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1 **Influence of type-I fimbriae and fluid shear stress on bacterial behavior and multicellular**
2 **architecture of early *Escherichia coli* biofilms at single-cell resolution**

3
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12 **Running title:** How fimbriae and fluid shear affect *E.coli* biofilm
13

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16 **Abstract**

17 Biofilm formation on abiotic surfaces in food and medical industry can cause severe contamination and
18 infection, yet how biological and physical factors determine cellular architecture of early biofilms and
19 bacterial behavior of the constituent cells remains largely unknown. In this study we examine the
20 specific role of type-I fimbriae in nascent stages of biofilm formation and the response of micro-colonies
21 to environmental flow shear at single-cell resolution. The results show that type-I fimbriae are not
22 required for reversible adhesion from plankton, but critical for irreversible adhesion of *Escherichia coli*
23 (*E.coli*) MG1655 forming biofilms on polyethylene terephthalate (PET) surfaces. Besides establishing a
24 firm cell-surface contact, the irreversible adhesion seems necessary to initiate the proliferation of *E.coli*
25 on the surface. After application of shear stress, bacterial retention is dominated by the 3D architecture
26 of colonies independent of the population and the multi-layered structure could protect the embedded
27 cells from being insulted by fluid shear, while cell membrane permeability mainly depends on the
28 biofilm population and the duration time of the shear stress.

29 **Keywords:** early biofilms; type-I fimbriae; irreversible adhesion; fluid shear stress.

30 **Importance**

31 Bacterial biofilms could lead to severe contamination problems in medical devices and food processing
32 equipment. However, biofilms are usually studied at a rough macroscopic level, thus little is known
33 about how individual bacterial behavior within biofilms and multicellular architecture are influenced by
34 bacterial appendages (e.g. pili/fimbriae) and environmental factors during early biofilm formation. We
35 apply Confocal Laser Scanning Microscopy (CLSM) to visualize *E.coli* micro-colonies at single-cell
36 resolution. Our findings suggest that type-I fimbriae are vital to the initiation of bacterial proliferation on
37 surfaces and that the responses of biofilm architecture and cell membrane permeability of constituent
38 bacteria to fluid shear stress are different, which are respectively regulated by the 3D morphology and
39 the population of micro-colonies.

40 **Introduction**

41 Biofilms are sessile communities of bacteria that grow on solid surfaces, embedded in an extracellular
42 polymeric substance (EPS) matrix (1-3). The biofilm formation generally proceeds via the following
43 steps (1, 4, 5): (i) Planktonic microbial cells approach and then reach the solid surface by Brownian
44 motion, hydrodynamic flow, and active swimming motion. (ii) Once reached, bacteria begin adhering to
45 surfaces. Initially, adherent bacterial cells are attached on the surface reversibly, and many detach to
46 resume the planktonic lifestyle due to the low activation energy for desorption. A fraction of these cells
47 then become irreversibly attached, which might be because bacterial appendages (flagella, fimbriae etc.)
48 overcome the physical repulsive forces of the electrical double layer (6). (iii) The adherent bacteria
49 divide and grow to form micro-colonies on the surface. (iv) Bacteria synthesize a matrix of extracellular
50 polymeric substances, allowing micro-colonies to further develop into mature biofilms, followed by (v)
51 the shed of part of the biofilm to release planktonic bacteria into the surrounding environment.

52 Biofilm formation is a key issue in food industry and in clinical setting. Surfaces infected by biofilms
53 in food-industrial equipment, medical devices and implants could commonly cause illnesses, lethal
54 infections and heavy costs in maintenance (7, 8). Furthermore, when organized into biofilms after
55 maturation, bacteria are resistant to many forms of stress, because EPS is designed to protect embedded
56 bacteria from antibiotics, disinfectants and environmental insults (9). The formation of biofilms
57 therefore has been intensively studied, especially in terms of dynamics of adhesion process and genes
58 involved in EPS production, as well as regulatory mechanisms and overall morphology of mature
59 biofilms (9, 10). Nevertheless, in order to provide a better insight into mechanisms underlying biofilm
60 formation, the nascent stages of biofilm development (i.e., adhesion and proliferation) need to be further
61 elaborated at single-cell resolution.

62 Macroscopic biofilm formation is dramatically affected by bacterial properties (e.g. cell appendages)
63 and environmental factors (e.g. fluid shear). For instance, deficient fimbriae would alter the morphology

64 of biofilms (11) or lead to a defective biofilm formation (12); fluid shear could cause biofilm breakage
65 (13) and deformation (14). However, at single-cell resolution, although the critical role of fimbriae/pili
66 in bacterial adherence to abiotic surfaces has been extensively studied over the past two decades, little is
67 understood about their effect on the proliferation stage of biofilm formation (step (iii)), and few studies
68 focus on the responses of early biofilms to fluid shear stress including cell membrane permeability and
69 multicellular architecture. Usually, the effect of shear stress on bacterial adhesion and macroscopic
70 biofilm morphology on abiotic substrates is investigated using bacteria suspended in the fluid, which
71 could reveal how the flow affects biofilm formation when surfaces are exposed to bacteria-existed
72 environment, e.g. ships in the sea and catheters in contaminated urine. These cells are allowed to attach
73 or grow on the immersed surface under different flow regimes, in which shear stress might: increase
74 bacterial adhesion due to mass transport, prevent bacterial attachment and remove bacteria that are
75 already bound (15-18). In contrast to our study, early biofilms grown on PET substrates were statically
76 incubated before the application of shear stress, thus rendering it possible to just focus on how fluid
77 shear influences bacterial behavior during the nascent stages of biofilm formation, which could mimic
78 cleaning in place (CIP) procedures for food processing equipment (19) and uncover how contaminated
79 surfaces of medical devices and implants are affected by flow fluids.

80 Previously we presented architectural transitions in *E.coli* MG1655 biofilms on PET surfaces over the
81 early stages of formation (steps (i) - (iii) on the list above) at single-cell resolution (20). These changes
82 revealed the dynamics, and the structural principle by which individual cells developed into micro-
83 colonies. We obtained the ‘growth curve’ of adhering cells, which exhibited distinct lag and log phases.
84 It is worth noting that the generation time in the log phase (step (iii)) was more than twice as long as that
85 of planktonic counterparts under the same incubation conditions, which was not due to the detachment
86 of the daughter cells back into the bulk medium - but purely due to extra expenditure for cells to remain
87 adhered on the surface. We found that the 3D cellular architecture of early biofilms could influence

88 biological processes (e.g. quorum sensing) and physical properties (e.g. interactions between cells and
89 cells/substrates) of the constituent bacteria. PET is the substrate of choice since it is ubiquitous in food
90 packaging and widely used in cardiovascular implants (e.g. vascular grafts) due to its excellent
91 physicochemical properties: good mechanical strength, stability in the presence of body fluids, and
92 relatively high biocompatibility (21, 22).

93 Here, we examine the influence of type-I fimbriae by comparing the growth of adherent *E.coli*
94 MG1655 mutants that lack type-I fimbriae - *E.coli* appendages facilitating the attachment, and the effect
95 of fluid shear stress on bacterial behavior and cellular architecture of *E.coli* micro-colonies. We will
96 clarify the specific role of type-I fimbriae in the initial adhesion of *E.coli* and in the subsequent growth
97 into colonies on surfaces, and find how cell behavior is affected when the collective architecture of
98 micro-colonies is challenged by fluid shear.

99 **Materials and Methods**

100 **Substrate.** PET plates with a thickness of 0.35 mm and low roughness ($Ra = 5 \pm 0.2$ nm) were
101 purchased from Goodfellow Cambridge Ltd. (Huntingdon, UK). The plates were cut into small pieces
102 (21.5 mm \times 8 mm) and cleaned ultrasonically in absolute ethanol for 15 min and then in deionized
103 purified water for 15 min. They were then dried with nitrogen.

104 **Bacterial strains, media, culture and mutant strain construction.** The wild-type *E.coli* MG1655
105 was cultured in Luria broth (LB) medium, and its mutants that lack the major subunits of type-I fimbriae
106 applied in this study to assess the biofilm behavior of cells with defective fimbriae was incubated in LB
107 medium with 100 μ g/mL kanamycin.

108 A single colony of a strain was inoculated into a test tube containing 5 mL of culture medium and
109 grown overnight at 37 °C, with agitation at 200 rpm. A 100 μ L of this culture was transferred into a fresh
110 tube of 5 mL medium and incubated with shaking until the stationary phase was reached (12 to 14 h) to

111 obtain the eventual microbial suspension, which contained $\sim 10^9$ colony-forming units per mL
112 (CFU/mL).

113 *E. coli* MG1655 $\Delta fimA::kan$ mutant strain (abbreviated as $\Delta fimA$ mutants in the text) was constructed
114 by oligo-directed mutagenesis (ODM) as described previously (23). Briefly, PCR was used to amplify a
115 kanamycin resistance cassette from the plasmid pBADkanFRT using oligonucleotides specific to the
116 cassette, which have 5' 60bp arms homologous to DNA directly flanking the gene of interest, using
117 primers 947 (aaagtgtacagaacgactgccatgtcgatttagaaatagtttttgaaaggaaagcagctagggataggcttacctcaagctc)
118 and 948 (atTTTTatcgacaagggtgggcatccctgccgtaatgacgtccctgaacctgggtaggatgacgatgacaagctcccccttcg).

119 *E. coli* MG1655 containing the pBAD λ Red plasmid was grown in LB medium containing 100 μ g/mL
120 ampicillin at 37°C, 200rpm to an OD₅₉₅ of 0.25. Expression of the red recombinase genes was induced
121 by the addition of L-arabinose to a final concentration of 0.2% w/v. Cultures were incubated further until
122 an OD₅₉₅ of 0.5 was reached, and electrocompetent cells were prepared. Purified PCR products were
123 used to electroporate the cells, and mutant colonies were selected on LB agar plates containing 50
124 μ g/mL kanamycin. Successful allelic replacement of *fimA* was confirmed by PCR and sequencing using
125 primers 1065 (ctatgagtcaaaatggcccca) and 1066 (agagcctgaataaagccggtt). The pBAD λ Red plasmid was
126 the cured by serial passage in the absence of antibiotic selection. Given that *fimA* is found within an
127 operon of 9 genes involved in the biogenesis of type-I fimbriae, any polar effects of the *fimA* deletion
128 would only influence other type-I fimbrial genes. Thus, we are confident that the observed phenotype is
129 due to the loss of type-I fimbriae.

130 **Bacterial adhesion and growth on PET surfaces.** The samples were prepared by following the
131 procedures reported previously (20), with slight modifications. Briefly, sterilized PET surfaces were
132 rinsed thrice with sterile water and then with LB medium. Each substrate was then placed vertically into
133 a test tube (diameter, 22 mm) containing stationary-phase *E. coli* MG1655 or $\Delta fimA$ mutant cells

134 suspended in LB culture at 37 °C for 1 h, to carry out the initial adhesion. Three of the samples were
135 subsequently taken out for Confocal Laser Scanning Microscopy (CLSM; LEICA TCS SP5) imaging
136 and another nine (three replicates for each fluid shear stress) were taken for fluid shear experiments. The
137 other ‘seeded’ PET plates with wild-type or mutant cells were subjected to the following incubation
138 process with refreshed LB medium.

139 The incubation process: The remaining ‘seeded’ PET plates were gently rinsed twice with 10 mL pre-
140 warmed fresh LB medium, before being individually immersed into a new tube containing 5 mL pre-
141 warmed fresh LB medium at 37 °C for 1 h. After each consecutive hour of incubation, three of the
142 samples were taken out for the CLSM imaging and another nine (three replicates for each fluid shear
143 stress) were taken for fluid shear experiments, but all other remaining plates were subjected to the
144 incubation process again.

145 In order to investigate the effect of quorum sensing and multiple-layer architecture on the response of
146 constituent bacteria in micro-colonies to the fluid shear stress, nine of the samples with ‘seeded’ *E.coli*
147 MG1655 on substrates were incubated with hourly-refreshed LB medium containing 1.56 mg/L (Z-)-4-
148 Bromo-5-(bromomethylene)-2(5H)-furanone (FC 30, Sigma-Aldrich, UK) for 8 h. FC 30 is a quorum
149 sensing inhibitor (signal antagonist), and it can compete with the quorum sensing signal molecules for a
150 common binding site on LuxR-type transcriptional activators, which are responsible to regulate
151 expression of target genes for the subsequent quorum sensing (24, 25). In our previous research (20), we
152 found at this concentration of FC 30, bacterial quorum sensing was inhibited but the growth rate was not
153 affected. These nine treated samples (three replicates for each fluid shear stress) were then challenged by
154 fluid shear.

155 **CLSM imaging.** In a comparison with the adhesion and growth of *E.coli* MG1655 on PET surfaces,
156 which have been previously elaborated (20), surface samples with mutant cells were gently washed with
157 tris-buffered solution (TBS) thrice, stained with the BacLight Live/Dead viability kit (Invitrogen, kit no.

158 L7007) for 15 min in the dark and then visualized using Confocal Laser Scanning Microscopy (LEICA
159 TCS SP5) with an oil-immersion objective lens at 40 × magnification, zoom 1:2.60 or 1:1.00. In CLSM
160 images, green and red stained cells could indicate membrane permeability status: green cells have intact
161 membranes, and red cells are membrane compromised (i.e. membranes are permeable or damaged).

162 **Fluid shear experiments.** A special vibration device was built to detach bacteria by a shear force
163 produced in oscillatory flow. This differs from other techniques (e.g. with rotating disk (26)) where the
164 shear force is consistent in one direction, enabling cells to adapt and develop resistance. In our case,
165 oscillations at high frequency create an essentially random force on ‘unprepared’ cells, and probe the
166 strength of their natural attachment. In order to expose bacteria to this shear force, samples were glued
167 onto a holder, which was vibrated horizontally in a dish filled with phosphate-buffered solution (PBS).
168 The dish was kept at 37 °C to produce optimal conditions for bacteria. The vibrational movement was
169 produced by a mechanical setup, which moves two magnets underneath the dish by a mechanism
170 converting rotational movement into a horizontal linear motion. The frequency of this motion can be
171 adjusted to different values between 5Hz and 25Hz. The moving magnets were locked to two reciprocal
172 magnets embedded in the sample holder, thus allowing the movement of the sample holder in the dish
173 without any leakage of liquid. The holder vibrated along one axis in the plane of the dish, because its
174 movement was constrained by a track-like excavation in the dish. The shear stress acting on the bacteria
175 on the surface, moving with respect to a stationary fluid above, is a function of vibration frequency. An
176 expression for this stress can be analytically calculated by considering the hydrodynamics around an
177 oscillatory plate in liquid, producing $f_{max} = -r\omega^{3/2}\sqrt{\rho\eta} \cdot A$, where f_{max} is the maximal force acting
178 on the cells during vibration, r is the amplitude of vibration, ω is the angular frequency of vibration
179 [rad/s], ρ and η are the density [kg/m³] and viscosity [Pa.s]. Finally, $A \approx 0.5 \mu\text{m} \times 3 \mu\text{m}$ is the average
180 surface area of the *E.coli* cell. In our experiments, we worked with the shear stress values of 0.58 Pa,

181 1.45 Pa and 3.30 Pa (referred to for convenience as low, medium and high stress in the following text),
182 which translate to the maximum force amplitude of 0.87 pN, 2.18 pN and 4.95 pN, respectively. These
183 shear stresses are comparable to the wall shear stress (WSS) in medical devices e.g. hemodialysis
184 vascular access (WSS applied mainly ranges from 0.1 to 3 Pa) (27) and central venous catheters (WSS
185 applied mainly ranges from 0 to 1.8 Pa) (28), and those generally used in CIP procedures for food
186 processing equipment (WSS applied in pipes mainly ranges from 0 to 3 Pa) (19).

187 The surface samples with 'seeded' *E.coli* MG1655 or $\Delta fimA$ mutant cells and those with bacterial cells
188 statically incubated after each consecutive hour were treated by fluid shear stresses, and then stained and
189 visualized by CLSM.

190 **Image processing.** Images were processed using ImageJ software (NIH, Bethesda, Maryland,
191 <http://rsbweb.nih.gov/ij/>) to quantitatively determine the number of wild-type/mutant cells on surfaces
192 (i.e. cell density, cells/mm²) and the cell size (i.e. area of the individual cell μm^2). For each sample, 20
193 fields of view were randomly chosen, and the whole measurement process was repeated three times
194 independently, i.e. a total of 60 values for each cell density. The fraction of cells remaining on substrates
195 is the ratio of cell density after shear stress was applied and the density in the control sample without
196 shear treatment. The fraction of cells with intact membranes is the ratio of the cell density of green cells
197 and that of total cells remaining on surfaces.

198 The above experiment process was repeated three times independently. All statistical analysis was
199 performed using ANOVA testing within Microsoft Excel (Microsoft Corp., Redmond, WA). Values were
200 reported in the text as mean value \pm standard deviation.

201 **Results**

202 **The adhesion and growth of $\Delta fimA$ mutants on surfaces.** After 1 h incubation of PET substrates with
203 $\Delta fimA$ mutant suspension (i.e. the start point of incubating surface samples with fresh LB medium,

204 labelled as 0 h), the CLSM images (Fig. 1) reveal that the densities of ‘seeded’ mutant cells and ‘seeded’
205 *E.coli* MG1655 cells are $(6.4 \pm 0.7) \times 10^2$ cells/mm² and $(6.1 \pm 0.9) \times 10^2$ cells/mm², respectively. It could
206 be seen that there is no significant difference observed in this initial adhesion between non-fimbriated
207 cells and wild-type cells. Following the first 2 h incubation of ‘seeded’ cells on substrates with sterile LB
208 medium, Fig. 2 illustrates that the numbers of *AfimA* mutants and of *E.coli* MG1655 cells on the surface
209 both decreased with incubation time. Meanwhile, over this 2 h incubation, the cell size of individual
210 adherent *AfimA* mutants increased from $2.1 \pm 0.3 \mu\text{m}^2$ to $5.6 \pm 0.4 \mu\text{m}^2$ ($P < 0.005$), as the wild-type cells
211 did from $2.3 \pm 0.4 \mu\text{m}^2$ to $5.7 \pm 0.5 \mu\text{m}^2$ ($P < 0.005$).

212 However, the behavior of adherent non-fimbriated *E.coli* was totally different from that of wild-type
213 cells hereafter: after 2 h, the apparent number of surface-bound wild-type cells stopped decreasing and
214 then began to exponentially grow on the surface, while the attached mutants continued detaching from
215 the surface with incubation time until adherent fimbriae mutants could hardly be seen in any field of
216 view at 6 h (Fig. 1), suggesting that non-fimbriated cells remained reversibly attached on the substrate
217 by purely physical interactions.

218 **The effect of fluid shear stress on micro-colonies.** To elaborate the effect of the shear on the cellular
219 response of early biofilms, the *E.coli* MG1655 growing on PET substrates which exhibits three stages -
220 ‘singles’ (only single cells could be observed in the field of view, i.e., lag phase), ‘single-layer clusters’
221 and ‘multi-layer colonies’, were treated with different fluid shear stresses. Figure 3 shows that apart
222 from the altered architecture resulting from bacterial detachment, the cellular response of constituent
223 bacterial cells in micro-colonies to shear stress should include cell membrane permeability.

224 Figure 4 summarizes bacterial detachment and cell membrane permeability due to the shear stress
225 treatment. It could be seen from Fig. 4a that higher shear stress acting on adhering bacteria led to lower
226 bacterial remaining population at the same incubation time point, which is consistent with previous

227 literature (15), and that the amount of cells remaining on surfaces increased with bacterial progressing
228 colonization. During the first 2 h incubation when the single cells were in the lag phase, all of attached
229 cells could be removed by high shear stress, while the fraction of remaining bacteria gradually rose from
230 $20\% \pm 8\%$ at 0 h to $55\% \pm 8\%$ at 2 h under medium shear stress, implying that reversibly ‘seeded’ wild-
231 type *E.coli* were being irreversibly immobilized on PET surfaces over this period. The ‘seeded’ mutant
232 cells on PET substrates (i.e. samples at 0 h) and the adherent mutants on surfaces after the first 1 h or 2 h
233 incubation, were flushed by fluid at the medium shear stress (1.45 Pa) for only 10 s. Compared with
234 wild-type *E.coli*, all of adherent mutants on each surface sample were removed after fluid shear
235 treatment, confirmed by the CLSM images (data not shown). From 3 to 6 h when adhering wild-type
236 bacteria were exponentially growing into single-layered micro-colonies, the bacteria staying on surfaces
237 after being treated by medium and high shear stresses respectively maintained at the level of
238 approximately 83% and 40%. After 7 h when multiple-layer architecture of colonies emerged, the
239 bacterial retention under medium and high shear sharply increased to above 92% and around 80%,
240 respectively. The effect of fluid shear on the bacterial cell membrane permeability is shown in Fig. 4b.
241 Similar to the detachment, Fig. 4b illustrates that higher shear stress also caused lower fraction of cells
242 with intact membranes at the same incubation time point. However, the cell membrane permeability kept
243 constant until the end of ‘single-layer clusters’ stage (6 h). When clustered bacteria developed into
244 multiple-layer colonies after 7 h, for surfaces exposed to medium and high shear stresses, the fractions
245 of cells with intact membranes on surfaces increased from the level of 45% to above 99% and from the
246 level of 30% to above 80%, respectively.

247 To determine whether the multiple-layer architecture or the larger population results in such increased
248 bacterial retention and cell membrane integrity after 6 h incubation, *E.coli* MG1655 growing on PET
249 substrates were incubated with LB culture medium containing quorum sensing inhibitor (FC 30) at a low
250 concentration for 8 h. Based on our previous study (20), the bacterial growth rate would not be affected

251 in this situation, i.e. the number of cells at each hour interval would be same as counterparts without
252 inhibition of quorum sensing, but the multi-layered structure could not form with suppressed quorum
253 sensing, and colonies were therefore remained as large single-layered clusters after 8 h incubation (Fig.
254 5). Compared with the 8 h-samples without FC 30 treatment, the fractions of quorum sensing-inhibited
255 bacteria remaining on surfaces after application of medium and high shear stress both decreased to their
256 levels at 'single-layer clusters' stage (i.e. around 83% and 40%, respectively), and the corresponding
257 fractions of cells with intact membranes respectively declined to $96\% \pm 1\%$ and $58\% \pm 4\%$, which were
258 still significantly higher than their corresponding samples with incubation of less than 6 h (both P values
259 were less than 0.005).

260 To characterize how duration time of shear stress influences bacterial detachment and cell membrane
261 permeability, the original exposure time (120 s) was either shortened to 40 s or extended to 360 s. This
262 effectively tests the total mechanical energy delivered to cells over this time period, as opposed to
263 merely testing the detachment force. The surface samples with *E.coli* MG1655 growing for 5 h and 8 h
264 were chosen for this test. For 5 h-incubation samples, as shown in Fig. 6a, with the increase of duration
265 time, the amount of remaining bacteria slightly declined in the presence of medium fluid shear, while it
266 dramatically fell off from $67\% \pm 5\%$ to $14\% \pm 5\%$ ($P < 0.005$) when high shear stress was applied.
267 Although the longer duration of medium shear stress could hardly stimulate bacterial detachment, it led
268 to a rapidly decrease in the fraction of cells with intact membranes from $65\% \pm 5\%$ to $15\% \pm 3\%$
269 ($P < 0.005$), as the high shear stress did from $44\% \pm 6\%$ to $5\% \pm 2\%$ ($P < 0.005$) (Fig. 6b); For 8 h-
270 incubation samples, Figure 6 shows that the stimulating effect due to the extension of acting time on the
271 detachment and cell membrane permeability is much smaller than that exhibited on the surface with
272 single-layered colonies.

273 Discussion

274 **The role of type-I fimbriae in *E.coli* early biofilm formation.** Type-I fimbriae are generally

275 considered to be necessary for the initial adhesion of *E. coli* on abiotic surfaces, since the fimbrial mutant
276 of *E. coli* was widely reported to be defective in the initial attachment on abiotic surfaces. For instance,
277 Pratt and Kolter incubated polyvinylchloride (PVC) surfaces with diluted bacterial suspension in LB for
278 24 h, and found that type-I fimbrial mutants of *E. coli* were hardly attached (12). Cookson et al.
279 demonstrated that compared with the wild-type *E. coli*, the adherence of type-I fimbrial mutants to
280 surfaces was reduced by 45% after incubation in LB broth for 48 h (29). However, such a long period of
281 culture in the closed system could introduce bacterial detachment from and reattachment back onto the
282 surface, thus rendering it ambiguous whether this change in adhesion is due to the defective adhering
283 ability of mutants or to the detachment after reversible adhesion. In this study, we measured the number
284 of adherent *E. coli* MG1655 Δ *fimA* mutant cells on PET surfaces over time and the external medium in
285 which the PET surfaces were incubated was refreshed each hour to maintain a constant culturing
286 environment. Thus, in our case the continuous recruitment of new cells from the incubation medium
287 onto surfaces was deliberately avoided, to allow us finding the specific role of type-I fimbriae in the
288 initial reversible or irreversible adhesion of *E. coli*, and in the subsequent growth on surfaces.

289 The adhesion assays in which PET substrates were incubated with bacterial suspensions indicate that
290 mutant cells, which lack the type-I fimbria structure, could reach a surface equally well, and then
291 normally undergo the initial attachment. That is, type-I fimbriae of *E. coli* appear to be unnecessary for
292 the ‘cell to surface’ phase of step (i) described in the Introduction. Moreover, the detachment over the
293 first 2 h incubation under unstressed condition reveals that the initially ‘seeded’ cells of wild-type and
294 non-fimbriated *E. coli* were both reversibly attached on PET surfaces with a low barrier for desorption.
295 Hence, the similar amounts of initially adherent cells of both strains suggest that type-I fimbriae may be
296 not an essential factor in the reversible adhesion of *E. coli* to surfaces. Instead, the bacterial surface and
297 the flagella may facilitate the reversible cell-surface contact.

298 The wild-type cells stopped detaching from the surface at the end of lag phase (0-2 h), and more than
299 half of cells could remain surface-bound after medium shear stress treatment, suggesting that after the
300 lag phase the adherent wild-type cells completed transition from reversible to irreversible adhesion.
301 However, the further detachment of adherent *ΔfimA* mutants with the increase of incubation time under
302 unstressed condition shows that non-fimbriated *E.coli* could not achieve a stable cell-surface attachment
303 (i.e., become irreversibly attached). Thus, we could demonstrate that type-I fimbriae are of importance
304 to the irreversible adhesion of *E.coli* on abiotic surfaces. It also indicates that the defect in the initial
305 attachment of fimbrial mutants described in the previous literature (12, 29) may be due to the
306 detachment after adhesion, not to the deficient ability in the reversible attachment.

307 During the lag phase, the adhered bacteria of both strains did not replicate but dramatically increased
308 in size. This is because the initially attached cells on the surface need to synthesize the enzymes and
309 factors needed for cell division under the new environmental conditions (20, 30). For example, the
310 average intracellular concentration of FtsZ (the major cytoskeletal protein during binary fission) in
311 *E.coli* was found to remain constant over the course of the cell cycle, thus bacteria in the lag phase
312 delayed division until they achieved a size with sufficient levels of FtsZ to replicate on surfaces (31, 32).
313 Compared with wild-type counterparts, surprisingly, reversibly attached non-fimbriated cells could not
314 form any cluster after the lag phase. Therefore, it appears that bacterial transition from reversible to
315 irreversible adhesion is quite vital to the initiation micro-colony formation. One possible explanation is
316 that the irreversible attachment mediated by type-I fimbriae would lead to an alteration in the
317 composition of outer membrane proteins of *E.coli* (33), which might trigger the subsequent growth and
318 colony formation. The *ΔfimA* mutants that lack type-I fimbriae could only maintain reversible
319 attachment, thus these adherent non-fimbriated *E.coli* cannot progress into the next stage to grow on the
320 surface.

321 From the above discussion, it is clear that irreversible adhesion is required to initiate the colonization
322 of adherent bacteria on substrates. The irreversibly adherent parent bacteria would divide by undergoing
323 elongation and subsequent separation during which the parent cell and the new daughter *E.coli* would
324 utilize type-I fimbriae to stay irreversibly bound to the surface. By this means, bacterial irreversible
325 attachment on the substrate could be passed on to the next generation. This may explain why the genes
326 encoding structural components of type-I fimbriae were found up-regulated in the biofilm cells (12, 34).
327 Moreover, this also reminds us of the issue that the doubling time in the log phase of attached *E.coli*
328 (~38 min) was more than twice as long as that of planktonic cells (~16 min) under the same incubating
329 condition, which was found in our previous research (20). Different from the proliferation of freely
330 swimming bacteria in liquid medium, newborn daughter cells of surface-bound bacteria may need some
331 time to complete irreversible attachment, thus this time would prolong the doubling time of sessile cells
332 growing on surfaces, which might be the reason for this issue.

333 **The responses of early biofilms to fluid shear stress.** The fluid flow acting parallel to a substratum
334 surface would generate shear stress. When the shear forces overcome the adhesive forces anchoring cells
335 onto the abiotic substrate, the fluid flow may cause adhering bacteria to slide and roll over the surface,
336 potentially detaching them from substrates (16, 35). It could be clearly seen from the 8 h-samples in Fig.
337 3 that the shear flow led to morphological changes in the cellular architecture of micro-colonies through
338 bacterial migration and detachment. The large compacted multi-layered colonies were migrated into
339 loose smaller groups under medium shear stress, and the constituent cells especially those in the upper
340 layer were detached from surfaces under high shear.

341 The fraction of remaining bacteria, which can quantitatively reveal interaction forces between bacteria
342 and substratum surfaces (5, 36), was strongly dependent on the growing phases of *E.coli* cells before
343 they were challenged by the fluid flow. When cells were in the ‘lag phase’, i.e. ‘singles’ stage, although
344 these adherent cells did not start to divide, their binding forces with surfaces were stronger with the

345 increase of incubation time; After the population entered the log phase, the resulting single-layered
346 colonies showed much higher ability to resist removal by shear than the single cells in the lag phase.
347 During the stage of ‘single-layer clusters’, we found the interaction between colonized bacteria and
348 substrates did not increase with the development of micro-colonies, different from bacteria-host tissue
349 interaction which increased with bacterial population during the initial growing on tissue surfaces (37).
350 Instead, it maintained at the same level under the given shear stress until the occurrence of multi-layered
351 colonies when the interaction significantly increased. Combined with the results regarding 8 h-samples
352 with quorum sensing inhibited (single-layered colonies containing an equal amount of cells with the
353 multi-layered still displayed the fraction of remaining cells shown in the stage of ‘single-layer clusters’),
354 it could be concluded that the attachment strength of early biofilms is concerned with the population but
355 with the 3D architecture of micro-colonies, and the multiple-layer structure of biofilms could protect the
356 embedded cells from being insulted by fluid shear, which might be due to the formation of EPS (3, 9).

357 The effect of duration time of shear stress on bacterial retention was found to rely on the relative level
358 of the given shear force and bacterial adhesive force. If the flow shear force was lower than the adhesive
359 force of most of bacteria in the micro-colonies, few loosely attached bacteria would be immediately
360 detached after application of the flow, but the rest of cells would tend to stay on surfaces independent of
361 the periods loading shear stresses. When the applied shear stress was quite high to remove the adhering
362 cells in the colonies, an increase of duration time of the shear would yield a higher bacterial removal
363 from surfaces.

364 Although bacteria are protected by a rigid cell wall composed of peptidoglycans, the external
365 hydrodynamic stress would result in damage inflicted on bacterial cell surface (38-40), however few
366 studies exist on bacterial membrane damage in biofilms caused by flow shear. In this work, by staining
367 cells with a viability kit, the response of cell membrane permeability in micro-colonies to fluid shear
368 was investigated. The cell membrane permeability after medium shear stress treatment kept constant

369 during 0-2 h, i.e., the permeability was not affected by bacterial transition from reversible to irreversible
370 adhesion, which reveals that this effect was independent of bacterial binding strength with substrates.
371 Different from the results on bacterial retention, the permeability was mainly dependent on the
372 population of colonies: The fraction of cells with intact membranes would be kept constant until the
373 amount of constituent cells in micro-colonies was above some critical level. Then the cell membrane
374 integrity after shear treatment increased with the population, even when the 3D architecture of colonies
375 was inhibited by quorum sensing inhibitor. It appears that the multi-layered architecture was able to
376 protect cell surface from damaging due to high flow shear. Moreover, cell membrane permeability in
377 early biofilms was found to be time-dependent regardless of the magnitude of shear stress in the
378 environment.

379 **Conclusions**

380 In this study, we clarify that the type-I fimbriae could facilitate the irreversible adhering process of
381 daughter cells during the exponential phase of early biofilm formation, and after surface-bound bacteria
382 enter the proliferation phase to form micro-colonies, they would be difficult to detach. When quorum
383 sensing is inhibited, the interaction between bacteria in micro-colonies and substrates and the cell
384 membrane of constituent cells are more vulnerable to high shear stress, which suggests that fluid flow
385 shear could be combined with the application of cell signaling inhibitor to remove bacterial
386 contamination on surfaces.

387

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391

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489

490 **Figure legends**

491 Fig.1 CLSM fluorescence images of *E.coli* $\Delta fimA$ mutant cells (labeled as $\Delta fimA$) and wild-type *E. coli*
492 cells (labeled as WT) on PET surfaces at each hour interval. 0 h represents the initial ‘seeded’ bacteria
493 after PET surfaces were incubated with bacterial suspension of $\Delta fimA$ mutants or wild-type cells for 1 h.
494 Cells with intact and compromised membranes are stained with green and red, respectively.

495

496 Fig.2 The number of $\Delta fimA$ mutants (black) and that of wild-type cells (blue) on PET surfaces after
497 incubation with refreshed external LB medium. The data at 0 h refer to the respective number of the
498 initially ‘seeded’ $\Delta fimA$ mutants and the wild-type *E.coli*. The wild-type results are presented in our
499 previous study (20), shown here for comparison.

500

501 Fig.3 CLSM fluorescence images of wild-type *E.coli* MG1655 on PET surfaces, which were incubated
502 with refreshed external LB medium for 2, 6 and 8 h, and then challenged for 120s with low, medium and
503 high shear stress, respectively. Scale bar: 25 μ m. Cells with intact and compromised membranes are
504 stained with green and red, respectively.

505

506 Fig.4 (a) The fraction of wild-type *E.coli* MG1655 remaining on PET surfaces after application of low,
507 medium and high shear stress for 120 s. (b) The corresponding fraction of cells with intact membranes
508 accounting for total cells remaining on surfaces. The data at 0 h refer to the initially ‘seeded’ *E.coli* after
509 fluid shear treatment.

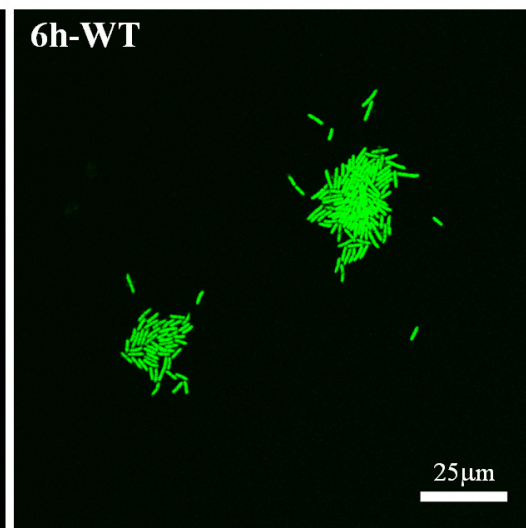
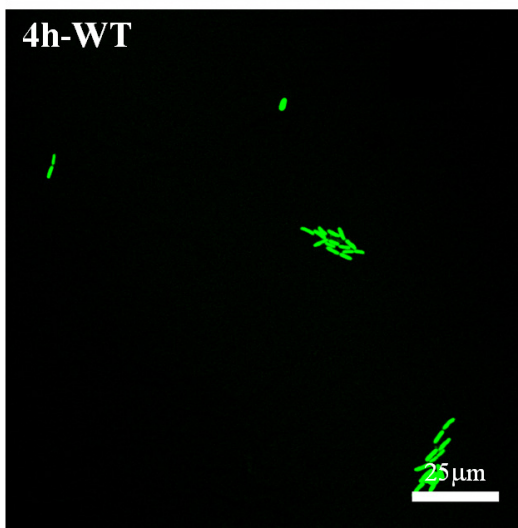
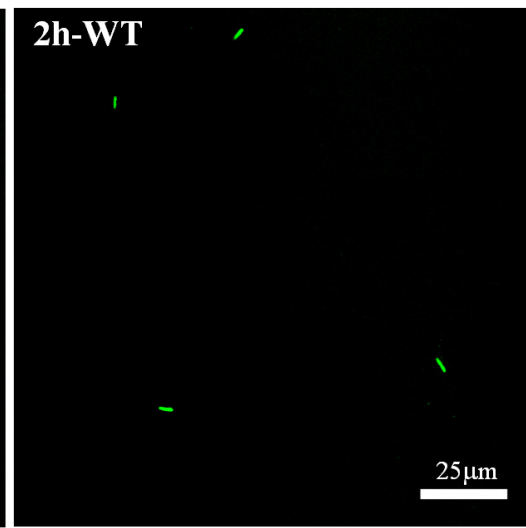
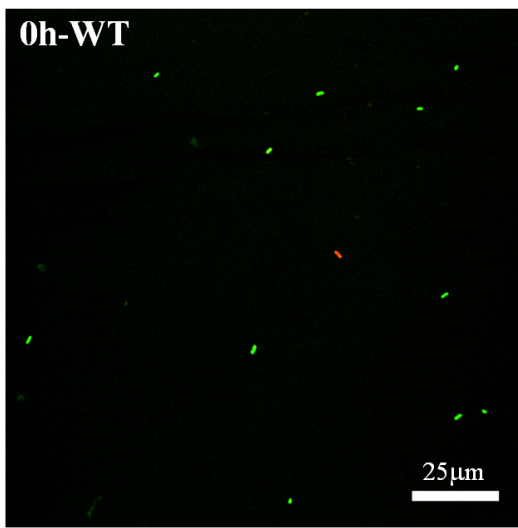
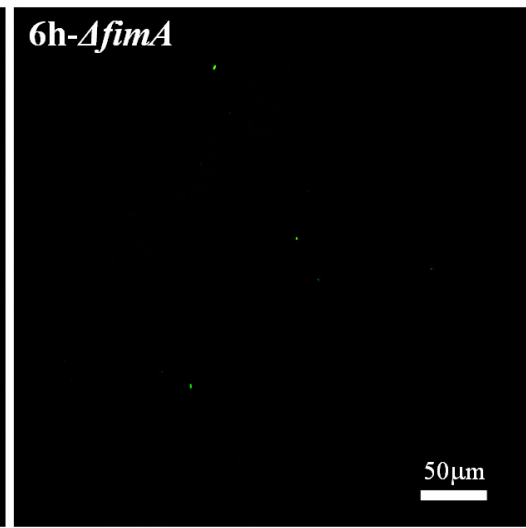
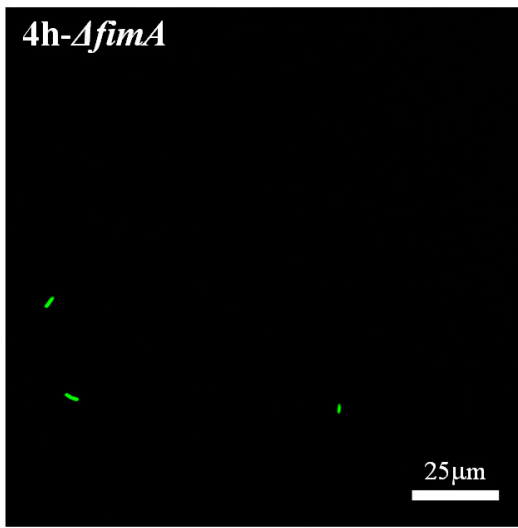
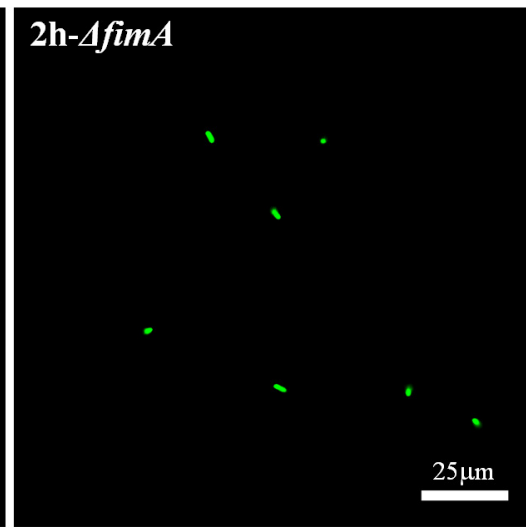
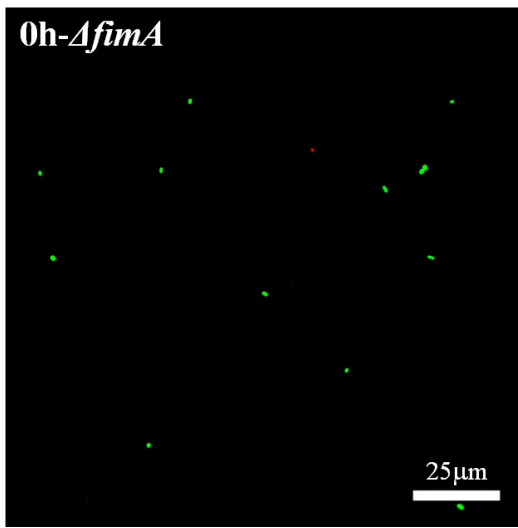
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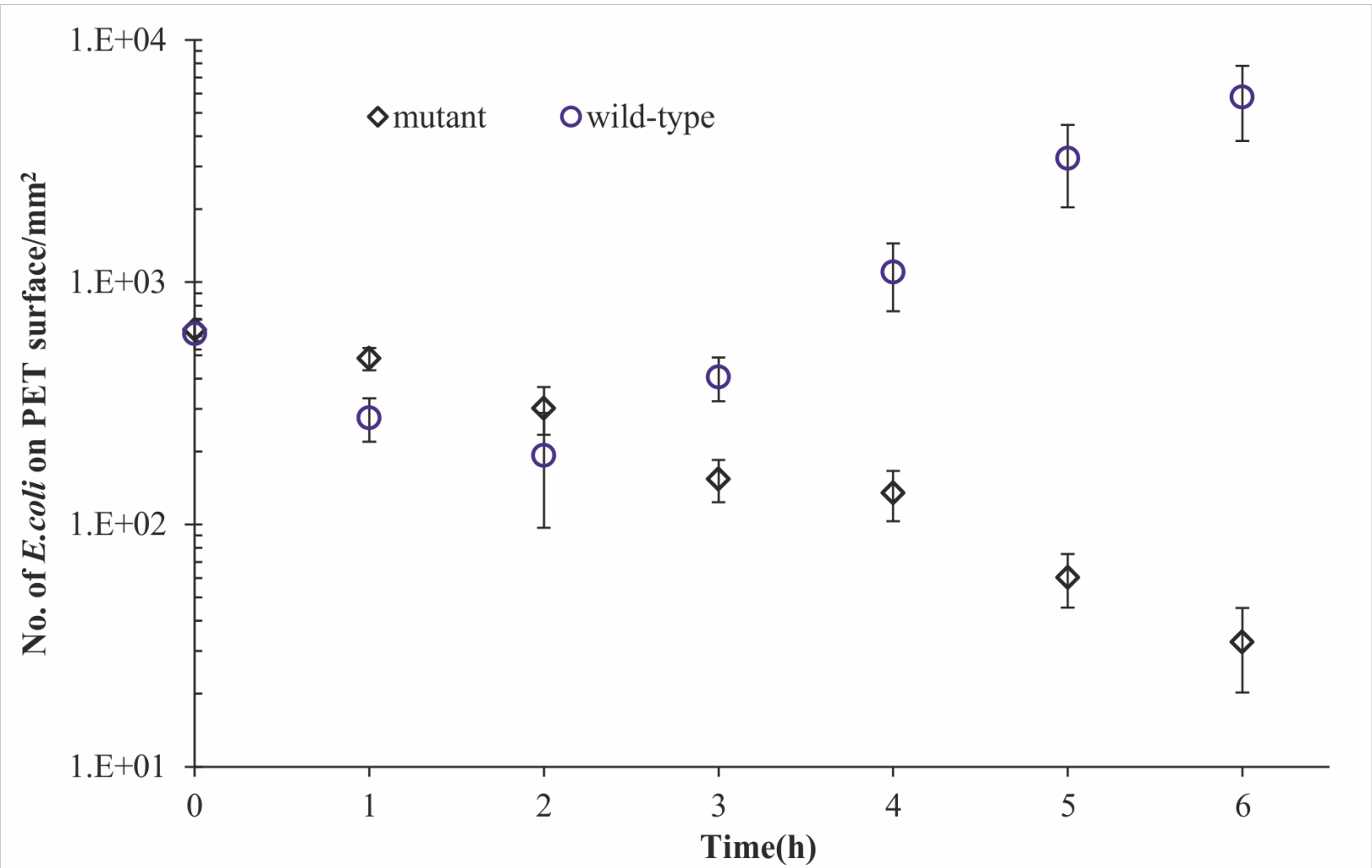
511 Fig. 5 CLSM fluorescence images of *E.coli* MG1655 cells, which grew on PET surfaces for 8 h with
512 quorum sensing inhibited by quorum inhibitor (FC30) and then were respectively treated by low,

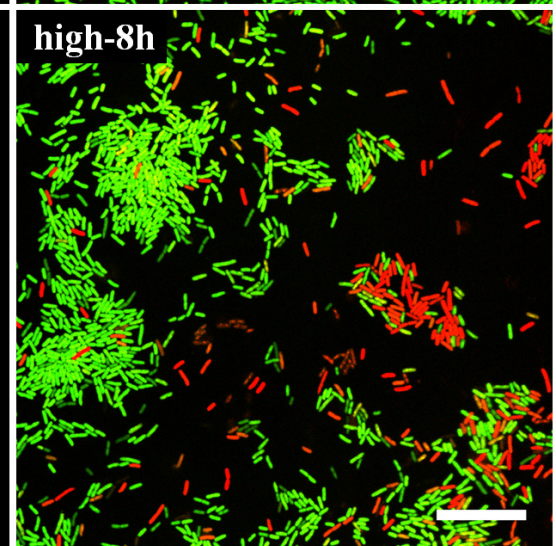
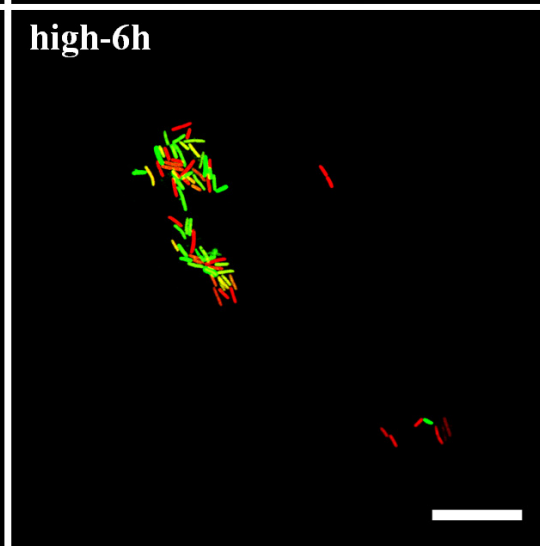
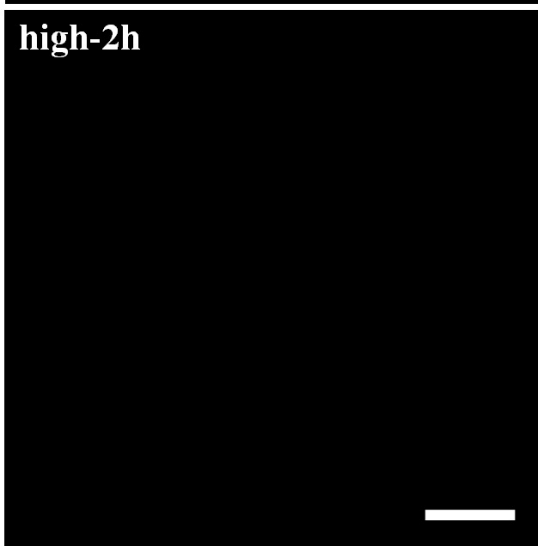
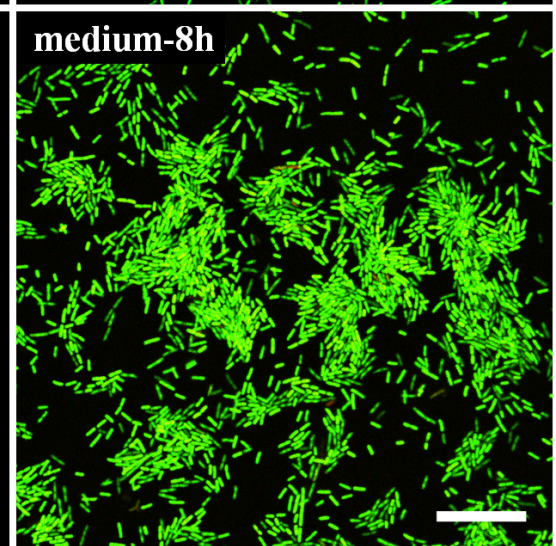
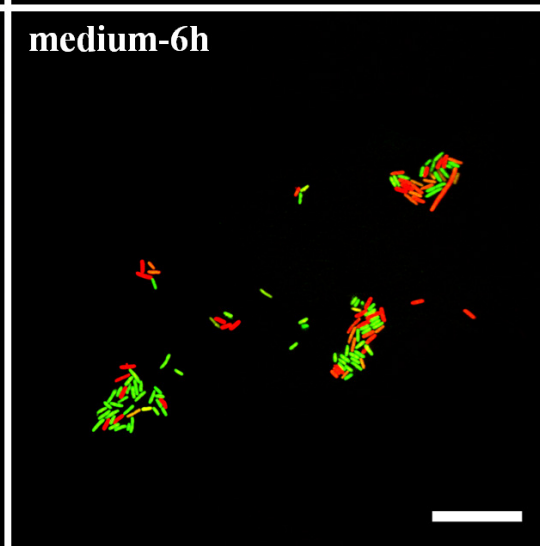
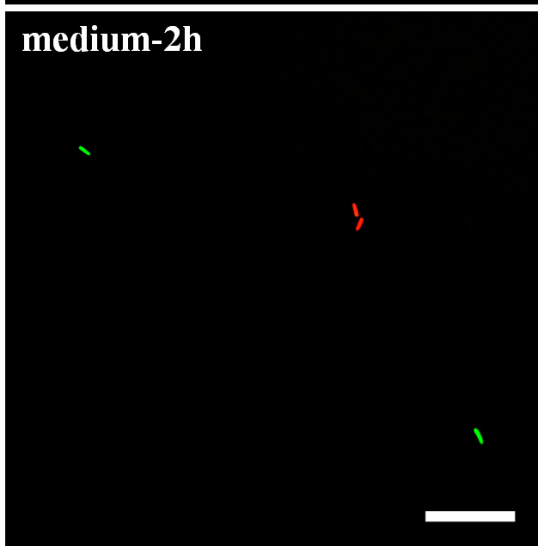
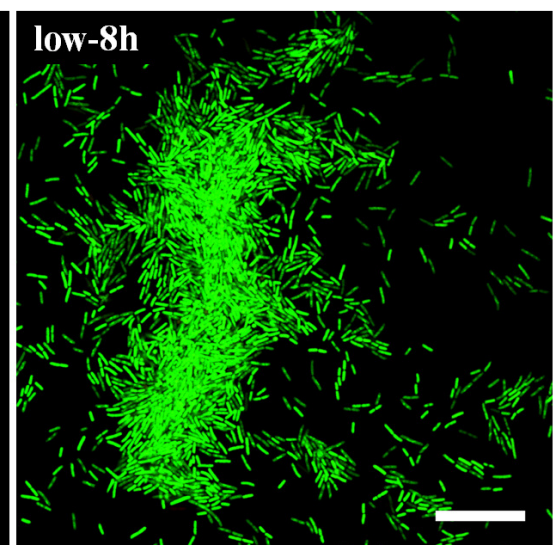
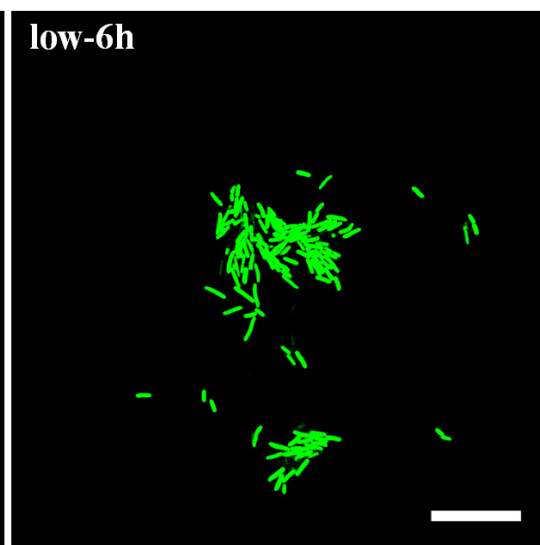
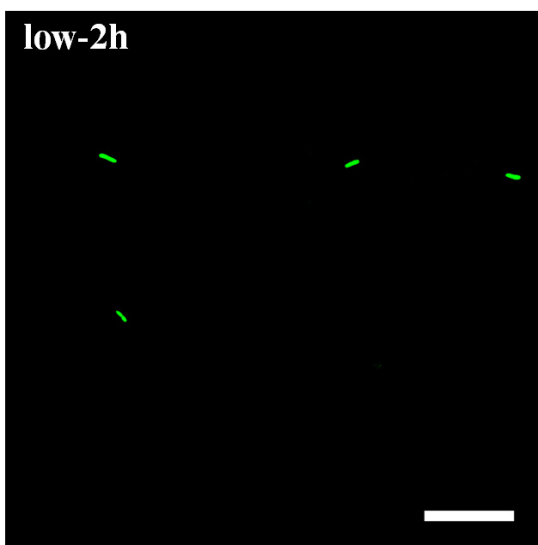
513 medium and high shear stress for 120 s. Control: bacteria grew on PET surfaces under unstressed
514 condition for 8 h without quorum sensing inhibited (the inset shows the upper layer of cells in the
515 multilayer colony, when they are put in focus using CLSM 3D-scanning-mode, and the inset scale bar is
516 10 μm). Cells with intact and compromised membranes are stained with green and red, respectively.

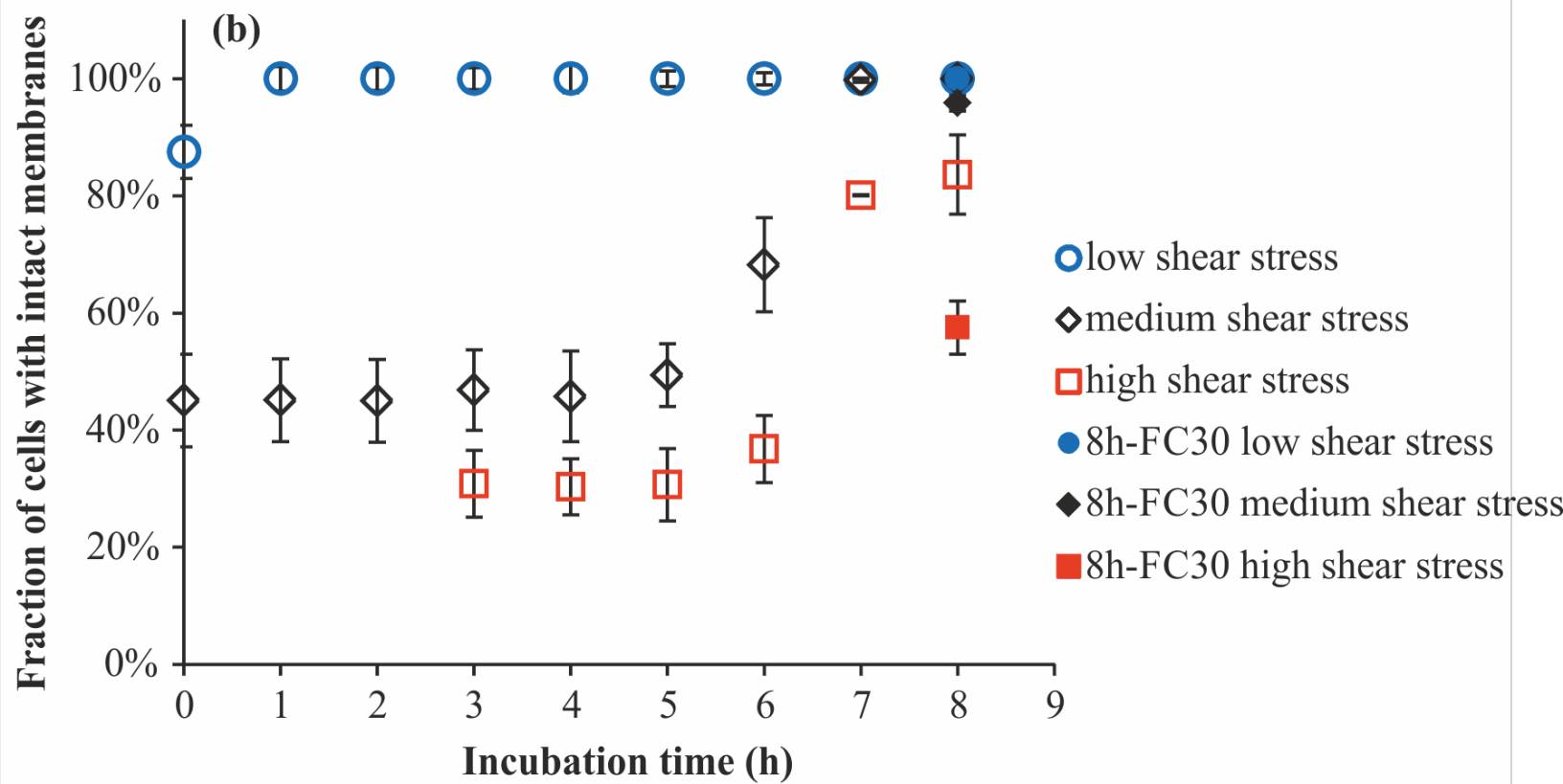
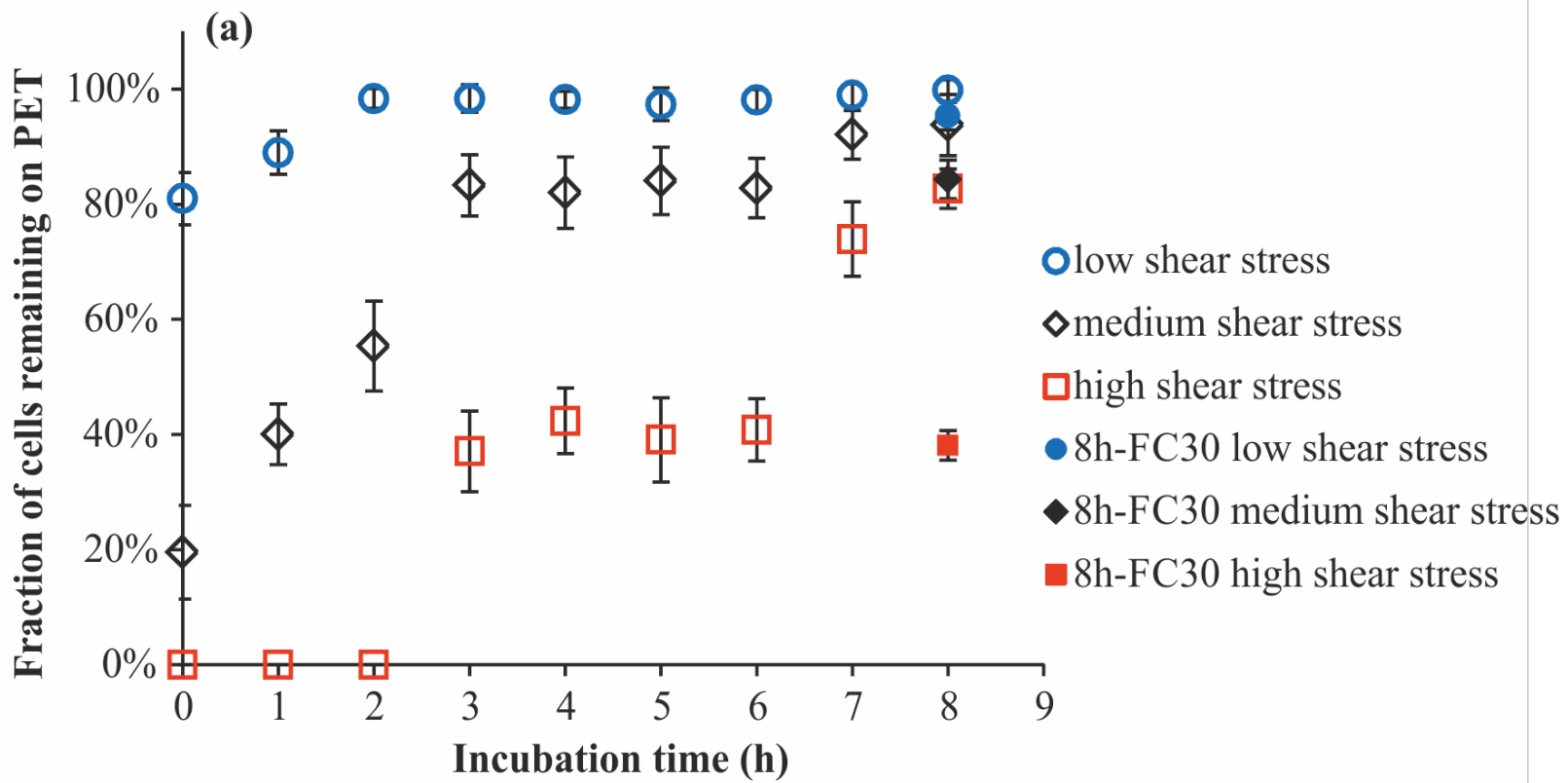
517

518 Fig.6 (a) The fraction of *E.coli* MG1655 remaining on PET surfaces after application of low, medium
519 and high shear stress for 40, 120 and 360 s. (b) The corresponding fraction of cells with intact
520 membranes accounting for total cells remaining on surfaces.

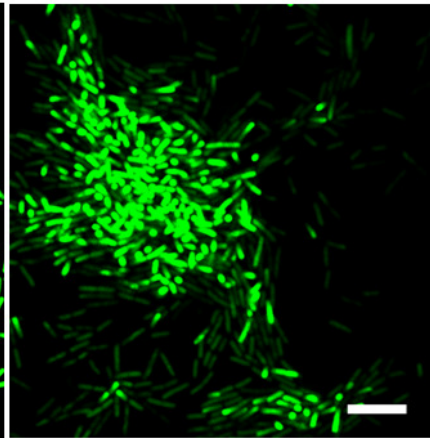




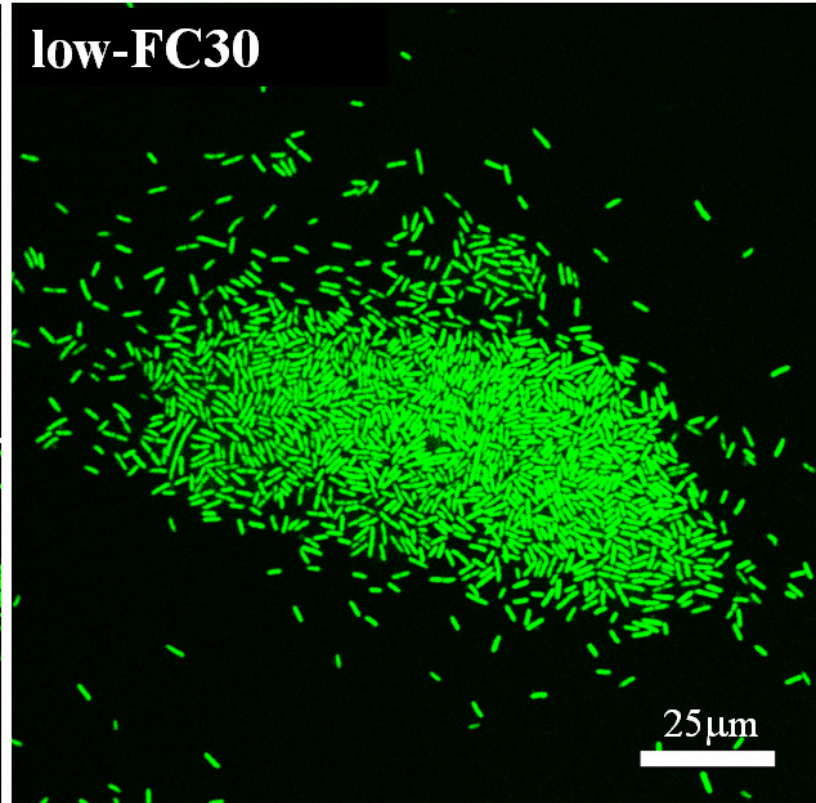




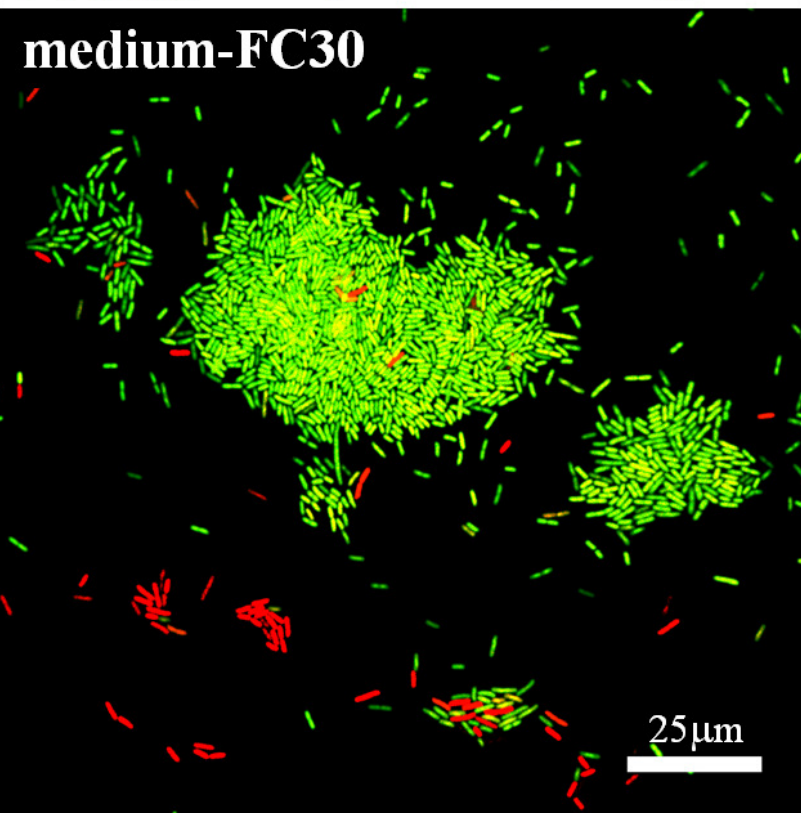
control



low-FC30



medium-FC30



high-FC30

