Biofilm disruption by an air bubble reveals heterogeneous age-dependent detachment patterns dictated by initial extracellular matrix distribution

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INTRODUCTION

Biofilms are surface-associated microbial communities encased in a self-secreted matrix of extracellular polymeric substances (EPS). Biofilms account for the largest fraction of bacterial biomass on the planet, and often have deleterious effects in natural, industrial and medical settings. In the environment, biofilms can mobilize heavy metals such as mercury and arsenic, causing stream or soil contamination. In industrial processes, the formation of biofilms is responsible for huge economic losses (in the billions of dollars yearly) resulting from biofouling and biocorrosion, which leads to equipment clogging and damage, and product contamination. In medicine, biofilms represent the major source of infections associated with catheters and implanted devices. Despite the importance of finding effective methods for biofilm removal in these and other applications, our understanding of biofilm development and in particular of the mechanisms responsible for biofilm detachment remains far from complete.

Detachment refers to the release of bacterial cells or clusters from the surface of the biofilm into the bulk fluid. Several factors can contribute to detachment, including matrix-degrading enzymes, nutrient levels, and quorum-sensing signals. Mechanical forces associated with fluid flow have also been investigated as potential approaches to remove adsorbed bacteria from surfaces, with a striking example involving the passage of bubbles or air plugs. Air bubbles remove bacteria from a surface when the three-phase line (separating the liquid, the air and the solid surface) contacts the cells. The capillary action of moving air-liquid interfaces is known to cause colloidal aggregation and to generate forces that tend to detach bacteria from surfaces in a broad range of environments, including in the oral cavity during eating, speaking, drinking and swallowing, in the eye and on contact lenses during blinking, and on rocks and ship hulls in aquatic systems. However, previous research on biofilm detachment by air bubbles has focused on endpoint measurements to quantify the net amount of biofilm removed, whereas the mode of biofilm disruption has remained unexplored.

Here we show that, for early-stage biofilms (when bacterial colonies are organized as monolayers), insult by mechanical forces results in a new phenomenon, whereby the passage of a long bubble opens regular holes in the biofilm but fails to completely remove it. We rationalize this finding in terms of the competition between dislodging shear forces and the spatially varying adhesion strength resulting from intrinsic heterogeneity in EPS distribution within the biofilm.

RESULTS AND DISCUSSION

We studied the formation and disruption of controlled Pseudomonas aeruginosa PA01 biofilm patches on the glass bottom of a
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Fig. 1  The passage of a long air bubble creates a characteristic pattern of holes in a biofilm. a Schematic of the microfluidic setup showing the geometry of the microchannel and the experimental method, in which P. aeruginosa bacteria preferentially attached to hydrophobic patches. After a specified growth time (4 h, 8 h, or 12 h), a controlled air bubble was injected in the channel at a mean flow speed of 250 μm s⁻¹. b Residual biofilm after the passage of the bubble, revealing a semi-regular pattern of holes formed in an 8 h-old biofilm, for different initial patch sizes (measuring 400² μm², 300² μm², 200² μm², and 100² μm²). Scale bars, 50 μm.

microfluidic channel (Fig. 1a). Biofilm patches formed by the preferential adhesion of P. aeruginosa cells to hydrophobic square patches, previously created on the glass by a microcontact printing (Supplementary Figs S1–S2; Methods) technique. A dilute bacterial suspension (optical density OD₆₀₀ ~ 0.2) was injected in the channel and incubated under quiescent conditions for 1 h, allowing cells to attach to the channel’s surfaces. Then, the bacterial suspension was replaced by a minimal culture medium (M63), which was flown continuously at 3 μl min⁻¹ (average flow velocity = 250 μm s⁻¹) to supply adhering cells with nutrients. Over the course of a few hours, P. aeruginosa cells progressively covered the surface of the hydrophobic patches. While some bacteria attached to the surface outside of the patches, most bacteria adhered onto the patches, where cell adhesion was greatly favored by the substrate’s strong hydrophobicity. The concentration of adhering cells could be controlled by varying the concentration of the chemical octadecyltrichlorosilane (OTS; see Methods) used in printing the patches.

To determine the effect of a mechanical insult on an early-stage biofilm, the patches were exposed to the controlled passage of a long bubble. The bubble was created by rapidly switching injection from medium to air, creating an approximately 2.5 mm-long bubble traveling at 250 μm s⁻¹ (Methods). The air bubble traveled over each patch in approximately 10 s, causing a dramatic and highly characteristic disruption of the original biofilm patch: the resulting biofilms were in the shape of a semi-regular pattern of holes, from which bacteria had been entirely removed, separated by ‘bacterial levees’, consisting of a concentrated monolayer of cells (Fig. 1b). Image analysis showed that holes, which varied in shape but displayed no obvious asymmetry associated with the flow direction, had an equivalent radius of 6.5–8 μm and the surface porosity of the end-state biofilm was ~68%. This pattern was highly consistent among different patches within the same microchannel and among replicate experiments. Experiments with patches of different size (100², 200², 300² and 400² μm²) showed that the pattern and its porosity were independent of patch size within the range explored (Supplementary Fig. S3). Moreover, a chemical analysis of the surface using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/EDX) showed that the OTS coating was stable even under high shear flows and confirmed that the holes in the biofilm resulted from the detachment of bacteria, rather than the detachment of the underlying OTS layer (Supplementary Fig. S4).

The adhesion properties of an early-stage biofilm are strongly dependent on the biofilm growth time. This is clearly revealed by comparing experiments in which the air bubble was injected after 4, 8 and 12 h of biofilm growth (Fig. 2). For 4 h-old biofilms, the long bubble reduced the surface coverage within patches by nearly two thirds, from 31.6% (±2.4%) to 12.7% (±0.6%). For 8 h-old biofilms, the reduction in surface coverage caused by the bubble was considerably smaller, from 44.6% (±0.9%) to 38.7% (±2.5%), while for 12 h-old biofilms, the reduction was essentially negligible, from 49.5% (±2.3%) to 47% (±2.4%), and the bubble produced no visible change of the biofilm structure. This age-based trend is supported by an analysis of the mean distance between individual cells in the biofilm before and after the passage of the bubble. The rearrangement of the 8 h-old biofilm is evident from the imaging (Fig. 2b and e) and is further supported by a considerable change in the fractal dimension for the 12 h-biofilm (Fig. 2g). Taken together, these measurements indicate that a long air bubble largely removed 4 h-old biofilms, primarily rearranged cells in 8 h-old biofilms without detaching them, and had little effect on 12 h-old biofilms.

Imaging of the hole formation dynamics at high temporal resolution (50 frames s⁻¹) revealed that the hole pattern resulted from the rupturing of the residual thin liquid film between the channel surface and the bubble at discrete locations. The ensuing movement of the contact line scraped bacteria outwards from the holes, to form “bacterial levees” between adjacent holes (Fig. 3; Supplementary Video 1). A long air bubble traveling over a solid surface remains separated from it by a thin layer of liquid, whose...
thickness depends on the capillary number, \( Ca = \mu U/\sigma \), which measures the relative importance of viscous forces and capillary forces. Here, \( \mu \) is the dynamic viscosity of the liquid, \( U \) is the bubble velocity, and \( \sigma \) is the interfacial tension between liquid and air. In our experiments, the bubble traveled at \( U = 250 \mu m \cdot s^{-1} \) in culture medium contaminated by the presence of bacteria and EPS on the walls of the channel; in these conditions, we estimated \( \mu = 10^{-3} \) Pa s and \( \sigma = 25-50 \times 10^{-3} \) Nm \(^{-1} \) (see ref. 19), resulting in \( Ca = (5-10) \times 10^{-6} \). For \( Ca < 1 \) the thickness of the liquid film, \( h \), follows Bretherton’s law, \( h/H \sim Ca^{2/3} \), where \( H = 50 \mu m \) is the height of the microchannel and the constant of proportionality is on the order of unity. This results in an estimated liquid film thickness of \( h \leq 0.1 \mu m \). Thus, the bubble would create on a flat surface a liquid film that is thinner than the thickness of a bacterium (\( \sim 1 \mu m \)) which would then quickly rupture through an evaporation-driven instability. An evaporation rate of \( 5 \times 10^{-5} \) kg m\(^{-2} \) s\(^{-1} \), determined assuming room temperature and 50% relative humidity (RH), indicates that a 0.1 \( \mu m \) thick water film evaporates in \( \sim 2 \) s (see ref. 21). A sensitivity analysis on RH, which is unfortunately unknown in our experiments, reveals that this conclusion is robust for even large variations in RH (for RH between 10 and 90%, the evaporation rate ranges from \( 10^{-5} \) to \( 10^{-4} \) kg m\(^{-2} \) s\(^{-1} \) and the evaporation time from 1 s to 4 s). The evaporation time is thus substantially smaller than the time taken by the entire bubble to pass over any given point on the bacterial film (\( \sim 10 \) s; the bubble’s length divided by its speed), indicating that the film can fully evaporate before the bubble has passed.

The evaporation timescale is consistent with the dynamics of hole opening (Fig. 3; Supplementary Video 1), suggesting that—by rapidly thinning the liquid layer—evaporation can lead to the deformation of its free surface in the voids between bacteria and to its ultimate rupture. The resulting three-phase contact line ruptures and moves radially outward from the point of rupture to minimize surface energy. This process is akin to “confined dewetting lithography”, where the thinning and rupture of a liquid film is used to arrange surface-residing colloidal particles.
into defined patterns. Because bacteria are thicker than the liquid film, they protrude above the air-liquid interface of the dewetting hole’s receding contact line: the resulting capillary force can dislodge individual cells, which are transported outwards by the receding contact line, until the latter becomes pinned when the accumulated bacteria form a levee between the opening hole and an adjacent hole (Fig. 3b).

An analysis of the time evolution of this process shows that the dynamics of hole formation is rather consistent among different holes within a single patch (Fig. 3c, d) and is characterized by a 3-step process: (i) a slow, initial opening of the hole, followed by (ii) a rapid, linear growth in time, and concluded by (iii) a slow, final growth. The overall process takes approximately 5 s and is therefore consistent with evaporation being the driver of the liquid film’s rupture and with an estimated film thickness of about 0.1 µm; this also means that van der Waals forces—significant only for film thicknesses below 10 nm—can be safely neglected. Although the evaporation of the thin liquid layer occurs regardless of the density of bacteria on the surface, this mechanism only works at intermediate adhesion strengths (8 h-old biofilm on the hydrophobic patch), whereas for younger biofilms, cells are swept away, and for older biofilms, the capillary force is insufficient to scrape cells across the surface. The critical role played by surface tension forces in the formation of holes is confirmed by experiments in which we used high flow rates (up to 100 µl min⁻¹), albeit for a short time, and did not observe any spatial re-arrangement of the bacteria as in the case of the passage of an air bubble. This result also shows that shear stress alone was not sufficient in our case to remove attached cells from the surface. Taken together, these results illustrate how the effect of an external insult depends strongly on the stage of biofilm development (here characterized by 1–2 layers of cells in the early stages), and that small variations in biofilm age (a few hours) for early-stage biofilms can have profound effects on their disruption by mechanical insults.

The “age” of the biofilm is not, of course, an absolute quantity, but rather depends on how fast bacteria attach to a substrate and develop micro-colonies. In our experiments, the formation of a biofilm on the hydrophilic substrate outside the patches occurs at a slower rate than on the hydrophobic patches: after 8 h the density of bacteria on the hydrophilic substrate is still relatively low and the capillary effect of the residual films after the passage of the air bubble is similar to the one observed for biofilms on the patches after 4 h (Fig. 2 and Supplementary Fig. S5; Supplementary Video 1). In addition, this result also shows that the detachment and spatial re-arrangement of cells on the surface is largely independent of the specific chemical nature (i.e., hydrophobic vs. hydrophilic) of the substrate and therefore is likely to be a more general phenomenon that may also occur on many natural surfaces.

A correlation analysis shows that the spatial distribution of EPS is the primary determinant of the local strength of adhesion, strongly suggesting that it is therefore responsible for the observed spatial patterns (Fig. 4). To quantify the distribution of EPS, we injected fluorescently labeled lectins (wheat germ agglutinin (WGA) conjugated with tetramethyl rhodamine isothiocyanate (TRITC)) in the microchannel at different times during the biofilm’s growth. Surprisingly, we found that, although bacteria quickly covered the surface of each patch so that after 8 h of growth the cell distribution is nearly uniform (Figs 2b and 4a), the distribution of EPS remained highly heterogeneous.
networks driven by twitching motility. The biofilm's location is important for its initial formation dynamics, but is retained as an underlying structure that controls the effect of a mechanical insult on surface colonization. Thus, this work supports the view that the underlying structure that controls the effect of a mechanical insult is initially uniformly colonized because they demonstrate that an environment in which a surface is initially colonized with more matrix is initially deposited are those where micro-colonies. Also inline with those earlier findings is the observed characteristic length-scale of the disruption pattern (≈10 μm). However, the results reported here add a new dimension to our understanding of biofilm disruption because they demonstrate that an environment in which a surface is initially uniformly colonized—which is different from the trail networks driven by twitching motility—can in fact hide an underlying structure that controls the effect of a mechanical insult on surface colonization. Thus, this work supports the view that the locations where more matrix is initially deposited are those where the biofilm is strongest and represent microscale strongholds that preferentially resist mechanical insult. Here, heterogeneity in the biofilm disruption by an air bubble

CONCLUSIONS

We have demonstrated that mechanical insults can result in partial removal of biofilms, depending on biofilm age, with the emergence of characteristically heterogeneous patterns. Correlation of these patterns with the spatial distribution of EPS abundance suggests that microscale heterogeneity in initial colonization and in later EPS production can have major consequences on the local strength of adhesion of biofilm cells, and ultimately result in a major rearrangement in the biofilm's organization after a mechanical insult. These findings point to the role of biofilm age and the associated heterogeneity in adhesion strength on biofilm removal, and should be considered in the design of shear-based or bubble-based removal strategies.

Biofilms are often assumed to be homogeneous and this view has affected biofilm models. However, the results presented here show that the time history of biofilm formation—and the associated spatial heterogeneity in the distribution of EPS and thus of the mechanical properties of a biofilm—is not only important for its initial formation dynamics, but is retained as memory in the system also for later macroscopic processes such as biofilm disruption by mechanical forces. This microscale heterogeneity implies the existence of strong differences in the mechanical microenvironment of cells in a biofilm, but likely also in the chemical microenvironment and possibly in the cells' physiological characteristics. One example could be the formation of biofilms in porous media and groundwater systems in which it
is common to have low flow rates and a multiphase flow. These micro-gradients may have multiple repercussions on biofilm dynamics, open new ecological niches, foster phenotypic heterogeneity and influence susceptibility to antimicrobials.

**METHODS**

**Materials**

OTS (CH₂(CH₂)₇SiCl₃) (97%), hexane (anhydrous, 99%), and fluorocarbon isoctylacrylate-bovine serum albumin (FITC-BSA) and TRITC-WGA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning.

Bacterial strain, growth conditions and device operation

The wild-type strain of *P. aeruginosa* PA01 was used for this study (courtesy of George O’Toole, Dartmouth University). For the preparation of the cell culture, cells from freezer stocks were inoculated in LB medium (10 g L⁻¹ NaCl, 5 g L⁻¹ yeast extract, 10 g L⁻¹ tryptone) at 30 °C under shaking (150 rpm). Cells were resuspended in fresh LB medium and incubated at 37 °C under shaking (180 rpm) up to OD₆₀₀ = 0.2, corresponding to early exponential phase. An aliquot of this cell suspension was injected and left folowing for 4 min wide, 50 µm tall microfluidic channel to allow cells to adhere to surfaces. Thereafter, continuous injection of M₆₃ minimal medium (which contains salts that supply nitrogen, phosphorus, and trace metals) and 0.5% glucose at a constant flow rate of 3 µl min⁻¹ began, and this flow rate was maintained for 4, 8 or 12 h, respectively, to produce biofilms of different ages. This flow rate corresponds to an average flow velocity of 250 µm s⁻¹ and a shear rate at the bottom surface of ~30 s⁻¹.

Air bubble generation

To introduce controlled air bubbles in the microchannel, we used a three-way valve: one inlet for the bacterial solution (used for initial injection), one inlet for the bacteria-free M₆₃ medium (used for 4 h, 8 h or 12 h), and one inlet for atmospheric air to generate air bubbles. The latter were introduced in the channel through the use of a syringe pump and valve.

Generation of patterned hydrophobic coatings

PDMS stamps were fabricated by curing the prepolymer on silicon masters patterned with SU-8 photoreist (SU-8 2050, MicroChem, MA, USA) using conventional soft photolithography. The masters used for patterning had recessed features, which resulted in PDMS replicas with protruding masters patterned with SU-8 photoresist (SU-8 2050, MicroChem, MA, USA) and had recessed features, which resulted in PDMS replicas with protruding还有特点。28 To assist in removal of cured PDMS from the SU-8 masters, had recessed features, which resulted in PDMS replicas with protruding hydrophobic patterns of masters patterned with SU-8 photoresist (SU-8 2050, MicroChem, MA, USA) and had recessed features, which resulted in PDMS replicas with protruding hydrophobic patterns of masters patterned with SU-8 photoresist (SU-8 2050, MicroChem, MA, USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning.

Microscopy and image analysis

Epifluorescence microscopy imaging was performed using an inverted microscope (Nikon TE-2000E) equipped with green fluorescent protein and red fluorescent protein filter sets. Images were acquired with ×40 and ×60 objectives and an Andor iXon CCD camera (50 frames s⁻¹) cooled to −65 °C. Image analysis was performed using built-in plugins of the ImageJ software (http://rsweb.nih.gov/ij/). Surface coverage, porosity and average hole radius of the biofilms were calculated using standard image processing techniques. The fractal dimension was computed using the box-counting method, as the slope of the linear fit of In(N) against In(s), where N is the number of boxes that cover the bacterial patches and s is the box size.

**ACKNOWLEDGEMENTS**

H.J. was partly supported through a Samsung Fellowship. We acknowledge support from a Gordon and Betty Moore Marine Microbial Initiative Investigator Award (#3783, to R.S.)

**AUTHOR CONTRIBUTIONS**

H.J., R.R. and R.S. designed research. H.J. performed experiments. H.J. and R.R. analyzed data. H.J., R.R. and R.S. wrote the paper.

**COMPETING INTERESTS**

The authors declare no competing interests.

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Supplementary Information accompanies the paper on the *npj Biofilms and Microbiomes* website (doi:10.1038/s41522-017-0014-5).