Strong Shear Flow Persister Bacteria Resist Mechanical Washings on the Surfaces of Various Polymer Materials

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Environmental bacteria persistently exist in hospitals and thereby often contaminate biomedical devices, which usually causes device-associated infections that have become a major cause of patient illness and death in the hospital. In this study, for the first time, the identification of strong shear flow persister (SSP) cells in *Pseudomonas aeruginosa* is reported. Unlike common persister cells that are highly tolerant to antibiotics, it is reported that the SSP cells can resist mechanical washings on the surfaces of various polymer materials and can form distinctive biofilms that are tolerant to high doses of aminoglycoside antibiotics. Most importantly, a general molecular mechanism is revealed by which an outer membrane protein crosslinks with polysaccharides to form gel-like adhesion complexes that can exert extremely strong adhesion strength (up to 50 N mm^{-2}). Therefore, these findings are urgently required for ongoing research focused on preparing antifouling biomedical materials.

Biomedical devices can extend the functionality of essential body systems to considerably enhance the quality of our lives,[1] thus becoming an indispensable part of modern medical care. Increasing use of biomedical devices in healthcare systems is always associated with a definitive risk of bacterial infections.[2] For examples, the majority of cases in urinary tract infection (95%), bloodstream infection (87%), or pneumonia (86%) are related to using catheters or mechanical ventilations, in which the opportunistic pathogen *Pseudomonas aeruginosa* has a notable impact in device-associated infections because of intrinsic tolerance to antibiotics and persistent existence in the hospital environments.[3] In addition, *P. aeruginosa* is likely to form biofilms that often cause antibiotic-resistant chronic infections.

In all cases of device-associated infections caused by pathogens, the adhesion of bacteria to the surface of the device is a vital step,[2a] which is supposed to be highly dependent on the physicochemical properties of materials used in the device,[4] such as wettability or surface microtopography. Because of the favorable biocompatibility and desired mechanical properties of synthetic polymer materials, they are currently widely used to produce biomedical devices.[5] For example, polydimethylsiloxane (PDMS) has been widely used to manufacture various implantable devices such as pacemakers, blood pumps, catheters, and esophagus replacements. During cleaning or operating these biomedical devices, shear flows with different shear strengths ranging from 10 to 2700 Pa are often encountered (**Figure 1**a).[6] For example, the shear flow caused by urination on the surface of urethral catheters usually manufactured using PDMS or polyvinyl chloride is weaker (ranging from 10 to 50 Pa) than that caused by arterial blood flow on artificial heart valves usually manufactured using polyethylene terephthalate or polytetrafluoroethylene (PTFE; ranging from 150 to 450 Pa). Investigating whether pathogenic bacterium can adhere to these polymer materials in the presence of mechanical washing is crucial to prevent the device-associated infections.

Here, we focused on examining whether a shearing of flow can be used to remove the opportunistic pathogen *P. aeruginosa* from the surfaces of various polymer materials commonly used produce biomedical devices. Using a combination of microfluidics, microscopy, molecular biology, and particle tracking techniques, we first established a high-throughput method that can be used to characterize bacterial adhesion and quantify the adhesion strength in single-cell scale. We observed that 15%–25% bacteria, that was be termed as strong shear flow persister (SSP) cells, persistently adhered to the tested surface despite the shear strength of 2000 Pa. The shear strength of 2000 Pa is close to the maximum shear strength of biomedical devices employed in real settings; for example, the dental water jet used for cleaning teeth. Second, we investigated how adhesion factors to facilitate *P. aeruginosa* to attach on the surfaces of various polymer materials using different adhesion factors mutants. We elucidated the general molecular mechanism underlying the high tolerance of SSP cells to mechanical washings on various surfaces: CdrA can crosslink with the polysaccharide Psl to form gel-like adhesion complexes in the bacterial wall, which can exert extremely strong adhesion strength on substrates. The adhesion strength mediated by CdrA–Psl adhesion complexes is close to the strongest adhesion strength found in nature[7] (arising from the glue of *Caulobacter crescentus*), that the chemical and biophysical basis for the impressive adhesion strength is still unknown. Moreover, we found that this phenotype in *P. aeruginosa* can quickly develop on surfaces to form distinctive biofilms that can tolerate high dose of aminoglycoside

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DOI: 10.1002/adbi.201700161

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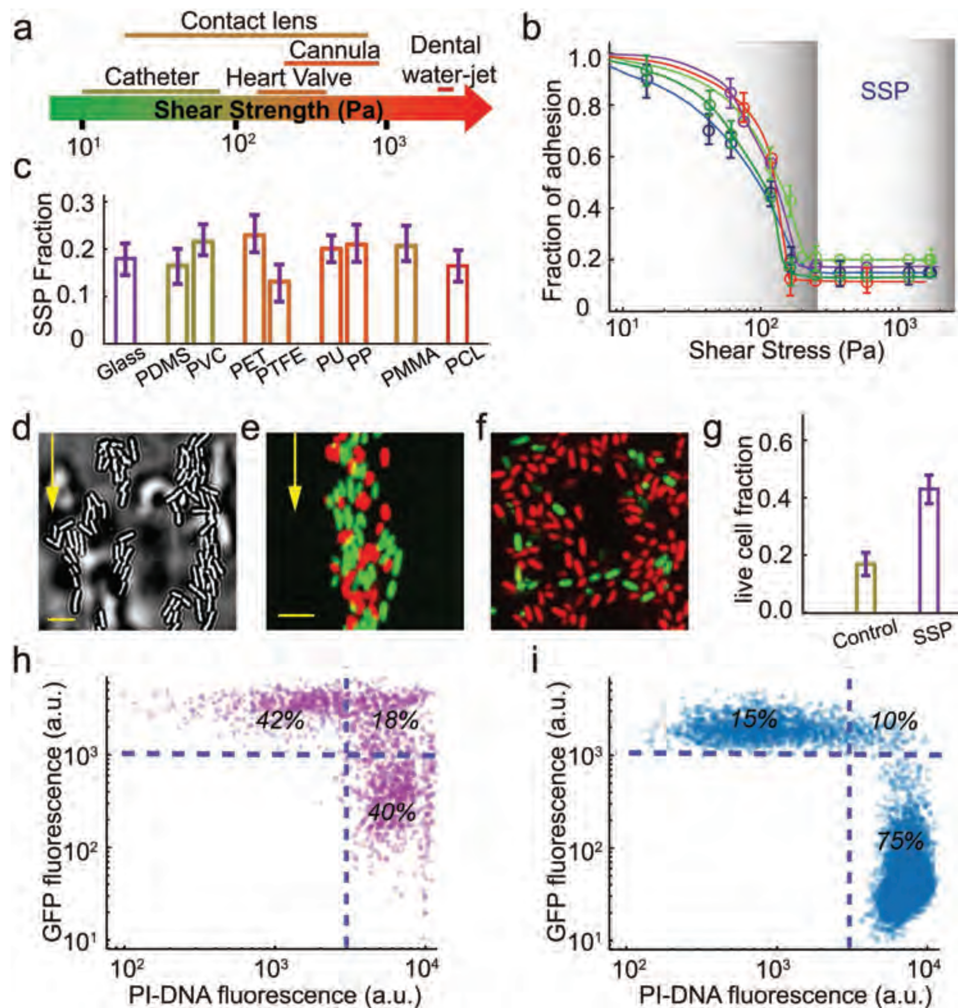

Figure 1. Strong shear flow persister (SSP) cells are identified in *P. aeruginosa* on the surfaces of various polymer materials. a) Typical shear strengths that can be used in cleaning or operating biomedical devices. b) Shear stress dependence of the fraction of cells (PAO1-GFP: GFP tagged PAO1) that can remain adhered to the surfaces of various materials: glass (blue); polytetrafluoroethylene (PTFE, red); polyurethane (PU, cyan); polypropylene (PP, purple); and polydimethylsiloxane (PDMS, green). The symbols, error bars, and lines represent mean values, standard obtained from ≥3 replicates, and spline lines, respectively. SSP cells represent the cells that remain adhered to the surface to resist strong washings (≥250 Pa). c) The fraction of SSP cells found on the surfaces of various polymer materials commonly used to produce biomedical devices, where colors indicate the magnitude of shear strengths that may be used in cleaning or operating biomedical devices (cell number ≥500, collected from ≥3 replicates). d) Representative bright-field image shows that SSP cells can form microcolonies on a glass surface in 480 min. The yellow arrow indicates the direction of the flow. Biofilms formed by SSP cells were highly tolerant to tobramycin. e,f) Representative confocal images, g) average fraction of live cells, or h,i) GFP (green) versus propidium iodide (PI, red) fluorescent intensity of multiple cells ($n \geq 2000$, collected from ≥3 replicates) of young biofilms (12 h) treated with 100 µg mL^{-1} tobramycin: (e, h) and (f, i) exhibit the biofilm formed by SSP cells or all surface-attached cells, respectively. Error bars represent standard obtained from ≥3 replicates. Scale bars for all images are 5 µm.

antibiotics. Thus, it is almost impossible to completely eliminate device-associated infections via mechanical washings and antibiotic treatments. Our discoveries are thereby expected to broadly impact to ongoing researches ranged from biomedical materials, anti-biofilms, to clinical treatments.

To examine whether *P. aeruginosa* cells can remain adhered to the surfaces of polymer materials to resist mechanical washings with different shear strengths, we directly washed cells attached to the surface of a microfluidic device and counted the fraction of the cells that remained adhered to the surface to resist washings. The microfluidic device precisely generated a laminar flow with adjustable shear strength ranging from 10 to 2000 Pa.[8] We found that the cells attached on the tested surface gradually decreased with increasing the strength of shearing ranged from 10 to 200 Pa, as shown in Figure 1b, indicating that using a flow can wash out a part of *P. aeruginosa* from the surfaces of various polymer materials. However, when the shear strength exceeds 200 Pa, we observed that 15–25% bacteria persistently adhered to the tested surface despite the shear strength of 2000 Pa (Figure 1b), indicating that a subpopulation of *P. aeruginosa* can persistently adhere to various surfaces (Figure 1b,c) to resist mechanical washings. Furthermore, we observed that the plateau region in the Figure 1b nearly started at 200 Pa for all tested materials, which enabled us to readily

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identify this subpopulation of *P. aeruginosa* by using a strong flow (>250 Pa) to wash all surface-attached cells. We termed this newly identified subpopulation of *P. aeruginosa* as SSP cells. Our results indicated the SSP cells can readily form on hydrophobic (PTFE) or hydrophilic surfaces (clean glass), indicating that the wettability of surface does not affect SSP formation. We demonstrated that 15–25% of SSP cells can be found on the surfaces of various polymer materials commonly used to produce biomedical devices (Figure 1c).

Next, we investigated whether SSP cells can form biofilms under a strong shear flow. We first removed non-SSP cells in *P. aeruginosa* from a glass surface using a strong shearing of flow (580 Pa) and then continuously cultured those rest cells (SSP) up to 24 h. We observed that (1) SSP cells normally divided in the place to form microcolonies (Figure 1d; Movie S1, Supporting Information) and further formed thick biofilms in 24 h (Figure S2b, Supporting Information); and (2) ≈62% daughters of SSP cells remain to resist the strong shear stress, which indicates that SSP cells are phenotypical reversible and SSP formation is not fully stochastic (Figure S3, Supporting Information). Note that the higher circulating rate in the microchannel led to SSP cells grow faster (90 min doubling time) than that of all surface-attached cells (130 min doubling time) grown under a lower circulating rate (Figure S2a, Supporting Information). In addition, we found that SSP cells did not use twitching motility during microcolony formation (Movie S1, Supporting Information) and that the orientation of cells in the young biofilm (Figure 1d) was slightly aligned to the direction of the flow. To more closely link our findings to those of clinical studies, we harvested the young biofilms (12 h) formed only by SSP cells or all surface-attached cells. Note that these young biofilms just developed to form thin layer (nearly monolayer) of cells that covered the surface rather to form 3D structures. Then, we treated these biofilms with the first-line antibiotic tobramycin for 8 h in absence of flow, which ensure that all cells in the biofilm can sufficiently react with the antibiotic. We stained the green fluorescent protein (GFP)-labeled cells in the biofilms with propidium iodide to visualize dead cells (red fluorescence; Figure 1e,f). After treatment with 100 μg mL^{-1} tobramycin, ≈42% cells survived in the biofilm formed by SSP cells (Figure 1e,h), which was twofold higher than the percentage of cells which survived in the biofilm formed by all surface-attached cells (Figure 1f,i). Our results indicated (Figure 1g; Figure S4, Supporting Information) that the biofilms formed by SSP cells are highly tolerant to high doses of tobramycin (up to 300 μg mL^{-1}), which is close to the maximum dose level used in clinical treatments.[9]

The surface adhesion of *P. aeruginosa* depends on several adhesion factors,[10] including surface appendages such as the flagellum, pilus, and fimbria; polysaccharides; and adhesion proteins. To investigate adhesion factors facilitating the adherence of SSP cells to various surfaces in order to resist mechanical washings, we performed screening experiments on glass surfaces by using 39 mutant strains (Table S1, Supporting Information). We screened almost all potential adhesion factors in *P. aeruginosa*. We observed that the mutant deficient in the expression of the outer membrane protein CdrA or in the production of the polysaccharide Psl did not form SSP cells on glass surfaces, whereas the mutant deficient in the expression of surface appendages and adhesion proteins or in the production of rhamnolipid, Pel, or alginate could still form SSP cells on glass surfaces. Furthermore, we observed that the mutant deficient in the expression of CdrA or in the production of Psl did not form SSP cells on all tested surfaces (Figure S5, Supporting Information). These results indicate that CdrA and Psl are essential adhesion factors for the formation of SSP cells on the surfaces of various polymer materials.

The importance of the protein CdrA and polysaccharide Psl for biofilm formation have already been identified.[11] They suggested Psl stiffens biofilms and increases biofilm toughness by binding to CdrA that is a likely cross-linker for the Psl components of the biofilm matrix, but the mechanism of how CdrA and Psl determines the bacterial adhesion at the single cell level is unknown. To explore the roles of CdrA or Psl in the formation of SSP cells, we analyzed the responses of single cells to washings on a glass surface through a single-cell tracking technique. We used a mutant strain (*ΔfliCΔpilA*) in following experiments to ensure that bacterial motility did not affect the response of single cells to washings. Note that double knouting out of fliC and pilA does not affect SSP formations (Table S1, Supporting Information). We observed that single cells responded differently to mechanical washings: (1) the cell first slid a certain distance (X_d) toward the direction of the flow and then eventually stuck on the surface (stick type) despite the shear flow (**Figure 2**a,d; Movie S2, Supporting Information); (2) the cell kept sliding on the surface (slide type) with an approximately constant velocity (V_s; Figure 2b,e and Movie S3, Supporting Information); and (3) the cell first slid a short distance (X_d') and then detached from the surface (detach type; Figure 2c,f and Movie S4, Supporting Information). Furthermore, we observed that (1) increasing the shear strength from 15 to 250 Pa gradually increased the detach-type cells from 3% to 66% and gradually reduced the slide- and stick-type cells from ≈6% to 0% and from 91% to 34%, respectively, and (2) further increasing the shear strength from 250 to 2000 Pa did not change the percentage of stick-type cells (Figure 2g), indicating that SSP cells are formed from stick-type cells.

We investigated the effect of CdrA or Psl on single cells in response to mechanical washings by using the mutant (*ΔfliCΔpilAΔcdrAB* or *ΔfliCΔpilAΔpslBCD*) deficient in the expression of CdrA or in the production of Psl, respectively. In addition to the observation that these mutants cannot form SSP cells, we observed that the stick-type cells completely disappeared and the slide-type cell percentage dramatically increased in the mutant *ΔfliCΔpilAΔcdrAB* cells (Figure 2h). By contrast, in the mutant *ΔfliCΔpilAΔpslBCD* cells, the slide-type cells completely disappeared (Figure 2i) and the stick- or detach-type cells did not slide any distance before getting stuck on the surface or detaching from the surface (Figure S6, Supporting Information). Psl can release from the outer membrane to stick on the substrate in the absence of CdrA (Figure S7b, Supporting Information); and the protein CdrA firmly attached on the cell surface when bacterial crawling on the substrate (Figure S8, Supporting Information). These findings indicate that CdrA or Psl has a distinct function in the formation of SSP cells: CdrA enables cells to grasp the surface, which does not allow surface-attached cells to slide under washings, whereas Psl enables cells to stick on the surface without any fixation,

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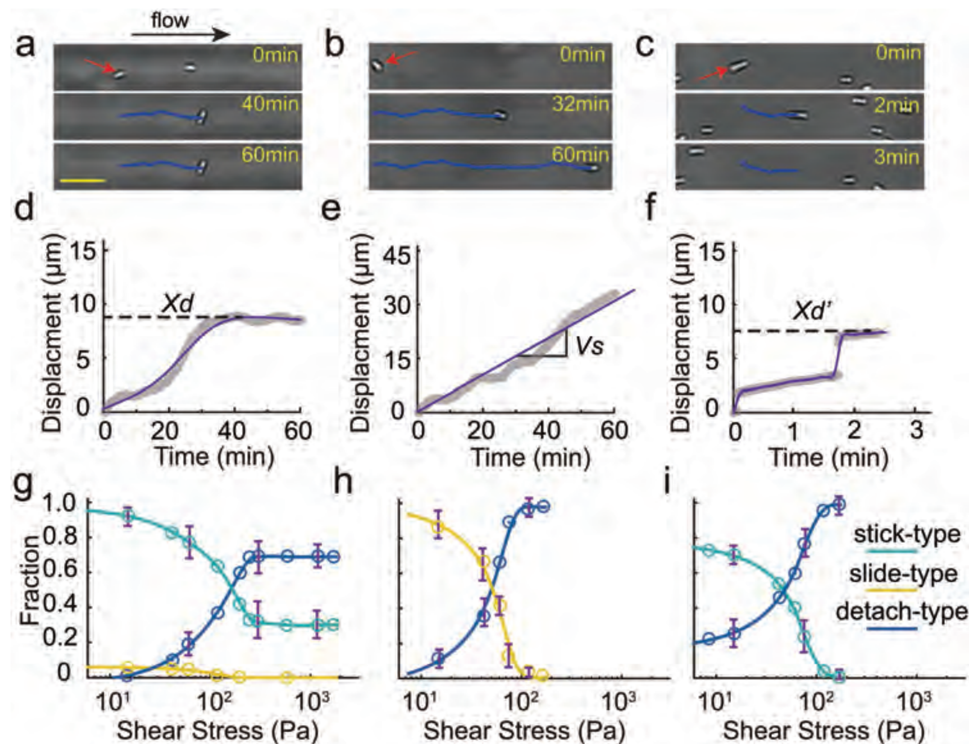

Figure 2. CdrA or Psl has a distinctive function in the formation of strong shear flow persister (SSP) cells. A single *P. aeruginosa* cell (*ΔfliCΔpilA*) differently responded to a shear flow of 15 Pa, including three distinctive types: a,d) stick type, b,e) slide type, and c,f) detach type, where (a–c) display representative bright-field images and (d, e) display time-dependent displacement of the bacterial centroid along the direction of the flow. Blue lines in (a–c) represent the bacterial trajectories in response to shearing. Symbols and lines in (d–f) represent the original and smoothing data, respectively. Shear-strength dependence of the stick type (cyan), slide type (yellow), and detach type (blue) in g) *ΔfliCΔpil A*, h) *ΔfliCΔpilAΔcdrAB*, and i) *ΔfliCΔpilAΔpslBCD* mutants, where symbols, error bars, and lines represent mean values, standard obtained from ≥3 replicates, and spline lines, respectively. Scale bars for all images are 5 μm.

which allows surface-attached cells to slide under surface washings.

To examine whether CdrA and Psl can generate a synergic effect to mediate the resistance of SSP cells to mechanical washings, we used a high-spatial-resolution confocal microscope to directly image immunofluorescently labeled CdrA and Psl on SSP cells. We observed that CdrA (green fluorescence) was typically scattered on the outer membrane and likely superimposed on Psl (red fluorescence) that was more scattered on the outer membrane (**Figure 3**a–d), suggesting that CdrA and Psl can form adhesion complexes on the outer membrane of SSP cells. In addition, we observed that >99% SSP cells (Figure 3i and Figure S9, Supporting Information) and ≈17% surface-attached cells (Figure 3j) expressed abundant CdrA on their outer membranes. The production of Psl did not significantly differ between SSP and surface-attached cells. These findings indicate that SSP cells are formed from *P. aeruginosa* cells highly expressing CdrA on their bacterial walls. To further confirm that CdrA and Psl can form adhesion complexes to mediate the resistance of SSP cells to washings, we endogenously cleaved CdrA to detach SSP cells from the surface by inducing the expression of lapG (PA1434).[12] We observed that adhesion complexes formed by CdrA and Psl remained on the surface (Figure 3e–h; Figure S10, Supporting Information).

We investigated how the adhesion complexes mediate the resistance of SSP cells to mechanical washings. We repeatedly sheared an SSP cell by using alternative strong and weak shear flows (Movie S5, Supporting Information). We observed that switching the shear strength from 23 to 61 Pa moved the centroid of the SSP cell to ≈0.25 μm along the direction of the flow, whereas converse switching of the shear strength caused the cell to move back (**Figure 4**a; Figure S11a, Supporting Information). The shear strength used in this experiment did not cause detectable elongations of the bacterial wall (Figure S11b, Supporting Information). This finding indicates that the adhesion complex formed by CdrA and Psl is a gel-like elastomer. This feature is in contrast with that of surface-attached cells solely mediated by Psl, in which the cell can always slide on the surface under washings with arbitrary shear strengths (Figure 4b), indicating that Psl is a viscous fluid. We estimated that the magnitude of the shear modulus for adhesion complexes formed by CdrA and Psl ranges from 1.5 to 38 kPa, and the magnitude of viscosity of Psl between the bacterial wall and the surface ranges from 28 to 140 Pa s. To further demonstrate that CdrA can crosslink with Psl to form a gel-like adhesion complex, we mixed purified Psl (C_{Psl} = 0.14 mg mL^{-1}) and mini-CdrA (a part of CdrA, C_{CdrA} = 0.31 mg mL^{-1}) solutions in vitro (Supporting Information). We observed that flocculation rapidly formed, resulting in 198-folds increasing of the scattering intensities of mixing solution (Figure 4c). The molecular mechanism underlying the high resistance of SSP cells to mechanical washings is schematically summarized in Figure 4.

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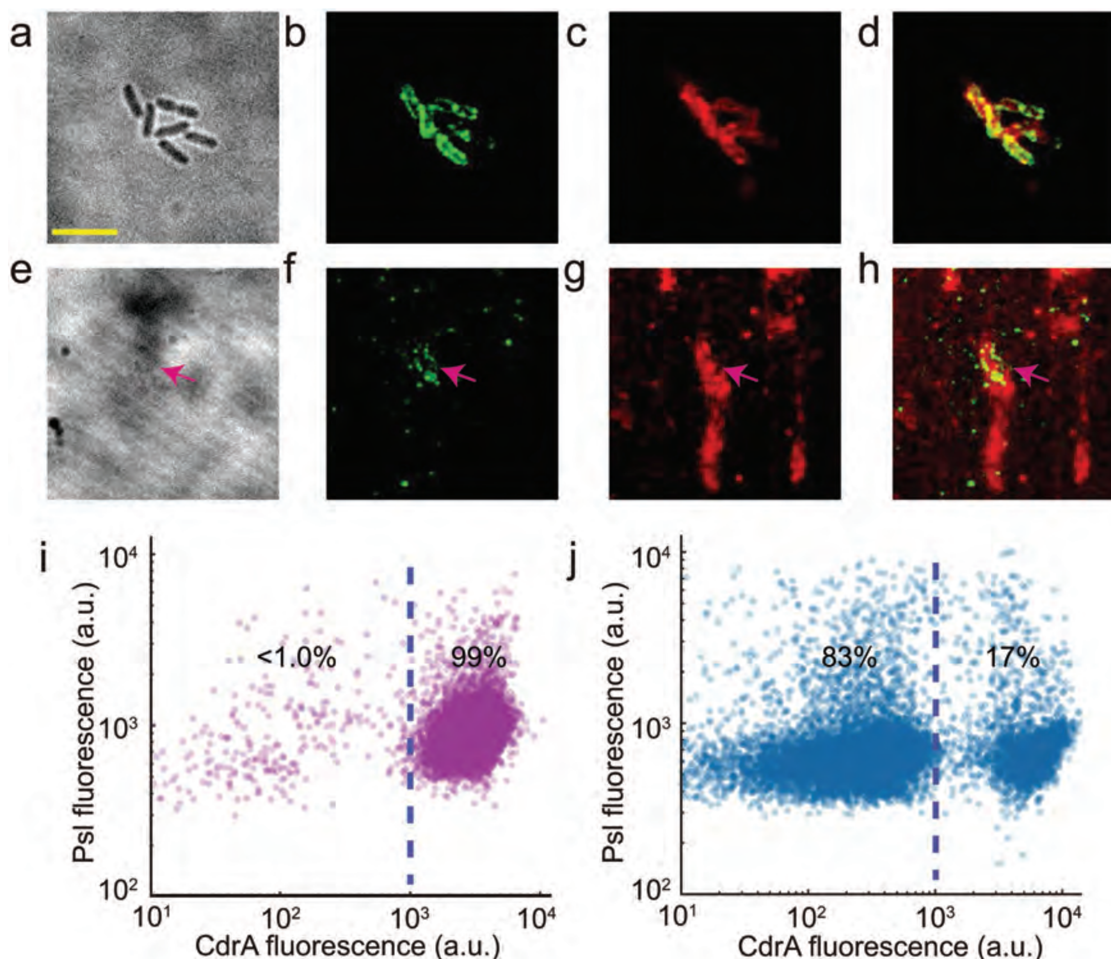

Figure 3. CdrA corsslinks with fluid-like Psl to form a gel-like adhesion complex that mediates the resistance of strong shear flow persister (SSP) cells to strong washings on various surfaces. Multichannel representative images show that (1) a–d) CdrA and Psl form adhesion complexes that can mediate the attachment of SSP cells on the surface and (2) e–h) inducing the expression of LapG can lead to the detachment of SSP cells from surfaces, where (a, e) are bright-field images displaying bacterial location; (b, f) are confocal images with an emission channel (green fluorescence) indicating the localization of HA-tagged CdrA; (c, g) are confocal images with an emission channel (red fluorescence) indicating the localization of Psl; and (d, h) are merged confocal images of the two channels. Red arrow indicates the position of the cell before detachment. Scale bars for all images are 5 µm. i,j) Psl (red) versus CdrA (green) florescent intensity of multiple cells ($n \geq 1000$) show SSP cells form due to the high CdrA expression in their outer membranes, where (i) and (j) arose from SSP cells and all surface-attached cells grown on the flow cell, respectively.

Our findings indicate that crosslinking of surface-adsorbed Psl by CdrA significantly reinforce the surface adhesion of *P. aeruginosa* mediated by Psl. This adhesion mechanism found in *P. aeruginosa* can be used to explain the transition of reversible to irreversible attachments of bacteria during biofilms formation;[13] namely, Psl solely mediate the reversible attachment of bacteria at the initial stage. Afterward, the elevation of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) level leads to the expression of CdrA at the second stage, which results in the irreversible attachments. Moreover, because Psl–CdrA adhesion complexes can mediate strong adhesion of *P. aeruginosa* on various abiotic surfaces, we speculate that *P. aeruginosa* can adhere to some biotic surfaces to resist strong shear flows. For example, a subpopulation of *P. aeruginosa* may persistently adhere to wound surfaces to resist clinical cleaning. However, this speculation warrants further investigation.

Psl is a neutral polymer with a flexible and relatively open conformation, where its molecular weight is typically higher than 15 kDa.[14] Psl is composed of repeating pentamers containing D-mannose, D-glucose, and L-rhamnose.[14a] The repeating units of D-mannose or D-glucose carries abundant hydroxyl groups that facilitate the adsorption of Psl on mineral surfaces through hydrogen bonding[15] or on zwitterionic surfaces through ion bridging,[16] whereas the repeating units of L-rhamnose carries[17] a group that is believed to facilitate the adsorption of Psl on hydrophobic surfaces through strong hydrophobic interactions. These chemicophysical properties readily allow the adsorption of Psl on various surfaces; however, they may not generate sufficiently strong adhesion strength that can mediate the resistance of surface-attached bacteria to strong shear flows. The crosslinking of surface-adsorbed polymers can greatly enhance the adhesion strengths of these polymers.[18] Thus, the crosslinking of surface-adsorbed Psl

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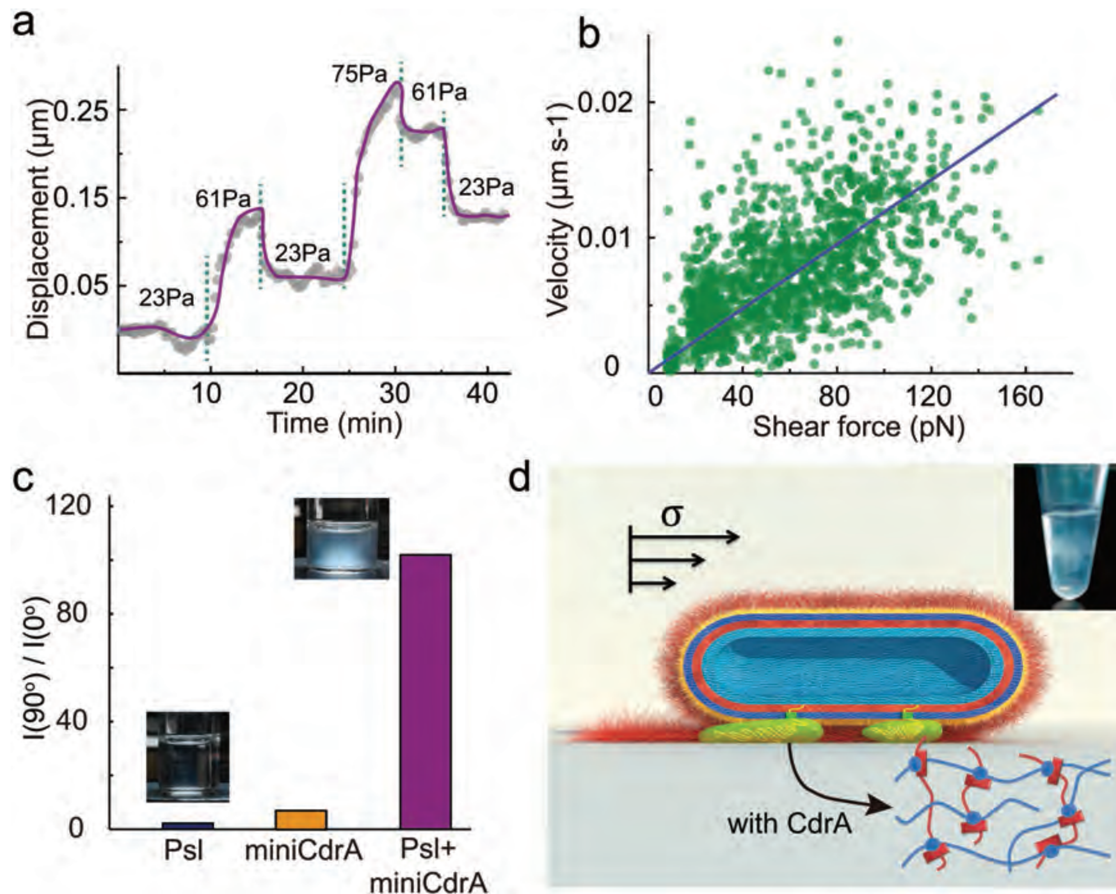

Figure 4. The properties of adhesion complex formed by Psl and CdrA. a) The alternative displacement of an SSP cell of *ΔfliCΔpilA* in response to flows with alternative shearing strengths, indicating that CdrA–Psl adhesion complexes are gel like. b) The sliding of a single *ΔfliCΔpilAΔcdrAB* cell along the direction of the flow with a velocity (V_s) show that the Psl layer sandwiched between the bacterial wall and substrate is fluid like, where the regression line arises from a linear fit (cell number ≥500). c) Representative photos or scattering intensities show that purified miniCdrA binds to Psl to form flocculation in vitro. d) Schematic representation of the molecular mechanism underlying the resistance of SSP cells to strong washings on various surfaces. The inserted image is the photo of the flocculation formed by miniCdrA and Psl. CdrA crosslinks with fluid-liked Psl to form a gel-liked adhesion complex that mediates the resistance of SSP cells to strong washings on various surfaces.

by CdrA may significantly reinforce the surface adhesion of *P. aeruginosa* mediated by Psl. This adhesion mechanism found in *P. aeruginosa* may differ with that found in *C. crescentus*, in which holdfasts can exert extremely strong adhesion strength exceeding 68 N mm^{-2}.[7] In addition, this methods may be suitable to other bacteria and fungi, and can also formed SSP cells.

We speculated the following two mechanisms can explain the tolerance of *P. aeruginosa* biofilms developed by SSP cells to high tobramycin doses: (1) biofilms developed by SSP cells produce a high amount of the extracellular polymeric substance (EPS) to enclose themselves, where the high EPS amount and thick EPS layer can provide more favorable protection to the biofilms to resist antibiotic attack.[19] This speculation is supported by the finding that SSP cells form due to increased CdrA expression in the outer membrane that has been positively related to intracellular c-di-GMP concentration.[12,20] Moreover, the high intracellular c-di-GMP concentration can lead cells to produce a high amount of EPS.[20] It reasoned that the biofilms or microcolonies formed from SSP cells may produce more polysaccharide to enclosed themselves, both including Psl and Pel, which may provide further protection for the cells to against aminoglycosides that consistent with results arose from Colvin,[21] (2) Abundant Psl–CdrA adhesion complexes exist in biofilms developed by SSP cells (Figure S13, Supporting Information), and the Psl–CdrA adhesion complexes can specifically bind to aminoglycoside antibiotics through hydrogen bonding[22] or nonspecifically suppress tobramycin activity by increasing local ionic strengths.[23] We examined whether abundant Psl can lead the high tolerance of tobramycin in *P. aeruginosa* biofilms using a mutant that overproduces Psl and deficient in expression of CdrA. Figure S12 (Supporting Information) indicated that overproducing of Psl in biofilms does not increasing their tolerance of tobramycin.

Experimental Section

Estimation of Mechanical Properties of CdrA–Psl Adhesion Complexes: The shear strength (S) generated by CdrA–Psl adhesion complexes for an SSP cell was estimated using the equations $= \sigma_{max} A/a$ where σ_{max} is the maximum shear strength that can cause detachment of SSP cells from the surface, A is the cross-sectional area of the bacterial wall, and a is the adhesion area mediated by CdrA–Psl adhesion complexes. a is

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related to the adhesion number (n_a) and the characteristic area a_0 of the adhesion complex according to the equation $a = n_a a_0$. sranged from 2 to 50 N mm^{-2} under the following condition: $2000 \le \sigma_{max} \le 10\,000$ Pa,[19] $A \approx 1$ µm^2, $2 \le n_a \le 10$ (data obtained from the analysis of the CdrA localization using 11 confocal images that typically contains 100 SSP cells), and $a_0 \approx 100$ nm^2 (data obtained from Busscher study.[24]) The characteristic length can be determined with a relation $a_0 \approx l^2$. The shear modulus (G) of the adhesion complex was estimated using the equation $G = \Delta\sigma a A/n_a l \Delta x$, where $\Delta\sigma$ is the difference between shear strengths provided by alternative flows (Figure 4a) and Δx is the displacement of the centroid arising from the alternative shearing. G ranged from 1.5 to 38 kPa under the following conditions: $\Delta\sigma = 40$ Pa and $0.05 \le \Delta x \le 0.25$ µm (Figure S11, Supporting Information; and cell number $n \ge 50$). The viscosity (η_p) of the Psl layer sandwiched between the bacterial wall and the substrate was estimated using the equation $\eta_p = \sigma l_p/V_s$, where l_p is the thickness of the Psl layer. η_p ranged from 28 to 140 Pa s under the following conditions: $10 \le l_p \le 50$ nm and $\sigma/V_s = 2.8$ Pa s nm^{-1} (Figure 4b).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

R.R.Z. and A.G.X. contributed equally to this work. The authors thank J. D. Shrout for providing the bacterial strain. This work was partially carried out at the USTC Center for Micro and Nanoscale Research and Fabrication. This work was supported by the National Natural Science Foundation of China (21474098, 21274141, and 21522406) and the Fundamental Research Funds for the Central Universities (WK2340000066, WK2030020023).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

antifouling materials, bacterial adhesion, biofilms, healthcare-associated infections, *Pseudomonas aeruginosa*

Received: August 16, 2017
Revised: September 21, 2017
Published online: November 21, 2017

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