (SCFA) are
40 critical for colonization resistance against *C. difficile* [3]. Protective metabolites produced by the
41 microbiota are slowly restored after completion of antibiotic treatment or fecal microbiota
42 transplant. However, more than 30% of patients that received antibiotics to treat their first CDI
43 relapse and 40% of the relapses are caused by the same strain that first infected the patient [1].
44 This suggests that *C. difficile* can persist in the gastrointestinal tract.

45 Based on current evidence, persistence and recurrence is thought to be mediated by sporulation,
46 biofilm formation, or a combination of the two [4][5]. Evidence for sporulation is based on the
47 ability of spores to enter epithelial cells [6] and the inability of a non-sporulating *spoOA*-
48 inactivated strain to cause relapses [7]. However, relapses in *C. difficile* infections are only
49 reduced when spore entry into epithelial cells is blocked [6] and *spoOA* controls other
50 physiological processes such as metabolism and biofilm-formation [8]. These findings strongly
51 suggest that persistence is not solely dependent on sporulation. Evidence suggesting a role for
52 biofilm formation in relapses includes the ability of *C. difficile* to incorporate into multi-species
53 biofilms formed by the gut microbiota [9][10] and biofilm-like structures are detectable on the

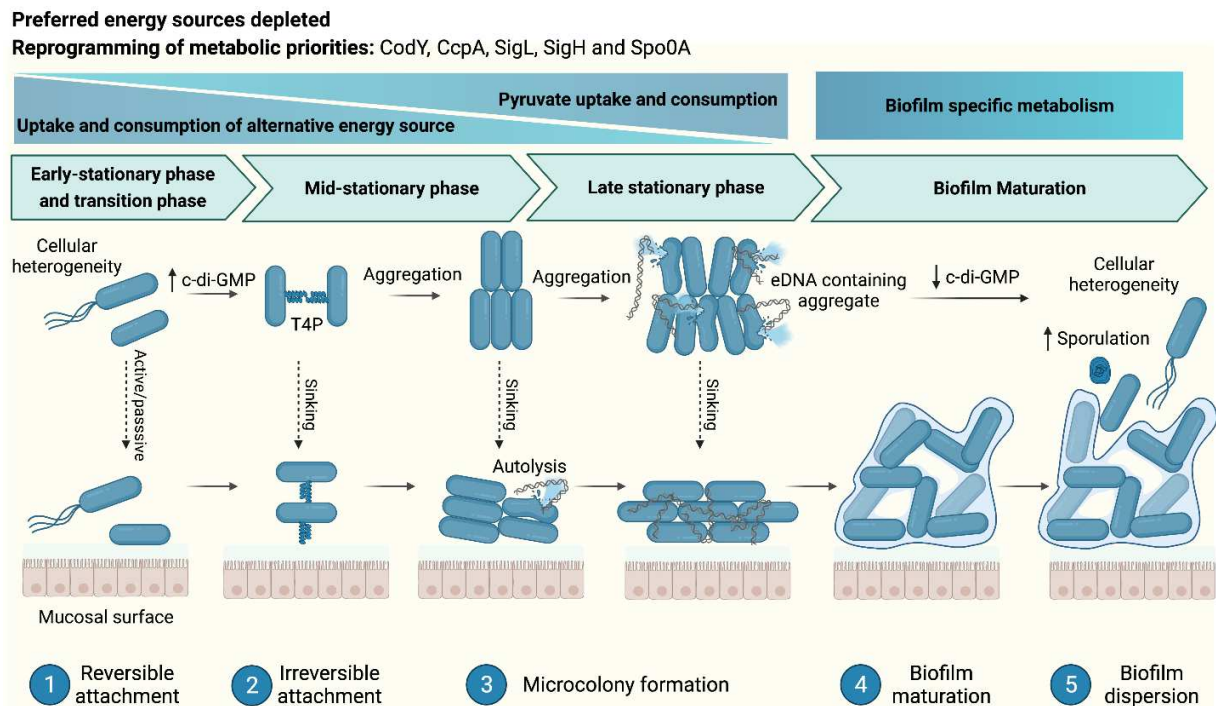
54 epithelium or mucus layer of infected animals [11][12][13]. Given the potential importance of
55 biofilm formation in *C. difficile* infection, researchers are trying to understand the genetic factors,
56 molecular events and the regulatory pathways leading to biofilm formation in *C. difficile*.

57 **The biofilm building blocks: general characteristics of *C. difficile* biofilms**

58 Using different models, growth conditions and technical approaches (reviewed in [5]), the genetic
59 factors, physiological processes, and matrix components of *C. difficile* biofilms were recently
60 identified. In a mature biofilm, extracellular DNA (eDNA) is the major component of the biofilm
61 matrix whereas polysaccharides and proteins are minor components [14][15][16][17]. However,
62 proteins play an important role during the early steps of biofilm formation [14][15]. The role of
63 eDNA in biofilm formation is to help *C. difficile* cells stick together in large aggregates or
64 structures (Figure 1). Release of eDNA from cells for biofilm formation is thought to occur by
65 prophage-induced lysis, sporulation, or autolysis during stationary phase [14][16][18] and current
66 evidence supports autolysis as the main mechanism contributing to eDNA. Indeed, the absence
67 of autolytic enzymes such as the lytic transglycosylases Cwp19 completely abolishes biofilm
68 formation [14]. Other autolysins are probably involved but have not been tested. Overall,
69 controlling cellular lysis is likely critical for biofilm formation in *C. difficile*.

70 The teichoic-like acid PS-II is detected in the biofilm matrix [14][15] although it is not clear how
71 PS-II is organized within the biofilm matrix. Cellulose might contribute to the biofilm matrix since
72 a secreted polysaccharide composed of acetylated glucose subunits is detected in culture
73 supernatants [19]. Moreover, the genome of *C. difficile* encodes for proteins predicted to
74 synthesize cellulose [20]. However, deletion of the *bscA* orthologue (*ccsA*), encoding a

75 glycosyltransferase involved in cellulose synthesis, did not significantly alter biofilm formed in
 76 the presence of DOCA [21]. Therefore, the identity and the exact role of the polysaccharides
 77 involved are not characterized.



78
 79 **Figure 1: Model of biofilm formation and regulation in *Clostridioides difficile*.** This model has two
 80 different modes of biofilm formation: aggregate biofilm and attached biofilm. Both modes are dependent
 81 on metabolic reprogramming following the depletion of the preferred energy source. This reprogramming
 82 is controlled by regulation factors such as CodY, CcpA, SigL, SigH and Spo0A and is required for *C. difficile*
 83 to use alternate source of energy such as glycine and cysteine, and eventually, pyruvate. This ensures that
 84 *C. difficile* maintains high viability during stationary phase while chromosomal DNA (extracellular DNA
 85 [eDNA]) is released by dying cells. Once enough eDNA has accumulated. *C. difficile* can then pass the
 86 “biofilm threshold”. In the aggregate biofilm, cyclic-di-GMP (c-di-GMP) production is increase in response
 87 to environmental cues or changes in cellular heterogeneity of the population. This leads to the production
 88 of type IV pilus (T4P) and auto-aggregation. As time progresses, the aggregates grow via auto-aggregation
 89 or increase presence of eDNA release by autolysis. Aggregates can sink and start or contribute to the
 90 development and maturation of an attached biofilm. (1) In the attached biofilm model, reversible
 91 attachment to surfaces is likely mediated by the flagellum, surface structures or general adhesins. (2) Once
 92 attached, signal transduction systems will induce c-di-GMP accumulation, which initiates the production
 93 of T4P and surface proteins such as CD630_2831 and CbpA. These newly synthesized adhesion molecules
 94 will provide stronger binding to the surface. (3) The tightly attached bacteria will multiply and start
 95 forming a microcolony. During microcolony formation, autolysis will release chromosomal DNA and will
 96 contribute to the biofilm matrix. (4) Maturation will lead to reorganization of the biofilm structure and

97 metabolism, including metabolic specialization of sub-populations. (5) As time progresses, subpopulations
98 will undergo different processes such as phase variation that will create cellular heterogeneity. This will
99 lead to sporulation, decrease c-di-GMP concentrations and will ultimately cause dispersion of the biofilm.

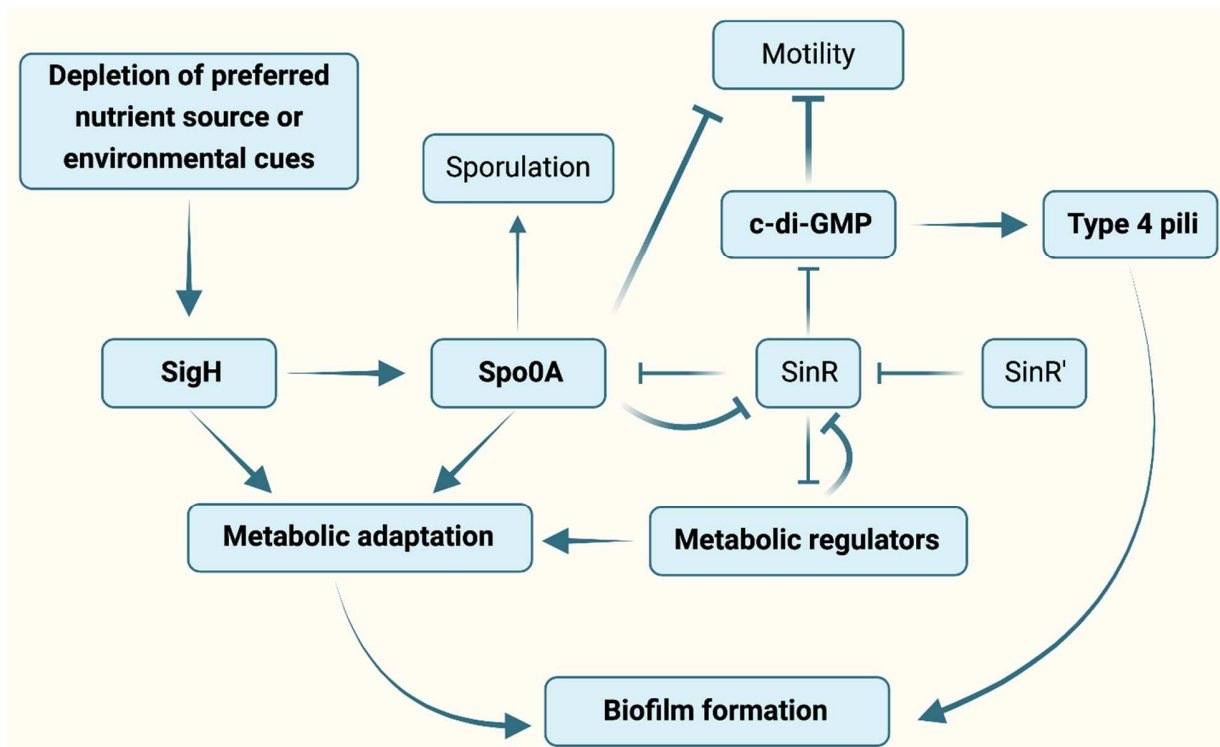
100 Events at the cell surface will also have an impact on biofilm formation. For example, S-layer
101 processing by the cysteine protease Cwp84 is critical for maintaining cellular hydrophobicity and
102 proper processing of surface proteins, which are important features for surface adhesion and
103 cell-cell attachment. Inactivation of Cwp84 can abolish or increase biofilm formation in a strain
104 dependent manner [15][22]. The most important surface structure appears to be the type IV pilus
105 (T4P) encoded by the *pilA1* locus [21], which is important for early steps in biofilm formation but
106 is dispensable during the later stages [23]. Among the other surface proteins, the fibronectin-
107 binding protein FbpA [24] and two collagen binding proteins, CbpA (CD630_31450) [25] and
108 CD630_28310 [16][26], contribute to biofilm formation. Finally, the flagellum is another
109 important adhesion factor although *C. difficile* can still form biofilms without flagellum and with
110 a non-motile flagellum [15][27].

111 Several genetic factors are indirectly involved in biofilm formation due to their pleiotropic effects
112 on *C. difficile* physiology. These include the RNA chaperone Hfq [28], the Ser/Thr kinase PrkC [29],
113 the protein chaperone DnaK [30] and the SOS-response regulator LexA [31]. For example,
114 absence of DnaK and LexA affects motility [30][31], and PrkC affects cell division and lysis [32].
115 These effects could explain the impact of DnaK, LexA or PrkC on biofilm formation. Therefore,
116 these should not be viewed as regulators of biofilm formation.

117 **How and when the biofilm building blocks are assembled: physiology and**
118 **regulation behind biofilm formation**

119 Based on recent advances in the *C. difficile* field and new biofilm formation models in bacteria
120 [33], we are proposing a model for surface-attached biofilm formation of *C. difficile* (see Figure
121 1) that incorporates two different modes of biofilm formation: aggregate biofilm and surface-
122 attached biofilm. Through this proposed model, a framework for the regulation of each biofilm
123 formation stage can be proposed as described in the next section.

124 **Pre-conditioning of planktonic cells:** Biofilms formed by *C. difficile* are typically measured 12h to
125 7 days after inoculation [8][17][23][34]. This indicates that biofilm formation is mainly induced in
126 the stationary phase. The transition from exponential phase to stationary phase requires
127 metabolic adaptation and reprogramming as preferred energy sources become depleted. This
128 adaptation and reprogramming are controlled by several regulators (see Figure 1 and 2) including
129 CcpA, CodY, and the sigma factors SigL and SigH, that are required for biofilm formation
130 [8][14][15][21][34][35]. Inactivation of the sporulation master regulator Spo0A results in
131 decreased biofilm formation in different growth conditions and models [8][14][15]. Spo0A is
132 controlled by SigH, the key sigma factor of transition phase, and also by SinR whose activity is
133 controlled by SinR'. The absence of SinR increased biofilm formation [34], but had no measurable
134 effect on DOCA-induced biofilms [21]. Based on the current literature, we think that SinR-SinR'
135 represses biofilm formation by rerouting Spo0A regulatory activity towards sporulation. This is
136 consistent with the fact that the sporulation cascade is probably not required since inactivation
137 of SigE or SigF had no measurable effect on DOCA-induced biofilm formation [14]. Therefore, the
138 role of SigH and Spo0A in controlling metabolic adaptation and decreasing motility is critical for
139 biofilm formation (Figure 2).



140
 141 **Figure 2: Proposed regulatory network controlling the biofilm-sporulation nod.** Under biofilm forming
 142 conditions, SigH and Spo0A control metabolic adaptation of cells entering a nutrient limited environment.
 143 Metabolic regulators and Spo0A inhibit SinR activity leading to up-regulation of c-di-GMP and Type 4 pili,
 144 and down-regulation of motility. Bolded font and line size indicate the dominant pathways.

145 Metabolic adaptation and reprogramming are required for *C. difficile* to stay metabolically active
 146 and viable during stationary phase. At the end of the exponential phase, preferred nutrient
 147 sources are depleted and this leads to the down-regulation of the preferred metabolic pathways
 148 and the up-regulation of alternative pathways [21][34][36]. Specifically, glycolysis and the
 149 pentose phosphate pathway are downregulated, and butanoate and propanoate fermentation
 150 become up-regulated (Figure 1) [21][34]. The latter process probably uses acetyl-CoA,
 151 oxaloacetate and pyruvate, which might be produced by glycine metabolism [37]. Furthermore,
 152 the Wood-Ljungdhal pathway and the glyoxylate shunt are important to generate energy to
 153 induce biofilm formation [34]. In the presence of DOCA, *C. difficile* relies on proteins degradation,
 154 peptides and amino acids uptake, and the oxidative Stickland fermentation pathways to produce

155 energy to drive biofilm formation [21]. Such metabolic adaptation and reprogramming are
156 dependent on the available nutrients and excreted metabolites. Overall, pyruvate, which is one
157 of the excreted metabolites, produced from the available precursors is a critical inducer of biofilm
158 formation [21][34][36]. The level of pyruvate is sensed by the two-component regulatory system
159 encoded by CD630_2601 and CD630_2602, which then probably induce expression of pyruvate
160 transporters including CstA [21].

161 Additionally, intracellular levels of the alarmone pGpp and cyclic-di-GMP (c-di-GMP) can also
162 increase during the transition to the stationary phase in response to changes in nutrient
163 availability [35][38][39][40], and for the latter, to induce biofilm formation during stationary
164 phase [34]. Overall, metabolic reprogramming prepares and improves the responsiveness of
165 planktonic cells to signals leading to biofilm formation. In the host, this reprogramming will be
166 influenced by the availability of nutrient such as mucus derived sugars [41][42] or microbiota-
167 derived metabolites [14][21].

168 **Attachment and aggregation:** Reversible attachment to a surface is an important event initiating
169 the biofilm formation cascade and, in *C. difficile*, this could be mediated by the flagellum. In
170 support of this, the absence of flagella rotation increased biofilm whereas complete absence of
171 the flagella did not [27][43]. Furthermore, highly motile clinical isolates formed more biofilm than
172 clinical isolates with no or low motility [44]. On the other hand, the absence of a flagellum also
173 influences auto-agglutination and sedimentation leading to an increase in biomass accumulation
174 in static biofilm models [15][27][43]. Adhesion via the flagellum is likely relevant for biofilms
175 formed in the gut as the interaction between the *C. difficile* flagellum and the *Fusobacterium*
176 *nucleatum* adhesin RadD enhanced biofilm formation [45].

177 Surface adhesion or nutritional signals will induce an increase in c-di-GMP level that turns off
178 flagellar motility via a type I riboswitch and induces production of the T4P via a type II riboswitch
179 (Figure 2) [39][46]. This would lead to a stronger attachment for cells at the surface (i.e.
180 irreversible attachment) or increase aggregation for planktonic cells [47]. Production of other c-
181 di-GMP controlled surface proteins, such as CD630_28310 and CbpA, will also increase surface
182 adhesion.

183 **Microcolony formation and maturation:** Once *C. difficile* irreversibly attaches, the cells will form
184 a larger cluster called microcolony. This process is mediated by cell division, large aggregate
185 sedimentation and production of the biofilm matrix. Cell division is supported by the metabolic
186 state of the bacteria and metabolic reprogramming controlled by regulatory factors such as CcpA,
187 CodY, SigL, SigH and Spo0A. Extracellular pyruvate and glycine metabolism, which is mediated by
188 glycine riboswitches [48], are probably also involved in this maturation steps [21][34]. This
189 reprogramming is required for a majority of *C. difficile* cells to maintain high viability while a small
190 fraction of the population releases chromosomal DNA, which accumulates in the biofilm. When
191 there is enough eDNA, *C. difficile* can then pass the “biofilm threshold”.

192 Control of cell lysis is critical at this point because it contributes eDNA to attached and aggregate
193 biofilms (Figure 1). As aggregate biofilms grow, these will sediment and start forming new or
194 contribute to pre-existing microcolony. Expression and production of extracellular
195 polysaccharides should also increase at this point. Recent studies suggested that *C. difficile* can
196 synthesize cellulose and produce an exopolysaccharide composed of acetylated glucose subunits
197 hypothesized to be cellulose but their roles remain unconfirmed [19][20].

198 Quorum-sensing molecules production should be at its peak during microcolony formation and
199 biofilm maturation as population density is reaching its peak and cells are in very close proximity.
200 The genome of *C. difficile* encodes two different quorum sensing systems: a LuxS/autoinduce-2
201 system and *agr* peptide system. The LuxS system affects biofilm formation under certain
202 conditions [15][18] but was dispensable in DOCA-induced biofilm formation [21]. LuxS is also
203 involved in sulfur metabolism and there is a lack of genetic evidence for an autoinducer-2
204 receptor in *C. difficile* [49][50]. Thus, the observed effect might be associated with metabolism
205 rather than quorum sensing. Strain 630 only has an incomplete *agr* type quorum sensing system
206 (*agr1: agrD1B1*) [51] which is not required for DOCA-induced biofilm formation [21]. However, a
207 complete (*agr2: agrACDB*) and an incomplete (*agr1: agrD1B1*) *agr* system are present in strain
208 R20291, whose role in biofilm formation is not known [51]. Therefore, there is still a need to
209 assess the role of quorum sensing in *C. difficile* biofilm formation.

210 Biofilm maturation leads to reorganization of the biofilm structure and metabolism, including
211 metabolic specialization of sub-populations. The creation of sub-populations typically leads to
212 division of labor or dispersion. Other mechanisms could also intervene within these sub-
213 populations as *C. difficile* also possess phase variation mechanisms that can influence flagellar
214 motility, and c-di-GMP phosphodiesterases (PDEs) expression, that affect colony morphology
215 [52][53][54]. Moreover, epigenetic regulation could also create population diversity as DNA
216 methylation by CamA affects biofilm formation [55].

217 **Dispersion:** Cell leaving the biofilm is an important step that is under studied in *C. difficile*.
218 Dispersion could be triggered by sporulation or decrease c-di-GMP levels, which could be driven
219 by an increase in phosphodiesterase activity. For example, the metalloprotease Zmpl is produced

220 when c-di-GMP levels are low [26][56]. This protease is exported to the cell wall, cleaves both
221 surface proteins CbpA and CD630_28310, and causes bacterial detachment from the surface
222 [26][56]. Other enzymes might also be exported to the cell surface to digest the eDNA (DNase)
223 or polysaccharides present in the matrix as *C. difficile* encodes a glycosyl hydrolase, CcsZ, within
224 the cellulose biosynthesis operon [20]. Sporulation could also cause biofilm collapse by
225 sequestering calcium from the biofilm matrix. This mechanism was recently identified in *Bacillus*
226 *subtilis* [57]. Furthermore, calcium is bound by eDNA, which is the major building block of the *C.*
227 *difficile* biofilm matrix and is important to maintain structural integrity of the biofilm [58].

228 **Conclusion**

229 We propose a biofilm formation model incorporating two different modes of biofilm formation:
230 aggregate biofilm and surface-attached biofilm. Our model is based on experimental data
231 generated using well plates and liquid medium; however, we think this model should help guide
232 future research on biofilm formation by *C. difficile*. We also must consider that some members
233 of the microbiota will influence *C. difficile* biofilm formation by providing nutrients (i.e., cross-
234 feeding) or helping adhesion (for examples see [42][45]).

235 Development of new tools for genetically modifying *C. difficile* strains [59], will allow molecular
236 dissection of the regulation cascade leading to biofilm formation and answer outstanding
237 questions in this important field (see Box 1).

238 **Conflict of interest statement.**

239 The authors have no conflict of interest to declare.

240

241 **Acknowledgements.**

242 The authors would like to acknowledge the Institut Pasteur (BD) and the University of
243 Saskatchewan, Saskatoon, Canada (YDNT). A special thanks to Jazmin Meza-Torres for her help
244 with the figures and to Jenny-Lee Thomassin for help with proofreading the manuscript.

245 **References**

- 246 1. Crobach MJT, Vernon JJ, Loo VG, Kong LY, Péchiné S, Wilcox MH, Kuijper EJ:
247 **Understanding *Clostridium difficile* colonization.** *Clin Microbiol Rev* 2018, **31**: e00021-17.
- 248 2. Abbas A, Zackular JP: **Microbe–microbe interactions during *Clostridioides difficile***
249 **infection.** *Current Opinion in Microbiology* 2020, **53**:19–25.
- 250 3. Theriot CM, Koenigsnecht MJ, Carlson PE, Hatton GE, Nelson AM, Li B, Huffnagle GB, Z.
251 Li J, Young VB: **Antibiotic-induced shifts in the mouse gut microbiome and metabolome**
252 **increase susceptibility to *Clostridium difficile* infection.** *Nat Commun* 2014, **5**:3114.
- 253 4. Frost LR, Cheng JKJ, Unnikrishnan M: ***Clostridioides difficile* biofilms: A mechanism of**
254 **persistence in the gut?** *PLoS Pathog* 2021, **17**:e1009348.
- 255 5. Meza-Torres J., Auria E., Dupuy B., Tremblay YDN: **Wolf in Sheep’s clothing: *Clostridioides***
256 ***difficile* biofilm as a reservoir for recurrent infections.** *Microorganisms* 2021, **9**:1922.
- 257 6. Castro-Córdova P, Mora-Uribe P, Reyes-Ramírez R, Cofré-Araneda G, Orozco-Aguilar J,
258 Brito-Silva C, Mendoza-León MJ, Kuehne SA, Minton NP, Pizarro-Guajardo M, et al.: **Entry**
259 **of spores into intestinal epithelial cells contributes to recurrence of *Clostridioides***
260 ***difficile* infection.** *Nat Commun* 2021, **12**:1140.

261 • Using different approaches, the authors showed that spores can enter epithelial cells and
262 survive antimicrobial treatments. Once the treatment is stopped, engulf spores causes a
263 relapse.

264 7. Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF,
265 Dougan G, Lawley TD: **The *Clostridium difficile* spo0A gene is a persistence and**
266 **transmission factor.** *Infect Immun* 2012, **80**:2704–2711.

267 8. Dawson LF, Valiente E, Faulds-Pain A, Donahue EH, Wren BW: **Characterisation of**
268 ***Clostridium difficile* Biofilm formation, a role for Spo0A.** *PLoS ONE* 2012, **7**:e50527.

269 9. Donelli G, Vuotto C, Cardines R, Mastrantonio P: **Biofilm-growing intestinal anaerobic**
270 **bacteria.** *FEMS Immunol Med Microbiol* 2012, **65**:318–325.

271 10. Normington C, Moura IB, Bryant JA, Ewin DJ, Clark EV, Kettle MJ, Harris HC, Spittal W,
272 Davis G, Henn MR, et al.: **Biofilms harbour *Clostridioides difficile*, serving as a reservoir**
273 **for recurrent infection.** *npj Biofilms Microbiomes* 2021, **7**:16.

274 11. Lawley TD, Clare S, Walker AW, Goulding D, Stabler RA, Croucher N, Mastroeni P, Scott P,
275 Raisen C, Mottram L, et al.: **Antibiotic treatment of *Clostridium difficile* carrier mice**
276 **triggers a supershedder state, spore-mediated transmission, and severe disease in**
277 **immunocompromised hosts.** *Infect Immun* 2009, **77**:3661–3669.

278 12. Buckley AM, Spencer J, Candlish D, Irvine JJ, Douce GR: **Infection of hamsters with the UK**
279 ***Clostridium difficile* ribotype 027 outbreak strain R20291.** *Journal of Medical*
280 *Microbiology* 2011, **60**:1174–1180.

- 281 13. Soavelomandroso AP, Gaudin F, Hoys S, Nicolas V, Vedantam G, Janoir C, Bouttier S:
282 **Biofilm structures in a mono-associated mouse model of *Clostridium difficile* infection.**
283 *Front Microbiol* 2017, **8**:2086.
- 284 14. Dubois T, Tremblay YDN, Hamiot A, Martin-Verstraete I, Deschamps J, Monot M, Briandet
285 R, Dupuy B: **A microbiota-generated bile salt induces biofilm formation in *Clostridium***
286 ***difficile*.** *npj Biofilms Microbiomes* 2019, **5**:14.
- 287 •• The authors demonstrated that deoxycholate induces biofilms formation in response to a
288 profound metabolic and this response was control by metabolic regulator. *C. difficile* was also
289 able to form dual-species biofilm with *Clostridium scindens* when these two species were
290 grown in the presence of cholate, which is transformed into deoxycholate by *C. scindens*.
- 291 15. Dhapa T, Leuzzi R, Ng YK, Baban ST, Adamo R, Kuehne SA, Scarselli M, Minton NP, Serruto
292 D, Unnikrishnan M: **Multiple factors modulate biofilm formation by the anaerobic**
293 **pathogen *Clostridium difficile*.** *Journal of Bacteriology* 2013, **195**:545–555.
- 294 16. Dawson LF, Peltier J, Hall CL, Harrison MA, Derakhshan M, Shaw HA, Fairweather NF,
295 Wren BW: **Extracellular DNA, cell surface proteins and c-di-GMP promote biofilm**
296 **formation in *Clostridioides difficile*.** *Sci Rep* 2021, **11**:3244.
- 297 17. Semenyuk EG, Laning ML, Foley J, Johnston PF, Knight KL, Gerding DN, Driks A: **Spore**
298 **formation and toxin production in *Clostridium difficile* biofilms.** *PLoS One* 2014
299 **9**:e87757.
- 300 18. Slater RT, Frost LR, Jossi SE, Millard AD, Unnikrishnan M: ***Clostridioides difficile* LuxS**
301 **mediates inter-bacterial interactions within biofilms.** *Sci Rep* 2019, **9**:9903.

- 302 19. Dannheim H, Will SE, Schomburg D, Neumann-Schaal M: ***Clostridioides difficile* 630Δ erm**
303 ***in silico* and *in vivo* - quantitative growth and extensive polysaccharide secretion.** *FEBS*
304 *Open Bio* 2017, **77**:602–615.
- 305 20. Scott W, Lowrance B, Anderson AC, Weadge JT: **Identification of the Clostridial cellulose**
306 **synthase and characterization of the cognate glycosyl hydrolase, CcsZ.** *PLoS ONE* 2020,
307 **15**:e0242686.
- 308 21. Tremblay YDN, Durand BAR, Hamiot A, Martin-Verstraete I, Oberkampf M, Monot M,
309 Dupuy B: **Metabolic adaption to extracellular pyruvate triggers biofilm formation in**
310 ***Clostridioides difficile*.** *ISME J* 2021, doi:10.1038/s41396-021-01042-5.
- 311 •• Using time-course transcriptomic and genetics, several metabolic regulatory factors and
312 molecular mechanisms were identified as contributing to biofilm formation. Extracellular
313 pyruvate was identified as a critical metabolite for the induction of biofilm formation.
- 314 22. Pantaléon V, Soavelomandroso AP, Bouttier S, Briandet R, Roxas B, Chu M, Collignon A,
315 Janoir C, Vedantam G, Candela T: **The *Clostridium difficile* protease Cwp84 modulates**
316 **both biofilm formation and cell-surface properties.** *PLoS ONE* 2015, **10**:e0124971.
- 317 23. Maldarelli GA, Piepenbrink KH, Scott AJ, Freiberg JA, Song Y, Achermann Y, Ernst RK,
318 Shirliff ME, Sundberg EJ, Sonnenberg MS, et al.: **Type IV pili promote early biofilm**
319 **formation by *Clostridium difficile*.** *Pathogens and Disease* 2016, **74**:ftw061.
- 320 24. Hennequin C, Janoir C, Barc M-C, Collignon A, Karjalainen T: **Identification and**
321 **characterization of a fibronectin-binding protein from *Clostridium difficile*.** *Microbiology*
322 2003, **149**:2779–2787.

- 323 25. Tulli L, Marchi S, Petracca R, Shaw HA, Fairweather NF, Scarselli M, Soriani M, Leuzzi R:
324 **CbpA: a novel surface exposed adhesin of *Clostridium difficile* targeting human collagen:**
325 **Collagen binding protein of *Clostridium difficile*. *Cell Microbiol* 2013, **15**:1674-1687.**
- 326 26. Arato V, Gasperini G, Giusti F, Ferlenghi I, Scarselli M, Leuzzi R: **Dual role of the**
327 **colonization factor CD2831 in *Clostridium difficile* pathogenesis. *Sci Rep* 2019, **9**:5554.**
- 328 27. Valiente E, Bouché L, Hitchen P, Faulds-Pain A, Songane M, Dawson LF, Donahue E, Stabler
329 RA, Panico M, Morris HR, et al.: **Role of glycosyltransferases modifying type B flagellin of**
330 **emerging hypervirulent *Clostridium difficile* lineages and their impact on motility and**
331 **biofilm formation. *Journal of Biological Chemistry* 2016, **291**:25450–25461.**
- 332 28. Boudry P, Gracia C, Monot M, Caillet J, Saujet L, Hajnsdorf E, Dupuy B, Martin-Verstraete
333 I, Soutourina O: **Pleiotropic role of the RNA chaperone protein Hfq in the human**
334 **pathogen *Clostridium difficile*. *J Bacteriol* 2014, **196**:3234–3248.**
- 335 29. Cuenot E, Garcia-Garcia T, Douche T, Gorgette O, Courtin P, Denis-Quanquin S, Hoys S,
336 Tremblay YDN, Matondo M, Chapot-Chartier M-P, et al.: **The Ser/Thr kinase PrkC**
337 **participates in cell wall homeostasis and antimicrobial resistance in *Clostridium difficile*.**
338 ***Infect Immun* 2019, **87**:e00005-19.**
- 339 30. Jain S, Smyth D, O'Hagan BMG, Heap JT, McMullan G, Minton NP, Ternan NG: **Inactivation**
340 **of the *dnaK* gene in *Clostridium difficile* 630 Δ erm yields a temperature-sensitive**
341 **phenotype and increases biofilm-forming ability. *Sci Rep* 2017, **7**:17522.**
- 342 31. Walter BM, Cartman ST, Minton NP, Butala M, Rupnik M: **The SOS response master**
343 **regulator LexA is associated with sporulation, motility and biofilm formation in**
344 ***Clostridium difficile*. *PLoS ONE* 2015, **10**:e0144763.**

- 345 32. Garcia-Garcia T, Poncet S, Cuenot E, Douché T, Gai Gianetto Q, Peltier J, Courtin P,
346 Chapot-Chartier M-P, Matondo M, Dupuy B, et al.: **Ser/Thr kinase-dependent**
347 **phosphorylation of the peptidoglycan hydrolase CwIA controls its export and modulates**
348 **cell division in *Clostridioides difficile***. *mBio* 2021, **12**:e00519-21.
- 349 33. Guzmán-Soto I, McTiernan C, Gonzalez-Gomez M, Ross A, Gupta K, Suuronen EJ, Mah T-
350 F, Griffith M, Alarcon EI: **Mimicking biofilm formation and development: Recent progress**
351 **in *in vitro* and *in vivo* biofilm models**. *iScience* 2021, **24**:102443.
- 352 •• Review that provides an excellent primer on recent development in biofilm formation
353 models and how to study biofilms.
- 354 34. Buckley AM, Ewin D, Moura IB, Wilcox MH, Douce GR: **Insights into the regulatory**
355 **mechanisms of *Clostridioides difficile* biofilm formation**. *bioRxiv* 2021 **02.19**:431970.
- 356 •• This study helps characterize the role of SinR as a repressor of biofilm formation and
357 identifies glycine riboswitch specifically expressed in biofilms.
- 358 35. Purcell EB, McKee RW, Courson DS, Garrett EM, McBride SM, Cheney RE, Tamayo R: **A**
359 **nutrient-regulated cyclic diguanylate phosphodiesterase controls *Clostridium difficile***
360 **biofilm and toxin production during stationary phase**. *Infect Immun* 2017, **85**:e00347-
361 17.
- 362 36. Brauer M, Lassek C, Hinze C, Hoyer J, Becher D, Jahn D, Sievers S, Riedel K: **What's a**
363 **biofilm?—How the choice of the biofilm model impacts the protein inventory of**
364 ***Clostridioides difficile*** . *Front Microbiol* 2021, **12**:682111.

365 •• The authors compared two different biofilm models using different omics approaches and
366 concluded that mechanisms required for biofilm formation in liquid culture are greatly
367 different than those required for colony biofilm on a solid medium.

368 37. Neumann-Schaal M, Jahn D, Schmidt-Hohagen K: **Metabolism the difficile way: The key**
369 **to the success of the pathogen *Clostridioides difficile***. *Front Microbiol* 2019, **10**:219.

370 38. Pokhrel A, Poudel A, Castro KB, Celestine MJ, Oludiran A, Rinehold AJ, Resek AM, Mhanna
371 MA, Purcell EB: **The (p)ppGpp synthetase RSH mediates stationary-phase onset and**
372 **antibiotic stress survival in *Clostridioides difficile***. *J Bacteriol* 2020, **202**:e00377-20

373 39. McKee RW, Harvest CK, Tamayo R: **Cyclic diguanylate regulates virulence factor genes**
374 **via multiple riboswitches in *Clostridium difficile***. *mSphere* 2018, **3**:e00423-18.

375 40. Poudel A, Pokhrel A, Oludiran A, Coronado EJ, Alleyne K, Gilfus MM, Gurung RK, Adhikari
376 SB, Purcell EB: **Unique features of magic spot metabolism in *Clostridioides difficile***.
377 *bioRxiv* 2021, **08.02**:454818.

378 • This study characterizes the enzyme responsible for alarmone synthesis and identify pGpp
379 as the only end-products of the tow enzyme.

380 41. Pereira, F.C.; Wasmund, K.; Cobankovic, I.; Jehmlich, N.; Herbold, C.W.; Lee, K.S.; Sziranyi,
381 B.; Vesely, C.; Decker, T.; Stocker, R.; et al. **Rational design of a microbial consortium of**
382 **mucosal sugar utilizers reduces *Clostridium difficile* colonization**. *Nat. Commun.* 2020,
383 **11**:5104.

384 42. Engevik, M.A.; Engevik, A.C.; Engevik, K.A.; Auchtung, J.M.; Chang-Graham, A.L.; Ruan, W.;
385 Luna, R.A.; Hyser, J.M.; Spinler, J.K.; Versalovic, J. **Mucin-degrading microbes release**

- 386 **monosaccharides that chemoattract *Clostridioides difficile* and facilitate colonization of**
387 **the human intestinal mucus layer. *ACS Infect. Dis.* 2020, **7**:1126–1142.**
- 388 43. Faulds-Pain A, Twine SM, Vinogradov E, Strong PCR, Dell A, Buckley AM, Douce GR,
389 Valiente E, Logan SM, Wren BW: **The post-translational modification of the *Clostridium***
390 ***difficile* flagellin affects motility, cell surface properties and virulence. *Molecular***
391 ***Microbiology* 2014, **94**:272–289.**
- 392 44. Pantaléon, V.; Monot, M.; Eckert, C.; Hoys, S.; Collignon, A.; Janoir, C.; Candela, T.
393 ***Clostridium difficile* forms variable biofilms on abiotic surface. *Anaerobe* 2018, **53**:34–**
394 **37**
- 395 45. Engevik, M.A.; Danhof, H.A.; Auchtung, J.; Endres, B.T.; Ruan, W.; Bassères, E.; Engevik,
396 A.C.; Wu, Q.; Nicholson, M.; Luna, R.A.; et al. ***Fusobacterium nucleatum* adheres to**
397 ***Clostridioides difficile* via the RadD Adhesin to enhance biofilm formation in intestinal**
398 **mucus. *Gastroenterology* 2021, **160**:1301–1314.e8.**
- 399 46. Bordeleau E, Purcell EB, Lafontaine DA, Fortier L-C, Tamayo R, Burrus V: **Cyclic di-GMP**
400 **riboswitch-regulated type IV pili contribute to aggregation of *Clostridium difficile*. *J***
401 ***Bacteriol* 2015, **197**:819–832.**
- 402 47. Soutourina OA, Monot M, Boudry P, Saujet L, Pichon C, Sismeiro O, Semenova E,
403 Severinov K, Le Bouguenec C, Coppée J-Y, et al.: **Genome-wide identification of**
404 **regulatory RNAs in the human pathogen *Clostridium difficile*. *PLoS Genet* 2013,**
405 **9:e1003493.**

- 406 48. Barrick JE, Corbino KA, Winkler WC, Nahvi A, Mandal M, Collins J, Lee M, Roth A, Sudarsan
407 N, Jona I, et al.: **New RNA motifs suggest an expanded scope for riboswitches in bacterial**
408 **genetic control.** *Proceedings of the National Academy of Sciences* 2004, **101**:6421–6426.
- 409 49. Dubois T, Dancer-Thibonnier M, Monot M, Hamiot A, Bouillaut L, Soutourina O, Martin-
410 Verstraete I, Dupuy B: **Control of *Clostridium difficile* physiopathology in response to**
411 **cysteine availability.** *Infect Immun* 2016, **84**:2389–2405.
- 412 50. Taggart MG, Snelling WJ, Naughton PJ, La Ragione RM, Dooley JSG, Ternan NG: **Biofilm**
413 **regulation in *Clostridioides difficile*: Novel systems linked to hypervirulence.** *PLoS*
414 *Pathogens* 2021, **17**:e1009817.
- 415 51. Martin MJ, Clare S, Goulding D, Faulds-Pain A, Barquist L, Browne HP, Pettit L, Dougan G,
416 Lawley TD, Wren BW: **The *agr* Locus Regulates Virulence and Colonization Genes in**
417 ***Clostridium difficile* 027.** *J Bacteriol* 2013, **195**:3672–3681.
- 418 52. Trzilova D, Anjuwon-Foster BR, Torres Rivera D, Tamayo R: **Rho factor mediates flagellum**
419 **and toxin phase variation and impacts virulence in *Clostridioides difficile*.** *PLoS Pathog*
420 2020, **16**:e1008708.
- 421 53. Anjuwon-Foster BR, Tamayo R: **A genetic switch controls the production of flagella and**
422 **toxins in *Clostridium difficile*.** *PLoS Genet* 2017, **13**:e1006701.
- 423 54. Sekulovic O, Mathias Garrett E, Bourgeois J, Tamayo R, Shen A, Camilli A: **Genome-wide**
424 **detection of conservative site-specific recombination in bacteria.** *PLoS Genet* 2018,
425 **14**:e1007332.
- 426 55. Oliveira PH, Ribis JW, Garrett EM, Trzilova D, Kim A, Sekulovic O, Mead EA, Pak T, Zhu S,
427 Deikus G, et al.: **Epigenomic characterization of *Clostridioides difficile* finds a conserved**

428 **DNA methyltransferase that mediates sporulation and pathogenesis. *Nat Microbiol*
429 2020, **5**:166–180.**

430 56. Corver J, Cordo' V, van Leeuwen HC, Klychnikov OI, Hensbergen PJ: **Covalent attachment**
431 **and Pro-Pro endopeptidase (PPEP-1)-mediated release of *Clostridium difficile* cell**
432 **surface proteins involved in adhesion: Attachment and release of *C. difficile* surface**
433 **proteins.** *Molecular Microbiology* 2017, **105**:663–673.

434 57. Nishikawa M, Kobayashi K: **Calcium prevents biofilm dispersion in *Bacillus subtilis*.** *J*
435 *Bacteriol* 2021, **203**:e0011421.

436 •• Sporulation is a process that requires large amount of calcium, and this process can lead
437 to biofilm dispersion. Adding calcium to the medium stabilize the biofilm structures during
438 sporulation.

439 58. Wang T, Flint S, Palmer J: **Magnesium and calcium ions: roles in bacterial cell attachment**
440 **and biofilm structure maturation.** *Biofouling* 2019, **35**:959–974.

441 59. Peltier J, Hamiot A, Garneau JR, Boudry P, Maikova A, Hajnsdorf E, Fortier L-C, Dupuy B,
442 Soutourina O: **Type I toxin-antitoxin systems contribute to the maintenance of mobile**
443 **genetic elements in *Clostridioides difficile*.** *Commun Biol* 2020, **3**:718.

444 •• Describes a new genetic tool that can be used to rapidly create marker less gene deletion.

445 **Box 1: Outstanding questions for future research**

- 446 1. What is the exact role of SinR and SinR' during biofilm formation?
- 447 2. Do sporulation- and prophage-mediated lysis contribute to biofilm formation?
- 448 3. Are quorum sensing systems required for biofilm formation?
- 449 4. Are the exopolysaccharides produced during biofilm formation and what are their role?
- 450 5. How is dispersion initiated and regulated?

- 451 6. What are the mechanisms behind the influence of the microbiota on *C. difficile* biofilm
452 formation (adhesion, cross-feeding, etc.)?
453 7. How do biofilms contribute to persistence of *C. difficile* in the gut and participate in
454 relapses
455