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

2

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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

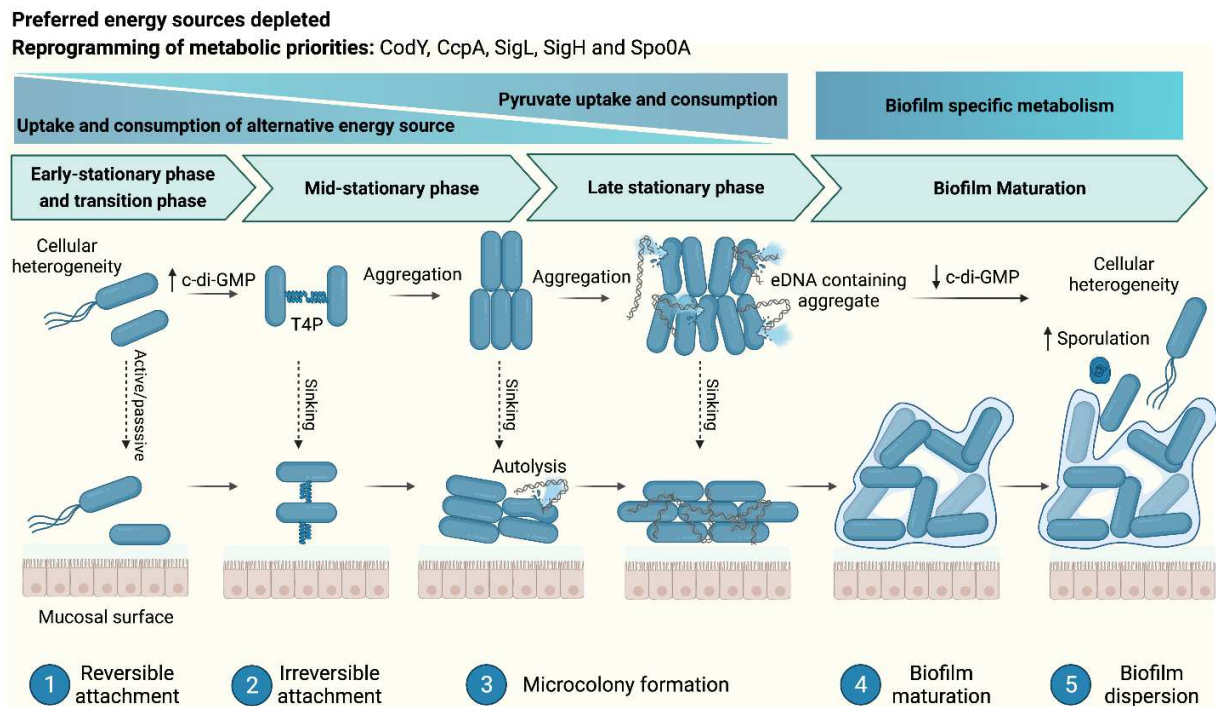
54 epithelium or mucus layer of infected animals [11][12][13]. Given the potential importance of  
55 biofilm formation in *C. difficile* infection, researchers are trying to understand the genetic factors,  
56 molecular events and the regulatory pathways leading to biofilm formation in *C. difficile*.

### 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].

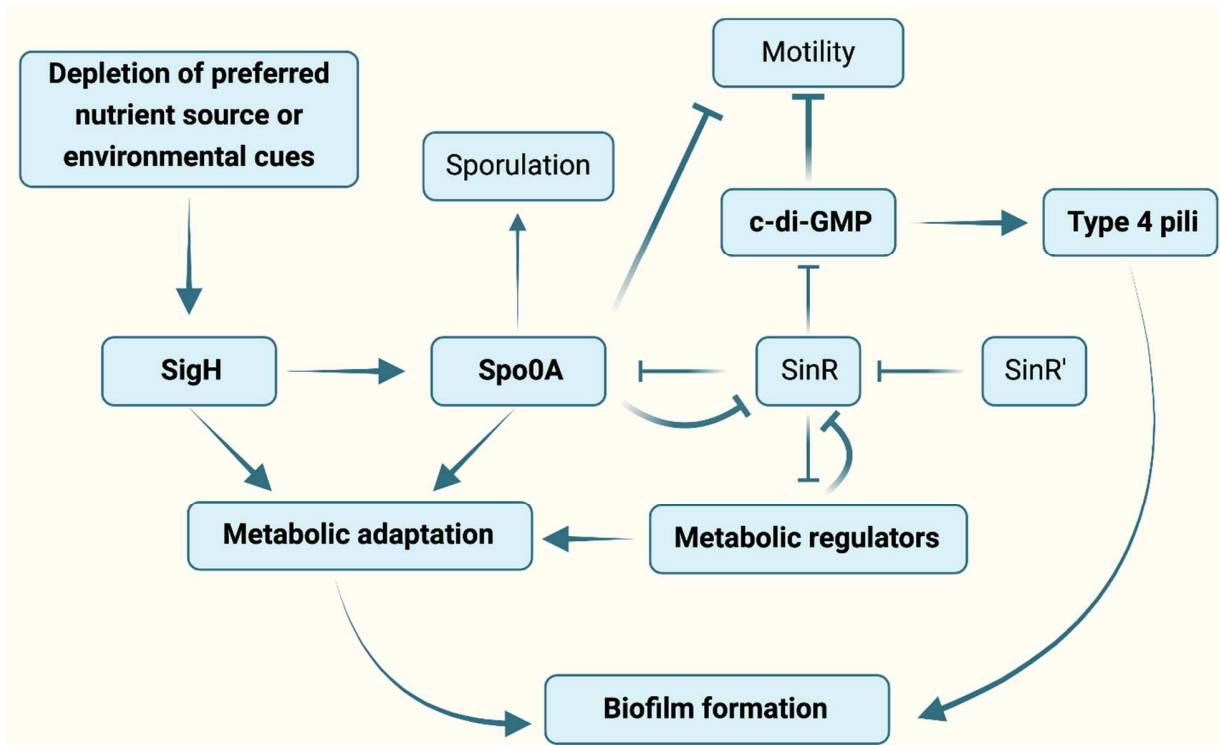
111 Several genetic factors are indirectly involved in biofilm formation due to their pleiotropic effects  
112 on *C. difficile* physiology. These include the RNA chaperone Hfq [28], the Ser/Thr kinase PrkC [29],  
113 the protein chaperone DnaK [30] and the SOS-response regulator LexA [31]. For example,  
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

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#### 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