The role of multicellular aggregates in biofilm formation

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Aggregates in biofilm

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Abstract

In traditional models of \textit{in vitro} biofilm development, individual bacterial cells seed a surface, multiply and mature into multicellular, three-dimensional structures. Much research has been devoted to elucidating the mechanisms governing the initial attachment of single cells to surfaces. However, in natural environments and during infection, bacterial cells tend to clump as multicellular aggregates, and biofilms can also slough off aggregates as a part of the dispersal process. This makes it likely that biofilms are often seeded by aggregates as well as single cells, yet how these aggregates impact biofilm initiation and development is not known. Here we use a combination of experimental and computational approaches to determine the relative fitness of single cells and pre-formed aggregates during early development of \textit{Pseudomonas aeruginosa} biofilms. We find that the relative fitness of aggregates depends markedly on the density of surrounding single cells, \textit{i.e.} the level of competition for growth resources. When competition between aggregates and single cells is low, an aggregate has a growth disadvantage because the aggregate interior has poor access to growth resources. However, if competition is high, aggregates exhibit higher fitness, because extending vertically above the surface gives cells at the top of aggregates better access to growth resources. Other advantages of seeding by aggregates, such as earlier switching to a biofilm-like phenotype and enhanced resilience towards antibiotics and immune response, may add to this ecological benefit. Our findings suggest that current models of biofilm formation should be reconsidered to incorporate the role of aggregates in biofilm initiation.
Introduction

Biofilms are three-dimensional communities of interacting unicellular organisms (1). In a biofilm of supposedly genetically-identical clones, the constituent cells develop differentiated patterns of gene expression and growth (2, 3). Differentiation is often linked to the positioning of cells in the biofilm structure, and the spatial location of cells also affects resource availability and intercellular contacts (4).

The initiation of *in vitro* biofilm formation has traditionally been thought to be due to random attachment of single cells to a surface; these cells then divide and develop into mature, three-dimensional biofilms (5, 6). However, when cells disperse to seed new biofilms, detachment can occur as the dispersal of single motile cells, or by the sloughing off of large aggregates of cells (7–9). Both single cells and multicellular aggregates can go on to initiate new biofilms. For aquatic biofilms, the enhanced stickiness and surface conditioning of planktonic multicellular aggregates has been shown to increase the attachment of bacteria to a surface in early biofilm initiation (10). Greater stickiness and surface conditioning may be considered a quasi-phenotypic physiological property resulting from greater content of organic polymers and colloids. Similarly, an increased tendency toward aggregation, likely a proxy for greater stickiness, has been associated with increased biofilm formation for *Pseudomonas aeruginosa* as well (11, 12).

Three-dimensional bacterial aggregates found in liquid batch cultures of *P. aeruginosa* can have diameters of 10-400 μm and can constitute up to 90 % of the total biomass of the culture (13). In contrast, individual *P. aeruginosa* cells are rod-shaped, of size ~1×2 μm. Thus, multicellular *P. aeruginosa* aggregates, when they attach to a surface, can extend significantly into the vertical dimension, away from the attachment surface. Moreover, aggregates are structurally and physiologically distinct from single cells. Yet, how these structural contrasts impact the seeding and
growth of new biofilms is not known. This constitutes a significant gap in our understanding, since a biofilm seeded from an aggregate may develop very differently from a biofilm seeded by single cells.

To investigate the influence of pre-formed aggregates on biofilm development, we performed computer simulations of biofilm development from aggregates and single cells using an individual-based model and subsequently measured the relative growth of *P. aeruginosa* single cells and aggregates during *in vitro* biofilm development in flow cells. Using biomass accumulation as a measure of growth fitness, we found that the relative fitness of aggregates was highly dependent upon the surrounding number and density of single cells on the surface, which we use as a proxy for the level of competition for growth resources. We found that when the initial surrounding density of single cells is low, aggregates are less fit than single cells, yet when the surrounding density of single cells is high, aggregates are fitter than single cells. Our results show that in highly-competitive environments the 3D configuration in which cells land on a surface can greatly impact upon their relative fitness, both in the earliest stages of biofilm development and during long-time development. Our work calls for a modification of the traditional model of biofilm development to take into account the impact of pre-existing cell aggregates. This opens new avenues to understanding the evolution and ecology of biofilms in the environment and in chronic infections such as cystic fibrosis, chronic wounds and implant related infections.
Results

**Simulated fitness of aggregates vs. single cells in the initial formation of biofilms**

To investigate the fate of initial aggregates versus single cells during biofilm development, we first carried out individual-based computer simulations, in which biofilms were grown from aggregates surrounded by competing single cells. By varying the density of single cells surrounding the aggregate we were able to vary the extent of competition in our simulations.

In our simulations, we use oxygen as the growth limiting resource (see Section “The growth limiting resource may be oxygen”). Figures 1A and B show the oxygen concentration profile after 30 h of growth in the low density (A) and high density (B) regimes. Comparing with Figures 1C and D, which show the growth rates of individual cells as a function of their position in the growing biofilm, it is clear that the oxygen concentration profiles are influenced by the morphology of the growing biofilms. In the growing biofilms, we see that oxygen is depleted in the deeper regions; this oxygen-deprived layer emerges because faster growing cells at the top (Figures 1C and D) consume oxygen faster than it can diffuse to the deeper regions. This in turn leads to further heterogeneity in individual cell growth rates (Figures 1C and D), resulting in two distinct layers of growth activity: an outer layer of metabolically active cells and an interior region of inactive cells. Simulation snapshots of biofilms formed after 10, 30 and 120 hours of simulated growth are shown in Figure S1.

After 120 hours of simulated growth, we compared the fate of cells that originated in aggregates with that of initially-unaggregated cells (see methods). To this end, we used the number of progeny per initiating cell, $N/N_0$, as a measure of fitness. Our simulation results show enhanced performance of the aggregates, compared to unaggregated cells, with increasing initial density. When the initiating density of surrounding single cells is low, initially-unaggregated cells show higher fitness than those in an aggregate (Figure 2A). However, when the initiating density of surrounding cells is high, cells in an
aggregate perform better than the initially unaggregated cells over long times (Figure 2B). This change in
the relative fitness reflects a decrease in the fitness of the single cells as their density increases, rather
than any substantial change in fitness of the aggregated cells (Figure S2).

The interplay between competition and spatial structure determines the relative fitness of aggregates

Our simulations show that the aggregate produces more progeny per initial cell than do its initially-
unaggregated counterparts only when competition for resources is high (and over longer times, which
corresponds to an increase in competition as cells on the surface multiply). Why does increased
competition favor the aggregate? Closer inspection of Figures 2A and B reveal that, in fact, the fate of
the aggregate is little affected by the increase in cell density on the surface. For instance \( N/N_0 \) for the
aggregate at 30 h decreases from \( \sim 14 \) (14.27) to \( \sim 11 \) (10.94) when going from low density to high
density. However, the decrease in \( N/N_0 \) for the initially-unaggregated cells over the same time period is
much larger (\( \sim 328 \) to \( \sim 15 \)). To analyze in more detail the fate of the aggregate, Figures 1E and F show
the average number of progeny produced by cells in the initial aggregate as a function of their initial
position. As shown previously (14), the initial position of a cell within an aggregate has a strong effect on
its number of progeny; cells in the interior produce fewer progeny that those initially located at the
upper edges (Figure 1E). With increasing density of surrounding cells on the surface (Figure 1F), we see
that cell fate is even more heterogeneous, with the most prolific cells now localized in the highest
portion of the aggregate. Thus, although the increased density of unaggregated cells on the surface
increases the level of competition for space and resources, our simulations reveal that the few cells
initially located at the top of the aggregate dominate the fate of the aggregate at all levels of
competition.
The relative fitness of aggregates and single cells depends on initial cell density

To test our in silico simulation predictions, we investigated experimentally the degree to which seeding with single cells versus preformed aggregates gave rise to different patterns of biofilm growth. We inoculated flow cells with an overnight culture containing both planktonic cells and aggregates. By varying the cell density of the inoculum from an OD of 0.001 to 0.1, we were able to vary the seeding density of single cells and thus the level of competition for growth resources on the coverslip surface of the flow cell. We imaged single cells and aggregates over the first 9 hours of growth and from this data obtained growth rates, based on change in biomass over time. Due to small variations in growth rates from experiment to experiment, we concentrated solely on the relative fitness of single cells and aggregates within the same experiment, and did not compare absolute growth rates between experiments. As predicted by our simulations, we found that the relative fitness of aggregates, compared to that of single cells, depends markedly on the density of seeding cells. At low inoculum density (OD 0.001), aggregates grew (0.1920±0.0126 div. h⁻¹) at a significantly slower rate than single cells (0.23±0.0159 div. h⁻¹) (P < 0.0001). At medium inoculum density (OD 0.01) there was no difference in growth rate between aggregates (0.2349±0.028 div. h⁻¹) and single cells (0.224±0.031 div. h⁻¹) (P = 0.414). At high inoculum density (OD 0.1), cells in aggregates grew faster (0.24±0.017 div. h⁻¹) than single cells (0.1795±0.04 div. h⁻¹) (P = 0.0029). Growth rates for cells in aggregates and single cells are plotted in Figure 3(A-C) and the results of exponential fits and significance tests are summarized in Table S1.

Although growth of single cells and aggregates over the first 9 hours of growth appears exponential, fitting with a single exponent makes the implicit assumption that all cells within an aggregate are growing at the same rate. To avoid this assumption, we also describe growth in terms of the number of progeny per initial cell, \( N/N_0 \). Here, \( N \) is the biomass after 9 hours of growth and \( N_0 \) is the initial biomass. We find that \( N/N_0 \) is greater for the aggregates in the high-density treatment (Figure 3D) and greater for single cells at low density (Figure 3F). Thus, the \( N/N_0 \) representation captures the same density-
dependent advantage for aggregates as the exponential growth rate representation. Our results therefore show that there is a relative disadvantage to growing in an aggregate at low competition and a relative advantage to growing in an aggregate at high competition.

To explore the dynamics of competition and the spatial distribution of cells within the biofilm over longer time periods, we followed the growth of aggregates and single cells in the flow cell up to 99 hours but focused primarily on the first 24 hours after inoculation. We found that areas that were seeded with an aggregate developed a corresponding large vertical protrusion above the surrounding biofilm lawn (Figure 4D). Areas that were initially seeded by only single cells developed into a much more homogeneous, unstructured lawn (Figure 4C).

Fitness of cells is enhanced by higher spatial positioning

Taken together, our simulation and experimental results suggest that height above the surface of the flow chamber may be a crucial factor in our experimental setup; for example, in our simulations, cells that are at the top of an aggregate produce more progeny. This suggests that, in general, cells positioned above the surface of the flow chamber should outperform cells that are positioned closer to the surface. To test this, we measured the growth of single cells positioned on a glass step 100 μm above the chamber surface, compared to that of single cells on the chamber surface. Indeed, we found that, regardless of initial density, cells positioned on the step (Figure S5; see Methods) grew faster than single cells on the chamber surface (P = 0.004, P = 0.0079 and P = 0.004, for OD 0.1, 0.01 and 0.001 respectively) (Figure 5; Table S2). Furthermore, we also performed a series of simulations in which we
eliminated the height advantage of the aggregate by surrounding it by a pre-grown layer of competitor
cells of equal height (see methods and supplementary material). As expected, the cells in the aggregate
no longer outperformed the single cells in these simulations.

Better substrate access at the top of aggregates can lead to a growth instability

Our simulations show that an aggregate contains a sub-population of slow-growing cells in its center and
a sub-population of fast-growing cells at the top of the aggregate; this differentiation arises due to
spatial gradients in the growth resource. Since cells at the top of an aggregate grow faster, this might
suggest that, over time, the shape of aggregates should become less spherical and more prolate
spheroid. We checked for this change in aspect ratio in our experiments by measuring the height and
width (at half height) of aggregates at the beginning of an experiment and six hours later. We then
calculated the fold change in aspect ratio over the 6 hour time period, $\Delta H/\Delta W$, where $\Delta H$ is the
fractional change in height and $\Delta W$ is the fractional change in width. A value of 1 for $\Delta H/\Delta W$ would
signify symmetrical growth in all directions, while a value greater than 1 would signify aggregates
becoming more prolate spheroid. To measure the fold change in aspect ratio, we used WT PAO1
aggregates and twitching-motility-knockout $\Delta$pilA PAO1 aggregates at high cell density (Figure 6). The
change in aspect ratio is greater than 1 for $\Delta$pilA, but not for twitching-motile WT. This suggests that, as
our model predicts, cells at the top of the aggregate are growing more quickly than cells in other parts of
the aggregate – but also that twitching-capable cells rearrange themselves to reduce the local, in-
aggregate cell density.
The limiting growth resource may be oxygen

We found no effect on our results upon varying the concentration of carbon source over four orders of magnitude, indicating that carbon is likely not the limiting growth resource. In contrast, measurement of oxygen concentrations in the inflow and outflow of our flow cells, we find that oxygen, the only electron acceptor present in our media, becomes limited within the first 9 hours of growth for our high-density inoculation. The area under the curve (AUC) for oxygen in the outflow media was 546.4, 854.7 and 874.3 for 0.1, 0.01 and 0.001, respectively, for the first nine hours of growth. Oxygen is thus very limiting for growth in our flow cells, which were inoculated with cells at OD 0.1. For flow cells inoculated at OD 0.01, oxygen is somewhat limiting. For the flow cells inoculated with at OD 0.001, oxygen levels in the outflow are not limiting within the 9 hour timeframe in which growth was measured. This strongly points towards oxygen as a growth-limiting resource in our experiments at high cell density (OD 0.1) and to a lesser extent at medium cell density (OD 0.01). As the outflow media content is close to 100% O₂ saturation for the flow chambers inoculated with low density over all 9 hours of measurement, we anticipate no significant competition for oxygen (Figure 7).

Discussion

Non-attached biofilm aggregates arise in the liquid phase of in vitro bacterial cultures and in natural liquid environments, and these aggregates are likely to often attach to surfaces. Despite this, little is known about which role aggregates play in biofilm development compared to single cells. This study examined the biofilm growth dynamics that arise when a biofilm is seeded from a preformed aggregate using both in silico simulation and a widely-used in vitro biofilm reactor system. Our simulations deliberately neglected many biological mechanisms, including exopolysaccharide production, cell-cell signaling and cell detachment. Thus, any phenomena that arise in our simulations can be attributed solely to cell growth, competition for growth resources diffusing from above, and mechanical
interactions between cells. Therefore, we attribute the change in relative fitness of the aggregates with
the level of competition to the interplay between the spatially-structured environment and the spatial
distribution of cells in the aggregate.

We found that aggregates have a fitness advantage over single cells when competition for resources is
high since the elevated position of cells at the top of the aggregate gives these cells better access to
growth resources. However, when competition is low the single cells have access to resources that is
comparable to that of cells at the top of the aggregate and better than that of cells in the aggregate
interior. As a result, at low competition single cells are fitter than aggregates. This shows that the
relative fitness of aggregates depends markedly on the density of surrounding single cells, i.e. on the
level of competition for growth resources. When competition between aggregates and single cells is
low, an aggregate has a net growth disadvantage because the aggregate interior has poor access to
growth resources. However, if competition is high, aggregates have a higher net fitness, because
extending vertically above the surface gives cells at the top of the aggregates better access to growth
resources. Our findings suggest that we should reconsider our models of biofilm formation to
incorporate the role of aggregates, because current models only focus on growth that is initiated from
individually-attached cells.

Upon comparing results for short, intermediate and long periods of biofilm growth we found that the
fitness of aggregates, relative to that of single cells, increases with time (Figure 2 and Figure S4). Thus,
the outcome of competition between initially-aggregated and single cells is time-dependent. As biofilms
develop, the descendants of aggregates tend to dominate (Figure 2 and Figure 4), since competition for
growth resources becomes more intense as the total biofilm biomass increases. This suggests that, in
the long term, if competition for growth resource is the sole pressure on cells, structures such as
aggregates that protrude into the third dimension, and thus have better access to growth resources, will always be favored over single cells and their descendants.

We know from previous investigations, and have confirmed here, that overnight liquid batch cultures of *P. aeruginosa* contain both single cells and multicellular aggregates (13, 15). These multicellular aggregates range in size from 10 μm to several hundred microns in diameter (13). After inoculating a flow cell with an overnight culture of PAO1, one typically finds some fields of view that are seeded only with single cells (Figure 4A) and other fields of view that are seeded with an aggregate surrounded by single cells (Figure 4B). We expect to see cells on the surface of an aggregate grow faster than those in the interior because the latter have restricted access to growth resources (4, 16).

Our *in vitro* flow cell experiments confirmed the findings from our simulations, showing competition-dependent fitness advantages for aggregates over single cells. The density of surrounding single cells determines the relative impact of the spatial distribution of cells in the aggregate. Under conditions of low competition, single cells and cells on the surface of an aggregate have free access to resources and can grow unrestricted, whereas cells in the aggregate interior have less access to resources and grow more slowly. This puts the cells in the aggregate at an overall fitness disadvantage when compared to single cells. However, as the level of competition among cells on the chamber coverslip surface increases, single cells on average produce fewer progeny. Cells at the top of aggregates are elevated above the level of the surrounding single cells, and are closer to untapped growth resources. In this scenario, cells within aggregates have a higher relative fitness than single cells.

In addition, our *in vitro* step experiment shows an enhanced growth rate for cells elevated above the surface of the flow chamber compared to single cells positioned on the surface. This supports our hypothesis that cells on the top of an aggregate have a growth advantage over single cells, due to their height. This growth advantage of the cells at the top of the aggregate compensates, under conditions of
high competition, for the slower-growing cells contained within the aggregates.

Our results suggest a new model for early biofilm development, in which seeding of the biofilm from pre-formed aggregates plays a major role (Figure 8). Our results also imply that, when competition for resources is the main selective force, structures that initially protrude above a surrounding lawn of cells will be favored. These results raise a number of evolutionary questions. Why might evolution favor the formation of multicellular aggregates, given that our simulations show that only cells at the top of aggregates produce large numbers of progeny and many of the constituent aggregate cells sacrifice their own fitness for the benefit of these cells? Aggregation may be maintained by kin selection, a process by which traits are favored because of their beneficial effects on the fitness of close relatives, such as those cells at the top of aggregates (17, 18). Previous simulation work has also shown height-related fitness advantages and kin selection during biofilm development, suggesting that strains of bacteria that produce aggregation-promoting extracellular polymeric substances (EPS) gain a fitness advantage in biofilms by pushing their progeny upwards into the medium (19). Our work supports this view, while also showing that such fitness advantages can arise by aggregation as well as by traits such as EPS production.

While our work suggests one possible advantage of aggregate formation, we note that cells in an aggregate may also incur other benefits, especially in an in vivo infection. Aggregates exhibit many of the same phenotypes as surface attached biofilms (15), meaning that they demonstrate increased antibiotic tolerance, resilience towards immune response, and a stabilized chemical environment (5, 7, 15, 20–23). In an infectious regime, detached, colonizing single cells may be more vulnerable than aggregates (24, 25). Thus, aggregates may provide bacteria with a protected mode of colonization of new niches in a hostile environment (15, 22). In fact, in ex vivo samples from chronic infections, single cells are rarely observed; instead, non-attached aggregates seem to be the norm (20, 26–28).
Conclusion

In conclusion, our results show that aggregates perform better than single cells during biofilm development when the biofilm is seeded at high cell density, corresponding to high initial competition, and that over long timescales, biofilm structures are likely to become dominated by progeny originating from aggregates. Our results call for a revision of the prevailing picture of *in vitro* biofilm development, to consider the role played by biofilm seeding by preformed aggregates. While our study has focused on the role of spatial structure in the development of bacterial biofilms, 3D growth of multicellular assemblies is a universal phenomenon within biology, from carcinogenesis to plant development. Therefore the phenomenon identified here, involving the interplay between the spatial structure of the growing cell assembly and of the surrounding growth resource field, may have wider implications for other biological processes and for understanding multicellular assembly in general.

Materials and Methods

Bacterial strains

All *P. aeruginosa* strains used in this study were in a PAO1 background, which was obtained from the University of Washington, Seattle, USA. To enable visualization with confocal microscopy, we tagged PAO1 Wild Type (PAO1 WT) with green fluorescent protein (GFP) by Tn7 transformation as described previously (29).

Growth conditions

We streaked all strains from frozen stock onto Lysogeny Broth (LB) (Fisher Scientific, USA) agar plates and incubated them overnight at 37°C. Colonies were inoculated into LB broth (Fisher Scientific, USA) and grown, shaking, overnight at 37°C. We determined the optical density (OD$_{600}$) of the overnight culture using a spectrophotometer (Genesys, USA) and the culture was then adjusted to the desired OD by dilution into M9 minimal medium (Serva, Germany) with 10% v/v A10 phosphate buffer (pH 6.7). We
supplemented the growth medium with 0.3 mM glucose (Fisher Scientific, USA) as a carbon source. The resulting bacterial suspensions, which contained a mixture of aggregates and single cells, were then used to initiate the growth of biofilms. To test whether glucose is the limiting resource, we also carried out experiments using 0.003 mM and 30 mM glucose.

We grew biofilms in standard flow cells as described by Tolker-Nielsen et al. (30) with modifications described in Hutchison et al. (31). We filled the flow cell system with pre-heated (37°C) growth media as described above, and each of three independent chambers of the flow cell were inoculated with 150-250 μl of diluted bacterial culture. We inoculated the flow cell by injecting bacterial culture into each chamber using a Luer-Lock connector. We left the inoculated flow cell static for one hour to allow bacteria to attach to the glass coverslip before flow was started. We maintained a laminar flow at 3 mL h⁻¹ with a Watson-Marlow 205S/CA pump (Watson-Marlow, USA) for the duration of the experiment. Once flow was started, any remaining suspended cells or aggregates were removed from the system and did not contribute significantly to biomass accumulation.

**Imaging biofilm growth**

Biofilms were grown and observed *in situ* on a confocal microscope (Zeiss Imager.Z2 microscope with LSM 710 CLSM running Zeiss Zen 2010 v. 6.0. (Zeiss, Germany)) for qualitative analysis and an inverted confocal microscope (Olympus FV1000, running Fluoview 3.1a software (Olympus, Japan)) for quantitative measurements of a time-series of z-stacks. For the latter, a programmable motorized stage was employed to cycle between several locations (n=10-35) in each of three independent sample chambers. This allowed us to study the growth dynamics of regions of the flow cell with different initial conditions in the same experiment, with adequate statistics for each initial condition, and allowed us to identify confidently which location initially contained an aggregate and which contained single cells only.
The microscope stage area was enclosed in an incubator chamber that maintained a constant temperature of 37°C.

Z-stacks of regions that initially contained a pre-formed aggregate and/or one or more single cells were recorded every three hours, using 488 nm excitation and a 505-525 nm emission filter. The growth of biofilms in flow cells is typically measured by measuring biomass (30, 32–34). We used the free, open-source software ImageJ (National Institute of Health, USA) to crop images to separate aggregates and their descendants from single cells and their descendants. Biomass was then measured and described in terms of pixels³, or voxels, in Matlab (MathWorks, USA) using in-house code (31). Growth in biomass as a function of time could be fitted with an exponential function. The specific growth rate, $\mu$, is given by the exponent in the expression biomass = $Ae^{\mu t}$, where $A$ is a biomass at $t_0$ and $t$ is time. $\mu$ represents the growth rate per unit of biomass and can be used as a proxy measure of cell fitness (which is ultimately a cell’s ability to propagate its genes to future generations (35)).

In this work we classified seeding structures as aggregates only if they were at least 5 μm high and had volume at least ten times that of a single cell. Structures intermediate in size between this and single cells were excluded from our analysis. Because we monitored biofilm growth continuously under the microscope, we would have been able to identify attachment of new cells to the growing biofilm from the overlying growth medium. This was not observed, either for aggregates or single cells.

**The effect of elevated position on growth**

We examined the effect that of height above the surface on the growth rate of cells within the biofilm. We broke glass coverslips and selected shards that had a sharp, pointed tip, to reduce the influence of fluid flow around the shard, and were small enough to fit inside the chamber. Using silicone sealant (3M, USA), we placed a shard in the sample chamber such that the tip pointed opposite to the direction of flow. Bacteria that attached to the shard were positioned about 100 μm above the surface of the sample...
chamber and were expected to experience similar hydrodynamic conditions as those experienced by
cells positioned at the very top of an aggregate. The net discharge flow in the sample chamber is
laminar, with a Reynolds number of ~3 describing effective transport integrated over the entire
chamber. Because laminar flow results in a fluid speed that varies with distance above the surface, we
also calculate Reynolds numbers at specific locations important to our experiments: the Reynolds
number for cells on top of the shard, on top of a typical aggregate and 1 micron off the surface is about
50, 30, and 0.03 respectively. These Reynolds numbers are consistent with non-turbulent flow. For
“shard” experiments, the inoculation and flow conditions were unchanged from our other experiments.
The imaging was modified slightly in that a 40x objective (Olympus, USA) was used rather than a 60x or
100x objective. The lower-magnification objective had a greater working distance, which facilitated
imaging 100 μm into the sample chamber. The analysis of images to determine growth rate was
performed as described above.

**Horizontal oxygen gradients**

We measured oxygen concentration in media as it entered and left the inoculated flow cell with 2 flow-
through sensor cells and the FireStingO2 (Pyroscience, Germany). Measurements were taken every 60
seconds. This provided us with time-resolved information on the percent oxygen saturation of the
ingoing and outgoing media, and thereby also allowed quantification of the drop in oxygen across the
flow cell as a function of biofilm growth time.

**Computer simulation algorithm**

We used the agent-based microbial simulation package iDynoMiCs (36) to model the growth of biofilms
that were seeded from pre-formed aggregates and single cells. In iDynoMiCs, bacterial cells are
represented as particulate agents that grow and divide as a result of consumption of nutrients (see
Supplementary Information). The growth and division processes lead to local mechanical stresses within
the biofilm that are relieved by a “shoving” algorithm. In the simulations, nutrients are represented by concentration fields, which change as a result of consumption and diffusion from above. These processes give rise to local concentration gradients that can strongly influence the growth dynamics and morphology of the developing biofilm (4, 37–40). As is common in computational biofilm studies (19, 36, 40–44), our simulations were performed in 2D for reasons of computational efficiency. However previous work shows that similar results are likely to be obtained in 3D simulations (14).

In our simulations, microbial growth kinetics were modeled using the Monod growth equation

\[
\frac{dx}{dt} = \mu_{\text{max}} \frac{[O]}{K_O + [O]} [X]
\]

where [X] is the local concentration of biomass, [O] is the local concentration of oxygen, \( \mu_{\text{max}} \) is the maximum specific growth rate, and \( K_O \) is the concentration of oxygen at which the growth rate is half the maximum (see Supplementary Information). The growth parameters \( \mu_{\text{max}} \) and \( K_O \) were assumed to be the same for all cells (i.e. both aggregated and single cells). We used growth parameters from previous empirical studies on \( P. \ aeuruginosa \), with oxygen as the single rate-limiting nutrient (Table 1). A bulk oxygen concentration of \( 6.64 \times 10^{-3} \) g L\(^{-1} \) (45) was used in all simulations, consistent with the saturation concentration of oxygen in water at 37°C. Using these parameters, our simulations produce biofilms several hundred micrometers in height after 120 hours of growth (Figure S1).

**Creation of initial simulation configurations**

To create the initial configurations for our simulations, circular aggregates of cells were generated by excision of a ~100-cell circular region from a biofilm that had previously been simulated (see Supplementary Material). This aggregate was placed on a surface and surrounded by single cells, at a given surface density, placed at random in regions of the surface that were not occupied by the
aggregate (Figure S1A). In the simulations presented here, the single cells on the surface form a layer that is only approximately one cell thick even at our highest density.

To ensure adequate statistical sampling of aggregate configurations, four independent aggregate configurations were generated, each of which was simulated for ten independent realizations of the distribution of surrounding single cells on the surface, for each cell density. Thus each simulation data point represents results averaged over 40 different simulations.

Simulation runs

For each value of the surface density of the single cells, we simulated up to 120 hours of biofilm growth starting from each of our 40 cell configurations. The growth of the aggregate, in terms of number of progeny per initial aggregated cell, was computed for each simulation run and the results were averaged over the 40 runs.

To assess the fitness of the single cells, initially seeded on the surface, we performed separate simulations in the absence of the aggregate, for each value of the single cell density. Analysing single cell and aggregate fitness in separate simulations mimics our experimental scenario, in which the growth of the initially unaggregated cells was monitored in regions many fields of view away from an aggregate. Cell growth, in terms of number of progeny per initial single cell, was also measured in these simulations.

As a control, we also performed simulations in which the height advantage of the aggregate was eliminated. To do this we surrounded the aggregate with a high density of surrounding cells on the surface (0.5 cell μm⁻¹), and disabled the growth of the aggregate until the surrounding cells grew to the height of the aggregate (see Figure S7A). At this point, we enabled growth of the aggregate cells and ran the simulation for 120 h. To assess the success of the aggregate vs the single cells, we subtracted the
time it took for the single unaggregated cells to reach the same height of the aggregate (15 h) from 120 h. The “fitness” measure for the red cells, again assessed in separate simulations, was then given by \( \frac{N_{105}}{N_0} \).

**Statistical Analysis**

Statistical significance of both experimental and simulation data was evaluated by a Mann-Whitney test. P-values <0.05 were considered significant. All tests were performed in GraphPad Prism 5 (GraphPad Software, USA).

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**Conflict of interest statement**

Authors declare no conflict of interest.

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Legends to figures

Figure 1

Biofilm morphology shapes the oxygen concentration profile, with the fittest cells those that are initially located at the top. A-B, oxygen concentration (g L\(^{-1}\)) in a sample simulation after 30 hours of growth: (A) Low density (0.01 cell $\mu$m\(^{-1}\)); (B) High density (0.5 cell $\mu$m\(^{-1}\)). C-D, growth rate ($\mu$) for resulting populations after 30 hours of growth: (C) Low density (0.01 cell $\mu$m\(^{-1}\)); (D) High density (0.5 cell $\mu$m\(^{-1}\)). E-F, 2D histograms representing the number of progeny, N, produced after 30 hours of growth by individual bacteria as a function of their initial location in the aggregate: (E) Low density (0.01 cell $\mu$m\(^{-1}\)); (F) High density (0.5 cell $\mu$m\(^{-1}\)). These distributions were averaged over 40 simulations for each aggregate. Note that the gradient in the number of progeny is so large that a log scale is used for visualization purposes.

Figure 2

Simulations reveal that aggregates are relatively fitter than single cells at high density of competing cells on the surface and over long times. A-B, Accumulated biomass normalized to initial biomass ($N/N_0$) after 10, 30 and 120 hours for single cells and for aggregates: (A) Low starting density (0.01 cells $\mu$m\(^{-1}\)); (B) High starting density (0.5 cells $\mu$m\(^{-1}\)). For biofilms that were initiated at low density, single cells produce more progeny than do cells in aggregates at all measured times. For biofilms that were initiated at high density, single cells are fitter for early growth but aggregated cells produce more progeny than do single cells after 120 hours.
In *in vitro* growth in flow cells, at low inoculum density, aggregates are less fit than single cells; at high inoculum density, aggregates are fitter than single cells. (A-C) Fitted exponential growth rates during the first 9 hours of growth for initially-aggregated and initially-single populations, starting with different cell densities in the inocula. (A) Inoculum optical density (OD) = 0.1. (B) Inoculum OD = 0.01. (C) Inoculum OD = 0.001. (D-F) Measured Biomass ratio N/N₀ after 9 hours of growth. (D) Inoculum OD = 0.1. (E) Inoculum OD = 0.01. (F) Inoculum OD = 0.001. Mean with SEM.

The presence of multicellular aggregates at the start of biofilm growth is reflected in the structure of the biofilm a day later. Shown are perspective projections created from confocal microscope z-stacks of *Pseudomonas aeruginosa* biofilms. (A) Single cells attached to the surface at 0 hours. (B) A preformed aggregate surrounded by single cells on the surface at 0 hours. (C) Biofilm descending from single cells from A (D) After 24 hours of growth, a large biofilm structure descending from the preformed aggregate shown in B, surrounded by biofilm descending from single cells.

Fitted exponential growth rates for the first 9 hours of growth of single cells of PAO1 either on the surface or elevated 100 μm above on a glass platform. The fractional relative fitness (w) is about 0.5 for all densities evaluated, indicating that cells on the step consistently have a growth advantage over cells on the surface. Low density = initial inoculum OD=0.001. Medium density = initial inoculum OD=0.01. High density = initial inoculum OD=0.1 Mean with SEM.
Figure 6

Change in aspect ratio for aggregates after 6 hours of growth at high competition (OD= 0.1). Mean change in aspect ratio for motile (WT PAO1) and non-motile (ΔpilA PAO1) aggregates. Mean with SEM

Figure 7

Oxygen content in inflow and outflow of media to the flow cells inoculated with either OD 0.001, 0.01 or 0.1 dilutions of bacterial cells. After inoculation the cells were left without flow for 1 hour. Before starting the flow at t₀. From t₀ to t₉, the AUC for outflow media was 546.4, 854.7 and 874.3 for 0.1, 0.01 and 0.001, respectively.

Figure 8

Proposed revision of biofilm development. The classical five stages of development in the presence of a pre-formed, multicellular aggregate. 1; the surface can either be seeded by single cells in a planktonic phenotype or by a preformed aggregate. 2; the single cells attach irreversibly, and the aggregated population grows. 3; the biofilm matures with complete matrix. Descendants from aggregate population reach out in an elevated structure. 4; the mature structured biofilm. The descendants of the aggregate towers several times higher than any surrounding structures descending from single cells. 5; Dispersal of single cells and sloughing off of biofilm aggregates.

Table 1

Parameters used in all agent-based simulations of biofilm growth.
Supplementary legends

Supplementary text

The materials and method section with additional information about \textit{in vitro} flow cell experiment using varied glucose concentration in the media to determine if glucose was a limiting growth factor, as well as additional information regarding the generation of circular aggregates for \textit{in silico} simulations and the \textit{in silico} simulations in general.

Figure S1

Simulation snapshots of biofilms seeded with a bacterial aggregate generated from a pre-grown biofilm: (A) Biofilm development involving initially aggregated cells (green) and surrounding single cells (red) at low density (0.01 cell $\mu m^{-1}$). The aggregate is also magnified (blue region) for visualisation purposes; (B) Generating bacterial aggregates of circular geometry. For the purposes of visualisation, the radius, $R$, in the schematic is much larger than the 20 $\mu m$ that was actually used.

Figure S2

Whether aggregates or single cells produce more progeny in the first 120 hours of growth depends on the starting density of cells. Shown are linear regressions based on simulated growth of either single cells or cells in an aggregate. $N/N_0$ gives the number of progeny per original cell as a function of growth time. (A) For a low-density inoculum (0.01 cells $\mu m^{-1}$), the slope of $N/N_0$ for aggregates is 2.247 ± 0.02149 hours$^{-1}$ and 37.77 ± 0.2769 hours$^{-1}$ for single cells. Here, aggregates grow faster than single cells. The growth of both aggregates and single cells are well-fit as linear functions of time ($r^2 = 0.9934$ and $r^2 = 0.9993$). (B) For a high-density inoculum (0.5 cells $\mu m^{-1}$) the slope of $N/N_0$ for aggregates is 1.377 ± 0.01345 hours$^{-1}$ and 0.7409 ± 0.0004667 hours$^{-1}$ for single cells. Thus, here, aggregates grow faster than
single cells. Note that the vertical axis in panel B covers a much smaller scale than in panel A. As above, the growth of both aggregates and single cells are well-fit as linear functions of time ($r^2 = 0.9931$ and $r^2 = 1.000$).

Figure S3

Two examples of the large structures resulting from a preformed aggregate of GFP-tagged *P. aeruginosa*. after 99 hours of growth in a flow cell. A, C cross-section/top down-view of two aggregates. B, D 3D projections of two aggregates. 630x.

Figure S4

The relative fitness of the aggregate increases with increasing competition. We measure the relative fitness of the aggregate as the ratio (number of progeny per initial cell for an aggregate) : (number of progeny per initial cell for single cells). Thus, ratios greater than 1 indicate that the aggregate is fitter than the single cells. Plotted is the relative fitness of the aggregate, at 10, 30, and 120 hours, as a function of the initial density of cells on surrounding cells.
Figure S5

Schematic drawing of flow chamber with a 100 μm glass platform with single cells attaching on the surface (green) and on top of the platform (blue).

Figure S6

Exponential growth rate during the first 9 hours of growth for aggregates or single cell population of PAO1 in M9 supplemented with either 0.3 or 30 mM glucose at an initial cell density of OD 0.01. Mean with SEM.

Figure S7

The aggregate produces fewer progeny per initial cell relative to that of the single cells when its height advantage is eliminated: (A) The growth represented by N/N₀ for aggregates and single cells grown at same height in computer simulations. Single cell growth was measured at the same height as aggregates over 105 hours. The initially unaggregated single cell population produces significantly more progeny per initial cell than the aggregate. Mean with SEM. (B) Simulation snapshot at 15 h of single cell growth (aggregated growth switched off); at 15 h the initially unaggregated competing cells (red) have reached same height as aggregated population (green).
Table S1

Exponential growth rates and $N/N_0$ of aggregates and single cells at initial densities of OD 0.001, 0.01 or 0.1. Mean with SD. Statistical P-values comparing aggregates and single cells are the results of Mann Whitney testing.

Table S2

Exponential growth rates of single cells on the surface or on a step 100 μm above the surface at initial cell densities of OD 0.1, 0.01 or 0.001. Mean exponential growth rates with standard deviation (SD). Statistical P-values comparing surface and step populations are the results of Mann Whitney testing.
Figures 1 to 8 Main Manuscript
A. **P = 0.0029**

B. *P = 0.4140*

C. **P < 0.0001**

D. *P = 0.0201*

E. *P = 0.7879*

F. **P < 0.0001**

3
Figures S1 to S9 Supplementary
Growth rate measured of cell on the top of platform (∙) and cell population on the surface (∙)

Flow of media

Glass platform

100 µm

Cells attached to the surface of the chamber

Cells attached on top of glass platform

S5
