The Inoculation Method Could Impact the Outcome of Microbiological Experiments

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ABSTRACT For the past 150 years, bacteria have been investigated primarily in liquid batch cultures. Contrary to most expectations, these cultures are not homogeneous mixtures of single-cell bacteria, because free-floating bacterial aggregates eventually develop in most liquid batch cultures. These aggregates share characteristics with biofilms, such as increased antibiotic tolerance. We investigated how aggregates develop and what influences this development in liquid batch cultures of *Pseudomonas aeruginosa*. We focused on how the method of inoculation affected aggregation by assessing aggregate frequency and size using confocal laser scanning microscopy. Several traditional methods of initiating an overnight bacterial culture, i.e., inoculation directly from frozen cultures, inoculation using agar-grown cells, or inoculation using cells grown in liquid cultures, were investigated. We discovered a direct link between the inoculation method and the size and frequency of biofilm aggregates in liquid batch cultures, with inoculation directly from a plate resulting in the most numerous and largest aggregates. These large aggregates had an overall impact on the cultures' subsequent tolerance toward tobramycin, indicating that the inoculation method has a profound impact on antibiotic tolerance. We also observed a mechanism whereby preformed aggregates recruited single cells from the surrounding culture in a "snowball effect," building up aggregated biomass in the culture. This recruitment was found to rely heavily on the exopolysaccharide Psl. Additionally, we found that both *Escherichia coli* and *Staphylococcus aureus* produced aggregates in liquid batch cultures. Our results stress the importance of inoculation consistency throughout experiments and the substantial impact aggregate development in liquid batch cultures may have on the outcomes of microbiological experiments.

IMPORTANCE Pure liquid cultures are fundamental to the field of microbiological research. These cultures are normally thought of as homogeneous mixtures of single-cell bacteria; the present study shows that this is not always true. Bacteria may aggregate in these liquid cultures. The aggregation can be induced by the method chosen for inoculation. The presence of aggregates can significantly change the outcomes of experiments by altering the phenotype of the cultures. The study found a mechanism whereby preformed aggregates are able to recruit surrounding single cells in a form of snowball effect, creating more and larger aggregates in the cultures. Once formed, these aggregates are hard to remove. Aggregates in liquid cultures may be an immense unseen challenge for microbiologists.

KEYWORDS aggregation, *Pseudomonas aeruginosa*, biofilms

Since the days of Robert Koch and Walter Hesse, cultivation in pure liquid cultures has been the mainstay for the study of bacteria (1). When working with a bacterial liquid batch culture, it is often assumed that the culture consists of a phenotypically

homogeneous population of single bacterial cells. This assumption is based on the fact that liquid cultures are generally thought to be initiated from clones of a single bacterial cell (1–3). With the traditional technique of streaking frozen stocks or liquid cultures onto a solid medium, researchers are able to select for colonies originating from, in theory, a single bacterium of a particular genotype or morphology and to examine cultures for contamination (3, 4). Subsequently, colonies may be selected and used to inoculate liquid cultures to study bacteria in a free-floating (planktonic) state (1, 4, 5).

Most undergraduate biology students have been taught the growth pattern of bacterial liquid batch cultures described by distinct growth phases (6–9). When cells are inoculated into fresh culture medium, they initially enter the lag phase. During the lag phase, cells adjust to the fresh medium by synthesizing proteins that prepare the cells for growth (9–14). This phase varies in length, depending on the growth state of the inoculated cells, and the entire population of the culture consists of the inoculum. When the cells have adjusted to the new environment, they start to increase in number through exponential growth (14–16). During this exponential phase, the rapid growth results in a rapid decrease in the proportion of the initial inoculated population. Following exponential growth, the growth rate decreases and eventually the population enters the stationary phase. In the stationary phase, cells either do not divide or divide at the same rate as they die, thereby maintaining a stable population number (17). Ultimately, the population enters a "death phase" in which the population numbers decrease (18). A basic assumption of batch bacterial growth is that the decrease in the proportion of the inoculated population eliminates all residues of the phenotypic state of the initial inoculum.

Recently, there has been an increased focus on investigating the degree of homogeneity of liquid batch cultures. Schleheck and colleagues showed how nonattached aggregates of the opportunistic pathogen *Pseudomonas aeruginosa* form spontaneously in liquid batch cultures (19). After 6 h of growth, 90% of the biomass of the cultures consisted of aggregated bacteria in spheres with diameters of 5 to 600 μm. With overexpression of the exopolysaccharide Psl, *P. aeruginosa* exhibited increased aggregation in liquid cultures (20). For *P. aeruginosa*, these studies have clinical significance because nonattached biofilm aggregates are proposed to play a central role in the bacterial life history, both in the environment and during infection (21–25). Nonattached aggregates present the same phenotypic characteristics as biofilms attached to a surface, including enhanced antibiotic tolerance and resilience toward the immune response, compared to single cells (25). Thus, the frequency of nonattached aggregates in liquid laboratory cultures has significant effects not only on population-level phenotypes such as antibiotic tolerance but also on the reproducibility of experiments within and between laboratories (25–27).

The ability to conduct *in vitro* microbiological experiments consistently has always been a fundamental aspect of microbiological research. Among many other benefits, the assumption that bacterial liquid batch cultures are relatively homogeneous has spurred the use of microbes as experimental systems in many other scientific fields, including physics, engineering, and evolution. However, differences in practices among laboratories may often lead to different results based on the same protocol. We have investigated how the method used to inoculate batch cultures influences the development of nonattached biofilm aggregates and subsequently the experimental outcomes. We discovered that the aggregated biomass of a liquid *P. aeruginosa* culture depends on the method of inoculation, with direct inoculation using agar-grown colonies leading to the highest level of aggregate biomass. The level of aggregation had a profound heritable effect on the phenotype of subsequent bacterial populations, including antibiotic tolerance. The data presented here emphasize the importance of consistency in experimental workflow to control the effects of unintended nonattached aggregates in experiments. Variations in the method of inoculation, intentional or not, may have downstream effects that influence the experimental conclusions.
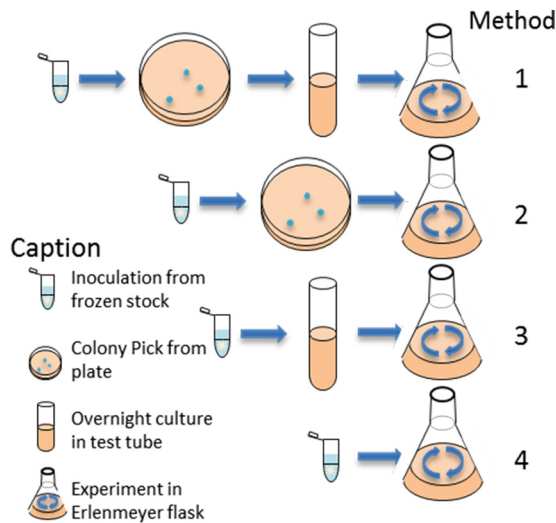
FIG 1 Schematic drawing of the four ways of inoculating liquid batch cultures. Method 1, frozen stock to a plate, a colony picked and added to LB for overnight culture in a test tube, and then inoculation for the experiment; method 2, frozen stock to a plate and then direct inoculation of a colony; method 3, frozen stock to LB for overnight culture in a test tube and then inoculation; method 4, direct inoculation from frozen stock.

RESULTS

Growth and aggregation in cultures inoculated by various methods. To examine the impact of inoculation method on aggregate growth and abundance in liquid culture, four different inoculation methods were evaluated after 4 and 18 h of growth, reinoculated, and then evaluated again after an additional 4 and 18 h (Fig. 1). Cultures inoculated with a lysogeny broth (LB) overnight culture from a single colony (method 1) or directly from a single colony from a plate (method 2) or from a LB overnight culture started from frozen stock (method 3) grew with comparable growth kinetics ($P = 0.49$). The culture inoculated directly from frozen stock (method 4) displayed an increased lag phase, compared to other cultures, over the first 7 h ($P = 0.003$) (see Fig. S2 in the supplemental material). After 24 h of growth, all four cultures had reached similar cell densities ($P = 0.75$).

Aggregation was evaluated by confocal laser scanning microscopy (CLSM) after 4 and 18 h in first-generation cultures (defined as the cultures that were initially inoculated by the various methods) (Fig. 2). Significantly more aggregated biomass was observed after 4 h in the cultures inoculated from a single colony from a plate (method 2), compared to cultures from a single colony grown in LB overnight before inoculation (method 1) ($P = 0.02$) and cultures inoculated from frozen stock grown in LB overnight before inoculation (method 3) ($P = 0.04$). Cultures inoculated directly from the frozen stock were highly variable in the abundance of aggregation but exhibited significantly
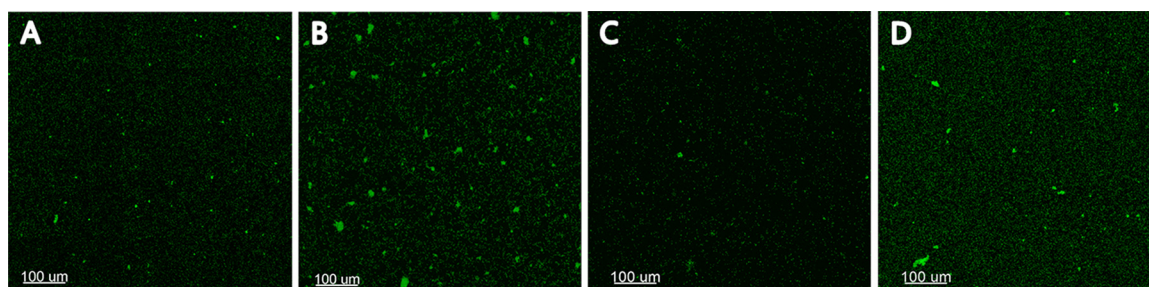
FIG 2 Representative images of first-generation *P. aeruginosa* liquid cultures after 18 h of growth when inoculated by the four test methods. (A) Method 1. (B) Method 2. (C) Method 3. (D) Method 4. Collected z-stack Easy 3D images are shown. Magnification, ×630.
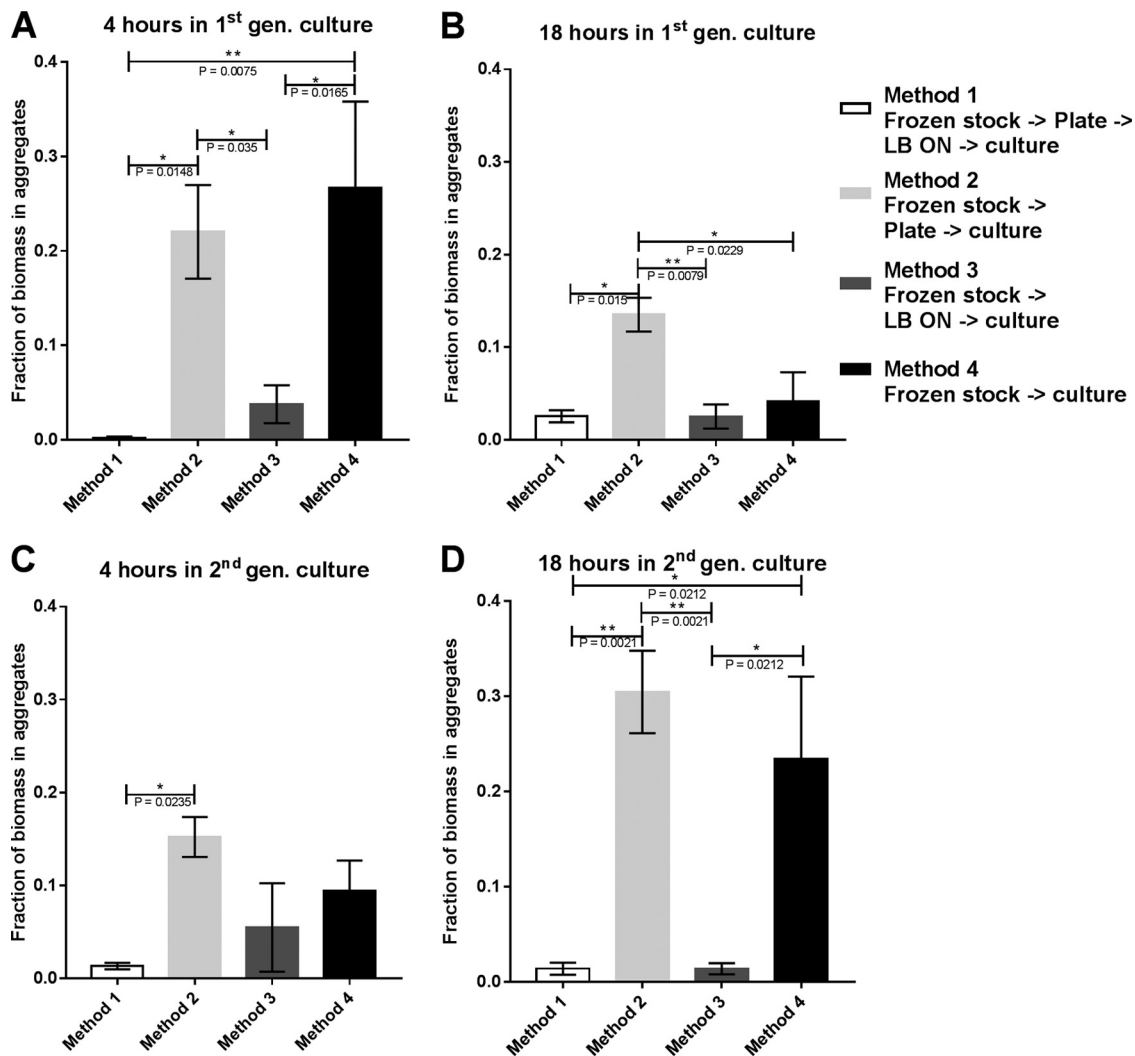
FIG 3 Fractions of total *P. aeruginosa* biomass in aggregates for cultures inoculated by the four different methods. (A) Aggregation in cultures after 4 h in first-generation cultures. (B) Aggregation in cultures after 18 h in first-generation cultures. (C) Aggregation in cultures after 4 h in second-generation cultures. (D) Aggregation in cultures after 18 h in second-generation cultures. Aggregates were defined as cell clusters with biomass containing a minimum volume of 250 μm^3. Biomass was normalized to the OD_{600} values of the cultures. Bars represent means with standard errors of the means (SEM). ON, overnight.

more aggregation than that found for methods 1 and 3 ($P = 0.001$ and $P = 0.02$, respectively) (Fig. 3A). After 18 h of growth, cultures inoculated from a plate (method 2) showed more aggregates than all other methods tested (Fig. 3B).

First-generation cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.005 in fresh medium and grown for an additional 4 h to create the second-generation culture. After 4 h, the second-generation cultures originally inoculated from a plate (method 2) contained significantly more aggregated biomass than did cultures inoculated with method 1 ($P = 0.02$) but not significantly more than cultures inoculated with method 3 or 4 ($P = 0.14$ and $P = 0.59$, respectively) (Fig. 3C). After 18 h of second-generation growth, cultures inoculated with method 2 had significantly more aggregated biomass than did cultures inoculated with methods 1 and 3 ($P = 0.002$ and $P = 0.002$, respectively). Cultures inoculated with method 4 displayed the same tendency to aggregate more than those inoculated with methods 1 and 3 ($P = 0.02$ and $P = 0.02$, respectively) (Fig. 3D). Thus, inoculation directly from an agar plate resulted in more aggregation in liquid cultures than did inoculation from a liquid LB overnight culture.

Tolerance toward tobramycin. To test whether the method of inoculation altered antibiotic tolerance, cultures were exposed for 24 h to tobramycin at a concentration
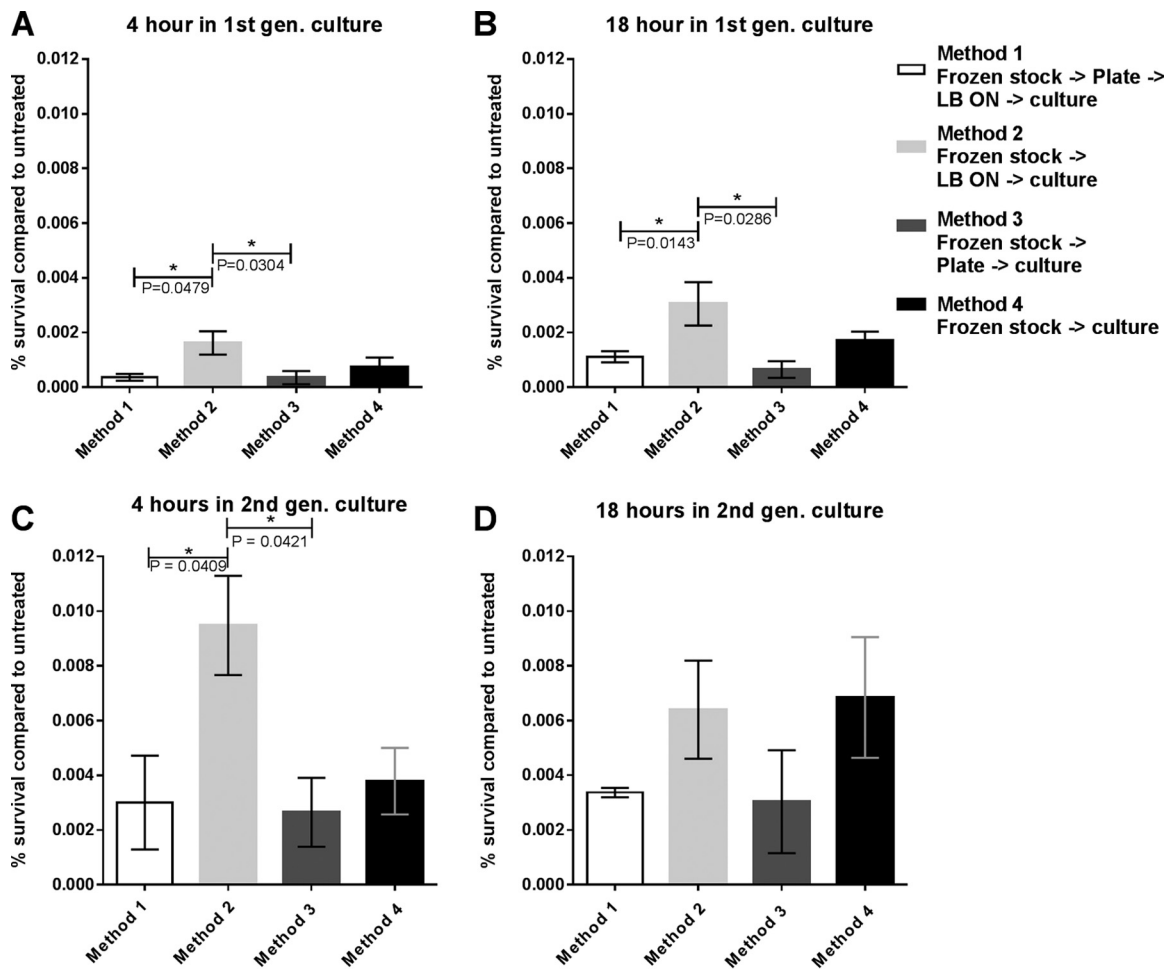
FIG 4 Survival of *P. aeruginosa* cultures treated with 10 μg ml^{-1} tobramycin for 24 h. (A) Survival in samples taken after 4 h in first-generation cultures. (B) Survival in samples taken after 18 h in first-generation cultures. (C) Survival in samples taken after 4 h in second-generation cultures. (D) Survival in samples taken after 18 h in second-generation cultures. The percent survival was based on CFU in treated versus untreated cultures. Bars represent means with SEM. ON, overnight.

10 times higher than the standard MIC determined by Etest for *P. aeruginosa* (25, 28). After 4 and 18 h of growth in both first- and second-generation cultures, samples were challenged with 10 μg ml^{-1} tobramycin for 24 h. Cultures treated after 4 h of growth in first-generation cultures, inoculated from plates (method 2), had significant higher survival rates than did cultures inoculated from LB overnight cultures (method 1 or 3) ($P = 0.048$ and $P = 0.03$, respectively). No increased survival in cultures initiated from a frozen stock (method 4), compared to those initiated from LB overnight cultures (method 1 or 3), was observed (Fig. 4A). After 18 h, the same relationship existed; plate-inoculated (method 2) cultures had significantly higher survival rates than did LB-overnight-culture-inoculated (methods 1 and 3) cultures ($P = 0.014$ and $P = 0.029$, respectively). Cultures inoculated from frozen stock inoculated (method 4) cultures had the same survival as seen in cultures inoculated from LB (method 1 and 3) (Fig. 4B).

In the second-generation culture, similar results were observed after 4 h of growth. The culture from plates (method 2) had an increased survival compared to the cultures from LB overnight (method 1 and 3) ($P = 0.041$ and $P = 0.042$, respectively). However, frozen stock inoculated (method 4) cultures did not show an increased survival compared to LB overnight inoculated (method 1 and 3) (Fig. 4C). After 18 h of growth in second-generation cultures, there was no significant difference in survival for any inoculation method (Fig. 4D).

Break up of aggregates by sonication. To test whether the disruption of pre-formed aggregates before inoculation could remove the differences in aggregation
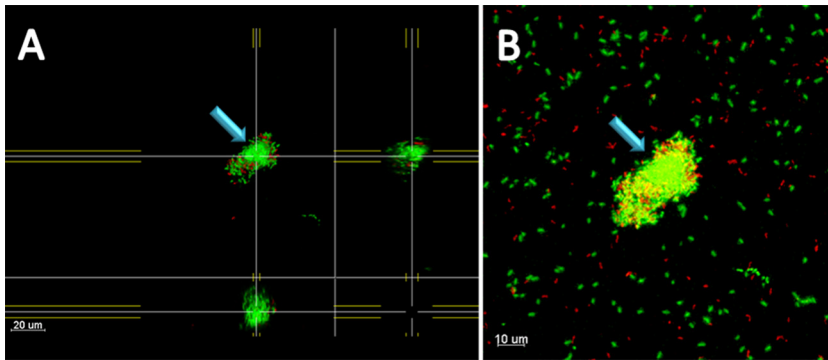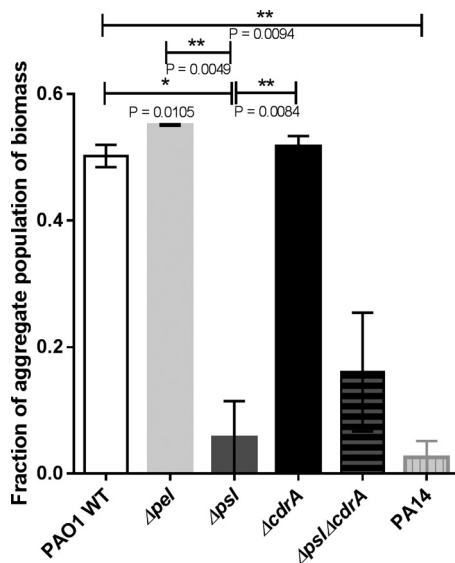
FIG 5 CLSM presentation of *P. aeruginosa* aggregates in a second-generation culture after 4 h of growth. The first-generation culture was inoculated only with GFP-expressing *P. aeruginosa* according to method 2. At reinoculation into a second-generation culture, the first-generation culture was diluted and mixed 1:1 with a primarily single-cell culture of *P. aeruginosa* expressing mCherry. The turquoise arrows mark a central core of GFP-expressing cells. This core is surrounded by mixed GFP- and mCherry-expressing cells. (A) Top-down and side views. (B) Compiled Easy 3D image of all slides in a z-stack. Magnification, ×630.

seen in the cultures, the inoculum was sonicated just prior to inoculation, according to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines for biofilm diagnostics (29). The sonication did not break up the preformed aggregates found in the inoculum from agar-grown colonies (method 2) to any significant degree ($P = 0.54$) (Fig. S3). The inoculated cultures grew for 18 h and were evaluated again. After 18 h, the cultures inoculated from a plate (method 2) had large visible aggregates observed with CLSM, as seen in cultures inoculated with nonsonicated inocula. The cultures inoculated from frozen stock (method 4) had some degree of aggregation as well. No preformed aggregates could be found in inocula from LB overnight cultures (methods 1 and 3) (Fig. S4). These data indicate that sonication appears unable to disrupt preformed aggregates.

Mixed-culture aggregates. To elucidate the mechanism of formation of aggregates in the second-generation cultures, a PAO1 green fluorescent protein (GFP)-expressing first-generation culture containing aggregates was diluted and mixed 1:1 with a PAO1 mCherry-expressing culture of primarily planktonic single cells. With CLSM, we were able to observe aggregates formed in the second-generation culture after 4 h of growth. In several aggregates, a clear core consisting exclusively of GFP-marked cells surrounded by a layer of mixed GFP- and mCherry-marked cells was visible; no cores consisting of mCherry-marked cells were observed (Fig. 5). This finding suggests that aggregates were formed either during the inoculation process or in the first-generation culture and that, when transferred to the second-generation culture, aggregates recruited planktonic cells from the surrounding culture.

Involvement of biofilm matrix components in aggregate formation. To investigate the mechanism underlying the observed recruitment of single cells around aggregates, we tested the ability of several *P. aeruginosa* strains with different deficiencies in extracellular matrix production to aggregate in liquid culture. A PAO1 strain deficient in production of the exopolysaccharide Psl showed a significant decrease in aggregation, compared to wild-type PAO1 and a PAO1 strain deficient in production of the exopolysaccharide Pel ($P = 0.0105$ and $P = 0.0049$, respectively). Deletion of the surface adhesin CdrA, which has been shown to associate with the Psl exopolysaccharide (30), did not affect the ability to aggregate, compared to the wild-type strain. A decrease in aggregation similar to the PAO1 *psl* mutant was also observed for *P. aeruginosa* strain PA14, a strain not able to produce Psl (31), indicating that the phenotype was not specific to strain PAO1 ($P = 0.0094$) (Fig. 6).

To further investigate the involvement of Psl exopolysaccharide in the aggregate recruitment of single cells in liquid cultures, a strain with inducible Psl production and Pel deficiency (Δpel P_{BAD}-*psl*) was created. Aggregation of *psl*-induced or noninduced

FIG 6 Fraction of *P. aeruginosa* biomass present as aggregates for strains with various deficiencies in extracellular matrix production. Cultures were inoculated according to method 2 and examined after 18 h of growth in a first-generation culture. The strains tested were PAO1 wild type (WT), PAO1 Δ*pel*, PAO1 Δ*psl*, PAO1 Δ*cdrA*, PAO1 Δ*psl* Δ*cdrA*, and PA14 wild type. Bars represent means with SEM.

cultures was similar after 4 h of growth; after 18 h of growth, however, there was significantly more aggregated biomass in the cultures in which Psl production was induced, compared to the uninduced cultures ($P = 0.037$). Using these induced and uninduced cultures as inocula showed that the aggregate phenotype was conserved in the second-generation cultures ($P = 0.0039$), indicating that the aggregate phenotype was heritable at least over a few generations (Fig. 7).

To test the involvement of extracellular DNA (eDNA) in recruitment of single cells and in the structural integrity of aggregates, cultures inoculated directly from a plate were treated with DNase. After 4 h of treatment, no difference in the degree of aggregation between DNase-treated and untreated cultures could be observed ($P = 0.38$). After 18 h, the cultures treated with DNase had significantly ($P = 0.0019$) less aggregated biomass, compared to untreated cultures (Fig. S5).

Stability of the aggregated population in continuous culture. How long is the aggregate phenotype able to persist? To answer this question, a long-term chemostat experiment was performed with a 1:1 mixture of GFP- and mCherry-marked cells and a dilution rate of 0.2 per hour (bacterial doubling time of 5 h). Both the GFP- and mCherry marked populations were inoculated using agar-grown colonies (method 2), in an effort to induce maximal initial aggregation. For the entire duration of the experiment (13 days), aggregates were present to a degree comparable to that of an 18-h-old batch culture. However, the amount of aggregated population decreased toward the end of the 13 days. This may be a result of the constant exchange of medium, with slow dilution of the aggregate population because the formation of aggregates was not able to keep up with dilution (Fig. 8A). By visual examination of the aggregates being produced in the culture, a clear pattern emerged. Most aggregates had clear zones of monocolored bacteria in the first 2 to 3 days (Fig. 8B) but, as the experiment progressed, aggregates began to appear more homogeneous and mixed between GFP- and mCherry-tagged strains (Fig. 8C). Thus, aggregates remained a stable part of the population for at least 7 to 9 days after inoculation.

Aggregation of other common pathogenic bacterial species in liquid culture. To test whether the observed link between the method of inoculation and the degree of aggregation is a general phenomenon, *Escherichia coli* and *Staphylococcus aureus* were tested in the aggregation assay. Inoculation from agar-grown colonies and LB overnight cultures (methods 2 and 3, respectively) were employed
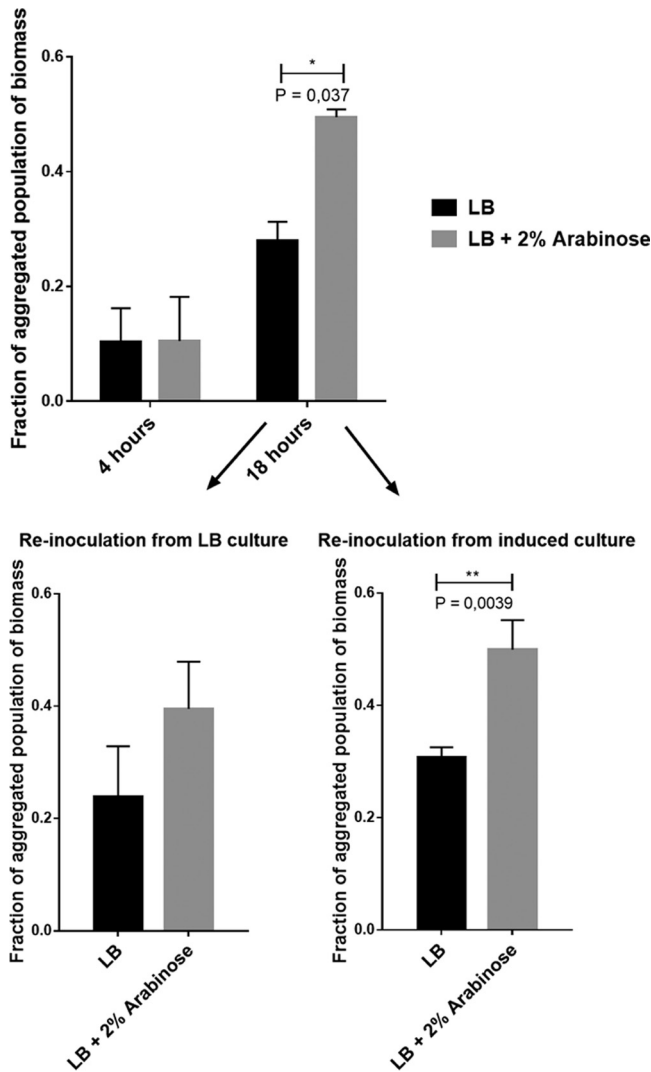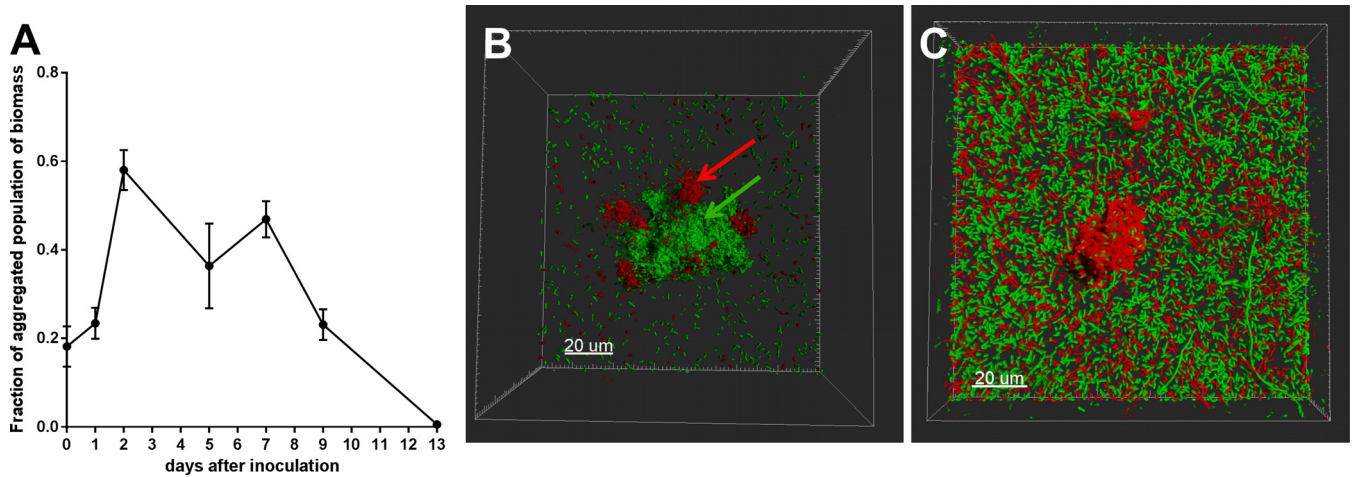
FIG 7 Fraction of aggregated biomass of the PAO1 Δ*pel* P$_{BAD}$-*psl* strain in cultures inoculated according to method 2, into LB with or without 2% arabinose (to induce *psl*). After 24 h of growth, cultures were reinoculated into fresh LB medium with or without 2% arabinose, creating four second-generation cultures. (Top) First-generation cultures sampled after 4 and 18 h. (Bottom left) Reinoculated cultures from the original noninduced (no arabinose) cultures. (Bottom right) Reinoculated cultures from the original induced (2% arabinose) cultures. Bars represent means with SEM.

as two extremes of aggregate induction, according to the results from *P. aeruginosa* experiments. *E. coli* showed increased aggregation, compared to PAO1, with a mean aggregated population fraction of approximately 0.7 after 4 h in first-generation cultures and approximately 0.2 in 18-h-old first-generation cultures. No significant correlation between the method of inoculation and the degree of aggregation was observed for *E. coli*. *S. aureus* exhibited the same link between inoculation directly from a plate and increased aggregation during early growth (4 h) as seen for *P. aeruginosa* cultures ($P < 0.0001$) (Fig. 9). Thus, aggregation in liquid cultures is not limited to *P. aeruginosa*.

To assess whether the recruitment of single cells to aggregates can occur between different species, tagged *P. aeruginosa* and *E. coli* were mixed as described above. Preaggregated *E. coli* cells were mixed with predominantly single-cell *P. aeruginosa*. After 18 h of combined growth, several aggregates with a monocolored center of *E. coli* could be found with a mixture of *P. aeruginosa* and *E. coli* surrounding the center (Fig. 10).

FIG 8 Findings from chemostat cultures grown for 13 days. (A) Fraction of aggregated biomass in samples taken on day 0, 1, 2, 5, 7, 9, and 13 postinoculation. Bars represent means with SEM. (B) Three-dimensional projection of an aggregate in chemostat culture after 2 days of growth. Red and green arrows indicate monocolored sections of the aggregate. (C) Three-dimensional projection of an aggregate in chemostat culture after 9 days of growth. The aggregate consists of a mixed population of red and green cells both originally inoculated directly from a plate, in an effort to distinguish between original aggregates (monocolor) and aggregates generated in chemostat culture (mixed colors). Magnification, ×630.

DISCUSSION

Microbiology has seen a resurgence as evolutionary biologists, physicists, and engineers have adopted microbial model systems for testing theories and producing and testing products. The traditional microbiology viewpoint is that test-tube-grown populations are relatively homogeneous and distinct from biofilm-grown bacteria. The result is that phenotypic differences, including enhanced tolerance toward antibiotics and host defenses, are frequently assumed to discriminate test-tube-grown planktonic cells from biofilms (32–34). Here we show that the method of inoculation of planktonic cultures has a profound effect on the phenotypes observed, including antibiotic tolerance.

The antibiotic tolerance of planktonic cultures depends on bacterial aggregation, which varies based on the method of inoculation. Indeed, the increased survival rates seen for *P. aeruginosa* cultures inoculated directly from colonies on plates are in accordance with the findings that these cultures had more aggregated biomass and
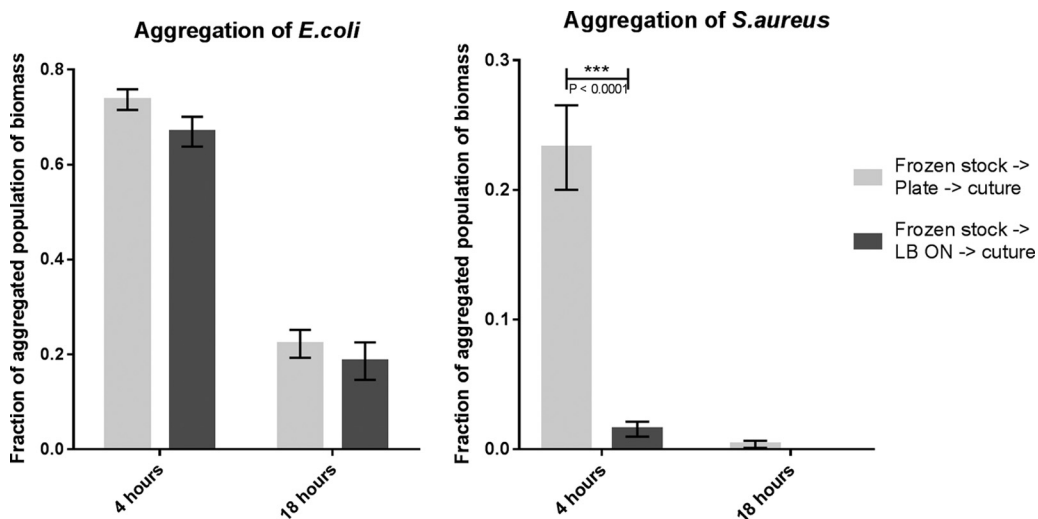
FIG 9 Fractions of aggregated biomass for cultures of *E. coli* and *S. aureus* inoculated using method 2 or method 3. Cultures were evaluated for aggregation after 4 and 18 h of growth. (Left) *E. coli*. (Right) *S. aureus*. Bars represent means with SEM. ON, overnight.
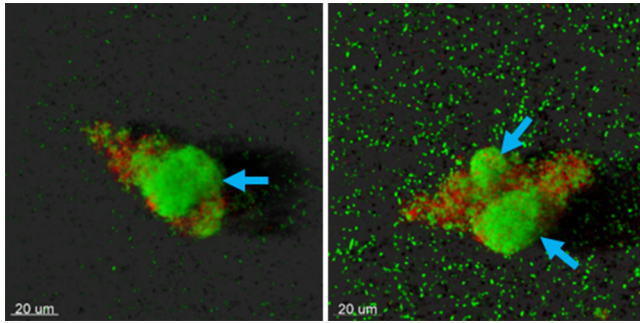
FIG 10 Two examples of three-dimensional projections of multispecies aggregates. GFP-tagged *E. coli* cells from aggregate-rich cultures were mixed with predominately single-cell *P. aeruginosa* cultures. After 18 h of growth, the mixed cultures contained large proportions of aggregates with a central core of green GFP-tagged *E. coli* (marked with blue arrows) surrounded by a mixture of green *E. coli* cells and red mCherry-tagged *P. aeruginosa* cells.

that tobramycin, an antibiotic known for killing actively growing cells (35), failed to kill parts of nonattached aggregates (25). Therefore, it is likely that the increased tobramycin tolerance of cultures inoculated using method 2 is a result of cells being protected either due to reduced metabolic activity in parts of the larger aggregated population or as a result of Psl providing a shielding effect against tobramycin (36, 37). This hidden difference in antibiotic tolerance among macroscopically identical cultures could have major effects on the outcomes of various studies; for example, this could be the case in susceptibility testing of antimicrobial agents, where aggregation in cultures may induce various fractions of tolerant cells.

Interestingly, the greater prevalence of aggregated biomass in second-generation cultures originally inoculated from a colony or directly from frozen stock was maintained even after ~1,000-fold dilution in fresh medium. This indicates that cultures first seeded with aggregates inherit the frequency of aggregation of the parent culture and maintain it over several generations. We propose that the maintenance of aggregate frequency in second-generation cultures is mediated by recruitment of planktonic cells to preformed aggregates, which results in larger aggregates that eventually seed smaller aggregates that again recruit planktonic cells, resulting in a snowball effect of aggregate formation. Our proposal is supported by the fact that we were able to find a core of the ancestral aggregate within aggregates produced in second-generation cultures, indicating that these core parts were inherited from the first-generation culture and recruited cells. The fact that our chemostat cultures maintained high aggregate frequencies for 7 days (33 generations) indicates that a failure to pay attention to the possible introduction of preformed aggregates may have important repercussions for both short- and long-term experiments.

Psl has been shown repeatedly to be crucial for the formation of surface-attached *P. aeruginosa* biofilms (30, 38, 39). Our results indicate a similar dependency in the formation of nonattached aggregates in the liquid phase. The Psl-associated adhesin CdrA was not required for aggregation, indicating that Psl does not need this adhesin for aggregation. In contrast to Psl, the exopolysaccharide Pel, which has been the focus of great interest for its involvement in *P. aeruginosa* biofilms (40, 41), was not involved in aggregate formation in our system. Thus, the exopolysaccharide Psl seems to be a central mediator for the recruitment of single cells around preformed aggregates introduced into a liquid culture. eDNA was reported previously to be critical for the integrity of biofilm aggregates (25). With removal of eDNA from cultures by DNase supplementation, we did not see any change in aggregation in the early hours of growth (4 h), compared to untreated cultures. In contrast to this, all aggregates were removed from the cultures in the late stages of growth (18 h). This finding suggests that eDNA is involved as an adhesive that maintains the cohesiveness of the aggregate structure, as well as Psl, but may not be as essential for the recruitment of single cells in young cultures.

The ability of *E. coli* to aggregate in liquid cultures was remarkable but was not affected by the method of inoculation. It is worth noting that, even with the method found to generate the least aggregation for *P. aeruginosa*, *E. coli* cultures could still contain close to 70% of the biomass in an aggregated state. This raises the question of when, if ever, an *E. coli* culture is a completely homogeneous mixture of single cells. One possible mediator of this high propensity to aggregate could be curli, which has been shown to play a major role in the ability of *E. coli* to form aggregates and to adhere to surfaces (42, 43). Although curli expression in most *E. coli* strains is recognized to reach a maximum during growth below 30°C, the K-12 strain that was used in this study produces curli at 37°C (43). Interestingly, we found that preformed *E. coli* aggregates were able to recruit a mixture of *E. coli* and *P. aeruginosa* single cells, creating multispecies aggregates. This finding reveals a completely new area of possible interactions between species of aggregated bacteria and leads to ideas of multispecies aggregated biofilms.

S. aureus aggregation after 4 h of growth was greater when cultures were inoculated from a plate than when they were inoculated from a LB overnight culture, as found with *P. aeruginosa*. In contrast to what was observed with *P. aeruginosa*, however, almost no aggregates could be found in 18-h-old *S. aureus* cultures. The dissolution of aggregates in older *S. aureus* cultures might be explained by the ability of *S. aureus* to secrete proteases in the late stationary phase, which enables cells to disperse from an aggregated state to a single-cell state. The secretion of protease K, for example, is regulated by the *agr* quorum-sensing (QS) system in *S. aureus* (44). Despite this possible late-stationary-phase dispersal, our results indicate effects of the method of inoculation on *S. aureus* in early-exponential-phase cultures similar to those seen for *P. aeruginosa*. Interestingly, Haaber et al. described previously how *S. aureus* aggregates recruit single cells from the surrounding liquid phase during exponential-phase and late-exponential-phase growth, in a manner similar to that we observed with *P. aeruginosa* (45). Therefore, our results from studies of *E. coli* and *S. aureus* indicate that aggregation in liquid culture is not limited to *P. aeruginosa*.

In conclusion, we have discovered a connection between the method of inoculation of bacterial liquid cultures and the degree of aggregation within those cultures. This variation in *P. aeruginosa* aggregation was associated with altered tolerance toward the antibiotic tobramycin. These differences in aggregation and tobramycin tolerance were inherited by next-generation cultures when they were initiated from dilutions of the first-generation cultures. Our results stress the importance of consistency throughout experimental procedures beginning with inoculation, and they indicate that differences in inoculation procedures can have phenotypic consequences for >30 generations of bacterial growth and thereby can affect the outcomes of experiments.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains used in this study were of the PAO1 strain background, from the University of Washington (Seattle, WA) (46). The strain *P. aeruginosa* PAO1 Δ*pelA* P$_{BAD}$-*psl* was created from *P. aeruginosa* PAO1 P$_{BAD}$-*psl* (47) by introduction of the *pelA* allelic exchange vector pMPELA (20), essentially as described in reference 48. The *E. coli* strain used was K-12 MG1655 (ATCC 47076). *S. aureus* strain RN4220, constitutively expressing GFP from pAH13, was used (49). To enable visualization with CLSM, the wild-type PAO1 strain was made to express GFP or mCherry by Tn*7* transformation (50). Other strains were stained using Syto9 (Molecular Probes, USA).

Growth conditions. All growth experiments were performed at 37°C. Experiments were performed in LB (BD, USA), and LB agar (2% [wt/vol]) plates were used for routine cultures. Frozen stocks (−80°C) consisted of 80% overnight cultures in LB and 20% glycerol (Sigma, USA). Liquid cultures were shaken at 180 rpm.

Growth experiments and aggregate evaluation. Erlenmeyer flasks were inoculated in four ways (Fig. 1), as follows. In method 1, frozen stock was streaked on LB plates and left to grow overnight. Subsequently, one colony was inoculated into LB, grown overnight, and used for inoculation of the Erlenmeyer flask. In method 2, frozen stock was streaked on LB plates and left to grow overnight. One colony was picked, suspended in 1 ml LB, and used for inoculation of the Erlenmeyer flask. In method 3, LB was inoculated directly from frozen stock, left to grow overnight, and used for inoculation of the Erlenmeyer flask. In method 4, frozen stock was directly suspended in 1 ml of LB before inoculation of the Erlenmeyer flask.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Pseudomonas aeruginosa		
PAO1	Wild-type strain	53
PAO1 Δ*pel*	Pel-deficient knockout mutant	30
PAO1 Δ*psl*	Psl-deficient knockout mutant	54
PAO1 Δ*cdrA*	CdrA-deficient knockout mutant	30
PAO1 Δ*psl* Δ*cdrA*	Psl- and CdrA-deficient knockout mutant	30
PAO1 Δ*pelA* P$_{BAD}$-*psl*	Pel-deficient knockout mutant with arabinose-inducible expression of *psl*	This study
PA14	Wild-type strain	
Escherichia coli MG1655	Wild-type strain derived from *E. coli* K-12	55
Staphylococcus aureus AH13	Strain derived from *S. aureus* RN4220, with constitutive expression of *gfp*	49
Plasmids		
pMPELA	*pelA* allelic exchange vector	20
pAH13	Plasmid for GFP expression in *S. aureus*	49

All four types of *P. aeruginosa* inocula were added to obtain a final OD$_{600}$ of 0.005 in the Erlenmeyer flask. Each 250-ml Erlenmeyer flask contained 100 ml LB medium. The four cultures were monitored by OD$_{600}$ measurements with a UV-1800 spectrophotometer (Shimadzu, Japan) every 30 min during the first 7 h of growth. At 4 and 18 h, samples were taken for microscopic evaluation. These cultures are referred to as first-generation cultures. Time points were chosen to represent exponential-phase cultures (4 h) and stationary-phase cultures (18 h). For microscopic evaluation, 50 μl of culture was applied to a counting chamber (μ-slide VI Flat; Ibidi, Germany). Chambers were scanned in z-stacks in a tile scan (5 by 5 fields of view; 674.56 μm by 674.56 μm by 40 μm) with a Zeiss Imager Z2 microscope with a LSM 880 CLSM system and the accompanying Zen 2010 version 6.0 software (Zeiss, Germany). Imaris image analysis software (Bitplane, Switzerland) was used to measure the size and frequency of aggregated biomass, using the Measuring Pro package. Dense collections of cells with a volume of at least 250 μm³ were considered to be aggregates. This threshold was chosen to enable sufficient discrimination from several single cells connected on the surface and dense three-dimensional structural aggregates (see Fig. S1 in the supplemental material). The average volume of a single bacterium measured in this way is ~4 μm³; therefore, 250-μm³ aggregates contain ~62 cells.

First-generation cultures were reinoculated into fresh medium after 24 h of growth in Erlenmeyer flasks. The 24-h-old cultures were diluted into fresh LB to a final OD$_{600}$ of 0.005 (~1,000-fold dilution) and grown for an additional 18 h, with samples taken after 4 and 18 h for microscopic evaluation of aggregate formation. These cultures are referred to as second-generation cultures.

Aggregation of strains deficient in Psl, Pel, and CdrA expression. PAO1 strains deficient in production of the exopolysaccharides Psl (Δ*psl*) and Pel (Δ*pel*) and the surface adhesin CdrA (Δ*cdrA*), as well as the Psl-deficient *P. aeruginosa* strain PA14, were tested. Based on results from previous experiments, method 2 (frozen stock then agar plate then inoculation) was chosen for inoculation, to achieve the greatest possible initial aggregated population. Strains were grown for 24 h in first-generation cultures and reinoculated into fresh medium in second-generation cultures before being evaluated for aggregation after 18 h of growth.

A PAO1 Pel-deficient strain with inducible Psl production (Δ*pel* P$_{BAD}$-*psl*) was also tested for aggregation. Cultures inoculated from plates, according to method 2, were grown in LB with or without 2% arabinose. After 24 h, the cultures were reinoculated into fresh LB either with or without 2% arabinose. Samples were evaluated for aggregation at 4 and 18 h of growth in both the first- and second-generation cultures.

To assess eDNA involvement in aggregation, DNase (Pulmozyme [dornase alpha]; Genentech, USA) was added at 0 h after inoculation of cultures directly from a plate, according to method 2. DNase was diluted to a final concentration of 90 U ml^{-1}, which was reported previously to disrupt *P. aeruginosa* biofilm aggregates (25).

E. coli and S. aureus aggregation. For evaluation of the ability of *E. coli* and *S. aureus* to aggregate in liquid batch cultures, Erlenmeyer flasks were inoculated according to methods 2 and 3, directly from agar plates or LB overnight cultures. Aggregation was evaluated in samples taken at 4 and 18 h from first-generation cultures, as described above.

Disruption of aggregates in inocula by ultrasonication. To disrupt preformed aggregates before introduction into cultures, inocula were treated with sonication (ultrasound) immediately prior to inoculation. Inocula were created based on the four previously described methods and subsequently were treated in a Branson 2510 ultrasonication bath (Branson, USA) with a program consisting of 5 min of degassing followed by 5 min of ultrasonication at 42 kHz (±6%) and 100 W. The level of aggregation was evaluated by microscopy in the inoculum before and after ultrasonication treatment, as well as in the resulting culture after 18 h of growth.

Antibiotic treatment of inoculated cultures. Liquid *P. aeruginosa* batch cultures inoculated according to the four methods described were grown for 24 h, with constant shaking (first-generation

cultures). The cultures were then reinoculated into second-generation cultures and grown for 18 h. At 4 and 18 h, two 15-ml aliquots of the cultures (both first- and second-generation cultures) were transferred to two new Erlenmeyer flasks. One flask was treated with 10 μg ml^{-1} tobramycin (Sigma), and one was left untreated as a control. The cultures were grown for an additional 24 h. One milliliter of culture was washed three times, degassed for 5 min, and sonicated before serial dilutions and plating to obtain CFU counts.

Recruitment and mixed cultures. Cultures of a GFP-producing *P. aeruginosa* strain were inoculated according to method 2 and grown for 24 h. Concurrently, overnight cultures of an mCherry-marked strain were inoculated according to method 3. The mCherry-producing overnight culture was diluted to an OD$_{600}$ of 0.005, regrown in LB to an OD$_{600}$ of 0.1, diluted, and regrown again for 3 h in fresh medium, to keep the formation of aggregates to a minimum. The first-generation GFP-producing culture and the primarily single-cell mCherry-producing culture were diluted to an OD$_{600}$ of 0.005 and mixed in a 1:1 ratio. The mixed cultures were grown for 4 h. Samples were subsequently taken for examination with CLSM.

Interspecies aggregation was tested by mixing, in a 1:1 ratio, a primarily single-cell mCherry-tagged *P. aeruginosa* culture with an *E. coli* culture tagged with GFP and inoculated according to method 2. Subsequent incubation samples were taken for examination with CLSM.

Aggregation in chemostat-grown cells. A chemostat was constructed based on the design of Whiteley et al. (51). A medium based on a trace metal minimal medium with 10% (vol/vol) A10 phosphate buffer (52) and 10% (vol/vol) LB was used. This medium resulted in an OD$_{450}$ of ~0.5. The chemostat contained 200 ml of culture, with a flow rate of 40 ml h^{-1}, resulting in a dilution rate of 0.2. The culture was aerated by continuously bubbling atmospheric air through it. The culture was continuously mixed with a magnetic stirrer set at ~200 rpm. The chemostat was inoculated with a 1:1 ratio of GFP- and mCherry-tagged cells taken directly from agar plates according to method 2. The culture was adjusted to a final starting OD$_{450}$ of 0.005. The chemostat was operated for 13 days, with samples being evaluated for aggregation at day 0, 1, 2, 5, 7, 9, and 13.

Statistical analysis. Statistical significance was evaluated by one-way analysis of variance (ANOVA) with multiple comparisons for quantitative parametric data. Quantification of the area under the curve was used for the evaluation of growth patterns. *P* values of <0.05 were considered significant. All tests were performed in GraphPad Prism 6 (GraphPad Software, USA). All quantitative experiments were performed with at least biological triplicates. Antibiotic treatment was evaluated using three technical replicates for each biological sample.

SUPPLEMENTAL MATERIAL

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