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1 *Clostridium difficile* forms variable biofilms on abiotic surface

2

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18 Keywords : *Clostridium difficile*; biofilm; motility

19 Abstract

20 *Clostridium difficile* can form biofilms. Thirty-seven strains were characterized for their  
21 ability to form a biofilm, adhesion on an inert surface and hydrophobicity. No correlation  
22 between the ability to form a biofilm and the strain virulence was highlighted. However, non-  
23 motile strains were not able to form a high biofilm.

24

25 *Clostridium difficile*, an anaerobic Gram-positive spore-forming bacterium, is responsible  
26 for a wide spectrum of infections ranging from mild/severe diarrhea to life-threatening  
27 pseudomembranous colitis. The evolution of *C. difficile* infection (CDI) depends on both strain  
28 virulence and host response. *C. difficile* clinical relevance has significantly increased since 2003  
29 with the emergence of hypervirulent PCR-ribotype 027 strains that have been involved in  
30 outbreaks, and that have been associated with severe disease in both North America and Europe.  
31 *C. difficile* causes at least 450,000 infections leading to 29,000 deaths per year in the United  
32 States of America (USA) [1]. The US Centers for Disease Control has designated this pathogen  
33 as the pre-eminent of three “Urgent Threats” to US healthcare. The increasing incidence of CDI  
34 and the high rate of recurrent disease have a major impact on healthcare costs. Because patient  
35 contamination occurs from *C. difficile* spores present in the environment, increased spore  
36 dissemination during outbreaks significantly complicates approaches to control infection [2, 3].  
37 Antibiotic exposure leading to dysbiosis of gut microbiota is with advanced age and  
38 hospitalization a major risk factor associated with CDI [4]. The major challenge is the  
39 recurrence of CDI. Up to 35% of patients may undergo a recurrence after the first CDI episode  
40 [5]. This rate increases after first recurrence. In case of relapse with the same *C. difficile* strain,  
41 reinfection or persistence of *C. difficile* in the intestine may occur. Persistence may be the result  
42 of germination/sporulation cycles or biofilm formation [6, 7]. Recently, *C. difficile* was  
43 proposed to form a biofilm structure in the caecum and colon in a mono-associated mouse  
44 model [7]. In addition, *C. difficile* is also able to reside in multicellular communities in mice,  
45 but also in human periodontal-disease-associated biofilm [6, 8]. Similarly, in vitro *C. difficile*  
46 is able to form a thick biofilm in the presence of *Fingoldia magna* [9]. In vitro, biofilm may  
47 protect *C. difficile* from different antibiotics, such as vancomycin and metronidazole [10, 11].  
48 In contrary, some antibiotics, such as surotomycin and fidaxomicin, were shown to be able to  
49 disrupt the biofilm structure [12]. Biofilm matrix is composed of eDNA, polysaccharides and

50 proteins. Toxins were found in the biofilm matrix and may facilitate infection [10, 13]. *C.*  
51 *difficile* has also been shown to sporulate in biofilm [13]. Moreover, spores were reported to be  
52 found preferentially within biofilm structures in a chemostat gut model [14]. *C. difficile* life in  
53 biofilm may therefore be crucial for its in vivo persistence. However, in vivo and in vitro  
54 biofilm studies of *C. difficile* are still poorly explored with respect to other bacterial species (for  
55 reviews [15, 16]) [15]. In vitro, only 7 different strains of *C. difficile* were described (mostly  
56 the 630 and the R20291) to form a qualitatively weak biofilm [13, 17-22]. Here, we tested 37  
57 strains from different origins to assess their ability to form in vitro biofilms to detect any  
58 correlation with strain characteristics.

59 All the strains used in this study were isolated from infected patients except 2 that came  
60 from poultry or rabbit (Table 1). They were all tested for their capacity to form a biofilm (72h  
61 at 37°C, in 24-well polystyrene plates) on abiotic surface as described in Pantaleon *et al.* [19]  
62 and quantified by crystal violet staining (Figure 1). Data are representative of three independent  
63 experiments each performed in triplicate. According to their ability to form a biofilm, strains  
64 were classed into three groups that are significantly different (Bartlett test of homogeneity of  
65 variances, <http://marne.u707.jussieu.fr/biostatgv/>): the low-biofilm formers ( $OD_{570} < 1.1$ ), the  
66 moderate-biofilm formers ( $1.1 < OD_{570} < 3.5$ ) and the high-biofilm formers ( $OD_{570} > 3.5$ ). The  
67 strain 94-1155 formed the highest biofilm with a mean  $OD_{570} = 10.759$  and the strain T5 formed  
68 the smallest biofilm with a mean  $OD_{570} = 0.218$ . 48.5% of the strains form a low-biofilm, 21.5%  
69 a medium-biofilm and 30% a high-biofilm. Three strains were isolated from patient with relapse  
70 (95-1078, 96-631 and 94-1155), and we note that they belong to the medium and high-biofilm  
71 formers. These results suggest that biofilm is highly variable among *C. difficile* strains.

72 To investigate whether the ability to form a biofilm may be due to their PCR-ribotype  
73 and sequence type (ST), they were determined for each strain (Table 1). The PCR-ribotype was  
74 performed according to the protocol of Bidet *et al* [23] and the multi-locus sequence type  
75 (MLST) was performed as previously described [24]. The toxinotype when known was also  
76 reported in Table 1. Some strains were closely related, according to their ST, ribotype and  
77 toxinotype. However, they have differences in their sequences and are therefore different:  
78 R20291/CD196 [25], E16/T61, E15/T11, T15/E25 [26]. No statistical test could be performed  
79 due to the low number of strains available for each typing method (ribotype and ST). However,  
80 our results suggest that biofilm is variable among *C. difficile* strains, e.g. ribotypes 005 or 020  
81 formed medium and high biofilms; strains belonging to ST19 formed low, medium and high  
82 biofilms. Similarly, our results suggest that there is no correlation between the *C. difficile* strain  
83 clinical symptoms during infection and the ability to form a biofilm.

84 The initial adhesion on an inert support may be involved in biofilm formation [27].  
85 Initial adhesion of the 37 strains was therefore assessed on an abiotic surface (24-well  
86 polystyrene plates, 1h at 37°C) as described in Pantaleon *et al.* (Figure 2) [19]. Data are  
87 representative of three independent experiments each performed in triplicate. The mean of  
88 adhesion for all the tested strains was 2.56% with a maximum of 8.89% (T6 strain) and a  
89 minimum of 0.41% (T42 strain). No correlation was observed between adhesion of the strains  
90 and their ability to form a biofilm (Pearson method: correlation coefficient = 0.01, pvalue =  
91 0.96).

92 The surface hydrophobicity properties of bacteria are also usually involved in the  
93 adhesion and the biofilm formation [28]. The properties of surface hydrophobicity of the 37  
94 *C. difficile* strains were studied using the hexadecane method [19] (Table 1). The mean of  
95 hydrophobicity was 18.74% with a maximum of 71.95% (VPI11186 strain) and a minimum of  
96 6.84% (CD1 strain). The three strains forming the smallest biofilms (T5, 4485 and T20) and  
97 the three strains forming the highest biofilms (T14, 3751 and 94-1155) had a similar surface  
98 hydrophobicity mean. This result suggests that the surface hydrophobicity of *C. difficile* do not  
99 correlate (Pearson method: correlation coefficient = 0.07, pvalue = 0.67) with their ability to  
100 form biofilms.

101 The motility of bacteria has also been described as a feature of biofilm formation [29].  
102 The motility of all strains was analysed using BHI agar 0.3% in test tubes. The agar medium  
103 was inoculated with one colony and placed in anaerobic chamber at 37°C for 24 to 48 hours.  
104 As expected, all the strains were motile except 6 (E1, T5, T20, IT1106, CD15 and 4485) which  
105 belong to the 078, 079 and 126 ribotypes. These six strains belong to the ST49 (Table 1). The  
106 strains E1, T5 and T20 have been sequenced [26] and they all possess the *fliC* and *fliD* genes,  
107 but not the F3 locus, which does code for the motor and basal body of the flagellum. The six  
108 non-motile strains belong to the low-biofilm former group. This result suggests that motility or

109 the flagellum presence is involved in biofilm formation after 3 days (Pearson method:  
110 correlation coefficient = 0.33, pvalue = 0.05). Other studies, using mutant strains affecting  
111 directly or indirectly either the flagellum or the motility have shown that biofilm was increased  
112 in the mutant in comparison with the parental strains [21, 22, 30]. This apparent discrepancy  
113 may be due to a difference in the global regulation due to the absence of the F3 region, and  
114 probably *fliC* expression which may also impact the regulatory mechanisms and formation of  
115 biofilm. Indeed, FliC has been proposed to be responsible for pleiotropic gene regulation  
116 [31].

117           In conclusion, our study presents the ability to form a biofilm of 37 different strains.  
118 The importance of motility in *C. difficile* biofilm was highlighted. However, no correlation  
119 between their ability to form a biofilm with neither the origin nor the clinical symptoms of *C.*  
120 *difficile* strains was observed.

121



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124

125 References

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224  
225

Table 1

Strains	Origin (geography)	Origin (ecology)	Symptoms	Biofilm biomass (OD <sub>570</sub> )	Strain typing			Hydrophobicity	Motility	Références
					MLST	PCR-ribotype	Toxinotype			
E23	Europe	human	SCDI	0.359	36	001 ; 001/072*	0	12.30%	+	<a href="#">[26]</a>
CD268	Italy	rabbit	/	0.648	33	001	ND	20.40%	+	Lab Collection
FM17	unknow	unknow	Unknown	0.314	19	002	ND	9.80%	+	Lab Collection
96-631	France	human	Relapse	3.509	21	003	ND	10.19%	+	Lab Collection
T15	France	human	Diarrhoea	1.15	19	005	0	15.51%	+	<a href="#">[26]</a>
E25	France	human	Sepsis, Col	2.917	19	005	0	30.18%	+	<a href="#">[26]</a>
94-1155	France	human	Relapse	10.759	19	005	ND	16.55%	+	Lab Collection
CD630	UK	human	PMC	1.039	27	012*	0*	18.50%	+	<a href="#">[32]</a>
E14	Europe	human	SCDI	3.803	33	014	0	14.19%	+	<a href="#">[26]</a>
P30	Italy	poultry	/	5.370	34	014/020*	0*	15.36%	+	<a href="#">[33]</a>
4684/08	Italy	human	Unknown	5.599	33	014/020*	0*	15.90%	+	<a href="#">[33]</a>
1064	Italy	human	Unknown	7.670	33	014/020*	0*	23.49%	+	<a href="#">[33]</a>
CD49	France	human	Carriage	0.607	33	014/020/077	ND	22.58%	+	Lab Collection
E16	France	human	Perf, Peri	0.586	1	015	0	13.19%	+	<a href="#">[26]</a>
T61	France	human	Diarrhoea	0.963	1	015	0	11.98%	+	<a href="#">[26]</a>
E24	France	human (community)	SS	1.675	33	020	0	16.57%	+	<a href="#">[26]</a>
T42	France	human	Diarrhoea	4.871	33	020	0	16.98%	+	<a href="#">Accession Number : PRJEB216</a>

CD196	France	human	CDI, PMC	1.630	3	027*	III*	22.59%	+	<a href="#">[26]</a>
R20291	UK	human	CDI	2.471	3	027*	III*	19.04%	+	<a href="#">[20]</a>
VPI11186	USA	Unknown	Unknown	1.884	16	038*	ND	70.00%	+	Lab Collection
E15	France	human	MG, SS, Col	0.4	5	075	III	18.67%	+	<a href="#">[26]</a>
T11	France	human	Diarrhoea	0.662	5	075	III	9.18%	+	<a href="#">[26]</a>
T20	Ireland	human	CDI	0.312	49	078	V	20.16%	-	<a href="#">[26]</a>
IT1106	Italy	human	Unknown	0.704	49	078*	V*	17.96%	-	<a href="#">[33]</a>
4485	France	human	PMC	0.264	49	078/126	ND	14.54%	-	Lab Collection
CD15	France	human (HIV+)	Diarrhoea	0.561	49	078/126	ND	23.29%	-	Lab Collection
T5	Italy	human	CDI	0.218	49	079	V	22.66%	-	<a href="#">[26]</a>
T6	Hungary	human	CDI,	4.255	33	095	0	11.61%	+	<a href="#">[26]</a>
E12	UK	human	SCDI	0.502	26	106	0	19.87%	+	<a href="#">[26]</a>
E1	Austria	human	SCDI	1.004	49	126	V	14.41%	-	<a href="#">[26]</a>
T14	Ireland	human	CDI	7.747	26	156	0	32.51%	+	<a href="#">[26]</a>
T10	Germany	human	CDI	3.115	36	165	0	11.13%	+	<a href="#">[26]</a>
VPI10463	USA	human	PMC	1.047	11	ND	0*	11.04%	+	<a href="#">[34]</a>
94-1458	France	human	Unknown	0.575	1	other	ND	19.86%	+	Lab Collection
95-1078	France	human	Relapse	2.517	~20	other	ND	18.50%	+	Lab Collection

Table 1: strains and characteristics

The upper part of the table reports the low-biofilm former, the middle part reports the medium-biofilm formers forming an intermediate biofilm and the lower part reports the high-biofilm formers. ND, not determined; CDI *Clostridium difficile* infection; SCDI Severe *Clostridium difficile* infection; Col, colectomy; HIV, human immunodeficiency virus; MG, Megacolon; Peri, Peritonitis; Perf, perforation; PMC, Pseudomembranous Colitis; SS, septic shock. \* corresponds to the characteristics of the strains described in other studies (see references in the text). other: PCR-ribotypes that are not among the following ,001, 015, 017, 023, 056, 081, 087, 014/020/077, 003, 019, 046, 050, 117, 005, 018, 106, 131, 012, 029, 053, 075, 002, 070, 078/126

## Figure legend

### Figure 1: ability of the 37 strains to form a biofilm

Biomass of the 37 strains after 72h of incubation. Strains are divided (dashed lines) in three categories, low, medium and high-biofilm formers.

### Figure 2: adhesion of the 37 strains on an inert surface

Percentage of initial adhesion of the 37 strains to a polystyrene inert surface. Error bars represent standard deviation. Strains are classed by low, medium and high-biofilm formers.