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

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

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

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

A single colony of a strain was inoculated into a test tube containing 5 mL of culture medium and grown overnight at 37 °C, with agitation at 200 rpm. A 100 μL of this culture was transferred into a fresh tube of 5 mL medium and incubated with shaking until the stationary phase was reached (12 to 14 h) to obtain the eventual microbial suspension, which contained ~10^9 colony-forming units per mL

(CFU/mL).

E. coli MG1655 *ΔfimA::kan* mutant strain (abbreviated as *ΔfimA* mutants in the text) was constructed by oligo-directed mutagenensis (ODM) as described previously (23). Briefly, PCR was used to amplify a kanamycin resistance cassette from the plasmid pBADkanFRT using oligonucleotides specific to the cassette, which have 5' 60bp arms homologous to DNA directly flanking the gene of interest, using primers 947 (aaagtgtacagaacgactgcccatgtcgatttagaaatagttttttgaaaggaaagcagctagggataggcttaccttcaagctc) and 948 (atttttatcgcacaagggtgggcatccctgcccgtaatgacgtccctgaacctgggtaggatgacgatgacaagctccccctttcg). *E.coli* MG1655 containing the pBADλRed plasmid was grown in LB medium containing 100 μg/mL ampicillin at 37˚C, 200rpm to an OD_{595} of 0.25. Expression of the red recombinase genes was induced by the addition of L-arabinose to a final concentration of 0.2% w/v. Cultures were incubated further until an OD_{595} of 0.5 was reached, and electrocompetent cells were prepared. Purified PCR products were used to electroporate the cells, and mutant colonies were selected on LB agar plates containing 50 μg/mL kanamycin. Successful allelic replacement of *fimA* was confirmed by PCR and sequencing using primers 1065 (ctatgagtcaaaatggcccca) and 1066 (agagcctgaataaagccgttt). The pBADλRed plasmid was the cured by serial passage in the absence of antibiotic selection. Given that *fimA* is found within an operon of 9 genes involved in the biogenesis of type-I fimbriae, any polar effects of the *fimA* deletion would only influence other type-I fimbrial genes. Thus, we are confident that the observed phenotype is due to the loss of type-I fimbriae.

Bacterial adhesion and growth on PET surfaces. The samples were prepared by following the procedures reported previously (20), with slight modifications. Briefly, sterilized PET surfaces were rinsed thrice with sterile water and then with LB medium. Each substrate was then placed vertically into a test tube (diameter, 22 mm) containing stationary-phase *E.coli* MG1655 or *ΔfimA* mutant cells suspended in LB culture at 37 °C for 1 h, to carry out the initial adhesion. Three of the samples were subsequently taken out for Confocal Laser Scanning Microscopy (CLSM; LEICA TCS SP5) imaging

and another nine (three replicates for each fluid shear stress) were taken for fluid shear experiments. The other 'seeded' PET plates with wild-type or mutant cells were subjected to the following incubation process with refreshed LB medium.

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

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

CLSM imaging. In a comparison with the adhesion and growth of *E.coli* MG1655 on PET surfaces, which have been previously elaborated (20), surface samples with mutant cells were gently washed with tris-buffered solution (TBS) thrice, stained with the BacLight Live/Dead viability kit (Invitrogen, kit no. L7007) for 15 min in the dark and then visualized using Confocal Laser Scanning Microscopy (LEICA TCS SP5) with an oil-immersion objective lens at 40 × magnification, zoom 1:2.60 or 1:1.00. In CLSM

images, green and red stained cells could indicate membrane permeability status: green cells have intact membranes, and red cells are membrane compromised (i.e. membranes are permeable or damaged).

Fluid shear experiments. A special vibration device was built to detach bacteria by a shear force produced in oscillatory flow. This differs from other techniques (e.g. with rotating disk (26)) where the shear force is consistent in one direction, enabling cells to adapt and develop resistance. In our case, oscillations at high frequency create an essentially random force on 'unprepared' cells, and probe the strength of their natural attachment. In order to expose bacteria to this shear force, samples were glued onto a holder, which was vibrated horizontally in a dish filled with phosphate-buffered solution (PBS). The dish was kept at 37 °C to produce optimal conditions for bacteria. The vibrational movement was produced by a mechanical setup, which moves two magnets underneath the dish by a mechanism converting rotational movement into a horizontal linear motion. The frequency of this motion can be adjusted to different values between 5Hz and 25Hz. The moving magnets were locked to two reciprocal magnets embedded in the sample holder, thus allowing the movement of the sample holder in the dish without any leakage of liquid. The holder vibrated along one axis in the plane of the dish, because its movement was constrained by a track-like excavation in the dish. The shear stress acting on the bacteria on the surface, moving with respect to a stationary fluid above, is a function of vibration frequency. An expression for this stress can be analytically calculated by considering the hydrodynamics around an oscillatory plate in liquid, producing $f_{max} = -r\omega^{3/2}\sqrt{\rho\eta} \cdot A$, where f_{max} is the maximal force acting on the cells during vibration, r is the amplitude of vibration, ω is the angular frequency of vibration [rad/s], ρ and η are the density [kg/m^3] and viscosity [Pa.s]. Finally, A≈ 0.5 μm ×3 μm is the average surface area of the *E.coli* cell. In our experiments, we worked with the shear stress values of 0.58 Pa, 1.45 Pa and 3.30 Pa (referred to for convenience as low, medium and high stress in the following text), which translate to the maximum force amplitude of 0.87 pN, 2.18 pN and 4.95 pN, respectively. These shear stresses are comparable to the wall shear stress (WSS) in medical devices e.g. hemodialysis

169 vascular access (WSS applied mainly ranges from 0.1 to 3 Pa) (27) and central venous catheters (WSS
170 applied mainly ranges from 0 to 1.8 Pa) (28), and those generally used in CIP procedures for food
171 processing equipment (WSS applied in pipes mainly ranges from 0 to 3 Pa) (19).

172 The surface samples with 'seeded' *E.coli* MG1655 or *ΔfimA* mutant cells and those with bacterial cells
173 statically incubated after each consecutive hour were treated by fluid shear stresses, and then stained and
174 visualized by CLSM.

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

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

186 **Results**

187 **The adhesion and growth of *ΔfimA* mutants on surfaces.** After 1 h incubation of PET substrates with
188 *ΔfimA* mutant suspension (i.e. the start point of incubating surface samples with fresh LB medium,
189 labelled as 0 h), the CLSM images (Fig. 1) reveal that the densities of 'seeded' mutant cells and 'seeded'
190 *E.coli* MG1655 cells are $(6.4 \pm 0.7)\times10^2$ cells/mm^2 and $(6.1 \pm 0.9)\times10^2$ cells/mm^2, respectively. It could
191 be seen that there is no significant difference observed in this initial adhesion between non-fimbriated
192 cells and wild-type cells. Following the first 2 h incubation of 'seeded' cells on substrates with sterile LB

medium, Fig. 2 illustrates that the numbers of *ΔfimA* mutants and of *E.coli* MG1655 cells on the surface both decreased with incubation time. Meanwhile, over this 2 h incubation, the cell size of individual adherent *ΔfimA* mutants increased from 2.1 ± 0.3 μm^2 to 5.6 ± 0.4 μm^2 ($P<0.005$), as the wild-type cells did from 2.3 ± 0.4 μm^2 to 5.7 ± 0.5 μm^2 ($P<0.005$).

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

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

Figure 4 summarizes bacterial detachment and cell membrane permeability due to the shear stress treatment. It could be seen from Fig. 4a that higher shear stress acting on adhering bacteria led to lower bacterial remaining population at the same incubation time point, which is consistent with previous literature (15), and that the amount of cells remaining on surfaces increased with bacterial progressing colonization. During the first 2 h incubation when the single cells were in the lag phase, all of attached cells could be removed by high shear stress, while the fraction of remaining bacteria gradually rose from 20% ± 8% at 0 h to 55% ± 8% at 2 h under medium shear stress, implying that reversibly 'seeded' wild-type *E.coli* were being irreversibly immobilized on PET surfaces over this period. The 'seeded' mutant

217 cells on PET substrates (i.e. samples at 0 h) and the adherent mutants on surfaces after the first 1 h or 2 h
218 incubation, were flushed by fluid at the medium shear stress (1.45 Pa) for only 10 s. Compared with
219 wild-type *E.coli*, all of adherent mutants on each surface sample were removed after fluid shear
220 treatment, confirmed by the CLSM images (data not shown). From 3 to 6 h when adhering wild-type
221 bacteria were exponentially growing into single-layered micro-colonies, the bacteria staying on surfaces
222 after being treated by medium and high shear stresses respectively maintained at the level of
223 approximately 83% and 40%. After 7 h when multiple-layer architecture of colonies emerged, the
224 bacterial retention under medium and high shear sharply increased to above 92% and around 80%,
225 respectively. The effect of fluid shear on the bacterial cell membrane permeability is shown in Fig. 4b.
226 Similar to the detachment, Fig. 4b illustrates that higher shear stress also caused lower fraction of cells
227 with intact membranes at the same incubation time point. However, the cell membrane permeability kept
228 constant until the end of 'single-layer clusters' stage (6 h). When clustered bacteria developed into
229 multiple-layer colonies after 7 h, for surfaces exposed to medium and high shear stresses, the fractions
230 of cells with intact membranes on surfaces increased from the level of 45% to above 99% and from the
231 level of 30% to above 80%, respectively.

232 To determine whether the multiple-layer architecture or the larger population results in such increased
233 bacterial retention and cell membrane integrity after 6 h incubation, *E.coli* MG1655 growing on PET
234 substrates were incubated with LB culture medium containing quorum sensing inhibitor (FC 30) at a low
235 concentration for 8 h. Based on our previous study (20), the bacterial growth rate would not be affected
236 in this situation, i.e. the number of cells at each hour interval would be same as counterparts without
237 inhibition of quorum sensing, but the multi-layered structure could not form with suppressed quorum
238 sensing, and colonies were therefore remained as large single-layered clusters after 8 h incubation (Fig.
239 5). Compared with the 8 h-samples without FC 30 treatment, the fractions of quorum sensing-inhibited
240 bacteria remaining on surfaces after application of medium and high shear stress both decreased to their

levels at ‘single-layer clusters’ stage (i.e. around 83% and 40%, respectively), and the corresponding fractions of cells with intact membranes respectively declined to 96% ± 1% and 58% ± 4%, which were still significantly higher than their corresponding samples with incubation of less than 6 h (both P values were less than 0.005).

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

Discussion

The role of type-I fimbriae in *E.coli* early biofilm formation. Type-I fimbriae are generally considered to be necessary for the initial adhesion of *E.coli* on abiotic surfaces, since the fimbrial mutant of *E.coli* was widely reported to be defective in the initial attachment on abiotic surfaces. For instance, Pratt and Kolter incubated polyvinylchloride (PVC) surfaces with diluted bacterial suspension in LB for 24 h, and found that type-I fimbrial mutants of *E.coli* were hardly attached (12). Cookson et al. demonstrated that compared with the wild-type *E.coli*, the adherence of type-I fimbrial mutants to

surfaces was reduced by 45% after incubation in LB broth for 48 h (29). However, such a long period of culture in the closed system could introduce bacterial detachment from and reattachment back onto the surface, thus rendering it ambiguous whether this change in adhesion is due to the defective adhering ability of mutants or to the detachment after reversible adhesion. In this study, we measured the number of adherent *E. coli* MG1655 *ΔfimA* mutant cells on PET surfaces over time and the external medium in which the PET surfaces were incubated was refreshed each hour to maintain a constant culturing environment. Thus, in our case the continuous recruitment of new cells from the incubation medium onto surfaces was deliberately avoided, to allow us finding the specific role of type-I fimbriae in the initial reversible or irreversible adhesion of *E.coli,* and in the subsequent growth on surfaces.

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

The wild-type cells stopped detaching from the surface at the end of lag phase (0-2 h), and more than half of cells could remain surface-bound after medium shear stress treatment, suggesting that after the lag phase the adherent wild-type cells completed transition from reversible to irreversible adhesion. However, the further detachment of adherent *ΔfimA* mutants with the increase of incubation time under unstressed condition shows that non-fimbriated *E.coli* could not achieve a stable cell-surface attachment (i.e., become irreversibly attached). Thus, we could demonstrate that type-I fimbriae are of importance

to the irreversible adhesion of *E.coli* on abiotic surfaces. It also indicates that the defect in the initial attachment of fimbrial mutants described in the previous literature (12, 29) may be due to the detachment after adhesion, not to the deficient ability in the reversible attachment.

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

From the above discussion, it is clear that irreversible adhesion is required to initiate the colonization of adherent bacteria on substrates. The irreversibly adherent parent bacteria would divide by undergoing elongation and subsequent separation during which the parent cell and the new daughter *E.coli* would utilize type-I fimbriae to stay irreversibly bound to the surface. By this means, bacterial irreversible attachment on the substrate could be passed on to the next generation. This may explain why the genes encoding structural components of type-I fimbriae were found up-regulated in the biofilm cells (12, 34). Moreover, this also reminds us of the issue that the doubling time in the log phase of attached *E.coli*

(~38 min) was more than twice as long as that of planktonic cells (~16 min) under the same incubating condition, which was found in our previous research (20). Different from the proliferation of freely swimming bacteria in liquid medium, newborn daughter cells of surface-bound bacteria may need some time to complete irreversible attachment, thus this time would prolong the doubling time of sessile cells growing on surfaces, which might be the reason for this issue.

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

The fraction of remaining bacteria, which can quantitatively reveal interaction forces between bacteria and substratum surfaces (5, 36), was strongly dependent on the growing phases of *E.coli* cells before they were challenged by the fluid flow. When cells were in the 'lag phase', i.e. 'singles' stage, although these adherent cells did not start to divide, their binding forces with surfaces were stronger with the increase of incubation time; After the population entered the log phase, the resulting single-layered colonies showed much higher ability to resist removal by shear than the single cells in the lag phase. During the stage of 'single-layer clusters', we found the interaction between colonized bacteria and substrates did not increase with the development of micro-colonies, different from bacteria-host tissue interaction which increased with bacterial population during the initial growing on tissue surfaces (37). Instead, it maintained at the same level under the given shear stress until the occurrence of multi-layered colonies when the interaction significantly increased. Combined with the results regarding 8 h-samples

with quorum sensing inhibited (single-layered colonies containing an equal amount of cells with the multi-layered still displayed the fraction of remaining cells shown in the stage of 'single-layer clusters'), it could be concluded that the attachment strength of early biofilms is concerned with the population but with the 3D architecture of micro-colonies, and the multiple-layer structure of biofilms could protect the embedded cells from being insulted by fluid shear, which might be due to the formation of EPS (3, 9).

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

Although bacteria are protected by a rigid cell wall composed of peptidoglycans, the external hydrodynamic stress would result in damage inflicted on bacterial cell surface (38-40), however few studies exist on bacterial membrane damage in biofilms caused by flow shear. In this work, by staining cells with a viability kit, the response of cell membrane permeability in micro-colonies to fluid shear was investigated. The cell membrane permeability after medium shear stress treatment kept constant during 0-2 h, i.e., the permeability was not affected by bacterial transition from reversible to irreversible adhesion, which reveals that this effect was independent of bacterial binding strength with substrates. Different from the results on bacterial retention, the permeability was mainly dependent on the population of colonies: The fraction of cells with intact membranes would be kept constant until the amount of constituent cells in micro-colonies was above some critical level. Then the cell membrane integrity after shear treatment increased with the population, even when the 3D architecture of colonies was inhibited by quorum sensing inhibitor. It appears that the multi-layered architecture was able to

protect cell surface from damaging due to high flow shear. Moreover, cell membrane permeability in early biofilms was found to be time-dependent regardless of the magnitude of shear stress in the environment.

Conclusions

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

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Figures

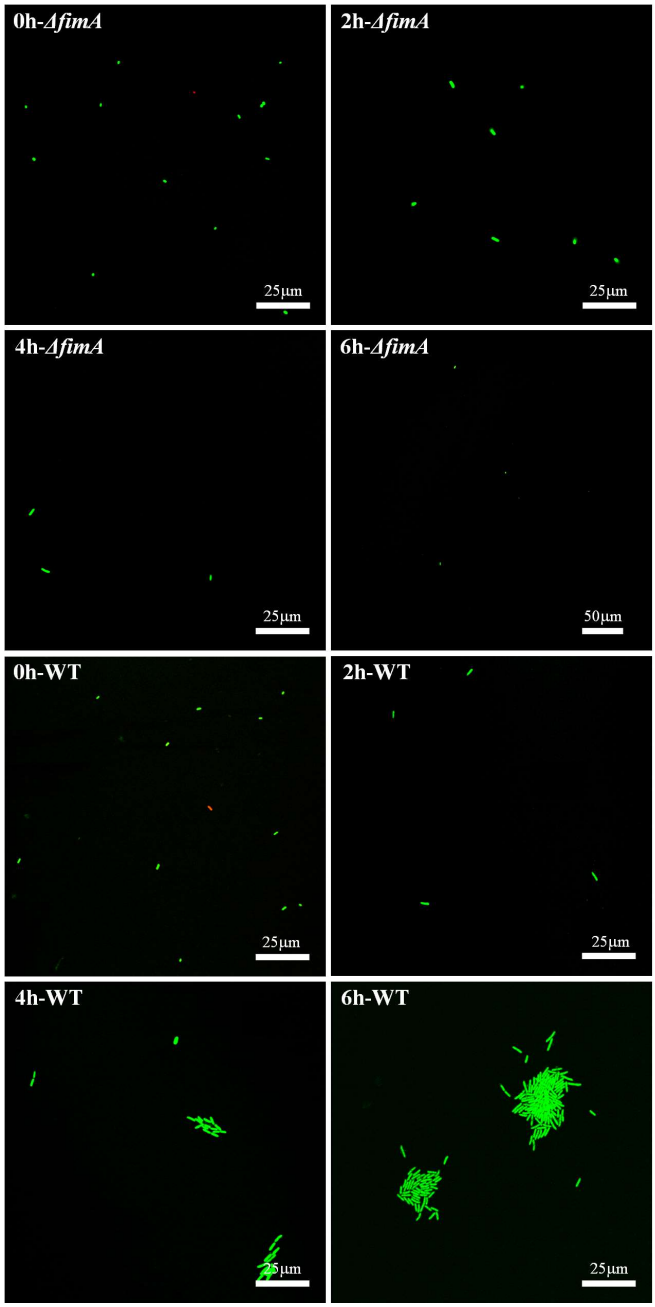
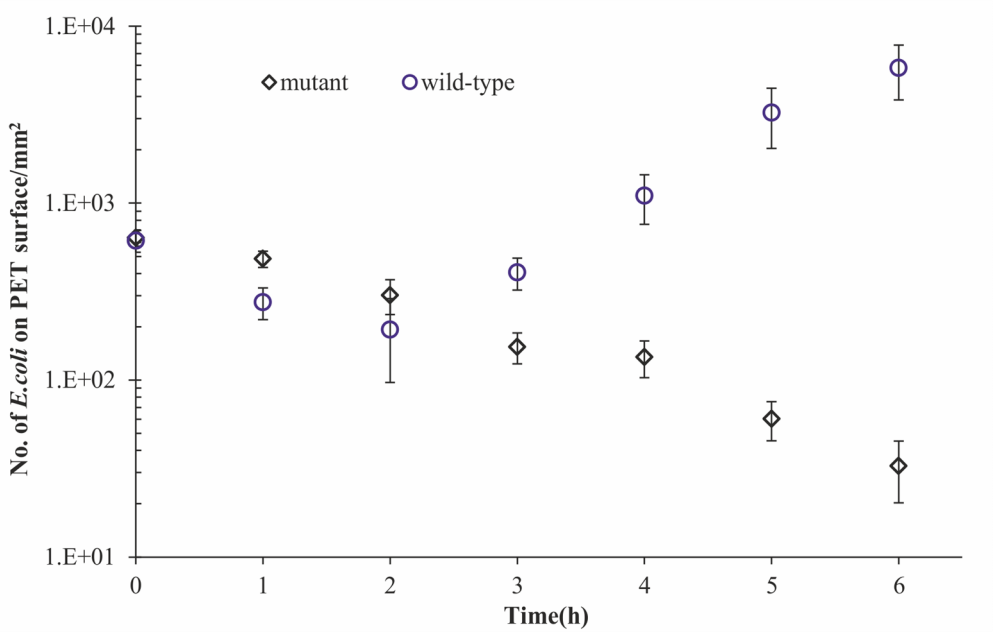


Fig.1 CLSM fluorescence images of *E.coli ΔfimA* mutant cells (labeled as *ΔfimA*) and wild-type *E. coli* cells (labeled as WT) on PET surfaces at each hour interval. 0 h represents the initial 'seeded' bacteria after PET surfaces were incubated with bacterial suspension of *ΔfimA* mutants or wild-type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



483

484 Fig.2 The number of *ΔfimA* mutants (black) and that of wild-type cells (blue) on PET surfaces after
485 incubation with refreshed external LB medium. The data at 0 h refer to the respective number of the
486 initially 'seeded' *ΔfimA* mutants and the wild-type *E.coli*. The wild-type results are presented in our
487 previous study (20), shown here for comparison.

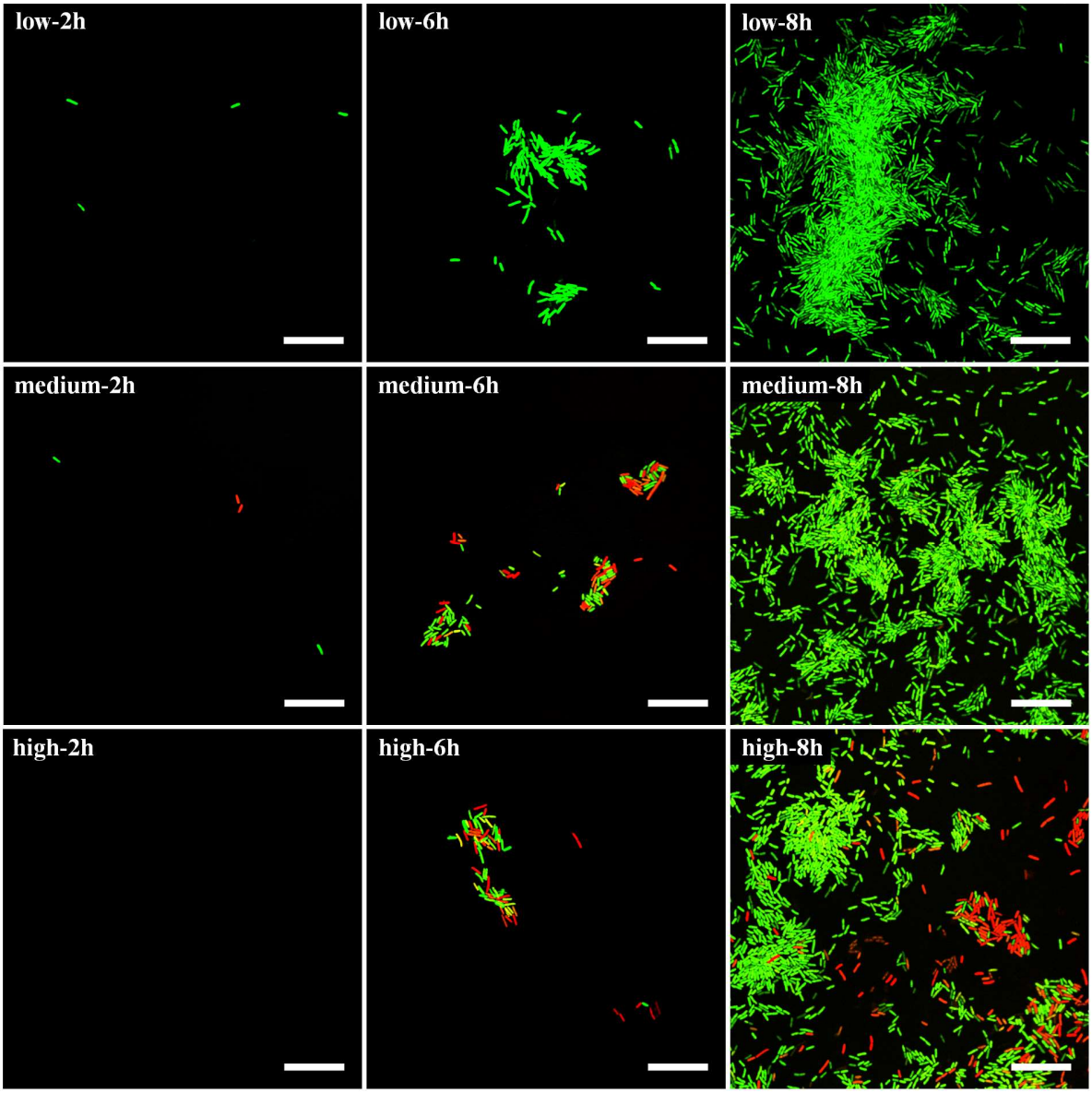
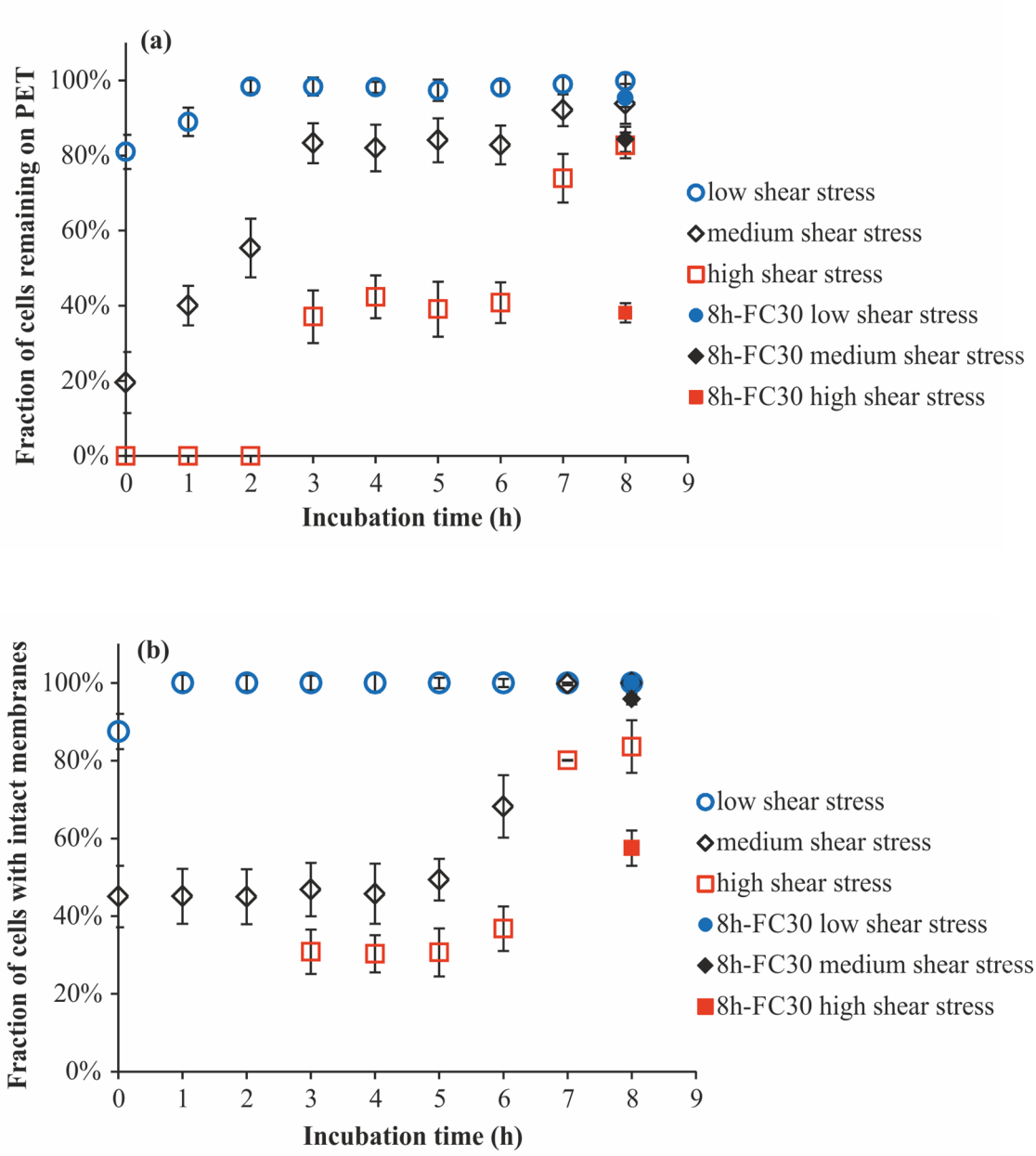


Fig.3 CLSM fluorescence images of wild-type *E.coli* MG1655 on PET surfaces, which were incubated with refreshed external LB medium for 2, 6 and 8 h, and then challenged for 120s with low, medium and high shear stress, respectively. Scale bar: 25 μm. Cells with intact and compromised membranes are stained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



493

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

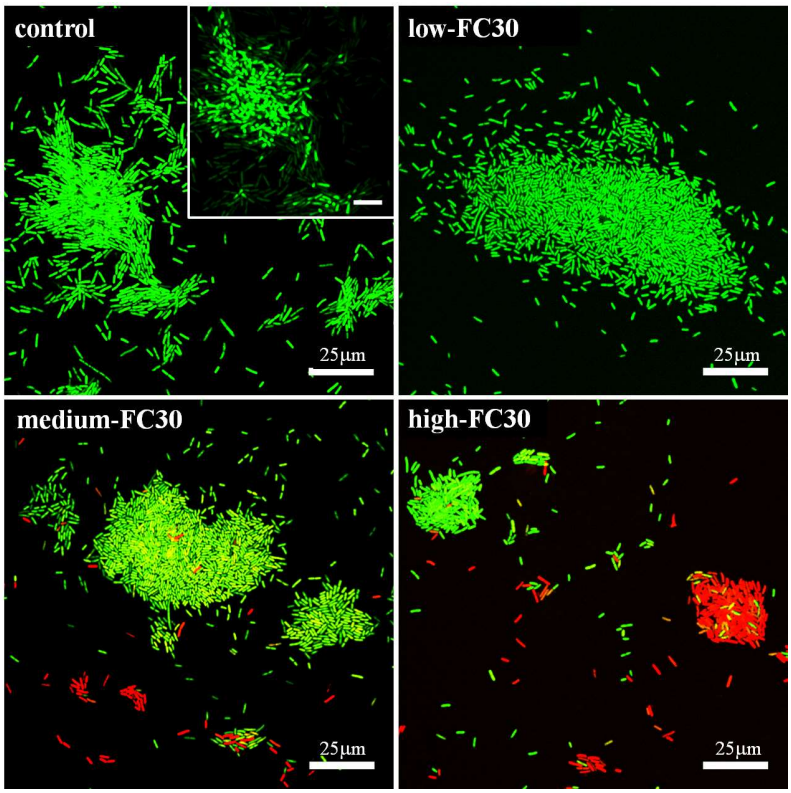
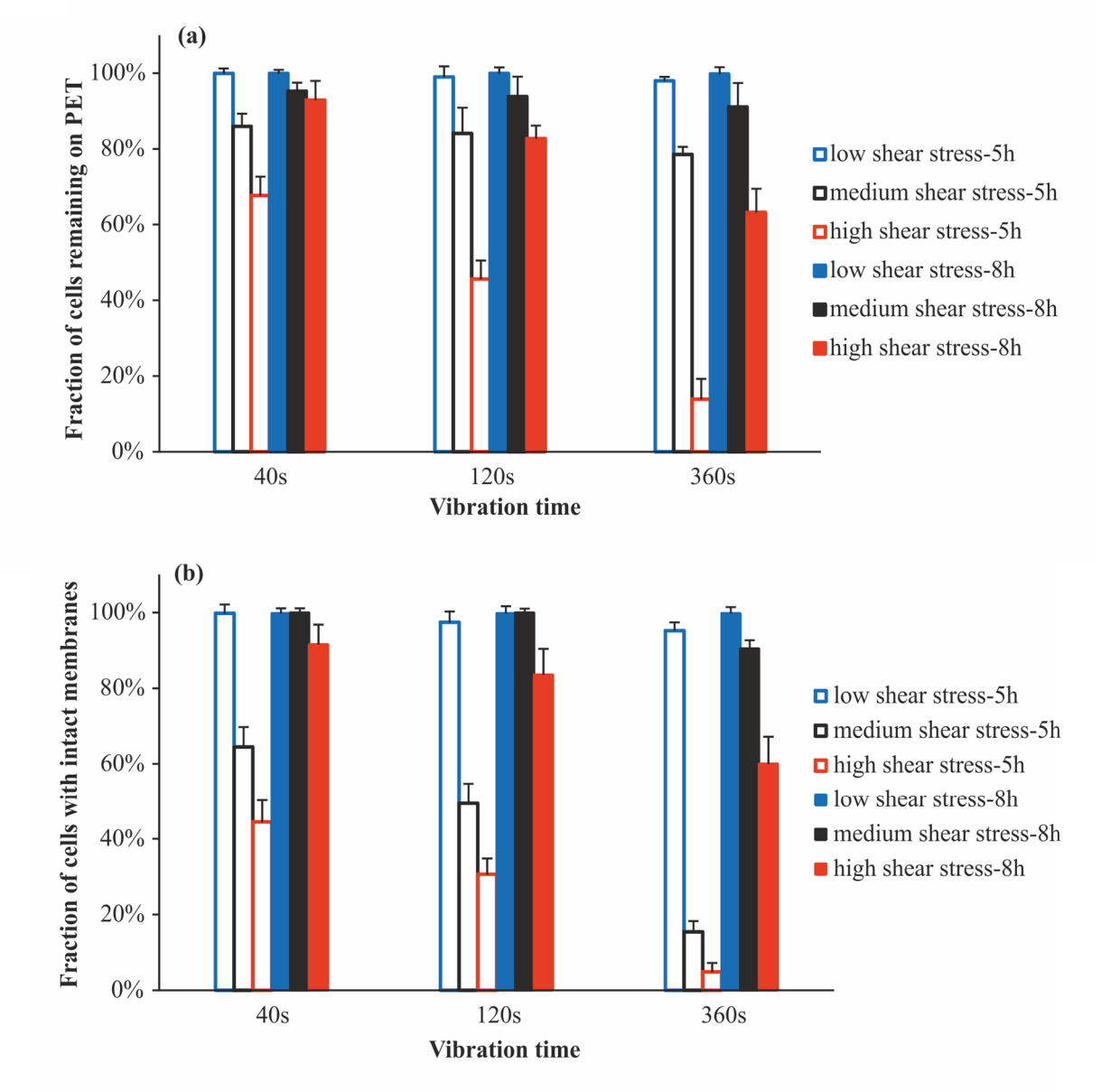


Fig. 5 CLSM fluorescence images of *E.coli* MG1655 cells, which grew on PET surfaces for 8 h with quorum sensing inhibited by quorum inhibitor (FC30) and then were respectively treated by low, medium and high shear stress for 120 s. Control: bacteria grew on PET surfaces under unstressed condition for 8 h without quorum sensing inhibited (the inset shows the upper layer of cells in the multilayer colony, when they are put in focus using CLSM 3D-scanning-mode, and the inset scale bar is 10 μm). Cells with intact and compromised membranes are stained with green and red, respectively.



505

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