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Thesis of
Rebecca J. Quinn

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science
Biological Sciences

Nova Southeastern University
Halmos College of Arts and Sciences

July 2020

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HALMOS COLLEGE OF ARTS AND SCIENCES

Disruption of pyoverdine synthesis in *Pseudomonas aeruginosa* through periodic disturbance of biofilm structure.

By

Rebecca J. Quinn

Submitted to the Faculty of
Halmos College of Arts and Sciences
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Biological Sciences
Nova Southeastern University
July 2020

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Abstract

Infections due to bacteria were once easily treated using antibiotics. However, the effective shelf life of antibiotics is diminishing due to the rise and spread of antibiotic resistant bacteria. Accordingly, novel approaches to treating infections are required. Pseudomonas aeruginosa is a multi-drug resistant, biofilm forming opportunistic pathogen. A determinant of P. aeruginosa's pathogenicity is pyoverdine, a siderophore that is used to sequester iron from the environment. After being produced by a bacterium, pyoverdine is secreted into the environment where it complexes with iron. The pyoverdine-iron complex is then returned to the bacteria through diffusion, where it drives the synthesis of additional pyoverdine. Accordingly, the relative positions of bacteria and pyoverdine are important to facilitate access to this pyoverdine-iron complex. Pyoverdine is critically important in the infection process as its removal or attenuation results in decreased infection severity. Interestingly, previous studies have suggested that periodically disrupting spatial structure of a bacterial population, through physical means, can disrupt the ability of bacteria to access diffusible molecules required for growth. Based on this previous work, we hypothesized that disrupting biofilms composed of P. aeruginosa biofilms using physical disturbance would prevent accessibility to pyoverdine, which would ultimately reduce its synthesis. To test this hypothesis, we periodically disturbed the spatial structure of a biofilm using a microplate reader. We found that a disturbance frequency of 6 shakes/hr, we were able to significantly reduce endpoint pyoverdine amount compared to the control frequencies of 0 shakes/hr and 30 shakes/hr. This observation was consistent when the density of biofilms was increased using medium with increasing concentrations of glucose. Interestingly, we observed periodic disturbance at 6 shake/hr could also disturb the ability of 20 different P. aeruginosa strains isolated from the clinic to produce pyoverdine; in some cases, the amount of pyoverdine increased, decreased, or stayed the same as a result of periodic disturbance. Overall, this suggests that the ability to perturb pyoverdine production through periodic disturbance is not limited to a single parameter space (one strain, one growth rate, one biofilm density), and instead may be observable in strains and environments that lead to diverse biofilm densities. As pyoverdine is required for the infection process, it is possible that our research might lead to novel strategies to reduce, or prevent, infections due to P. aeruginosa.

Keywords: Pseudomonas aeruginosa, pyoverdine, disturbance, biofilm

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Introduction

Antibiotic resistance as a pressing medical problem

Infections due to bacteria were once easily treated using antibiotics. However, it is well-established that the effective shelf life of antibiotics is diminishing as bacteria acquire new mechanisms that confer resistance [1, 2]. Further confounding this issue is the rapid spread of antibiotic resistant bacteria in that they are growing in their global distribution. For example, bacteria that carry extended spectrum β-lactamases, such as *Pseudomonas aeruginosa* and *Escherichia coli*, can spread rapidly in the clinic and have been associated with causing significant mortality and morbidity [3]. In the past decade, there has been a general lack of investment from pharmaceutical companies in the identification and development of new antibiotics, which is further confounding this issue. This lack of investment is often attributed to the large costs associated with drug discovery and regulatory approval processes coupled with the rapid pace at which bacteria apparently develop resistance [4]. When taken together, this has created the 'perfect storm' and has resulted in antibiotic resistant bacteria leading to extended hospital stays [5] and increased mortality rates [6].

In a natural setting, antibiotic resistance occurs as natural interactions between antibiotic producing microbes and antibiotic susceptible microbes co-evolve. Stated more simply, there is an arms race between antibiotic producing and antibiotic susceptible microbes. These interactions create a selective pressure that drives the evolution and selection of antibiotic-resistant individuals, which eventually dominate the population. Often, selection by an antibiotic (e.g., the ribosome, cell wall, DNA gyrase) drives a corresponding response. Mutations to susceptible genes can render the antibiotic ineffective while continuing to allow the cell to divide. In fact, the arms race between antibiotic producing and antibiotic susceptible microbes has likely always been occurring in natural systems. Genes encoding resistance to several antibiotics including β-lactam and glycopeptide antibiotics have been sequenced from 30,000 year old permafrost sediments [7]. This principle can be extended to antibiotics that are used in the clinic where the use of aminoglycoside antibiotics can drive structural changes in the ribosome [8], and the use of fluoroquinolones can also drive changes in DNA gyrase [9]. As in a natural setting, this ultimately results in resistance.

One inherent drawback of the use of antibiotics that target a specific molecular structure is the intense selective pressure that is placed on that structure. This has resulted in the use of

combinatorial treatment strategies, such as the simultaneous use of multiple antibiotics. The notion here is to layer selective pressures on two or more druggable targets. In theory, increasing the targets of selection should reduce the likelihood of favorable mutations to confer sufficient resistance, thus reducing the probability of evolving effective resistance. However, the mechanisms by which antibiotics interact is complex, and can often result in antagonistic effects, thus reducing overall efficacy. Furthermore, interactions between antibiotics can lead to adverse complications in the patient. Thus, combinatorial treatment while evolutionarily salient is not always plausible, especially in the clinical practice.

There has also been increased interest in limiting the use of antibiotics in general. For example, the general use of antibiotics to treat unresponsive viral infections at the request of concerned patients has now been limited [10]. In the agricultural setting, small doses of antibiotics are often used to increase growth of livestock while reducing feeding rate [11]. Larger doses are also used to maintain animal health [4]. This setting drives the selection of antimicrobial resistance in livestock [12]. Moreover, antibiotics consumed by animals are often excreted into the environment [4]. Such excreted antibiotics can then enter sewage systems and wastewater systems. As above, selection pressure from these antibiotics can correspondingly lead to the development of resistance in soil microbes. If such genetic determinants are found on conjugative plasmids they can then be passed to their pathogenic counterparts. Due to the prevalence of antibiotic resistant strains of bacteria, there is growing public attention to the consequences of antimicrobial resistance or AMR [13]. Many governments have developed national AMR action plans to reduce antimicrobial use, particularly in humans [13]. These policies include a wide range of policies recommended by the WHO [13]. Educational approaches such as awareness campaigns and guidelines were commonly used across all regions [13]. These educational approaches commonly target individual prescribers, such as physicians, instead of altering healthcare structures to reduce the overuse and misuse of antibiotics [13]. In addition to educational approaches, improving sanitation and preventing the spread of infection is another necessary measure that can be taken to reduce the use of antibiotics [14]. Preventing infection from occurring in the first place reduces the need for subsequent treatment [14]. Additionally, limiting the use of antibiotics in agriculture and aquaculture—particularly antibiotics being used to promote growth or prevent infection—would also limit antibiotics entering the environment [14]. Reduction of antibiotic pollution through animal, human, and chemical waste is also necessary to prevent the development of

antibiotic resistant bacteria [14]. Overall improvement of global surveillance of drug resistance and antimicrobial consumption in both humans and animals can provide an early warning system for emerging threats and improve patient health [14]. Additionally, rapid diagnosis of patients should also reduce antimicrobial prescriptions that are unnecessary [14] and promoting the development of vaccines and alternatives to antimicrobials would also serve to limit use of antibiotics [14]. Improving the number of people, the pay, and the recognition of people working in infectious disease could allow for a greater ability to combat antimicrobial resistant bacteria as well, allowing for more research [14].

Overall, there is an imperative need to determine novel strategies to control growth and spread of bacteria involving imposing multiple selective pressures that reduce the potential of evolved resistance in bacterial populations.

Pseudomonas aeruginosa

P. aeruginosa is a ubiquitous, Gram-negative, biofilm forming, pathogenic bacteria, that causes infections in humans with compromised immune systems (e.g., transplant patients, individuals who are HIV positive), in individuals with cystic fibrosis and other lung diseases, people with traumatized corneas, burn patients, and long-term intubated patents [15]. The elderly and those who have been in hospitals for extended periods of time are also susceptible to infection by *P. aeruginosa*. Though the vast majority of the human population is not typically susceptible to *P. aeruginosa* infections, this bacterium is able to adapt quickly in hosts that are susceptible leading to long lasting and serious *P. aeruginosa* infections.

This bacterium can grow and thrive in a variety of different environments, including the soil and the clinic, and is also widespread, which makes those susceptible to infection more likely to come in contact with this pathogen. The bacteria, which are considered commensal on the host body, can be transmitted through a breach in host tissue barriers or suppression of the host immune system [15]. The ability for this bacterium to penetrate past these barriers allows it to become an opportunistic pathogen. The global overuse of antibiotics has led to *P. aeruginosa's* resistance across a wide variety of antibiotics including many multi-drug resistant strains. As infections caused by *P. aeruginosa* are resistant to multiple antibiotics, they can lead to persistent infections causing secondary fungal infections, extensions of hospital stay, therapeutic failure, and premature death of cystic fibrosis patients.

Many naturally occurring and clinically acquired strains of *P. aeruginosa* are resistant to multiple antibiotics including penicillin G, β-lactams, aminopenicillin, first and second generation cephalosporins, piperacillin, tazobactam, cefepime, ceftazidime, aminoglycosides, quinolones, carbapenems, colistin, and fosfomycin, which leads to severe and persistent infections in those individuals who contract an infection [16]. For example, *P. aeruginosa* is the most prevalent of all cystic fibrosis-related pathogens and it has the greatest impact on mortality and morbidity. Outbreaks involving strains with resistance to multiple antibiotics are common in hospitals and intensive care units leaving those patients susceptible to contracting a secondary infection. Overall, given its prevalence in the environment and its ability to resist multiple antibiotics, developing strategies to reduce or eliminate infections due to *P. aeruginosa* is important.

Biofilm structure & importance

Biofilms are highly organized, structured communities of bacteria attached to one another and to a surface [17]. A biofilm is made up of surface-associated microbial cells enclosed in an extracellular polymeric substance (EPS) matrix [18]. This EPS matrix in *P. aeruginosa* is made up of exopolysaccharides, nucleic acids, and proteins [19]. Biofilms are considered to be robustly associated with a surface and not able to be removed by gentle rinsing [18]. In *P. aeruginosa,* activation of quorum-sensing [20] and the acquisition of sufficient iron [21] stimulates biofilm formation [22]. Towards the former, *P. aeruginosa's* quorum-sensing system involves several autoinducers and the cognate receptors. One of these systems, the LasI/R system, produces and detects autoinducers that are important for the formation of biofilms. For example, when LasR is bound to its autoinducer, it activates expression of a number of target genes including virulence factors such as elastase which is a structural gene [22-24]. This gene is critical in the formation of biofilms as they encode for the LasR protein which is a transcriptional activator. The resulting production of pyoverdine to sequester sufficient iron subsequently drives the subsequent formation of biofilms [25]. In the *LasR* system, *LasR* binds to the promoter region of the *psl* operon, suggesting regulation of *psl* expression [26]. In addition, two more systems *RhlR* (which is controlled by the *LasR* system) and the *PQS* system, which is based on quinolone signals are also present. The *rhl* system enhances *pel* polysaccharide biosynthesis [26]. Both *psl* and *pel* serve as a primary structure scaffold for biofilm development, particularly in early stages of formation [26]. The *rhl* system also controls swarming motility, which is implicated in early biofilm establishment

[26]. Mutations in the PQS system result in reduced biofilm formation and the reduction of the production of virulence factors which is implicated to play a role in biofilm development [15]. Biofilms composed of *P. aeruginosa* are commonly found in prosthetic devices, medical implants, and heart, lung, and bladder infections [27].

Biofilm formation in *P. aeruginosa* involves two sequential events [19]. First, a bacterial cell contacts the surface it will form an attachment to and forms a reversible attachment [19]. Second, this cell then begins to produce the EPS matrix [19] using genes primarily found in the *pel* operon [28]. This EPS matrix is credited to structuring the mature biofilm [19]. Biofilm architecture can be influenced by nutritional cues, motility, and quorum sensing [22]. For example, P. *aeruginosa* biofilms grown in media where glucose is used at the carbon source take on the mushroom shaped topology [22]. Otherwise, with lactoferrin nutrients, biofilms take on a flat topology formed by sheet-like layers [22].

Bacteria growing in biofilms and in planktonic conditions have shown to have distinct antibiotic susceptibilities, growth rates and gene expression profiles. Cells within the biofilm have a slower growth rate than their planktonic counterparts, which may be due to oxygen and nutrient limitation within the biofilm [17]. This slowed growth reduces the efficacy of certain, but not all antibiotics, including β-lactam antibiotics that rely on active cell wall synthesis to result in cell death. In addition to slow growth, antibiotics can often fail to reach cells embedded inside the biofilm at inhibitory concentrations. Such sub-inhibitory concentrations of antibiotics can contribute to the evolution of antibiotic resistance as described above. Finally, due to their high density, bacteria often express different genes in biofilms in comparison to their planktonic counterparts [18]. These include virulence factors that are regulated through quorum sensing.

Biofilms of *P. aeruginosa,* in particular, have shown to have a higher resistance to antibiotics, such as penicillin G, β-lactams and second generation cephalosporins. *P. aeruginosa* infections are often found growing in iron limiting conditions in a biofilm [27]. *P. aeruginosa* biofilms, like other Gram-negative bacterium, grows slowly in these iron limiting conditions [29]. Cells growing at a slower rate, such as those in the biofilm, take up and process antibiotics slower. This is due in part to the antibiotic's inability to penetrate the EPS matrix of the biofilm [27] In addition, virulence factors such as pyoverdine and pyocyanin production, hydrogen cyanide, elastase, rhamnolipids, protease production, and lipopolysaccharide production, are present when cells are in biofilm state, also reduce susceptibility to antibiotics. This includes bacteria that have

been dislodged from the biofilm and placed in the planktonic state due to the continued presence of virulence factors. As the growth rate of these dislodged cells increases, their susceptibility to antibiotics increases [6].

Spatial structure, including biofilms, has been previously shown to allow sequestration of important small molecules [30]. *P. aeruginosa* is no different. Important growth factors, such as pyoverdine, and autoinducers involved in quorum sensing accumulate faster in biofilms. This owed to the spatial distribution of the cells, and the diffusion of the small molecules. Although small molecules will diffuse away from a biofilm, they will accumulate faster inside the biofilm allowing rapid synthesis by a large population of cells. The accumulation of these autoinducers and small molecules in the biofilm play a role in activating the aforementioned virulence factors in *P. aeruginosa.* One of these small molecules which accumulates in the biofilm and leads to the activation of virulence factors is the siderophore pyoverdine, produced by *P. aeruginosa* to scavenge for iron from the surrounding host matrix.

Pyoverdine – a siderophore required for infections

In environments with a scarcity of free iron, such as most areas in the human host, bacteria will use siderophores to sequester iron from the host. Pyoverdine is a naturally fluorescent diffusible peptide siderophore produced by *P. aeruginosa* strains to sequester iron from the environment [31]. Pyoverdine is commonly studied in *P. aeruginosa* due to its green fluorescent color making it clearly visible in colony growth. Once produced, pyoverdine is transported out of the cell where it can bind to iron in the environment. Once pyoverdine is released into the extracellular environment, it diffuses and forms a complex with iron [31]. After acquiring iron, pyoverdine-iron complexes are taken up through receptor proteins and the iron is released and used within the cell for incorporation into proteins. The pyoverdine peptide can then be re-transported out of the cell where is can harvest additional iron.

The production of pyoverdine in *P. aeruginosa* has often been considered as cooperative behavior [15, 32] and qualifying as a public good (27) as individuals within the colony benefit from pyoverdine but do not all share equally in the costs of its production. However, the distribution of pyoverdine is limited locally within *P. aeruginosa* biofilms thus allowing for those cells producing the pyoverdine to disproportionately benefit from its production as well as limit its exploitation by non-producing strains. In many of the habitats where *P. aeruginosa* is

commonly found, outside of the biofilm iron-seeking siderophores would not get back to the producing cells making its production paradoxical. Taken together, biofilm formation helps both sequester pyoverdine in a local environment benefiting all biofilm cells, while also bias the distribution of pyoverdine around producing cells thus stabilizing cooperation within the collective.

Pyoverdine is an essential virulence factor in *P. aeruginosa*. Studies performed in mice have demonstrated that removing pyoverdine through gene deletion significantly attenuates pathogenicity. In these pyoverdine deficient strains, under iron starved conditions they produced about 40% less pyoverdine than a wild type strain [33]. These pyoverdine deficient strains were then used to infect mice resulting in no fatalities [33]. In another study involving mice, the level of pyoverdine production and accumulation in the lungs was correlated with the severity of disease, supporting the correlation between virulence and pyoverdine [34, 35]. Accordingly, pyoverdine is required for establishing infection and for subsequent biofilm formation.

Pyoverdine has a complex bidirectional regulatory relationship with biofilm formation in *P. aeruginosa* [36]. Pyoverdine is responsible for obtaining extracellular iron, which is essential for biofilm development under iron starved conditions [36]. Similarly, pyoverdine production is regulated by biofilm formation when it is not in an iron-starved state [36]. Iron deficiency in *P. aeruginosa* leads to the expression of genes involved in iron acquisition, including the siderophores pyochelin and pyoverdine, iron transporters *haem* and *feo*, exoenzymes to cleave iron-bound host proteins, and other redox enzymes and toxins [15]. Pyoverdine is initially synthesized in the cytoplasm by non-ribosomal peptide synthetases including PvdL, PvdI and PvdD [37-39]. It is then subsequently transported into the periplasmic space by the ABC transporter PvdE where it matures [40]. Matured pyoverdine is then transported into the extracellular environment using the PvdRT-OpmQ efflux pump [41]. Once in the extracellular environment, pyoverdine will complex with ferric iron (Fe^{3+}) forming ferripyoverdine [42], which is subsequently transported across the periplasmic membrane into the periplasmic space using the FpvA/B transporter [43, 44]. Once in the periplasmic space, iron is released from ferripyoverdine, which is subsequently transported across the cell membrane using the FpvDE transporter [45]. Upon disassociation from ferric iron, pyoverdine will be recycled as it is re-transported into the extracellular environment [46]. In addition to sequestering iron from the environment, ferripyoverdine has additional downstream effectors that are important in biofilm formation [47]

and virulence [48, 49]. Binding of ferripyoverdine to the FpvA transport protein results in the activation of an alternative sigma factor called PvdS [50]. Activation of PvdS leads to the expression of virulence factors, including exotoxin ToxA and the endoproteinase PrpL. It also leads to additional synthesis of pyoverdine, thus forming a positive feedback loop [30]. The positive feedback loop can be attenuated if sufficient iron is internalized. In short, intracellular iron binds to Fur, which represses PvdS [51] as well as the subsequent production of pyoverdine [52]. Fur also plays a role in the regulation of biofilm formation [53] and the expression of virulence factors [51].

Given importance of pyoverdine in growth, expression of virulence factors, and biofilms production, there is increased interest in developing mechanisms to interfere with its functionality [30]. Compromising biofilm development by either genetic (such as mutations in genes that contribute to biofilm formation like *pel* biosynthesis and PQS synthesis) or chemical disruption (with novel inhibitors such as meta-bromo-thiolactone, 2-aminoamidazole derivatives, and zingerone) [20, 54, 55] decreased pyoverdine biosynthesis in *P. aeruginosa* [30]. Flourinated pyrimidines strongly attenuate *P. aeruginosa* pathogenesis, likely by compromising RNA metabolism. Pyoverdine production could be limited by 5-flurouracil, which also temporarily restricts bacterial growth, and 5-fluorouridine [56]. Gallium, a transitional metal, can also be used to disrupt bacterial Fe metabolism through being a substitute for iron and inhibiting iron dependent processes [57]. In addition, a study found four novel compounds which were able to quench more than half of pyoverdine fluorescence, LK10, LK11, LK12, and LK 31 [35]. Overall, methods to disrupt pyoverdine functionality and expression in *P. aeruginosa* are highly desirable as they may attenuate pathogenicity of this important opportunistic pathogen.

Perturbing spatial structure as a mechanism to break up cooperation

Previous studies have shown that disrupting spatial structure, including through physical means, can disrupt cooperation and influence growth. Recently, Wilson et al. demonstrated that intermediate frequencies of spatial disturbance of spatially structured populations could manipulate cooperation in *E. coli* [58]. Guided by a mathematical model, the authors revealed that spatial disturbance at intermediate frequencies resulted in a trade-off between accessing small molecules required for cooperation and survival and accessing nutrients, which impacted growth. When access to both was limited, the ability to use the small molecules required for cooperation

was disrupted. Given the diffusible and essential nature of pyoverdine to cell growth, we reasoned that periodically disrupting the spatial structure of a biofilm would reduce the access to pyoverdine. This would occur as disrupting the spatial structure would serve to decorrelate the relative positions of bacteria and pyoverdine, including bacteria removed from the biofilm state. Reducing access to pyoverdine would subsequently reduce the synthesis of additional pyoverdine by attenuating the positive feedback loop driven by iron acquisition. By placing the selective pressure on multiple aspects of cell physiology (pyoverdine synthesis, iron acquisition, biofilm formation) there is the potential that this represents a new way to disrupt pyoverdine functionality without driving resistance.

Questions and hypotheses:

The overarching goal of this project is to explore the ability to perturb pyoverdine production in *P. aeruginosa* using physical disruption of biofilms. My hypothesis is pyoverdine production in *P. aeruginosa* will be reduced when the structure of biofilms are disrupted using physical force. Within this overarching goal, we propose three aims: (1) determine how periodic physical disturbance using intermittent shaking changes pyoverdine production in *P. aeruginosa,* (2) determine if changes in biofilm robustness affects how periodic physical disturbance influences pyoverdine synthesis, (3) determine the effect of periodic disturbance on *P. aeruginosa* strains isolated from the clinic. We believe our research is significant as physical disruption of pyoverdine production will affect biofilm robustness and virulence of *P. aeruginosa*. This line of research could subsequently lead to the development of new mechanisms to combat *P. aeruginosa* infections in humans. We believe our research is innovative as, unlike previous studies (which used starvation techniques [59, 60] or multiple antibiotics [61, 62]), we propose to use physical force, which may be less prone to evolved resistance as it imposes selection over several potential targets.

Methods and Materials

Strains and growth conditions

P. aeruginosa strain PA14 was used as the wildtype strain in this study. Additional clinical isolates were obtained from BEI Resources (Table 1). Where indicated, we also used a strain of P. aeruginosa PA14 that lacks the ability to form biofilms (-pel, [63]). All experiments were performed in modified King's A medium [2% peptone, (Fisher Scientific, Waltham, MA), 0.5% potassium sulfate, (Acros Organics, Fisher Scientific), 17 mM magnesium chloride (Alfa Aesar, Parkridge Road Ward Hill, MA) with and without different concentrations of carbon source (0%, 1% or 2% glucose (VWR, Radnor, PA)). Single colonies of P. aeruginosa isolated from Luria-Bertani (LB) agar medium (MP Biomedicals, Solon OH) were shaken overnight (250 RPM and 37oC) in 3 mL of liquid LB medium contained in culture tubes (Genesee Scientific, Morrisville, NC). As indicated in the text, gallium nitrate (Acros Organics) was added to a final concentration of 10uM gallium modified King's A medium. We used the Innovotech (Edmonton, AB, Canada) MBEC Biofilm Inoculator to grow biofilms [64]. We grew overnight cultures of P. aeruginosa, washed the cells in fresh King's A medium, and diluted 1000-fold them into fresh modified King's A medium. We then placed 150 µL of culture into the wells of the biofilm plate and allowed the biofilm to form by shaking the plate at 110 RPM at 25oC for 24 hours.

Biofilm staining

We followed the protocol as outlined in [65]. Briefly, we grew biofilms as described above. After 24 hours, we washed the pegs (which remained attached to the lid of microplate) with 200 µL of fresh modified King's A medium for 10 seconds to remove any unadhered cells. Next, the washed pegs were placed in 125 µL of 0.1% Crystal violet (Acros Organics) for 10 minutes. We then washed the stained pegs in 200 µL of ddH2O four times to as to remove any excess crystal violet. Finally, we transferred the pegs to 200 µL of 30% acetic acid (Fisher Scientific) for 10 minutes to remove crystal violet from the biofilms. The amount of solubilized crystal violet was measured using optical density (OD) at 555 nm in a Victor X4 plate reader (Perkin Elmer, Waltham, MA). OD555 values were blanked using acetic acid.

Dispersal of cells from the biofilm due to shaking

We grew biofilms in modified King's A medium as described above. After 24 hours of growth, we removed the plate, and washed the pegs for 10 seconds in 200 µL of fresh King's A medium to remove any unadhered cells. The washed biofilms were placed in 200 µL of fresh modified King's A medium, and the plate was placed in a Victor X4 plate reader pre-set to 25°C. The biofilms were then shaken once at the amplitude indicated and were subsequently removed from the plate reader. We then measured the number of colony forming units in the King's A medium surrounding the plate as previously described [66].

Shaking experiments

We grew biofilms in modified King's A medium as described above. After 24 hours of growth, we removed the plate, and washed the pegs for 10 seconds in 200 µL of fresh King's A medium. The washed biofilms were placed in 200 µL of fresh modified King's A medium, and the plate was placed in a Victor X4 plate reader pre-set to 25oC. The plate was then periodically shaken (fast setting, frequency = 4800 mm/min, 10 seconds per shake, linear shaking feature which shakes the plate along the x-axis) at the frequency indicated in the results. Note that the Victor X4 plate reader does not have a continuous shaking function. Thus, a shaking frequency of 30 shakes/hr was used as a continuous shaking control. After 24 hours of growth in the plate reader, we removed the lid from the plate and measured cell density (OD600) and the concentration of pyoverdine (excitation: 405 nm, emission: 460 nm) using the Victor X4 microplate reader. Pyoverdine (arbitrary units) was normalized by OD600.

Measuring expression of green fluorescent protein

We prepared competent *P. aeruginosa* PA14 as described previously [67]. We transformed these cells with plasmid pAB1 [68], which contains an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible copy of enhanced green fluorescent protein (GFP), and confers ampicillin resistance. To measure the effect of periodically disturbing the spatial structure of the expression of GFP, we grew biofilms of these GFP expressing bacteria as described above. After washing the pegs to remove any unadhered cells, we placed the biofilms into fresh modified King's A medium that contained, or did not contain as a control, 1 mM IPTG (Thermo Scientific, Lithuania). We

then disturbed the spatial structure of these biofilms as described above. After 24 hours, we quantified cell density (OD_{600}) and GFP (λ_{excite}: 488 nm, $\lambda_{emission}$: 510 nm) using the Victor X4 microplate reader. GFP was normalized by OD_{600}.

Growth rate

We grew *P. aeruginosa* overnight as described above. The following day, strains of *P. aeruginosa* were washed once in modified fresh King's A medium. They were then diluted 1/100 into fresh King's medium with different percentages of glucose. 200 μL of diluted cells were then placed in the wells of a 96 well plate, which was subsequently shaken at 25°C at 250 RPM. After 4 hours of growth, we then measured OD_{600} every hour for a total of five additional hours using a microplate reader. To determine growth rate, we plotted a linear line through a plot of OD_{600} vs time and determined the slope. All lines had R^2 values greater than 0.9.

Statistical analysis

We used unpaired t-tests (with unequal variance between the samples) or one-way ANOVA as indicated in figure legend. T-test analysis was performed using Microsoft Excel (Redmond, WA). ANOVA and linear regression were performed using JASP (Amsterdam, the Netherlands).

Results

An experimental approach to disturbing the spatial structure of biofilms.

First, we established a method to grow and periodically disturb the spatial structure of *P. aeruginosa* biofilms. We used the MBEC Biofilm inoculator plates to grow biofilms (Figure 1 [49]). The MBEC Biofilm Inoculator plates are 96 well plates with pegs adhered to the inside surface of the plates lid. This system allows biofilms to form on the peg while it sits inside a well containing growth medium. In addition to the biofilm that forms on the peg, bacteria in the planktonic state grow in the liquid media surrounding the peg. The biofilms adhered to the pegs can be washed and transferred to different growth medium by removing the lid of the original 96 well plate and transferring the lid into a new 96 well plate. We confirmed the growth of biofilms on the pegs by first growing biofilms in the MBEC Biofilm Inoculator plate for 24 hours and used crystal violet staining to confirm the presence of the biofilm grown (Fig. 2).

We then disrupted the spatial organization of the biofilm through periodic disturbance of its spatial structure. Here, we relied on previous work [58] that demonstrated the shaking setting of a microplate reader perturbs the spatial organization of structured bacterial populations, such as the population of a biofilm. We hypothesized shaking a biofilm would remove cells from the biofilm and transition them into the surrounding liquid medium and into the planktonic state. For confirmation of this transition, we measured the density of bacteria in the biofilm and planktonic states both before and after a single shaking event (Fig. 3). Using this single shaking event, we observed this method both decreased the density of bacteria in the biofilm while also increased the density of bacteria in the planktonic state (Fig. 3). We also observed that increasing the amplitude of a single shaking event increased the density of bacteria in the planktonic state (Fig. 4). This produced a positive linear relationship between shaking amplitude and the amount of bacteria in the planktonic state (R^2=0.99, linear line, Fig. 4). Overall, these observations demonstrate that a single shaking event was sufficient to perturb the spatial structure of a biofilm.

Periodically disturbing the spatial structure of biofilms alters the amount of pyoverdine per cell.

In an iron limited environment, *P. aeruginosa* requires pyoverdine for growth [69]. *P. aeruginosa* uses pyoverdine for the import of iron which results in cell division. The increase in biomass via cell division also leads to the production of additional pyoverdine; in addition, the

import of iron through ferripyoverdine also increases pyoverdine synthesis through PvdS [70]. The relationship between pyoverdine synthesis and growth follows a positive feedback loop in an iron limiting environment.

To disrupt the positive feedback loop, we changed the distribution of cells in both the biofilm and planktonic states. We hypothesized that changing this positive feedback loop would disrupt the ability of bacteria in the biofilm to access ferripyoverdine. To disrupt this positive feedback loop, we grew biofilms of *P. aeruginosa* using the MBEC inoculator system for 24 hours. We then used the linear shaking function of a plate reader for an additional 24 hours to perturb the spatial organization of the biofilm. After these 24 hours, pyoverdine was quantified and normalized by cell density (OD_{600}). We observed that frequencies of 6 (6/hr) and 12 (20/hr) shakes per hour reduced the normalized pyoverdine in comparison to both unshaken and 30 shakes per hour control (this 30 shakes per hour control in our system was used as a surrogate for near continuous shaking (Fig. 5)). We also determined that no other shaking frequencies (aside from the aforementioned 6/hr and 12/hr) differed significantly from both control conditions (unshaken and 30/hr). We verified the majority of pyoverdine we measured was located in the planktonic bacteria and not within the biofilm by comparing the normalized pyoverdine of the planktonic bacteria and also the normalized pyoverdine located in the biofilm by resuspending the biofilm bacteria in media and measuring (Fig. 6). We verified that the reduction of pyoverdine at 6 shakes/hr was not due to general perturbations in either transcription or translation through an eGFP-expressing *P. aeruginosa* (Fig. 7). This GFP-expressing *P. aeruginosa* was grown under the same conditions and normalized by cell density generally increased as function of shaking frequency (Fig. 7).

Next, we examined the influence of biofilm formation on the reduction in pyoverdine at 6 shakes/ hr. This was investigated in a strain of *P. aeruginosa* that does not form biofilms (-*pel*) (Fig. 8). The strain was cultured as described above and disturbed at various shaking frequencies. We observed no significant decrease or increase in the amount of normalized pyoverdine across all measured frequencies (Fig. 8).We also observed an overall reduction in the total amount of pyoverdine produced, confirming previous observations that biofilm formation enhances pyoverdine synthesis [69]. These results also suggest that the reduction of pyoverdine production at 6 shakes/hr is dependent on the ability to form a biofilm.

We then perturbed the production of pyoverdine using the element gallium. Previous work has indicated that gallium binds to pyoverdine with high affinity, thus disrupting both its

functionality and discouraging production [57]. When we grew wild-type *P. aeruginosa* in the same way as previously described and included gallium in the medium, we observed a significant reduction in overall normalized pyoverdine (Fig. 9). In addition, there was no significant reduction in normalized pyoverdine relative to the unshaken (0/hr) and 30/hr control conditions compared to all other shaking frequencies tested. These results suggest a reduction in pyoverdine at 6 shakes/hr is dependent on pyoverdine functionality. Overall, our analysis suggests that, a reduction in normalized pyoverdine at 6 shakes/hr is dependent on the ability of *P. aeruginosa* to both form a biofilm and to use pyoverdine to acquire iron (Fig. 9).

Increasing the percentage of glucose in growth medium increases the range of shaking frequencies that reduce amount of pyoverdine synthesized.

Given that biofilm functionality appeared to be critical in the observed decrease of pyoverdine synthesis at 6/hr, we then sought to perturb the ability of *P. aeruginosa* to make biofilms. Previous work [71] has indicated that increasing the percentage of glucose in growth medium increases the ability of *P. aeruginosa* to form biofilm. However, it does not appear to alter growth rate (Fig. 10). Thus, this perturbation could separate the effects of biofilm formation and basal growth rate owing to central carbon metabolism. Given this information, we examined how periodic shaking affects pyoverdine synthesis when biofilms increased in robustness by increasing the concentrations of glucose in the growth medium.

We determined that we could increase the amount of pyoverdine synthesized by undisturbed biofilms by increasing the amount of glucose in the medium (Fig. 11). Furthermore, and as previously reported [71] increasing growth had no apparent effect on growth rate (Fig. 10). Next, we tested the influence of periodic disturbance on pyoverdine production in medium with increasing percentages of glucose. Here, we focused on a shaking frequency of 6/hr as it resulted in the greatest decrease in pyoverdine synthesis as shown in Fig. 11. We observed that as the concentration of glucose was increased in the medium, the total amount of pyoverdine consistently decreased in the 6/hr condition as compared to the unshaken (0/hr) and 30/hr conditions. While the total amount of pyoverdine produced in the 6/hr condition generally increased as a function of glucose concentration, the difference in pyoverdine between the control conditions was statistically equivalent across conditions. Overall, we observed that the shaking frequency of 6/hr

consistently resulted in decreased pyoverdine as the amount of glucose increased in the growth medium. Thus, periodic shaking influenced pyoverdine production even for robust biofilms.

Pyoverdine production is significantly affected by intermediate concentration of glucose across a large number of P. aeruginosa strains isolated from the clinic.

The above findings led to further investigate whether these relationships could be replicated in 20 different *P. aeruginosa* strains isolated from the clinic. These 20 different strains represent a wide range of biofilm forming capabilities (Fig. 12) and a wide range of growth rates (Fig. 12). This diverse set of strains allowed us to examine if the whether the effects of periodic disturbances on biofilm formation are generalized across a diverse set of physiological parameters.

Biofilms grown from all 20 strains of *P. aeruginosa* were subsequently disturbed at the frequency of 6 shakes/hr, as 6 shakes/hr consistently resulted in a significant reduction of normalized pyoverdine produced across different percentages of glucose (Fig. 12). Furthermore, the undisturbed (0/hr) condition served as the baseline control as there was no significant difference in pyoverdine production between 0/hr and 30 shakes/hr ($P > 0.360$ within all percentages of glucose, two-tailed t-test).

Using crystal violet, we observed a significant and positive linear correlation between initial biofilm density and corresponding change in biofilm density as a result of shaking. The denser the biofilm produced by a strain in an undisturbed condition, the greater the decrease in density as a result of shaking (Fig. 12).

Next, we plotted the amount of pyoverdine produced under undisturbed conditions (0 shakes/hr) as a function of the amount of pyoverdine produced when biofilms were disturbed at 6 shakes/hr for all 21 strains of *P. aeruginosa* studied in this thesis (20 strains from the clinic and our wildtype PA14 strain). These two endpoint measurements depict an observable, significant, and positive linear correlation for strains grown in medium with increasing percentages of glucose. Using this analysis, we demonstrate amount of variation in bacterial growth attributed to glucose in the medium (R^2) was substantially less in 1% glucose medium ($R^2 = 0.45$) than for bacteria grown in either 0% or 2% glucose ($R^2= 0.8$ and $R^2=0.94$, respectively) (Fig. 13).

In further investigation, we examined the change in biofilm density due to shaking using crystal violet staining and tracked resulting changes in density of planktonic bacteria using OD_{600}. To determine the effective change in each of these measurements, we subtracted the measurements

taken at 6 shakes/hr from the measurements taken for the undisturbed condition (Fig. 15 & 16). With this analysis, a negative value indicates an increase in each metric as a result of shaking, with positive values demonstrating the opposite. Overall, we found that as the percentage of glucose in the medium was increased, the difference in biofilm density due to shaking significantly increased. In addition, we observed a biphasic relationship between the change in the density of bacteria in the planktonic state due to shaking. The change in density of planktonic bacteria in 1% glucose media was significantly greater than the change of planktonic bacteria in 0% glucose and of 2% glucose media. Across a diverse set of *P. aeruginosa* strains, we found that when we included 1% glucose in the growth medium, a significant difference in the distribution of bacteria compared to the 0% and 2% glucose media conditions was observed.

A decrease in the amount of pyoverdine at intermediate glucose concentration is largely owing to reduction in biofilm density.

Next, we examined how shaking differentially alters pyoverdine production across the spectrum of individual strains grown in 1% glucose media. We observed a substantial increase in the number of strains that had a statistically significant change in pyoverdine in medium containing 1% glucose compared to the 0% glucose and 2% glucose media (Fig. 14). Overall, these changes were diverse, with pyoverdine increasing in some instances whereby decreasing in other.

Strains were subsequently grouped with one group involving those that had significant changes in pyoverdine due to shaking and the other containing those that did not have significant changes in pyoverdine due to shaking. We also examined the change in biofilm density and the change in bacteria density in the planktonic state as described previously. In 1% glucose medium, we observed a significant reduction in biofilm density due to shaking between the strains that were significantly affected by or not significantly affected by shaking (Fig. 18). We also examined the relationship between difference in pyoverdine amount between undisturbed and 6 shakes/hr as a function of growth rate. No linear correlation was found with any condition (Fig. 17). We examined the change in biofilm density as a result of shaking at 6 shakes/hr as a function of percentage of glucose in the growth medium and found that there was only a statistically significant decrease in biofilm density in media with 1% glucose (Fig. 19). We also observed, in contrast to the above, that across glucose conditions, there was no significant difference in the density of bacteria in the planktonic state (Fig. 20). Both of these observations together indicate that changes

in pyoverdine production attributed to shaking are largely dependent upon the ability of shaking to significantly affect biofilm structure.

Discussion

Previous studies have shown that disrupting the structure of a spatially organized population of bacteria, including through physical means, can disrupt cooperation and influence growth [58]. Given this backdrop, we explored the ability to disrupt the spatial structure of *P. aeruginosa* biofilms to disrupt pyoverdine production. This was accomplished by establishing a method using the MBEC Biofilm assay plate coupled with the shaking feature of a microplate reader. This allowed us to be able to measure, and perturb, the distribution of bacteria in both biofilm and planktonic states (Fig. 1). Using the set up as described, we confirmed the presence of biofilms using crystal violet staining (shown in fig. 2). Once it was determined *P. aeruginosa* biofilms could be produced, we determined whether shaking these biofilms effectively dislodge cells from the biofilm. Using colony forming units, we determined that shaking was able to remove cells from the biofilm and transfer them into planktonic state (fig. 3). Along this line, we observed both a decrease in CFUs in the biofilm and an increase in cells in planktonic media after shaking in comparison to measurements taken prior to shaking. In addition, when the density of bacteria in the planktonic state was normalized by biofilm density, we continued to observe an increase in the density of bacteria in planktonic state after shaking. Finally, we examine the impact that increased shaking amplitude had on the ability to transfer bacteria from the biofilm state to the planktonic state. We found that as shaking amplitude increased, the density of bacteria in the planktonic state increased (Fig. 4). Overall, we established that bacteria can be experimentally transitioned from the biofilm state to the planktonic state thus perturbing the spatial organization of this closed system. As pyoverdine is freely diffusible, altering the positions of bacteria in both states decouples the distribution of pyoverdine and bacteria. In all, our experiments demonstrate the potential to disrupt the ability of *P. aeruginosa* to access pyoverdine through periodically applied physical force.

After determining we could dislodge cells from the biofilm using the designed shaking protocol, we explored the impact of shaking frequency (number of shakes/ hr over 24 hours) on the amount of pyoverdine present in the medium surrounding the biofilm. Importantly, we confirmed that the majority of pyoverdine produced in our closed experimental system could be measured in the liquid medium surrounding the peg whereupon the biofilm grew. This was expected as each pyoverdine molecule is freely diffusible from the producing cell. Thus, even for

bacteria in the biofilm state, most of the pyoverdine ultimately ends up in the surrounding medium (Fig. 6). Focusing on measuring the amount of pyoverdine in the surrounding liquid medium, we tested a wide range of shaking frequencies in growth medium containing 0% glucose and measured endpoint pyoverdine after 24 hours of disruption (fig. 5). Across this spectrum of frequencies, we found the 6 shake/hr frequency resulted in significantly lower amounts of endpoint pyoverdine in comparison to both the undisturbed (0) shakes/hr condition and the nearly continuous 30 shakes/hr condition. In theory, as the frequency of shaking increases, an increasing amount of bacteria are removed from the biofilm and transferred into the surrounding medium. In the undisturbed condition, or without shaking, we would expect that the vast majority of bacteria would be in the biofilm state and that bacteria in the surrounding medium would be present as a result of sloughing off of the biofilm or active dispersal mechanisms. In this condition, because the biofilm is not disturbed, we would expect that the amount of pyoverdine would be high owing to a highly spatially organized population, which facilitates access to pyoverdine. In contrast, in the 30 shakes/hr condition, the near continuous disturbance of the biofilm would result in a large amount of bacteria in the planktonic state, with a relatively small biofilm. While bacteria under this condition would not benefit from increased access to pyoverdine owing to spatial organization, they would instead benefit from increased access to nutrients. Based on a previous publication, this would drive increased growth, which would increase the amount of pyoverdine synthesized due to increased total cell density. This would effectively compensate for a reduction in access to pyoverdine via spatial organization, thus allowing pyoverdine synthesis to remain high.

Between these two extremes, different frequencies of shaking serves to alter the relative densities of both populations; increasing shaking should increase the amount of bacteria in the planktonic state. Based on this notion, we suggest the shaking frequency of 6 shake/hr condition presents a ‘sweet spot.’ Here, the population of cells in the biofilm state is not sufficiently dense resulting in less pyoverdine build up within the biofilm. This results in these cells having less access to pyoverdine. In addition, the shaking is not frequent enough to transition sufficient bacteria into the planktonic state to take advantage of the potential of increased growth rate. Thus, cells in the planktonic state do synthesize additional pyoverdine to compensate for reduced spatial organization in the system. Overall, this results in a general reduction in the total amount of pyoverdine synthesized by the population.

Providing evidence towards our hypothesis, we performed three important controls. First, we used a *gfp* expressing strain of *P. aeruginosa* to determine that the reduction at 6 shakes/hr was specific to pyoverdine, and not additional protein products that could be perturbed by changing translation (Fig. 7-9). Consistent, with our hypothesis, we did not observe a significant decrease in normalized GFP at 6 shakes/hr as compared to the unshaken and near continuously shaken controls (Fig. 7). Next, to confirm that the observed decrease in pyoverdine was due to changes to the structure of a biofilm, we utilized a strain of PA14 (*-pel*) that does not form biofilms. We did not observe a reduction at 6 shakes/hr and noted that overall pyoverdine synthesis declined (Fig. 8). This led to two important conclusions. First, and consistent with previous publications, biofilm formation is required for robust pyoverdine synthesis. Second, it demonstrated that the reduction in pyoverdine at 6 shakes/hr requires the formation of a biofilm. Finally, we tested the ability of pyoverdine synthesis to be perturbed by gallium. As noted above, gallium binds with high affinity to pyoverdine and disrupt its ability to acquire iron and drive the synthesis of additional pyoverdine. We observed that the addition of gallium to growth medium results in a significant reduction in normalized endpoint pyoverdine compared to the wild type strain grown without gallium (Fig. 9). Furthermore, a reduction at 6 shakes/hr was not observed. This suggested that pyoverdine functionality was required to observe a reduction of pyoverdine amount at 6 shakes/hr. Overall, these control experiments provide evidence to three important assumptions of our hypothesis and mechanism describe above; 1) the reduction in pyoverdine at 6 shakes/hr is not due to general perturbation of transcription and translation, 2) biofilm formation is required to reduce pyoverdine at 6 shake/hr and 3) functional pyoverdine is required for the observed decrease at 6 shakes/hr.

A previous study found that increasing glucose in the growth medium increased the density of biofilms but did not apparently increase growth rate of cells in the planktonic state. Indeed, we confirmed the latter by observing that increasing glucose produced no significant effect on growth rate of bacteria grow in liquid culture (Fig. 10). Thus, we could increase biofilm density without changing basal growth rate by increasing the amount of glucose in the growth medium. In all three tested glucose concentrations (0%, 1%, and 2%), the 6 shakes/hr condition resulted in significant reductions of pyoverdine at (Fig. 11). In addition, we also observed increased amounts of endpoint pyoverdine in both the 1% glucose media and 2% glucose media compared to the 0% glucose media. This suggests that in line with a previous publication, that we were indeed increasing

biofilm density as our data presented above showed that increasing biofilm density increased pyoverdine production [71]. Moreover, it demonstrates that we could influence pyoverdine production through non-genetic intervention. Overall, these data suggests that the ability to perturb pyoverdine production through periodic disturbance is not limited to a single parameter (one strain, one growth rate, one biofilm density) and instead may be observable in strains and environments that lead to diverse biofilm densities.

Based upon this observation, we extended our analysis to 20 different strains isolated from a clinic. After characterizing the ability of these strains to form biofilms, and their growth rate, we concluded that by using these strains, we could test our hypotheses over a wide range of growth parameters. Note that due to the different environments from which these strains were isolated, they all likely have different pyoverdine synthesis rates, and genetic differences. We note that the former was challenging to measure after repeated attempts whereas the latter is likely true based on a preliminary analysis of genomic sequence (not shown).

In general, we observed the greatest change to pyoverdine amounts in the 1% glucose condition (Fig. 14). This was evidenced a reduced R^2 value between pyoverdine produced in the undisturbed and 6 shakes/hr condition and the large number of individual strains that showed a significant change in pyoverdine owing to shaking. When we examined the change in the distribution of bacteria grown in medium with increasing glucose, we observed that 1) as the concentration of glucose in the medium increased, the amount of bacteria in the biofilm owing to shaking increased and 2) the amount of bacteria in the planktonic state owing to shaking was greatest when the medium contained 1% glucose. Moreover, when we examined changes in the density of bacteria in the biofilm and planktonic states owing to shaking, we found that when the medium contained 1% glucose, there was a significant decrease in the amount of bacteria in the biofilm state owing to shaking (Fig. 15).

Based on these observations, we propose the following to account for the large change in pyoverdine amount owing to shaking in bacteria grown in medium with 1% glucose. When bacteria are grown in 0% glucose, they form overall weak biofilms. In these weak biofilms, perturbation with shaking only has a modest effect as the biofilms are less dense. In contrast, when growing in medium with 2% glucose media, bacteria produce robust biofilms, which are more resilient to perturbation through shaking. Thus, in both conditions, the number of strains with altered pyoverdine amounts as a result of shaking is relatively low. In contrast when bacteria were

grown in medium with 1% glucose, these biofilms are at an intermediate point between weak and robust. In this state, the biofilms are more easily perturbed, which results in a significant reduction in biofilm density. This coincides with a significant increase in the density of bacteria in the planktonic state. Because changing glucose does not change growth rate (across percentages of glucose examined in this study), bacteria in planktonic state and in 1% glucose have no additional advantage relative to their 0% and 2% glucose counterparts. Thus, although these bacteria can access additional nutrients, some strains cannot compensate for the reduction in pyoverdine accessibility owing to a reduction in biofilm structure. Thus, the amount of pyoverdine synthesized under this condition can decrease.

While the aforementioned hypothesis can account for reduction in a pyoverdine owing to shaking, it cannot explain instances of increased pyoverdine production. Indeed, a large number of strains showed an observable increase in the amount of pyoverdine synthesized owing to shaking (Fig. 18). These individual reaction norms are interesting and warrants further study. Initial possible changes include different pyoverdine expression rates, changes in the efficiency of pyoverdine to uptake and import iron, or other changes related to biofilm formation. Further research on this topic will continue in the lab using a bioinformatics approach to examine genetic differences in strains and explore how those differences impact changes in biofilm density and endpoint pyoverdine in the 6 shake/hr condition.

In future studies, we suggest investigating how periodic disturbance to biofilm structure impacts quorum sensing and the expression of virulence factors in *P. aeruginosa*. As quorum sensing is mediated by several diffusible metabolites, it is likely that periodic disturbance will also alter the relative position of these metabolites and bacteria, and thus could impact quorum sensing regulated expression of virulence factors. Moreover, as shaking clearly affected biofilm structure, it would be interesting to examine the combination of shaking and the application of pyoverdine inhibitors or antibiotics. Of particular note, shaking could increase the ability of these drugs to penetrate the biofilm, which would serve to augment their affect.

Literature Cited

1. Austin, D.J., K.G. Kristinsson, and R.M. Anderson, *The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance.* Proceedings of the National Academy of Sciences, 1999. **96**(3): p. 1152-1156.
2. Pepper, J.W., *DEFEATING PATHOGEN DRUG RESISTANCE: GUIDANCE FROM EVOLUTIONARY THEORY.* Evolution, 2008. **62**(12): p. 3185-3191.
3. Rawat, D. and D. Nair, *Extended-spectrum β-lactamases in Gram Negative Bacteria.* Journal of global infectious diseases, 2010. **2**(3): p. 263-274.
4. Limmathurotsakul, D., et al., *'Antibiotic footprint' as a communication tool to aid reduction of antibiotic consumption.* Journal of Antimicrobial Chemotherapy, 2019. **74**(8): p. 2122-2127.
5. Mulvey, M.R. and A.E. Simor, *Antimicrobial resistance in hospitals: How concerned should we be?* Canadian Medical Association Journal, 2009. **180**(4): p. 408-415.
6. Tacconelli, E., et al., *Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis.* The Lancet Infectious Diseases, 2018. **18**(3): p. 318-327.
7. D'Costa, V.M., et al., *Antibiotic resistance is ancient.* Nature, 2011. **477**(7365): p. 457-461.
8. De Stasio, E.A., et al., *Mutations in 16S ribosomal RNA disrupt antibiotic–RNA interactions.* The EMBO Journal, 1989. **8**(4): p. 1213-1216.
9. Marcusson, L.L., N. Frimodt-Møller, and D. Hughes, *Interplay in the selection of fluoroquinolone resistance and bacterial fitness.* PLoS pathogens, 2009. **5**(8): p. e1000541-e1000541.
10. Low, D., *Reducing antibiotic use in influenza: challenges and rewards.* Clinical Microbiology and Infection, 2008. **14**(4): p. 298-306.
11. Ferber, D., *WHO Advises Kicking the Livestock Antibiotic Habit.* Science, 2003. **301**(5636): p. 1027.
12. Witte, W., *Selective pressure by antibiotic use in livestock.* International Journal of Antimicrobial Agents, 2000. **16**: p. 19-24.
13. Rogers Van Katwyk, S., et al., *Government policy interventions to reduce human antimicrobial use: A systematic review and evidence map.* PLoS medicine, 2019. **16**(6): p. e1002819-e1002819.
14. O' Neil, J., *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations.* Review on Antimicrobial Resistance, 2016: p. 84.
15. Lee, J. and L. Zhang, *The hierarchy quorum sensing network in Pseudomonas aeruginosa.* Protein & Cell, 2015. **6**(1): p. 26-41.

16. Yayan, J., B. Ghebremedhin, and K. Rasche, *Antibiotic Resistance of Pseudomonas aeruginosa in Pneumonia at a Single University Hospital Center in Germany over a 10-Year Period.* PloS one, 2015. **10**(10): p. e0139836-e0139836.
17. Gellatly, S.L. and R.E. Hancock, *Pseudomonas aeruginosa: new insights into pathogenesis and host defenses.* Pathog Dis, 2013. **67**(3): p. 159-73.
18. Donlan, R.M., *Biofilms: microbial life on surfaces.* Emerging infectious diseases, 2002. **8**(9): p. 881-890.
19. Vasseur, P., et al., *The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation.* Microbiology, 2005. **151**(3): p. 985-997.
20. O'Loughlin, C.T., et al., *A quorum-sensing inhibitor blocks Pseudomonas aeruginosa virulence and biofilm formation.* Proceedings of the National Academy of Sciences, 2013. **110**(44): p. 17981-17986.
21. Banin, E., M.L. Vasil, and E.P. Greenberg, *Iron and Pseudomonas aeruginosa biofilm formation.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11076-81.
22. Karatan, E. and P. Watnick, *Signals, regulatory networks, and materials that build and break bacterial biofilms.* Microbiol Mol Biol Rev, 2009. **73**(2): p. 310-47.
23. Fuqua, C., S.C. Winans, and E.P. Greenberg, *Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators.* Annu Rev Microbiol, 1996. **50**: p. 727-51.
24. Gambello, M.J. and B.H. Iglewski, *Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression.* J Bacteriol, 1991. **173**(9): p. 3000-9.
25. Reinhart, A.A. and A.G. Oglesby-Sherrouse, *Regulation of Pseudomonas aeruginosa Virulence by Distinct Iron Sources.* Genes (Basel), 2016. **7**(12).
26. Rasamiravaka, T., et al., *The formation of biofilms by Pseudomonas aeruginosa: a review of the natural and synthetic compounds interfering with control mechanisms.* BioMed research international, 2015. **2015**: p. 759348-759348.
27. Evans, D.J., et al., *Susceptibility of Pseudomonas aeruginosa and Escherichia coli biofilms towards ciprofloxacin: effect of specific growth rate.* J Antimicrob Chemother, 1991. **27**(2): p. 177-84.
28. Ueda, A. and T.K. Wood, *Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in Pseudomonas aeruginosa through tyrosine phosphatase TpbA (PA3885).* PLoS pathogens, 2009. **5**(6): p. e1000483-e1000483.
29. Evans, D.J., et al., *Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle.* J Antimicrob Chemother, 1990. **25**(4): p. 585-91.

30. Kang, D., et al., *Pyoverdine, a siderophore from Pseudomonas aeruginosa, translocates into C. elegans, removes iron, and activates a distinct host response.* Virulence, 2018. **9**(1): p. 804-817.
31. Julou, T., et al., *Cell–cell contacts confine public goods diffusion inside Pseudomonas aeruginosa clonal microcolonies.* Proceedings of the National Academy of Sciences, 2013. **110**(31): p. 12577-12582.
32. Dockery, J.D. and J.P. Keener, *A mathematical model for quorum sensing in Pseudomonas aeruginosa.* Bulletin of Mathematical Biology, 2001. **63**(1): p. 95.
33. Meyer, J.M., et al., *Pyoverdin is essential for virulence of Pseudomonas aeruginosa.* Infect Immun, 1996. **64**(2): p. 518-23.
34. Kang, D., et al., *Pyoverdine-Dependent Virulence of Pseudomonas aeruginosa Isolates From Cystic Fibrosis Patients.* Frontiers in Microbiology, 2019. **10**(2048).
35. Kirienko, D.R., D. Kang, and N.V. Kirienko, *Novel Pyoverdine Inhibitors Mitigate Pseudomonas aeruginosa Pathogenesis.* Frontiers in Microbiology, 2019. **9**(3317).
36. Kang, D., K.E. Turner, and N.V. Kirienko, *PqsA Promotes Pyoverdine Production via Biofilm Formation.* Pathogens, 2017. **7**(1).
37. Mossialos, D., et al., *Identification of new, conserved, non-ribosomal peptide synthetases from fluorescent pseudomonads involved in the biosynthesis of the siderophore pyoverdine.* Molecular microbiology, 2002. **45**(6): p. 1673-1685.
38. Lehoux, D.E., F. Sanschagrin, and R.C. Levesque, *Genomics of the 35-kb pvd locus and analysis of novel pvdIJK genes implicated in pyoverdine biosynthesis in Pseudomonas aeruginosa.* FEMS microbiology letters, 2000. **190**(1): p. 141-146.
39. Merriman, T.R., M.E. Merriman, and I.L. Lamont, *Nucleotide sequence of pvdD, a pyoverdine biosynthetic gene from Pseudomonas aeruginosa: PvdD has similarity to peptide synthetases.* Journal of bacteriology, 1995. **177**(1): p. 252-258.
40. Yeterian, E., et al., *Synthesis of the siderophore pyoverdine in Pseudomonas aeruginosa involves a periplasmic maturation.* Amino acids, 2010. **38**(5): p. 1447-1459.
41. Hannauer, M., et al., *An efflux pump is involved in secretion of newly synthesized siderophore by Pseudomonas aeruginosa.* FEBS letters, 2010. **584**(23): p. 4751-4755.
42. Albrecht-Gary, A.-M., et al., *Bacterial iron transport: coordination properties of pyoverdin PaA, a peptidic siderophore of Pseudomonas aeruginosa.* Inorganic Chemistry, 1994. **33**(26): p. 6391-6402.
43. Poole, K., et al., *Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene fpvA of Pseudomonas aeruginosa.* Journal of Bacteriology, 1993. **175**(15): p. 4597-4604.

44. Ghysels, B., et al., *FpvB, an alternative type I ferripyoverdine receptor of Pseudomonas aeruginosa.* Microbiology, 2004. **150**(6): p. 1671-1680.
45. Ganne, G.r., et al., *Iron release from the siderophore pyoverdine in Pseudomonas aeruginosa involves three new actors: FpvC, FpvG, and FpvH.* ACS chemical biology, 2017. **12**(4): p. 1056-1065.
46. Imperi, F., F. Tiburzi, and P. Visca, *Molecular basis of pyoverdine siderophore recycling in Pseudomonas aeruginosa.* Proceedings of the National Academy of Sciences, 2009. **106**(48): p. 20440-20445.
47. Meyer, J.-M., et al., *Pyoverdin is essential for virulence of Pseudomonas aeruginosa.* Infection and immunity, 1996. **64**(2): p. 518-523.
48. Lamont, I.L., et al., *Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa.* Proceedings of the National Academy of Sciences, 2002. **99**(10): p. 7072-7077.
49. Bonneau, A., B. Roche, and I.J. Schalk, *Iron acquisition in Pseudomonas aeruginosa by the siderophore pyoverdine: an intricate interacting network including periplasmic and membrane proteins.* Scientific Reports, 2020. **10**(1): p. 1-11.
50. Beare, P.A., et al., *Siderophore-mediated cell signalling in Pseudomonas aeruginosa: divergent pathways regulate virulence factor production and siderophore receptor synthesis.* Molecular microbiology, 2003. **47**(1): p. 195-207.
51. Reinhart, A.A. and A.G. Oglesby-Sherrouse, *Regulation of Pseudomonas aeruginosa Virulence by Distinct Iron Sources.* Genes, 2016. **7**(12): p. 126.
52. Cornelis, P., S. Matthijs, and L. Van Oeffelen, *Iron uptake regulation in Pseudomonas aeruginosa.* Biometals, 2009. **22**(1): p. 15-22.
53. Banin, E., M.L. Vasil, and E.P. Greenberg, *Iron and Pseudomonas aeruginosa biofilm formation.* Proceedings of the National Academy of Sciences, 2005. **102**(31): p. 11076-11081.
54. Richards, J.J., T.E. Ballard, and C. Melander, *Inhibition and dispersion of Pseudomonas aeruginosa biofilms with reverse amide 2-aminoimidazole oroidin analogues.* Organic & Biomolecular Chemistry, 2008. **6**(8): p. 1356-1363.
55. Kumar, L., S. Chhibber, and K. Harjai, *Zingerone inhibit biofilm formation and improve antibiofilm efficacy of ciprofloxacin against Pseudomonas aeruginosa PAO1.* Fitoterapia, 2013. **90**: p. 73-8.
56. Kirienko, D.R., A.V. Revtovich, and N.V. Kirienko, *A High-Content, Phenotypic Screen Identifies Fluorouridine as an Inhibitor of Pyoverdine Biosynthesis and Pseudomonas aeruginosa Virulence.* mSphere, 2016. **1**(4): p. e00217-16.

57. Kaneko, Y., et al., *The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity.* The Journal of clinical investigation, 2007. **117**(4): p. 877-888.

58. Wilson, C.E., et al., *Cooperation and competition shape ecological resistance during periodic spatial disturbance of engineered bacteria.* Scientific Reports, 2017. **7**(1): p. 440.

59. Huynh, T.T., et al., *Glucose starvation-induced dispersal of Pseudomonas aeruginosa biofilms is cAMP and energy dependent.* PloS one, 2012. **7**(8): p. e42874-e42874.

60. Schleheck, D., et al., *Pseudomonas aeruginosa PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation.* PLoS One, 2009. **4**(5): p. e5513.

61. Gunderson, B.W., et al., *Synergistic Activity of Colistin and Ceftazidime against Multiantibiotic-Resistant Pseudomonas aeruginosa in an In Vitro Pharmacodynamic Model.* Antimicrobial Agents and Chemotherapy, 2003. **47**(3): p. 905-909.

62. Cabot, G., et al., *Pseudomonas aeruginosa Ceftolozane-Tazobactam Resistance Development Requires Multiple Mutations Leading to Overexpression and Structural Modification of AmpC.* Antimicrobial Agents and Chemotherapy, 2014. **58**(6): p. 3091-3099.

63. Friedman, L. and R. Kolter, *Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms.* Molecular Microbiology, 2004. **51**(3): p. 675-690.

64. Ceri, H., et al., *The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms.* Journal of clinical microbiology, 1999. **37**(6): p. 1771-1776.

65. Merritt, J.H., D.E. Kadouri, and G.A. O'Toole, *Growing and analyzing static biofilms.* Current protocols in microbiology, 2005. **Chapter 1**: p. Unit-1B.1.

66. Smith, R., et al., *Programmed Allee effect in bacteria causes a tradeoff between population spread and survival.* Proceedings of the National Academy of Sciences, 2014. **111**(5): p. 1969-1974.

67. R. Irani, V. and J. J. Rowe, *Enhancement of Transformation in Pseudomonas aeruginosa PAO1 by Mg 2+ and Heat*. Vol. 22. 1997. 54-6.

68. Walters, M.C., et al., *Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.* Antimicrobial agents and chemotherapy, 2003. **47**(1): p. 317-323.

69. Peek, M.E., et al., *Pyoverdine, the Major Siderophore in <i>Pseudomonas aeruginosa</i>, Evades NGAL Recognition.* Interdisciplinary Perspectives on Infectious Diseases, 2012. **2012**: p. 843509.

70. Beare, P.A., et al., *Siderophore-mediated cell signalling in Pseudomonas aeruginosa: divergent pathways regulate virulence factor production and siderophore receptor synthesis.* Mol Microbiol, 2003. **47**(1): p. 195-207.
71. She, P., et al., *Effects of exogenous glucose on Pseudomonas aeruginosa biofilm formation and antibiotic resistance.* MicrobiologyOpen, 2019. **8**(12): p. e933.

Table 1: Strains used in this study.

Strain name	Source
PA14	American Type Culture Collection
PA14 (-*pel*)	Dr. Roberto Kolter
PA + eGFF	This study
EnvKY1	BEI Resources
PAK	
Shr42	
PA1651	
PA1414	
MRSN1344	
PA1400	
MX0560	
MRSN994	
MRSN1380	
MRSN317	
MRSN1356	
MRSN17849	
MRSN16847	
MRSN16383	
MRSN16344	
MRSN1601	
MRSN552	
MRSN1583	
MRSN1388	

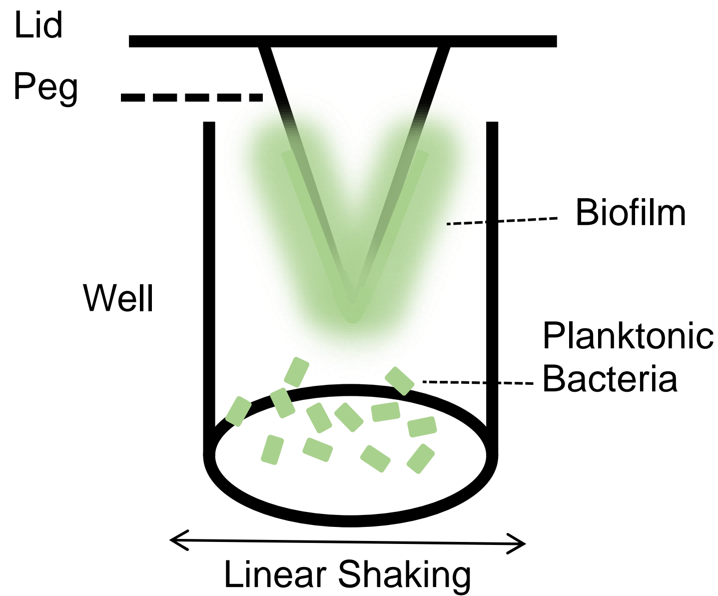

Figure 1: An experimental approach to disturbing the spatial structure of biofilms. We used the Innovotech MBEC Biofilm Inoculator to grow biofilms. After growing biofilms, we used the linear shaking function of a plate reader to disturb the distribution of bacteria in the biofilm. In general, bacteria will be removed from the biofilm and enter the planktonic state. The distribution of bacteria already in the planktonic state will also be perturbed.

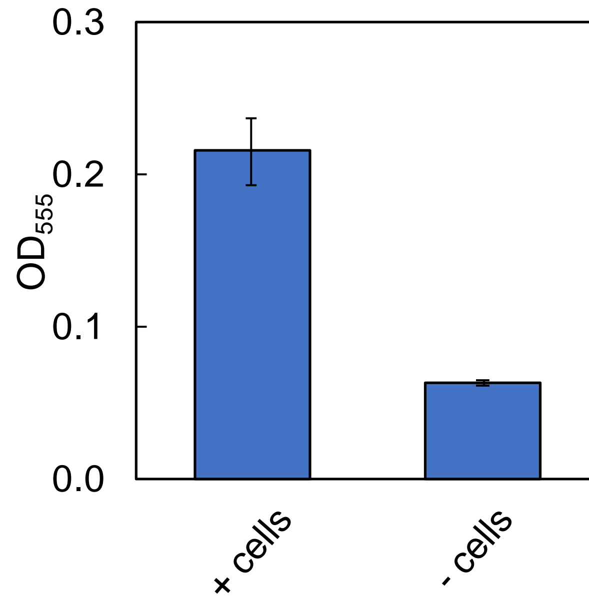

Figure 2: Using crystal violet (measured at OD_{555}), we confirmed the presence of biofilms on the peg of the MBEC Biofilm Inoculator using our growth procedure (see *Methods*). Standard deviation from 5 biological replicates (two-tailed t-test, $P = 0.0001$).

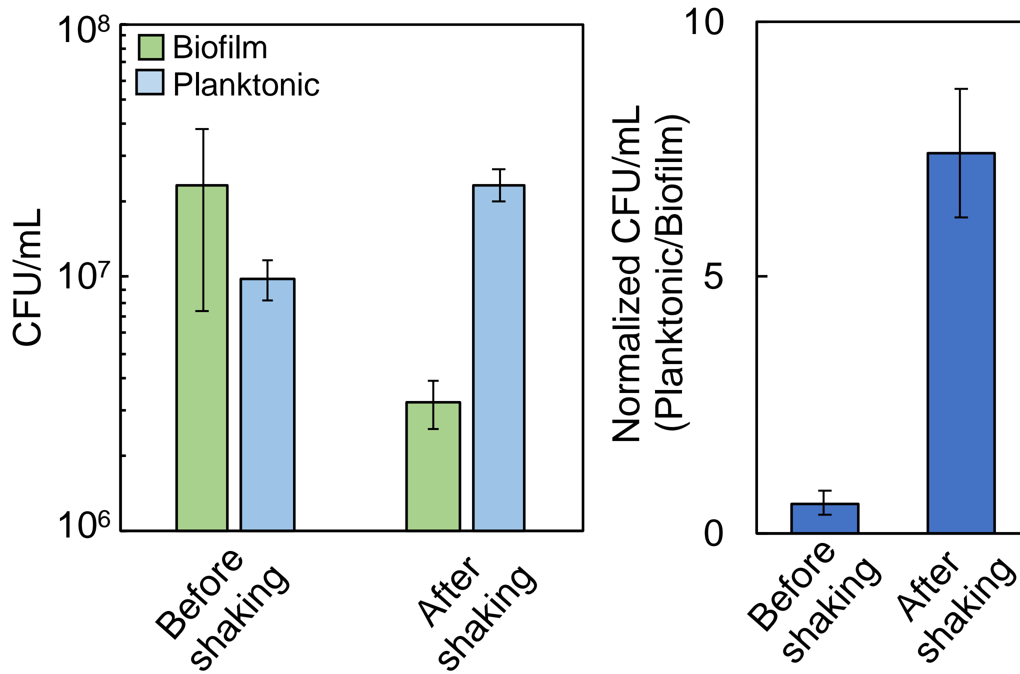

Figure 3: The density of bacteria in the biofilm and planktonic states before and after a single shaking event. Left panel: absolute cell density (biofilm, P = 0.21, planktonic, = 0.015, two-tailed t-test). Right panel: planktonic cell density normalized by biofilm cell density (P = 0.014, two-tailed t-test). Standard deviation from three biological replicates.

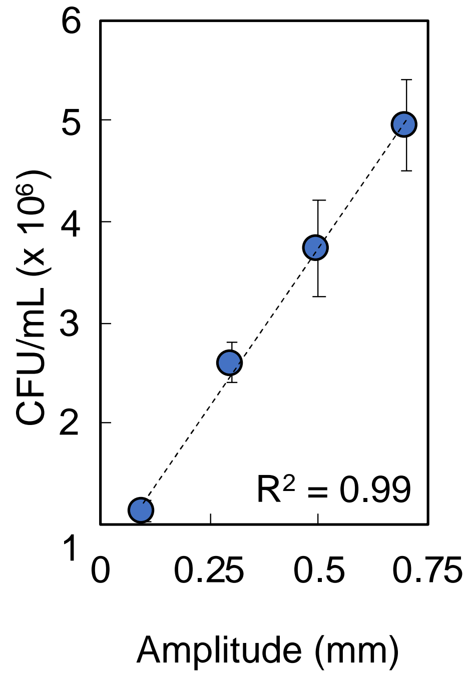

Figure 4: The density of bacteria in the planktonic state as the amplitude of shaking is increased ($P < 0.02$ for all comparisons, two-tailed t-test). R^2 reported from the fit of a linear line. Standard deviation from three biological replicates.

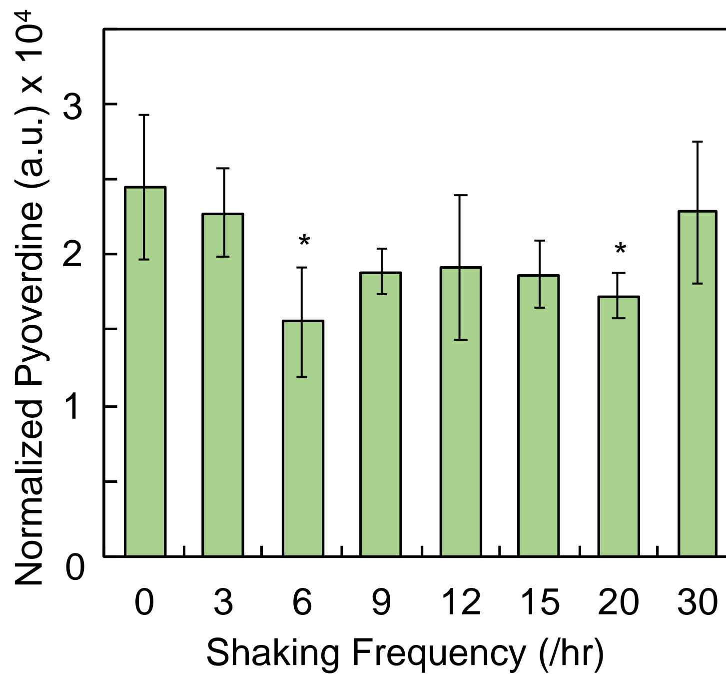

Figure 5: Periodically disturbing biofilms of *P. aeruginosa* strain PA14 can reduce the amount of pyoverdine. The amount of pyoverdine in growth medium with 0% glucose after periodically disturbing biofilms. In both panels, pyoverdine (measured using $\square_{excite}$ = 405 nm and $\square_{emit}$ = 460 nm, a.u.) normalized by OD_{600} and biofilms were disturbed for 24 hours. Average from a minimum of 4 replicates. * indicates statistical difference between unshaken and 30 shakes per hour using two-tailed t-test ($P < 0.016$).

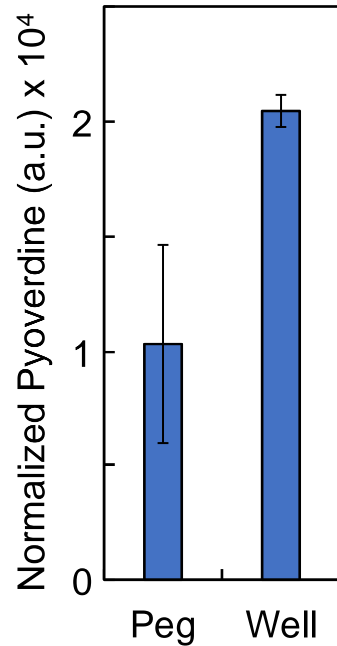

Figure 6: The quantity of pyoverdine in liquid medium and in cells growing in a biofilm on the pegged lid of the MBEC biofilm inoculator plates. We grew biofilms over 24 hours in media with 0% glucose and measured pyoverdine in both the planktonic bacteria as well as in the biofilm by resuspending the biofilm in media to measure it. Pyoverdine (measured using λ_{excite} = 405 nm and λ_{emit} = 460 nm, a.u.) normalized by OD_{600}. The majority of pyoverdine was found in liquid media in the well surrounding the peg (two-tailed t-test, P = 0.008). Standard deviation from five replicates.

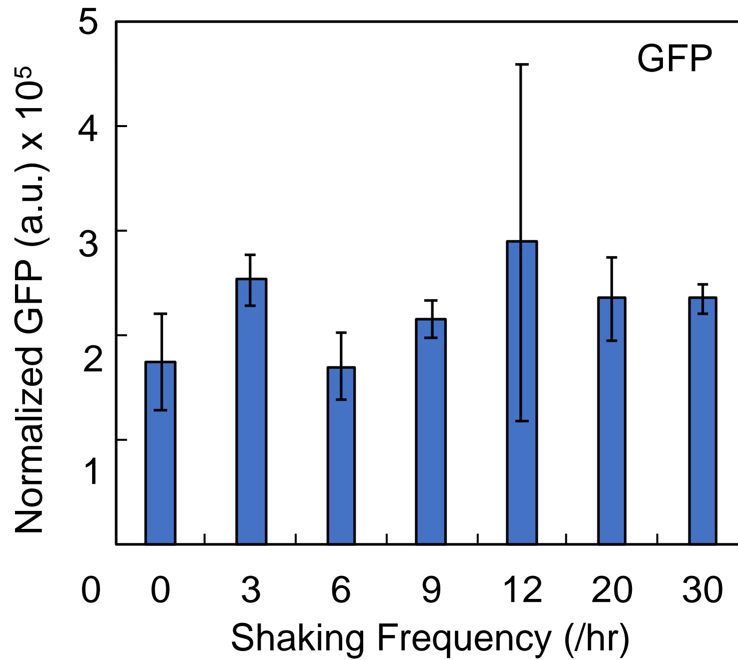

Figure 7: The amount of green fluorescent protein (GFP) in growth medium after periodically disturbing the spatial structure of biofilms containing *P. aeruginosa* expressing *gfp* for 24 hours. GFP (a.u.) normalized by cell density (OD_{600}). We did not observe a significant change in normalized GFP across between frequencies of unshaken (0 shakes/hr) and 6 shakes/hr (two-tailed t-test, P =0.84). We did observe a significant decrease in GFP when 30 shakes/hr was compared to 6 shakes/hr (P = 0.0002, two-tailed t-test) and the unshaken control (0 shakes/hr, P = 0.018). Standard deviation from four replicates. GFP and cell density measured from liquid medium in well.

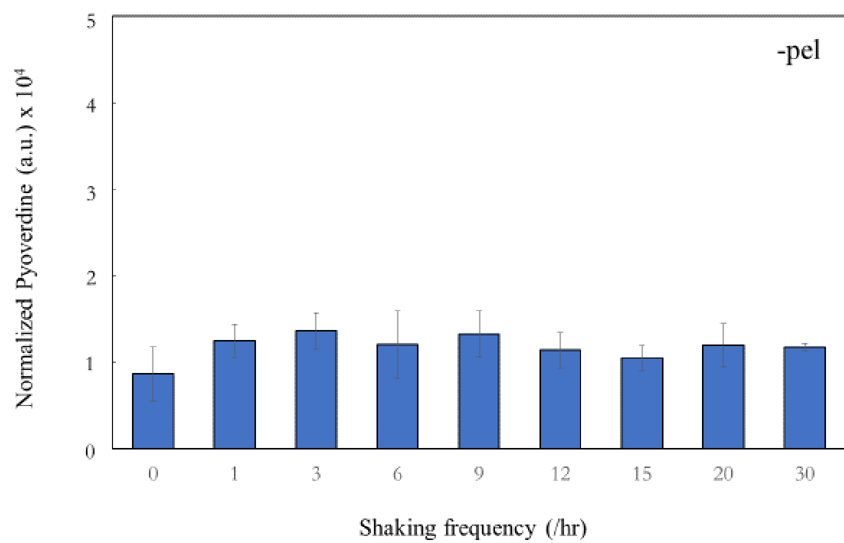

Figure 8: The effect of periodic shaking on normalized pyoverdine in a strain of *P. aeruginosa* (*-pel*) that lacked the ability to form biofilms. Standard deviation from 5 replicates.

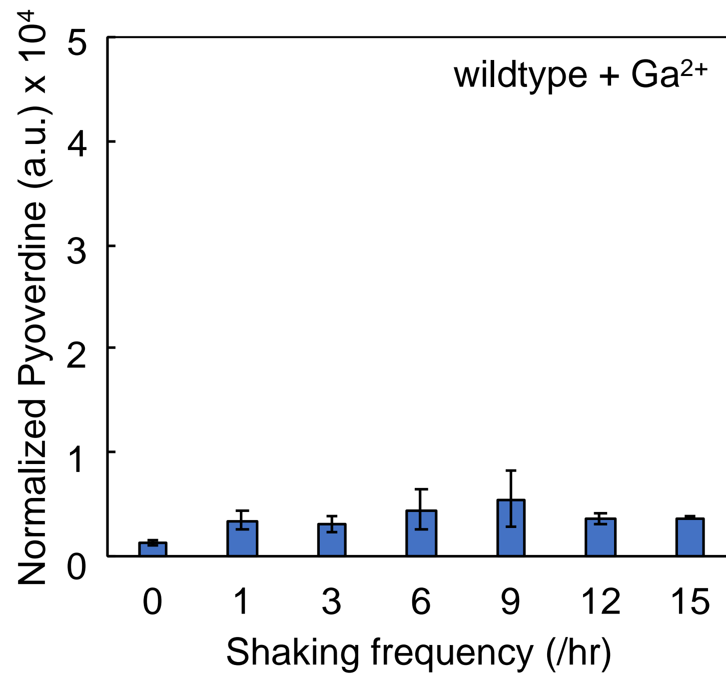

Figure 9: Inclusion of 10uM of gallium in the growth medium reduced the amount of pyoverdine synthesized. Furthermore, it did not result in a reduction of pyoverdine due to periodic shaking. Standard deviation from 5 replicates.

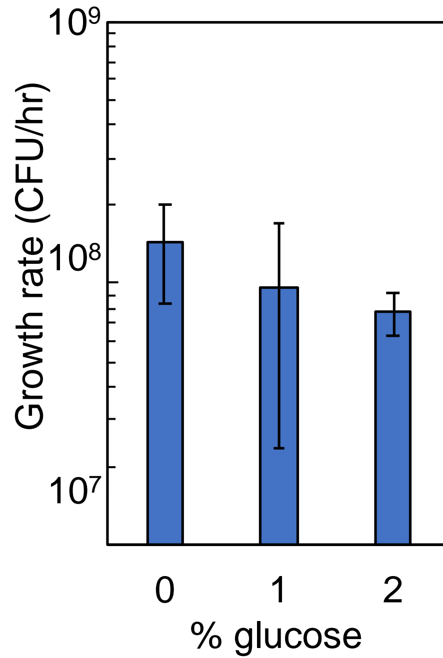

Figure 10: Average growth rate of *P. aeruginosa* strain PA14 in medium with increasing glucose as measured using CFU. Growth rate measured using CFU as described in *Methods*. Increasing glucose did not alter the average growth rate ($P \geq 0.15$, two-tailed t-test, all comparisons). Standard deviation from a minimum of 3 biological replicates.

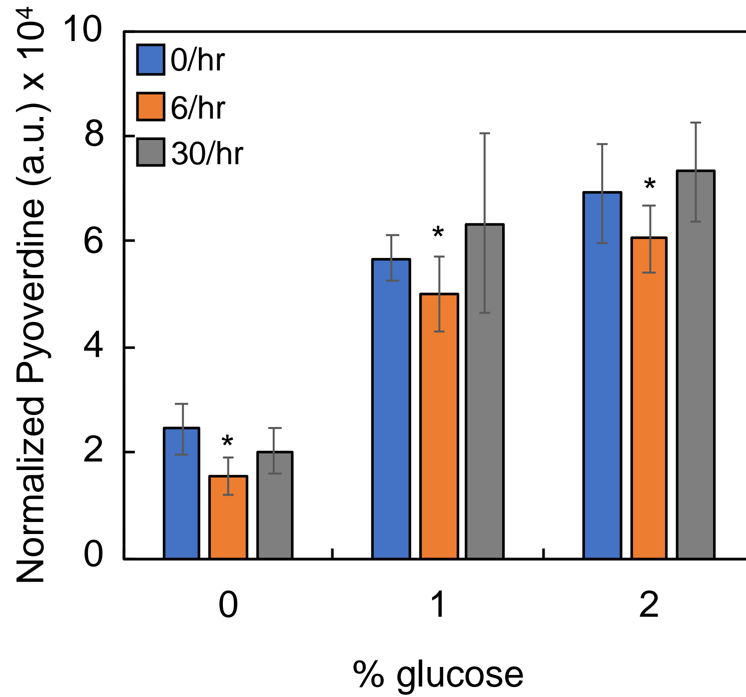

Figure 11: The amount of pyoverdine in growth medium with increasing percentage of glucose after periodically disturbing biofilms. * indicates statistical difference between unshaken and 30 shakes per hour using two-tailed t-test ($P \leq 0.014$, two-tailed t-test, all conditions).

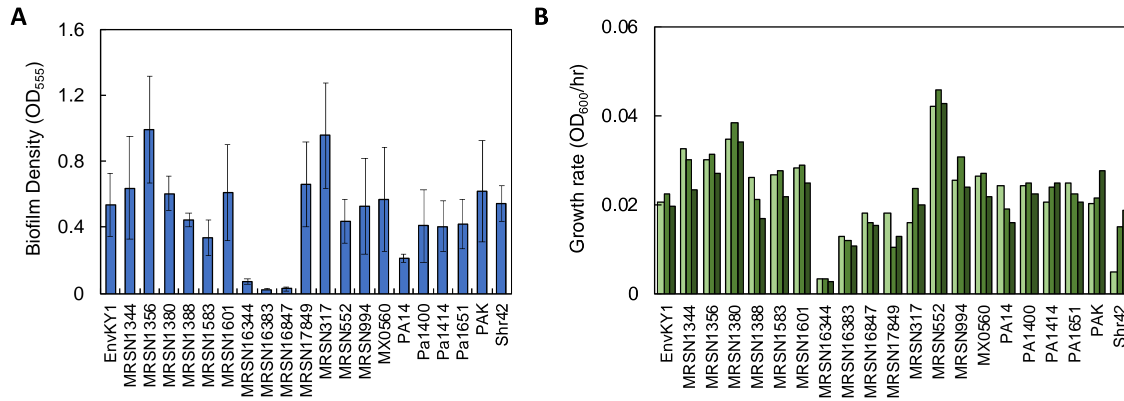

Figure 12: Growth characteristics of *P. aeruginosa* strains isolated from the clinic. A) The density of biofilms in the absence of shaking of different strains of *P. aeruginosa* as measured using crystal violet (OD_{555}). Standard deviation from three biological replicates. B) Average growth rate of *P. aeruginosa* strains as measured using OD_{600} in medium with increasing percentages of glucose. Standard deviation from three biological replicates.

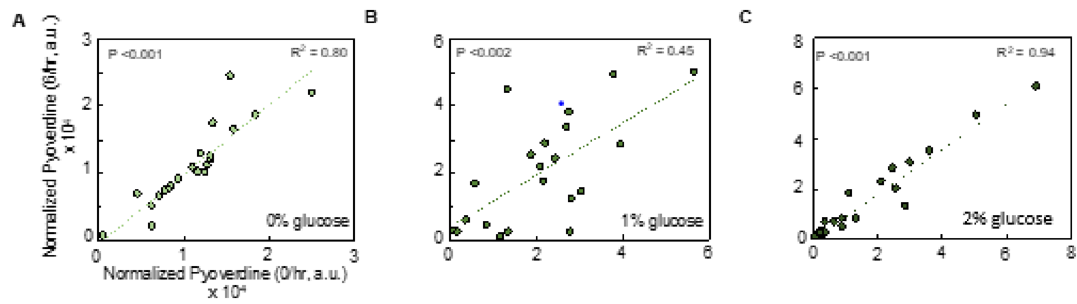

Figure 13: An intermediate percentage of glucose in the growth medium decreases the strength of a linear relationship between pyoverdine in an undisturbed and a periodically disturbed condition. Linear correlation between pyoverdine produced under unshaken (0 shakes/hr) and periodically disturbed (6 shakes/hr) conditions in medium with 0% glucose. For panels A-C, average plotted from six biological replicates. Indicated P value is from a linear regression analysis. R^2 reported after plotting a linear line through the data.

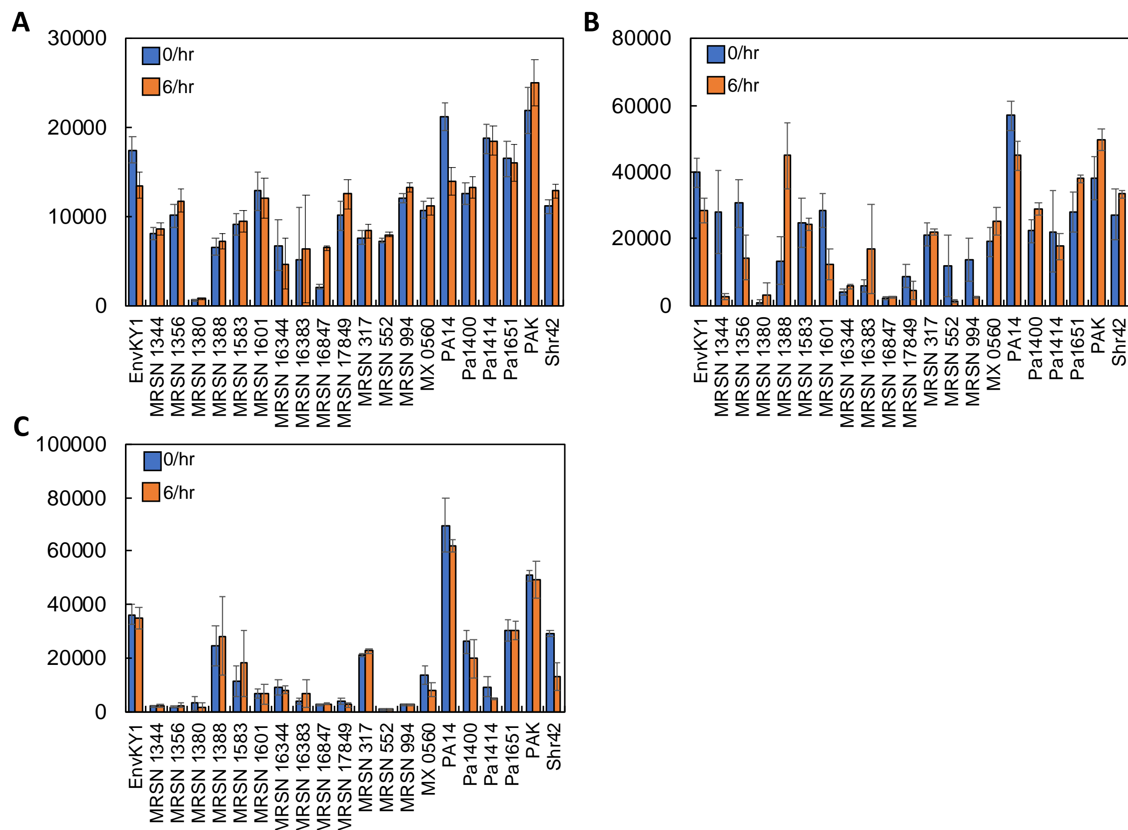

Figure 14: The average amount of pyoverdine synthesized by strains of *P. aeruginosa* in the undisturbed and 6 shake/hr conditions in medium with increasing percentages of glucose. In each panel (A = 0% glucose, B = 1% glucose, C = 2% glucose) standard deviation from 6 replicates. Pyoverdine normalized by cell density (OD_{600}).

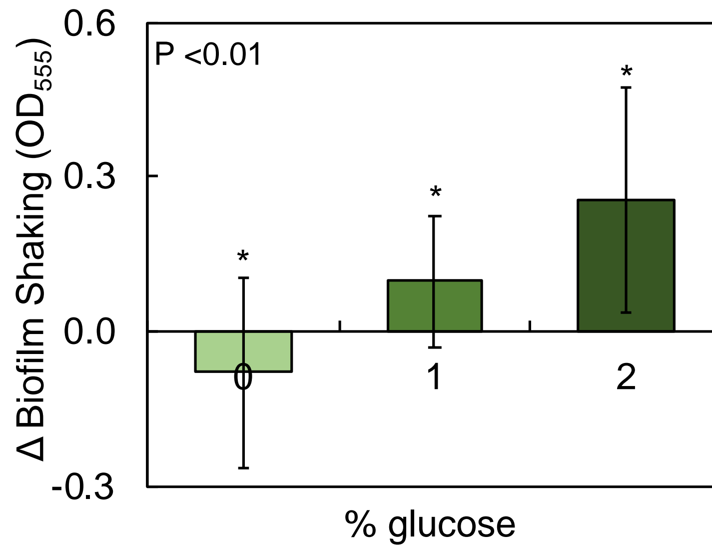

Figure 15: Change in biofilm density as a result of shaking at 6 shakes/hr as a function of percentage of glucose in the growth medium. Biofilm density measured at OD_{555} after staining with crystal violet. In panels D and E, we subtracted the 6 shakes/hr condition from the unshaken (0/hr) condition. * indicates significantly different using a two-tailed t-test ($P < 0.009$, all conditions compared).

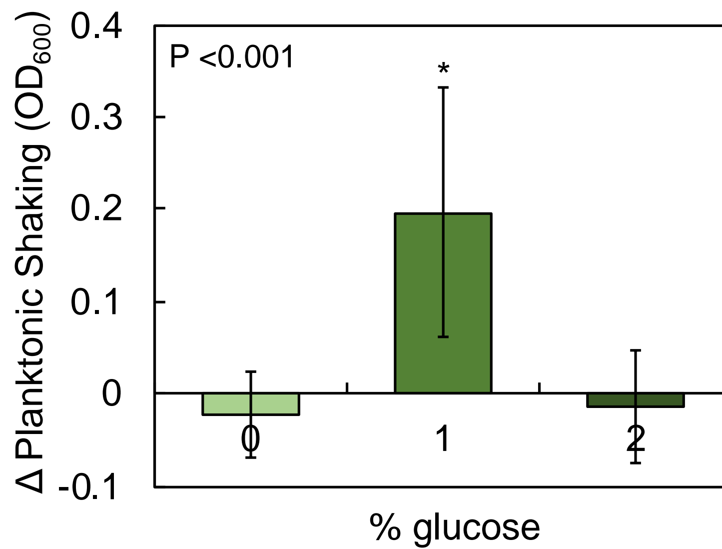

Figure 16: Change in the density of planktonic bacteria as a result of shaking at 6 shakes/hr as a function of percentage of glucose in the growth medium. Bacteria in the planktonic state measured using OD_{600}. To calculate change in biofilm density we subtracted the 6 shakes/hr condition from the unshaken (0/hr) condition. * indicates significantly different using a two-tailed t-test (0% glucose vs. 1% glucose, 1% glucose vs. 2% glucose, $P < 0.001$, 0% glucose vs. 2% glucose, $P = 0.64$).

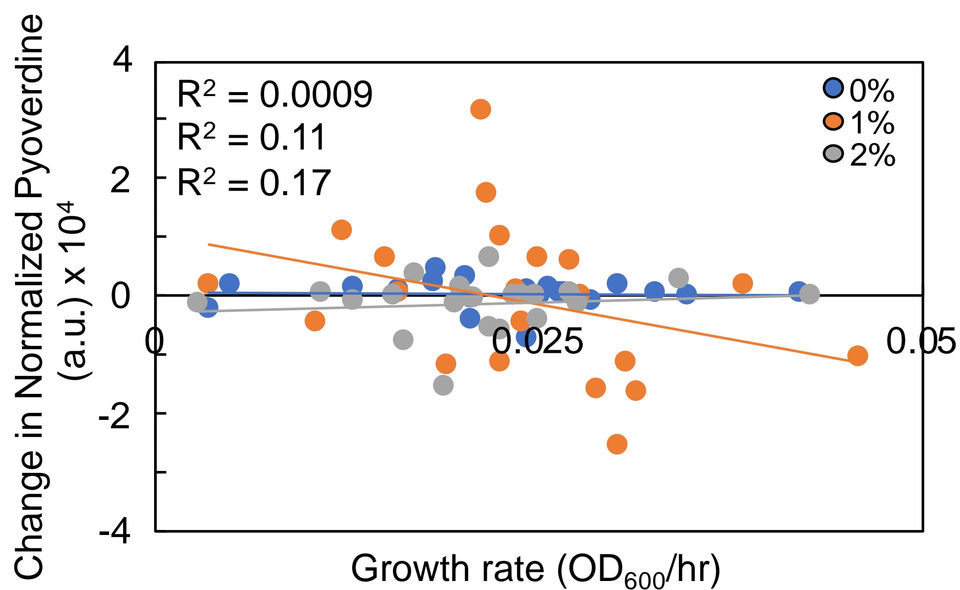

Figure 17: The relationship between difference in pyoverdine amount between undisturbed and 6 shakes/hr as a function of growth rate. No linear correlation was found with any condition. R^2 values from fit of linear line. Standard deviation from three replicates.

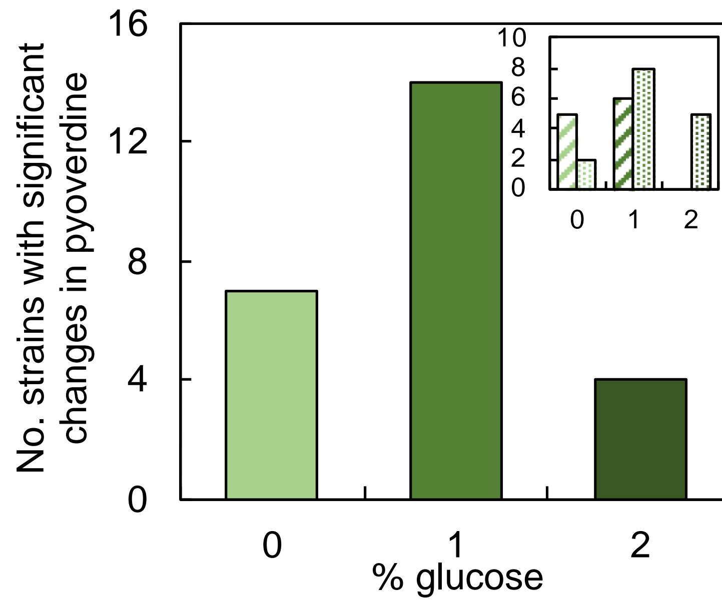

Figure 18: A significant reduction in biofilm density at intermediate percentage of glucose can result in a significant change in pyoverdine density. Number of *P. aeruginosa* strains that had a significant decrease in the amount of pyoverdine as a result of periodic disturbance at 6/hr. Significance determined using a two-tailed t-test comparing 0/hr and 6/hr within each percentage of glucose. Insert: Number of strains that had a significance increase (striped bars) or decrease (dotted bars) in the amount of pyoverdine.

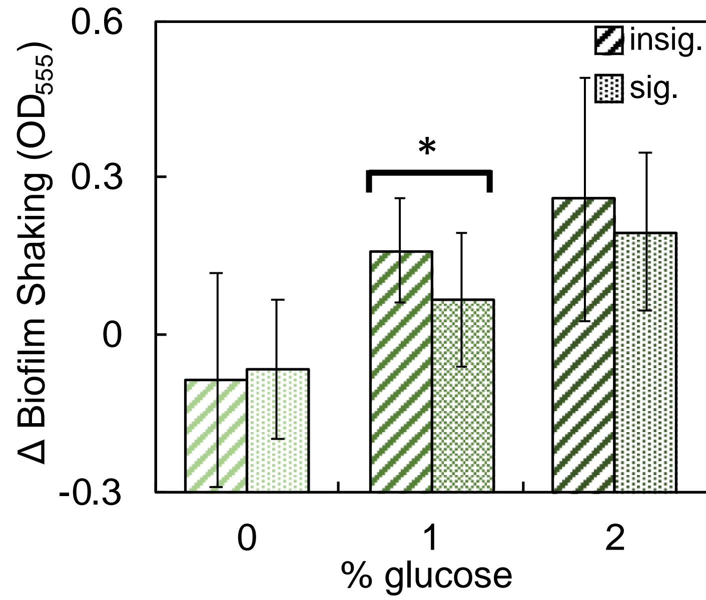

Figure 19: Change in biofilm density as a result of shaking at 6 shakes/hr as a function of percentage of glucose in the growth medium. Strains with a significant change in pyoverdine post shaking are indicated with dotted bars; strains with an insignificant change in pyoverdine indicated with striped bars. Biofilm density measured at OD_{555} after staining with crystal violet. * indicates statically significant decrease in pyoverdine using a one-tailed test (P = 0.049).

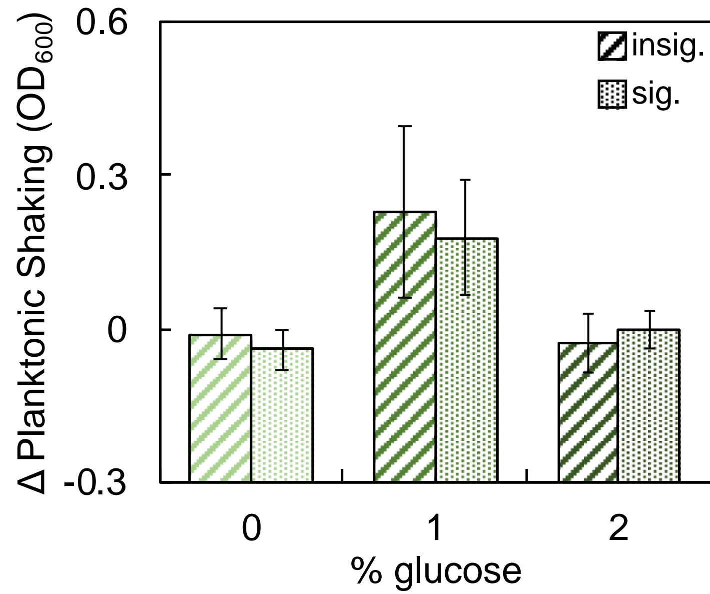

Figure 20: Change in the density of planktonic bacteria as a result of shaking at 6 shakes/hr as a function of percentage of glucose in the growth medium. Strains with a significant change in pyoverdine post shaking are indicated with dotted bars; strains with an insignificant change in pyoverdine indicated with striped bars. Bacteria in the planktonic state measured using OD_{600}.