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

IMPORTANCE Bacterial biofilms could lead to severe contamination problems in medical devices and food processing equipment. However, biofilms are usually studied at a rough macroscopic level; thus, little is known about how individual bacterium behavior within biofilms and the multicellular architecture are influenced by bacterial appendages (e.g., pili/fimbriae) and environmental factors during early biofilm formation. We applied confocal laser scanning microscopy (CLSM) to visualize *Escherichia coli* microcolonies at a single-cell resolution. Our findings suggest that type I fimbriae are vital to the initiation of bacterial proliferation on surfaces. We also found that the fluid shear stress affects the biofilm architecture and cell membrane permeability of the constituent bacteria in a different way: the onset of the biofilm is linked with the three-dimensional morphology, while membranes are regulated by the overall population of microcolonies.

KEYWORDS early biofilms, type I fimbriae, irreversible adhesion, fluid shear stress

Biofilms are sessile communities of bacteria that grow on solid surfaces and are embedded in an extracellular polymeric substance (EPS) matrix (1–3). 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, the bacteria begin adhering to the surfaces. Initially, adherent bacterial cells are attached to the surface reversibly, and many detach to resume the planktonic lifestyle due to the low activation energy for

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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 microcolonies on the surface. (iv) Bacteria synthesize a matrix of extracellular polymeric substances, allowing microcolonies to further develop into mature biofilms, followed by (v) the shedding of part of the biofilm to release planktonic bacteria into the surrounding environment.

Biofilm formation is a key issue in the food industry and in clinical settings. Surfaces infected by biofilms in food industrial equipment, medical devices, and implants could commonly cause illnesses, lethal infections, and heavy costs for maintenance (7, 8). Furthermore, when organized into biofilms after maturation, bacteria are resistant to many forms of stress, because the EPS is designed to protect the embedded bacteria from antibiotics, disinfectants, and environmental insults (9). The formation of biofilms has therefore been intensively studied, especially in terms of the dynamics of the adhesion process and the genes involved in EPS production, as well as the regulatory mechanisms and the overall morphology of mature biofilms (9, 10). Nevertheless, to provide better insight into the mechanisms underlying biofilm formation, the nascent stages of biofilm development (i.e., adhesion and proliferation) need to be further elaborated at a 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 could alter the morphology of biofilms (11) or lead to defective biofilm formation (12); fluid shear could cause biofilm breakage (13) and deformation (14). However, at the single-cell resolution, although the critical role of fimbriae/pili in bacterial adherence to abiotic surfaces has been extensively studied over the past 2 decades, little is understood about their effect on the proliferation stage of biofilm formation (step iii), and few studies have focused on the response of early biofilms, including cell membrane permeability and multicellular architecture, to fluid shear stress. Typically, the effects of shear stress on bacterial adhesion and macroscopic biofilm morphology on abiotic substrates are investigated using bacteria suspended in the fluid, which could reveal how the flow affects biofilm formation when the surfaces are exposed to a bacterium-containing 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 polyethylene terephthalate (PET) substrates were statically incubated before the application of shear stress, thus rendering it possible to focus only 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 *Escherichia coli* strain MG1655 biofilms on PET surfaces during the early stages of formation (steps i to iii on the above-described list) at a single-cell resolution (20). These changes revealed the dynamics and the structural principle by which individual cells developed into microcolonies. 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 the extra expenditure of the cells to remain adhered to the surface. We found that the three-dimensional (3D) cellular architecture of early biofilms could influence the 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, including 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 attachment—and the effects of fluid shear stress on bacterial behavior and cellular architecture of *E. coli* microcolonies. 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 determine how cell behavior is affected when the collective architecture of microcolonies is challenged by fluid shear.

RESULTS

Adhesion and growth of $\Delta fimA$ mutants on surfaces. After 1 h of incubation of PET substrates with a $\Delta fimA$ mutant suspension (i.e., the starting point of incubating surface samples with fresh LB, labeled 0 h), the confocal laser scanning microscopy (CLSM) images (Fig. 1) reveal that the densities of “seeded” mutant cells and “seeded” *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. There was no significant difference observed in this initial adhesion between nonfimbriated cells and wild-type cells. Following the first 2 h of incubation of seeded cells on substrates with sterile LB, the numbers of both $\Delta fimA$ mutants and *E. coli* MG1655 cells on the surfaces decreased with incubation time, as illustrated in Fig. 2. Meanwhile, over this 2 h of incubation, the cell size of individual adherent $\Delta fimA$ 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 did from $2.3 \pm 0.4 \mu\text{m}^2$ to $5.7 \pm 0.5 \mu\text{m}^2$ ($P < 0.005$).

Thereafter, the behavior of adherent nonfimbriated *E. coli* cells was totally different from that of wild-type cells: after 2 h, the apparent numbers of surface-bound wild-type cells stopped decreasing and they 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 nonfimbriated cells remained reversibly attached to the substrate by purely physical interactions.

Effect of fluid shear stress on microcolonies. To elaborate on the effect of shear stress on the cellular response of early biofilms, the *E. coli* MG1655 cells growing on PET substrates, which exhibit three stages, namely, “singles” (only single cells could be observed in the field of view, i.e., lag phase), “single-layer clusters,” and “multilayer 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 microcolonies to shear stress includes cell membrane permeability.

Figure 4 summarizes the results of bacterial detachment and cell membrane permeability due to the shear stress treatment. It can be seen in Fig. 4a that a higher shear stress acting on adhering bacteria led to a smaller bacterial population remaining at the same incubation time point, which is consistent with previous literature (15), and that the amount of cells remaining on the surfaces increased with progressing bacterial colonization. During the first 2 h of incubation when the single cells were in the lag phase, all of the attached cells could be removed by a high shear stress, while the fraction of bacteria remaining rose gradually from $20\% \pm 8\%$ at 0 h to $55\% \pm 8\%$ at 2 h under medium shear stress, implying that reversibly seeded wild-type *E. coli* cells were being irreversibly immobilized on PET surfaces during this period. The seeded mutant cells on PET substrates (i.e., samples at 0 h) and the adherent mutants on surfaces after the first 1 h or 2 h of incubation were flushed by the fluid at the medium shear stress (1.45 Pa) for only 10 s. Compared with that of the wild-type *E. coli* cells, all of the adherent mutants on each surface sample were removed after fluid shear treatment, which was confirmed by the CLSM images (data not shown). From 3 to 6 h, when adhering wild-type bacteria were growing exponentially into single-layered microcolonies, the bacteria staying on the surfaces after being treated by medium and high shear stresses were maintained at the levels of approximately 83% and 40%, respectively.

FIG 1 CLSM fluorescence images of *E. coli* $\Delta fimA$ mutant cells and wild-type (WT) *E. coli* cells on PET surfaces at each hour interval. The time 0 h represents the initial seeded bacteria after PET surfaces were incubated with a bacterial suspension of $\Delta fimA$ mutants or wild-type cells for 1 h. Cells with intact and compromised membranes are green and red, respectively.

After 7 h, when the multiple-layer architecture of colonies emerged, the bacterial retentions under medium and high shear stresses increased sharply to higher than 92% and around 80%, respectively. The effect of fluid shear on the bacterial cell membrane permeability is shown in Fig. 4b. Similar to the detachment, Fig. 4b illustrates that higher shear stress also resulted in a smaller fraction of cells with intact membranes at the same incubation time point. However, the cell membrane permeability remained constant until the end of the single-layer clusters stage (6 h). When the clustered bacteria developed into multiple-layer colonies after 7 h, for surfaces exposed to the medium and high shear stresses, the fractions of cells with intact membranes on the

FIG 2 Numbers of $\Delta fimA$ mutants (black) and wild-type cells (blue) on PET surfaces after incubation with refreshed external LB. The data at 0 h refer to the respective numbers of the initially seeded $\Delta fimA$ mutants and the wild-type *E. coli* cells. The wild-type results were presented in our previous study (20) and are shown here for comparison.

surfaces increased from 45% to more than 99% and from 30% to more than 80%, respectively.

To determine whether the multiple-layer architecture or the larger population resulted in increased bacterial retention and cell membrane integrity after 6 h of incubation, *E. coli* MG1655 cells growing on PET substrates were incubated with LB culture medium containing a quorum sensing inhibitor, FC 30 [(Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone], at a low concentration for 8 h. On the basis of the results from our previous study (20), the bacterial growth rate should not be affected in this situation, i.e., the number of cells at each hour interval would be the same as that for the counterparts without inhibition of quorum sensing, but the multilayered structure could not form with suppressed quorum sensing, and the colonies therefore remained as large single-layered clusters after 8 h of incubation (Fig. 5). Compared with that from the 8-h samples without FC 30 treatment, the fractions of quorum sensing-inhibited bacteria remaining on the surfaces after the applications of both medium and high shear stresses 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 declined to $96\% \pm 1\%$ and $58\% \pm 4\%$, respectively, which were still significantly larger than their corresponding samples with incubations of less than 6 h (both *P* values were less than 0.005).

To characterize how the duration 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 during this time period as opposed to merely testing the detachment force. The surface samples with *E. coli* MG1655 cells growing for 5 h and 8 h were chosen for this test. For the 5-h incubation samples, as shown in Fig. 6a, with the increase in duration, the amount of remaining bacteria declined slightly in the presence of medium fluid shear, while it dramatically fell off from $67\% \pm 5\%$ to $14\% \pm 5\%$ ($P < 0.005$) when high shear stress was applied. Although the longer duration of medium shear stress could hardly stimulate bacterial detachment, it led to a rapid decrease in the fraction of cells with intact membranes from $65\% \pm 5\%$ to $15\% \pm 3\%$ ($P < 0.005$), as the high shear stress did from $44\% \pm 6\%$ to $5\% \pm 2\%$ ($P < 0.005$) (Fig. 6b). For the 8-h incubation samples, Fig. 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.

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

DISCUSSION

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* to abiotic surfaces, since the fimbrial mutant of *E. coli* was widely reported to be defective in the initial attachment to abiotic surfaces. For instance, Pratt and Kolter incubated polyvinylchloride (PVC) surfaces with a 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 for 48 h (23). However, such a long period of culture in the closed system could introduce bacterial detachment from and reattachment to the surface, thus rendering it ambiguous whether this change in adhesion is due to the defective adhering ability of mutants or to their detachment after reversible adhesion. In this study, we measured the number of adherent *E. coli* MG1655 Δ *fimA* 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 culture environment. Thus, in our case, the continuous recruitment of new cells from

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

the incubation medium onto the surfaces was deliberately avoided, enabling us to find the specific roles of type I fimbriae in the initial reversible or irreversible adhesion of *E. coli* and in the subsequent growth on surfaces.

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

The wild-type cells stopped detaching from the surface at the end of the lag phase (0 to 2 h), and more than half of the cells remained surface bound after medium shear stress treatment, suggesting that after the lag phase, the adherent wild-type cells completed the transition from reversible to irreversible adhesion. However, the further detachment of adherent $\Delta fimA$ mutants with the increase of incubation time under an unstressed condition shows that nonfimbriated *E. coli* could not achieve a stable surface attachment (i.e., become irreversibly attached). Thus, we demonstrated that type I fimbriae are of importance to the irreversible adhesion of *E. coli* on abiotic surfaces. The data also indicate that the defect in the initial attachment of fimbrial mutants described in the previous literature (12, 23) may be due to the detachment after adhesion, not to a deficient ability in the reversible attachment.

FIG 5 CLSM fluorescence images of *E. coli* MG1655 cells, which grew on PET surfaces for 8 h with quorum sensing inhibited by a quorum inhibitor (FC 30) and then were treated with low, medium, and high shear stress for 120 s. Control, bacteria grew on PET surfaces under an unstressed condition for 8 h without quorum sensing inhibition. The inset shows the upper layer of cells in the multilayer colony, when they were put in focus using CLSM 3D scanning mode; scale bar, 10 μ m. Cells with intact and compromised membranes are green and red, respectively.

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, 24). 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 (25, 26). Surprisingly, compared with their wild-type counterparts, reversibly attached nonfimbriated cells could not form any cluster after the lag phase. Therefore, it appears that the bacterial transition from reversible to irreversible adhesion is quite vital to initiate microcolony formation. One possible explanation is that the irreversible attachment mediated by type I fimbriae leads to an alteration in the composition of outer membrane proteins of *E. coli* (27), which might trigger the subsequent growth and colony formation. The $\Delta fimA$ mutants that lack type I fimbriae could only maintain reversible attachment; thus, these adherent nonfimbriated *E. coli* cells cannot progress to 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 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 to be upregulated in the biofilm cells (12, 28). 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

FIG 6 (a) Fractions of *E. coli* MG1655 cells remaining on PET surfaces after application of low, medium, and high shear stress for 40, 120, and 360 s. (b) Corresponding fractions of cells with intact membranes accounting for the total cells remaining on surfaces.

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 the irreversible attachment; thus, this time would prolong the doubling time of sessile cells growing on surfaces, which might be the reason for this issue.

Response of early biofilms to fluid shear stress. The fluid flow acting parallel to a substratum surface generates shear stress. When the shear forces overcome the adhesive forces anchoring the cells to the abiotic substrate, the fluid flow may cause adhering bacteria to slide and roll over the surface, potentially detaching them from the substrates (16, 29). It can be clearly seen from the 8-h samples in Fig. 3 that the shear flow led to morphological changes in the cellular architecture of microcolonies through bacterial migration and detachment. The large compacted multilayered colonies migrated into loose smaller groups under medium shear stress, and the constituent cells, especially those in the upper layer, were detached from the surfaces under high shear stress.

The fraction of remaining bacteria, which can quantitatively reveal interaction forces between bacteria and substratum surfaces (5, 30), 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., the 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 a much greater ability to resist removal by shear stress than the single cells in the lag phase. During the stage of single-layer clusters, we found that the interaction between the colonized bacteria and the substrates did not increase with the development of microcolonies, different from the bacterium-host tissue interaction, which increased with size of the bacterial population during the initial

growth on tissue surfaces (31). Instead, it was maintained at the same level under the given shear stress until the occurrence of multilayered colonies, when the interaction significantly increased. Combined with the results regarding the 8-h samples with inhibited quorum sensing (in which single-layered colonies containing amounts of cells equal to that of the multilayered colonies still displayed the fraction of remaining cells shown in the stage of single-layer clusters), it can be concluded that the attachment strength of early biofilms is linked not with the population size but with the 3D architecture of the microcolonies, and the multiple-layer structure of biofilms can protect the embedded cells from insults from fluid shear, which might be due to the formation of the EPS (3, 9).

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

Although bacteria are protected by rigid cell walls composed of peptidoglycans, the external hydrodynamic stress can inflict damage on the bacterial cell surface (32–34); however, few studies exist on bacterial membrane damage in biofilms caused by flow shear. In this work, by staining the cells with a viability kit, the response of cell membrane permeability in microcolonies to fluid shear was investigated. The cell membrane permeability after medium shear stress treatment remained constant during 0 to 2 h, i.e., the permeability was not affected by the bacterial transition from reversible to irreversible adhesion, which reveals that this effect was independent of the bacterial substrate binding strengths. Different from the results on bacterial retention, the permeability was mainly dependent on the population size of the colonies. The fraction of cells with intact membranes remained constant until the amount of constituent cells in microcolonies was above some critical level. Then, the cell membrane integrity after shear treatment increased with the size of the population, even when the 3D architecture of colonies was inhibited by a quorum sensing inhibitor. It appears that the multilayered architecture was able to protect the cell surface from damage 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 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 microcolonies, detachment is difficult. When quorum sensing is inhibited, the interactions between the bacteria in the microcolonies and the substrates and the cell membranes of constituent cells are more vulnerable to high shear stress, which suggests that fluid flow shear could be combined with the application of a cell signaling inhibitor to remove bacterial contamination on surfaces.

MATERIALS AND METHODS

Substrate. PET plates with a thickness of 0.35 mm and low roughness ($R_a = 5 \pm 0.2$ nm) were purchased from Goodfellow Cambridge Ltd. (Huntingdon, UK). The plates were cut into small pieces (21.5 mm by 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, medium, culture, and mutant strain construction. The wild-type *E. coli* MG1655 strain was cultured in Luria broth (LB), and its mutant strain that lacks 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 with 100 μ g/ml kanamycin.

A single colony of a strain was inoculated in a test tube containing 5 ml of culture medium and grown overnight at 37°C with agitation at 200 rpm. One hundred microliters 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 $\sim 10^9$ CFU per ml.

The *E. coli* MG1655 $\Delta fliA::kan$ mutant strain was constructed by oligonucleotide-directed mutagenesis (ODM) as described previously (35). Briefly, PCR was used to amplify a kanamycin resistance cassette

from the plasmid pBADkanFRT using oligonucleotides specific to the cassette, which have 60-bp 5' arms homologous to the DNA directly flanking the gene of interest, namely, primers 947 (AAAGTGAC AGAAGCTGCCCCATGTCGATTAGAAAATAGTTTTTGAAAGGAAAGCAGCTAGGGTAGGCTTACCTTCAAG CTC) and 948 (ATTTTATCGCACAAAGGTGGGCATCCCTGCCGTAATGACGTCCCTGAACCTGGGTAGGATGA CGATGACAAGCTCCCCCTTCG). *E. coli* MG1655 cells containing the pBAD λ Red plasmid were grown in LB containing 100 μ g/ml ampicillin at 37°C with shaking at 200 rpm to an optical density at 595 nm (OD₅₉₅) of 0.25. The expression of the red recombinase gene was induced by the addition of L-arabinose to a final concentration of 0.2% (wt/vol). The cultures were incubated further until an OD₅₉₅ of 0.5 was reached, and electrocompetent cells were prepared. Purified PCR products were used for the electroporation of the cells, and mutant colonies were selected on LB agar plates containing 50 μ g/ml kanamycin. The successful allelic replacement of *fimA* was confirmed by PCR and sequencing using primers 1065 (CTATGAGTCAAAATGGCCCCA) and 1066 (AGAGCCTGAATAAAGCCGTTT). The pBAD λ Red plasmid was cured from cells 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. Each substrate was then placed vertically in a test tube (diameter, 22 mm) containing stationary-phase *E. coli* MG1655 or Δ *fimA* mutant cells suspended in LB and cultured at 37°C for 1 h, to carry out the initial adhesion. Three of the samples were subsequently taken out for CLSM (Leica TCS SP5), 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.

The remaining seeded PET plates were gently rinsed twice with 10 ml prewarmed fresh LB before being individually immersed in new tubes containing 5 ml prewarmed fresh LB at 37°C for 1 h. After each consecutive hour of incubation, three of the samples were taken out for CLSM 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.

To investigate the effect of quorum sensing and multiple-layer architecture on the response of constituent bacteria in microcolonies to the fluid shear stress, nine of the samples with *E. coli* MG1655 cells seeded on substrates were incubated with hourly refreshed LB containing 1.56 mg/liter (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 for regulating the expression of target genes for the subsequent quorum sensing (36, 37). In our previous research (20), we found at this concentration of FC 30 that 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. In a comparison of the adhesion and growth of *E. coli* MG1655 on PET surfaces, which were previously elaborated (20), surface samples with mutant cells were gently washed with tris-buffered saline (TBS) thrice, stained with the BacLight Live/Dead viability kit (no. L7007; Invitrogen) for 15 min in the dark, and then visualized using confocal laser scanning microscopy (Leica TCS SP5) with an oil-immersion objective lens at $\times 40$ magnification with a zoom of 1:2.60 or 1:1.00. In CLSM images, green and red stained cells indicate the 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 a rotating disk [38]), where the shear force is consistent in one direction, enabling the cells to adapt and develop resistance. In our case, oscillations at a high frequency create an essentially random force on “unprepared” cells and probe the strength of their natural attachment. To expose the bacteria to this shear force, samples were glued on a holder, which was vibrated horizontally in a dish filled with phosphate-buffered saline (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 5 Hz and 25 Hz. The moving magnets were locked to two reciprocal magnets embedded in the sample holder, thus enabling 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 $f_{\max} = -r\omega^{3/2}\rho\eta \cdot A$, 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), and ρ and η are the density (kg/m³) and viscosity (Pa $\cdot$ s), respectively. Finally, $A \approx 0.5 \mu\text{m}$ by $3 \mu\text{m}$ 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, respectively), which translate to the respective maximum force amplitudes of 0.87 pN, 2.18 pN, and 4.95 pN. These shear stresses are comparable to the wall shear stress (WSS) in medical devices, e.g., with hemodialysis vascular access (WSS applied ranges mainly from 0.1 to 3 Pa) (39) and central venous catheters (WSS applied

ranges mainly from 0 to 1.8 Pa) (40), and with those generally used in CIP procedures for food processing equipment (WSS applied in pipes ranges mainly from 0 to 3 Pa) (19).

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

Image processing. Images were processed using ImageJ software (NIH, Bethesda, MD; <https://rsbweb.nih.gov/ij/>) to quantitatively determine the numbers of wild-type/mutant cells on surfaces (i.e., cell density [cells/mm²]) and the cell size (i.e., area of the individual cell [μ m²]). For each sample, 20 fields of view were randomly chosen, and the whole measurement process was repeated three times independently, for a total of 60 values for each cell density. The fraction of cells remaining on substrates is the ratio of the cell density after shear stress was applied and the density in the control sample without shear treatment. The fraction of cells with intact membranes is the ratio of the cell density of green cells and that of the total cells remaining on surfaces.

The above-described experiment process was repeated three times independently. All statistical analyses were performed using analysis of variance (ANOVA) testing within Microsoft Excel (Microsoft Corp., Redmond, WA). Values are reported in the text as mean values $\pm$ standard deviations.

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