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Periodic Spatial Disturbances Alter the Expression of Quorum Sensing Virulence Factors in Pseudomonas Aeruginosa

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By

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen associated with severe acute and chronic illnesses. Current antibiotic-based approaches fail to effectively treat *P. aeruginosa* infections due to the effectiveness and robustness of the quorum sensing signaling system (QS). Pathogenic bacteria, such as *P. aeruginosa*, employ this population density-dependent communication mechanism to confer antimicrobial resistance, propagate infection, and coordinate the expression of virulence factors, through the production and detection of autoinducing signaling molecules (AI). As such, there is a growing interest in developing novel non-antibiotic-based techniques to attenuate the pathogenicity of *P. aeruginosa* by disrupting the functionality of its QS system. Previous studies suggest that periodic disturbances to the biofilm structures of bacteria performing QS alter the distribution of bacteria and autoinducer, thus attenuating the expression of its QS-regulated genes; but this has yet to be explored in *P. aeruginosa.* Accordingly, we examined the effect of periodic spatial disturbances on the expression of QS-regulated virulence genes in *P. aeruginosa*. We found that periodically disturbing biofilms composed of *P. aeruginosa* at various frequencies and amplitudes alter the distribution of bacteria in the biofilm and surrounding planktonic states, with higher frequencies and amplitudes greatly increasing the distribution of cells into the planktonic state. Moreover, qRT-PCR analysis suggests periodic disturbances decrease the expression of QS-transcription regulators and effector virulence factors across all major QS systems in planktonic state cells compared to an undisturbed control (0/hr). Finally, the range of disturbance frequencies that resulted in a reduction in the expression of QS-transcription regulators was dependent on the amplitude of the disturbance; at an intermediate amplitude of 0.3mm, a reduction in the expression of these critical genes occurred over a broader range of shaking frequencies relative to smaller or larger amplitudes. This suggests that an optimal combination of physical and high shear forces favors this reduction at intermediate disturbance amplitudes. Comparative gene expression analysis of wildtype PA14 and QS knockout strains ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR suggests that periodic disturbances alter QS stability and function in *P. aeruginosa*. Overall, these results establish that disrupting the spatial structure of *P. aeruginosa* biofilms has an effect on QS-regulated gene expression and suggest that modulating shaking frequency and amplitude of disturbance could lead to novel non-antibiotic-based approaches to mitigate pathogenesis, cooperation, and virulence of *P. aeruginosa*.

Keywords

Autoinducer, biofilm, gene expression, pathogenicity, shear force

List of Figures and Tables

Introduction

Infectious Diseases: A Serious Global Health Threat

Infectious diseases are a major cause of mortality around the world. Up until 2019, infectious diseases were historically responsible for 10.6% of all deaths in the United States [1] and accounted for approximately a quarter of worldwide mortalities each year [2, 3]. As of 2020, with the emergence of the Coronavirus disease 2019 outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [4] the mortality rate estimate caused by infectious agents has increased by 90%, with over 5.8 million deaths worldwide attributed to COVID-19 alone. Furthermore, incidence rates of coinfections with COVID-19 and complications arising from extended hospitalizations have further contributed to the ever-increasing number of deaths associated with infectious diseases [5-7]. In addition, prevalent communicable infectious diseases, such as bacteremia, HIV, influenza, and pneumonia, are still among the major causative agents contributing to high morbidity and mortality rates worldwide [8-10]. Furthermore, bacterial infections account for nearly 80% of infections in humans, ranging from rhinosinusitis, cystic fibrosis, non-healing chronic wounds, and implantable device-related infection [11]. Given the effect on death rates, life expectancy, and radical global life changes arising from pandemic infectious diseases, such as COVID-19 (hospital burden, mask mandates, travel policy, education, communication, global health, etc.), it is imperative to understand the underlying mechanisms by which infectious agents cause disease to find effective methods to attenuate virulence and pathogenicity and to develop novel therapeutic interventions.

Pathogenesis arises from a delicate interaction between the invading agent and the robustness of the host immune system [12]. A pathogenic organism is one that has the ability to cause disease in a host, where pathogenicity is thus measured in terms of ‘virulence’ [1]. The effect of a compromised immune system and bacterial toxins can render the host under-equipped to adequately combat invading pathogens [13-15]. Pathogenic organisms in particular are especially adept at evading the immune system and modulating the expression of the virulence factors to damage host tissue and alter their environment to promote colonization and establishing infection [1-3]. Alkaline protease is a toxic enzyme commonly secreted by several classes of bacteria infiltrating the lungs of cystic fibrosis patients used to inhibit the complement system and activate

the epithelial sodium channel to decrease overall mucociliary clearance, thus facilitating bacterial colonization in host lungs [14]. Infectious agents can also produce decoy proteins, such as Flavivirus nonstructural protein 1 (NS1) secreted by cells infected by Dengue virus, which binds competitively to Mannose-Binding Lectin, thus preventing the virus from being degraded through the complement pathway [16].

In contrast, individuals who are immunocompromised or who have an immune system deficiency are at higher risk of fatal, and usually opportunistic, infections. Azathioprine, a known immunosuppressant drug used to prevent kidney transplant rejection, inhibits CD28 costimulatory signals needed by T-lymphocytes for the production of cytokines, such as IL-2, thus limiting the proliferation of other T-cells, natural killer cells, and B-cells [17]. A limited number of functional lymphocytes and cytokines means the host defense system is unaware and unequipped to fight off pathogens, and as such, immunocompromised individuals are at much higher risk of opportunistic infection. As such, understanding pathogenesis and other mechanisms of infection are crucial, as it can provide clearer insights on how to disrupt and prevent further infections.

Antibiotics: Evolutionary Consequences

Another contributing factor to the pathogenicity of infectious agents is antibiotic resistance (AMR). Antibiotics are the most commonly prescribed antimicrobial therapy. Several classes of antibiotics target different aspects of bacterial cells, such as beta-lactams, which disrupt cell wall synthesis, fluoroquinolones, which inhibit replication and nucleic acid synthesis, and aminoglycosides which inhibit protein synthesis [18].

However, multi-drug resistance and antimicrobial resistance (AMR) have been described in both Gram-positive and Gram-negative bacteria, most notably in *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus,* and *Pseudomonas aeruginosa*. This increasing emergence of AMR pathogens poses a great threat to public health. According to the Centers for Disease Control and Prevention (CDC) [10], AMR cost an estimated 20 billion dollars in excess medical spending related to healthcare treatment costs and extended hospital stays, which has led to a net loss of 35 billion dollars each year [12, 19]. Furthermore, AMR infections affect over 2 million people each year. Ineffective treatment is especially harmful to vulnerable patients on

dialysis, undergoing chemotherapy, or that are immunocompromised, who are at higher risk of serious illness or death from contracting infections [20].

Several factors contribute to the development of AMR. Inappropriate antibiotic use is known to drive the evolution of resistance, which can occur through spontaneous mutations or horizontal gene transfer of resistance genes. As a result, previously non-pathogenic organisms can develop pathogenicity islands in their genome containing genetic markers from mobile genetic elements, such as plasmids and prophages, thus conferring virulence genes needed for pathogenicity, resistance, and survival. Remarkably, acquired AMR mechanisms include limiting drug uptake, having efflux pumps, inactivating drugs, and modifying drug targets on the cell [21]. Subinhibitory antibiotic concentrations have also been shown to cause changes in antibiotic-induced gene expression, increasing virulence, and mutagenesis, and even promoting the diversification of AMR bacterial strains in *P. aeruginosa* and *Bacteroides fragilis* [1, 20, 22, 23]. Furthermore, the limited availability of novel antibiotics further promotes the development of AMR. Nearly all novel antibiotics discovered since 1928 have conferred resistance. There is a lack of financial investment and academic research in the development of novel antibiotics, as the microbial-resistance arms race is no longer seen as profitable by major pharmaceutical companies [22, 24, 25]. As such, having limited novel treatment options promotes the use of otherwise infective antibiotics against these rapidly mutating virulent strains.

As such, further understanding of how bacteria modulate mechanisms of pathogenicity and resistance is therefore essential for the development of novel countermeasures against disease. Developing treatments and therapies against infectious agents should therefore not rely on antibiotics, and instead should focus on targeting other mechanisms involved in pathogenesis to circumvent the threat of antimicrobial resistance

Quorum Sensing: Bacterial Crosstalk

Many bacteria share a similar mechanism of cellular communication known as ‘quorum sensing’ (QS) to modulate bacterial behaviors in relation to cell density. Communication is mediated by small diffusible extracellular signaling chemicals called autoinducers (AIs), where the specific AIs used in QS tend to be both strain and species-specific [26]. The release and detection of AIs promote transcription of target genes, which include key factors in bacterial

growth, motility, and virulence [27]. In addition to activating the expression of downstream elements, AIs also increase the production of the signaling molecules themselves, thus generating a positive-feedback loop to further amplify their regulatory effect.

The use of QS has different effects on communal fitness depending on cell density. At low cell density, AIs diffuse away and do not meet the threshold needed for detection. As such, QS has limited use in low cell populations since limited signal detection and inefficient extracellular factor production do not contribute to communal fitness [28]. On the other hand, at high cell density, AIs concentration accumulates readily, thus enabling detection and subsequent response. As such, the production of extracellular components by QS is only beneficial when the population is sufficiently large to sustain crosstalk effectively [29-31]. Interestingly, the benefit of producing intracellular factors, such as those required for metabolism, is not dependent on population density, as non-social factors do not likely change the overall fitness of the entire population [28, 29, 32].

Furthermore, bacteria use QS to regulate cellular behaviors including aggregation and motility, cellular competence, exoenzyme secretion, immune evasion, and metabolism [19, 31, 33]. Pathogenic bacteria depend on QS systems and the production of their respective AIs to regulate the expression of virulence genes and the formation of biofilms [34]. Gram-positive bacteria, such as *S. aureus* and *Clostridium difficile,* use autoinducing peptides (AIPs) to induce QS. Gram-negative bacteria, such as *P. aeruginosa* and *Xanthomonas campestris*, use many different classes of AIs including acyl-homoserine lactones (AHLs), quinolones, and fatty acids [35]. By orchestrating QS and AI production, pathogenic bacteria are able to control the dynamics of the population and coordinate the expression of virulence until the infection is rigorously established in the host.

Finally, external factors, such as environmental stress and starvation can also trigger the expression of QS-virulence effectors. In environments with limited nutrient availability, bacteria alter their metabolic state to undergo the 'stringent response'. In *Escherichia coli*, amino acid starvation leads to the buildup of guanosine 3’,5’-bidiphosphate (ppGpp), which inhibits the synthesis of stable RNA, thus limiting bacterial growth and proliferation to promote survival. In *P. aeruginosa,* the stringent response induces QS prematurely to enable access to nutrients during infection. Increased concentration of ppGpp instead promotes the expression of AIs, promoting

the expression of QS-virulence genes needed to produce cytotoxic metabolites and tissue-degrading enzymes [29, 36].

Biofilms Shield Bacteria and Promote Aggregation of AIs

The formation of a biofilm is a critical step for bacteria to establish colonization in a new environment [33]. Bacteria form anchored biofilms encased in a self-generated extracellular polymeric secretion (EPS) which allows them to propagate in soil, water reservoirs, or a human host. Biofilms shield bacteria from different environmental stressors that would otherwise impede growth, such as desiccation, host immune response, or competitive interactions with other microbial competitors [33, 37]. Thick EPS walls also enhance antibiotic resistance by reducing the diffusion of antibiotics into the biofilm, thus lowering the effective concentration of the antibiotic, limiting drug uptake, and promoting the formation of metabolically dormant persistent cells [37-39]. Additionally, increased cell density owing to biofilm formation can further facilitate horizontal gene transfer, conferring evolution and spread of antibiotic resistance genes [38]. Overall, the interplay between QS, biofilm formation, and propensity for resistance, ensures bacterial survival in even the most unviable environments.

There are four main steps in bacterial biofilm formation: adhesion, replication, EPS production, and dispersion [33, 40]. Bacteria express adhesion molecules on the cell wall which promote attachment to surfaces and the formation of a monolayer. Following adhesion, cells undergo proliferation and begin generating polymeric EPS structures. Interestingly, in several bacterial systems, the production of EPS, and the subsequent formation of a biofilm, is potentiated by QS. As the biofilm grows and matures, the cells continue to communicate using QS, further increasing the production of necessary biofilm growth and dispersion molecules such as rhamnolipids and elastase [41, 42]. Owing to the highly structured and dense population of bacteria that populates a biofilm, AIs accumulate more rapidly as they are partially prevented from diffusing away from the biofilm. In some instances, biofilms can increase the rate at which QS-regulated gene expression occurs. Finally, a subset of cells will disperse from the biofilm, ensuring bacterial survival by allowing bacteria to propagate and colonize new areas after nutrients have been depleted in the local site.

P. aeruginosa: An Opportunistic Pathogen with a Well Characterized, Hierarchal QS System

P. aeruginosa is a pervasive and ubiquitous motile Gram-negative bacterium. It is found in a diverse range of habitats as it is a highly adaptable facultative anaerobic organism. While it is a part of normal skin flora in humans, *P. aeruginosa* is a notorious opportunistic pathogen and is a causative agent in a wide variety of infections. Specifically, this bacterium causes approximately 20% of all nosocomial infections and accounts for over 90,000 deaths annually [43]. *P. aeruginosa* is a causative infectious agent in severe illnesses including dermatitis, urinary tract infections, bone infections, and systemic bacteremia [34]. *P. aeruginosa* also is highly associated with burn wound infections, chronic lung infections, and mortality in individuals with cystic fibrosis [34, 44]. In nature, *P. aeruginosa* will form a mushroom-shaped biofilm composed of EPS, which is separated by distinct channels that permit water flow and nutrient diffusion [40]. Thriving in moist environments, *P. aeruginosa* can also easily form biofilms in inorganic settings, namely medical equipment such as ventilator tubes and catheters [11, 43], which poses a serious risk for patients with co-morbidities and individuals in critical care [32].

The QS network in *P. aeruginosa* is organized in a hierarchy that involves four main AI and their respective receptors (Fig. 1). The four main classes of AIs are N-(3-oxododecanoyl)-homoserine lactone (**OdDHL)**, N-butyryl homoserine lactone (**BHL**), 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone Signal, **PQS**) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing Signal, **IQS**) [34]. Together, these four AIs regulate the transcription of virulence factors in a hierarchal yet interconnected manner.

The ***las* system** includes *las* genes and regulators. While Las Receptor (LasR) shares similar homology to LuxR, which regulates bioluminescence in *Vibrio fischeri,* [45] its role in *P. aeruginosa* QS is integral to the orchestration of the system. As the "QS-conductor", activation of **LasR** initiates the complex and highly integrated QS-network in *P. aeruginosa* (Fig.1). The multimerization and formation of the **LasR-OdDHL** complex generates a positive-feedback loop by activating transcription of AI genes (*lasI*), QS-receptors genes (*rhlR, pqsR*) and effector genes (*lasA, lasB, aprA,* etc.) [29].

The second system, **RhlR-BHL**, then dimerizes and in a similar manner forms another positive feedback loop, as it activates transcription of *rhlI* as well as its regulon (*rhlA, rhlB, hcnA*),

further promotes transcription of *las* system (*lasI*), while also inhibiting transcription of QS-receptor *pqsR* [34].

The third system, **PqsR**, is activated by Las-OdDHL, inhibited by the RhlR-BHL system, and regulates transcription of the biosynthesis operon *pqsABCD* and as well as transcription of PQS conversion enzymes. **PQS** further activates the transcription of *rhlI* and *rhlR*, modulating the production of BHL and pushing the system towards the *rhl* quorum sensing [34, 46].

The fourth signal, **IQS**, is tightly regulated by the *las* system. However, certain environmental stress conditions, such as during phosphate depletion, shift the dominance of the *las* system towards IQS, thus upregulating *rhl* and PQS systems. Furthermore, disruption of IQS synthesis gene cluster *ambBCDE* results in the downregulation of PQS and BHL signaling [34, 42]. However, due to the novelty of this recent finding, the extent of the role of IQS in QS is still unknown [34].

This complex, hierarchically organized QS network regulates the expression of the vast majority of virulence factors in *P. aeruginosa* which have different roles in establishing infection. Some important virulence genes and their products include hydrogen cyanide (*hcnABC*) a toxin that enables the arrest of cellular respiration of host tissue and cells; metalloenzyme elastase B (*lasB*) which catabolizes elastin and other EPS proteins to promote iron acquisition, rhamnosyl-transferase (*rhlA)* which promotes biosynthesis of rhamnolipids used in motility, biofilm formation, and as biosurfactants [47], and pyocyanin (*phzM/phzABCD*) a cytotoxic phenazine that promotes colonization and immune evasion by neutrophil damage [34]. Together, the transcription of these suit of genes drives the process of infection [34]. Accordingly, there is increased interest in developing novel tools to disrupt the functionality of this network to attenuate the severity of *P. aeruginosa* infections.

Disturbance of QS in *P. aeruginosa*

Microbial communities are relatively flexible and hardy and are able to respond and adapt to disturbances in their environment [48]. The extent of this response is highly dependent on the bacterial community's ability to cooperate, which can be altered using chemical, biological, and physical forces [19, 41, 48].

Previous work has demonstrated that periodically disturbing the spatial structure of an organized population of bacteria can affect their ability to communicate and activate QS [19, 41, 48]. As previously stated, spatial structures, such as biofilm communities, allow for the accumulation of AIs. Higher concentrations of AI are thus found in areas of greater cell density in the biofilm and AI concentration then decreases further away from the biofilm. In other words, this establishes a concentration gradient of AI. Perturbing a structurally organized system such as this would displace both bacteria and AI alike. Thus, it can serve to decorrelate the concentration of AI and bacteria, which can reduce the effective concentration of AI sensed by any one bacterium, and therefore reduce the effectiveness of QS in the regulation of cooperative bacterial behaviors. This has been illustrated by the mathematical model formulated by Wilson et al to determine said effects on microbial cooperation, in which periodic spatial disturbances (i.e. shakes) resulted in a trade-off between access to AIs in the system and access to nutrients [28, 41]. Therefore, disturbing the spatial distribution of bacteria on a biofilm perturbs their ability to properly partake in QS, which would disrupt the consequent expression of downstream elements. Perturbing *P. aeruginosa* biofilms as a means to attenuate its pathogenicity could result in a promising and novel approach to limiting the attributed pathogenic effects of bacterial infections and preventing the recurrence of the disease.

Question and Hypothesis

The goal of this project was to examine the effects of periodic physical disturbances on the expression of QS-regulated virulence genes in *P. aeruginosa.* **The central hypothesis was that spatial disturbances uncouple the effective concentration of cells in biofilm and AI in the environment, thus causing a decrease in their ability to express virulence genes and promote virulence factors.** To address this hypothesis, three main aims were proposed: (1) to investigate the effect of periodic spatial disturbances on the distribution of biofilm and planktonic cells in *P. aeruginosa*, (2) to determine the effect of increasing shaking frequencies (/hr) on QS-regulated gene expression in *P. aeruginosa*, (3) to determine the effect of increasing shaking amplitudes (mm) on the expression of QS-virulence genes in *P. aeruginosa*. Current medical treatments for many bacterial infections still rely heavily on the use of antibiotics, which is a major contributing factor in the pathogenicity of infectious agents such as *P. aeruginosa*. As such, this research will shed light on some key aspects pertaining to virulence and attenuation of bacterial signaling

systems of *P. aeruginosa*. By providing a better understanding of the physical conditions that reduce its ability to perform quorum sensing, this research may lead to novel, and promising approaches to mitigate virulence that do not rely on the use of antibiotics and antimicrobial drugs.

Materials and Methods

Inoculating *P. aeruginosa* PA14

P. aeruginosa strain PA14 was used throughout this study (Table 1). Additionally, knockout strains of PA14 (ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR) were obtained from B. Bassler and were used to further examine gene expression [49]. Overnight cultures were initiated from single colonies isolated from Luria broth (MP Biomedical, Solon, OH) agar plate containing 1% agar (Alfa Aesar, Ward Hill, MA). To prepare an overnight culture, single colonies were inoculated into 3 mL Luria broth media [50] into 16 mL culture tubes (Genesee Scientific, Morrisville, NC), and were grown for 24 hours at 37°C at 250 revolutions per minute (RPM).

Growing *P. aeruginosa* Biofilms

Biofilms were grown using an MBEC (Minimum Biofilm Eradication Concentration) Assay Biofilm Inoculator Plates (Innovotech, Edmonton, AB, Canada). Overnight cultures were washed with King's A medium (KA) [51] and diluted 1:1000 into fresh KA media. 175μL of this culture was added to the 96-well MBEC microplate. To reduce evaporation during overnight incubation, the wells surrounding the culture were filled with 200 μL of autoclaved deionized water (DI H_2O) and the plate edges were sealed with two layers of parafilm. In addition, a beaker filled with ~100 mL of ddH_2O was also added to the incubator. The MBEC plate was then incubated at 37°C for 24 hours at 110 rpm. Additionally, biofilms were grown for 48 and 72 hours to confirm the development of the biofilm population. Growth media components can be found in Table 2.

Measuring Cell Density of *P. aeruginosa*

To measure the density of bacteria in the biofilm state, biofilms were grown as described above. The lid of the 96-well MBEC plate was then carefully removed, and the pegs were washed with 200 μL KA media for 10 seconds. Pegs on the MBEC lid containing biofilms were placed in

125 μL of 0.1% m/w crystal violet (Acros Organics, NJ, USA) in 95% ethanol (Fisher Bioreagents) and incubated at room temperature for 10 minutes. The MBEC lid was then sequentially washed four times in 200 μL of ddH_2O for 10 seconds. Excess crystal violet was removed from the biofilms by incubating the pegs in 200 μL of 30% acetic acid (Fisher Chemical) for 10 minutes. Absorbance was measured using optical density at 555 nm (OD_{555}) in a Victor X4 plate reader (Perkin Elmer, Waltham, MA). Background absorbance was measured using 30% acetic acid and was subtracted from all measurements. The density of bacteria in the planktonic state was measured using OD_{600} [52]. Background absorbance was measured using bacteria-free KA medium and was subtracted from all measurements to normalize measurements.

Periodic Disturbance of *P. aeruginosa* Biofilms

Biofilms were grown in KA medium as described above. After 24 hours, the pegs were washed in 200 μL of fresh KA media to remove unadhered cells. Biofilms were then placed in 180 μL fresh KA media and the plate was placed in a Victor X4 Perkin Elmer microplate reader pre-set at 37°C. The biofilms were then shaken at the frequency indicated in the text (0/hr, 3/hr, 6/hr, 12/hr, 15/hr) using a shaking amplitude of 0.3 mm (10 seconds per shaking event, linear shaking feature along the x-axis) for a total of 24 hours.

RNA Extraction

RNA extraction was performed using Qiagen RNeasy Mini Kit following the RNA Protect Bacteria Reagent Handbook 01/2015 with minor modifications. *P. aeruginosa* was grown as described above. 175 μL of media was collected from each well from the MBEC plate, placed in a 1.5 mL microcentrifuge tube, and was centrifuged for 2 minutes at 12,000 RPM. Cell pellets were washed and resuspended in 175 μL of fresh KA media and centrifuged again at 12,000 RPM for 2 minutes. The supernatant was discarded. The pellet was resuspended in 30 μL of 10 mg/mL lysozyme solution (MP Biomedicals) in 1X Tris-EDTA (Thermofisher) and vortexed every 2 minutes incubating at room temperature for a total of 20 minutes. Total RNA was then extracted following the manufacturer's recommended protocol, including the optional in-column DNase digestion stem using RNase-Free DNAse kit (Qiagen). An additional DNase step was performed after extraction using Qiagen DNAse Max Kit using the QuickStart protocol.

cDNA Synthesis

cDNA was synthesized using the Bio-Rad Reverse Transcription Supermix (Hercules, CA) following the manufacturer's recommended protocol. Reaction mixes were placed in Bio-Rad C1000 Touch Thermal Cycler, and cDNA synthesis was performed using the following protocol: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, and inactivation for 1 minute at 95°C.

qRT-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was performed using the iTaq Universal SYBR Green Supermix (BIORAD) kit following the manufacturer's instructions. Primers used for gene detection are listed in Table 3 and used at a 1:10 dilution. RT-qPCR pipetting super mix consisted of 5μL iTaq SYBR green, 3.5 μL RNA/DNAse-free water, 0.5 μL forward primer, 0.5 μL reverse primer, and 0.5 μL loading template (positive control cDNA, negative control RNA, or negative RT control water), for a total of 10 μL per well in an optical 8-tube strip. RT-qPCR was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System. The parameters for each RT-qPCR cycle were as follows: were 95°C for 3 minutes, 95°C for 10 seconds, 63°C for 30 seconds, (repeat 39X), 95°C for 5 seconds. A total of 40 cycles followed by melt-curve analysis was performed. Gene expression fold change was determined using normalized average threshold cycle (C_q) with the comparative C_t method [53] using the following equation:

$$\text{Gene Fold Change} = 2^{-\Delta\Delta C_T}\begin{bmatrix}(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ experimental group} \\ -\ (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ control group}\end{bmatrix}$$

All C_t values were normalized using the housekeeping gene *rpoD*. Additionally, C_q values were analyzed using the ΔC_t method [53].

Agarose Gel Electrophoresis

Amplicon specificity and size were confirmed using 1% agarose (Apex Bioresearch, Houston, TX) gel electrophoresis performed in 1X Tris-Acetate-EDTA (Fisher Scientific). Each gel contained 5 μL of SYBR Safe (Invitrogen, Carlsbad, CA). Electrophoresis was performed at

100 volts for 40 minutes. Band size was estimated using Gene Ruler 100bp plus DNA ladder (Fisher Scientific).

Statistical Analysis

All statistical tests were performed in JMP Pro 16 (SAS Institute Inc., Cary, NC) and RStudio 1.3.1073 (RStudio, PBC, Boston, MA). A Shapiro-Wilk test was used to assess normality. Mann–Whitney U test analysis was performed to determine significant differences in non-parametric datasets. Welch's t-tests were performed to determine significant differences in normally distributed datasets. P-values are indicated in the figure legend or in the text. Significant differences ($p < 0.05$) are indicated with an asterisk (*).

Results

Establishing Biofilms in the MBEC System

We first determined the ideal experimental conditions to grow robust *P. aeruginosa* biofilm structures using MBEC Biofilm Inoculator plates. The MBEC Biofilm Inoculator plate is composed of a 96-well plate and a lid comprised of 96 rounded polystyrene pegs. When submerged in liquid growth medium containing bacteria, the pegs act as an attachment surface enabling the aggregation and subsequent formation of a biofilm structure from the surrounding planktonic population of cells (Fig. 2). To this end, we grew *P. aeruginosa* biofilms for 24, 48, and 72 hours in two different environments: nutrient-rich, and nutrient-deprived (Fig. 3). Mature biofilms were established after 48 hours, with the nutrient-rich population growing significantly larger biofilms than the nutrient-deprived population (ANOVA, nutrient group p-value = 0.000411, time p-value = 2.23e-05, nutrient group and time p-value = 0.001084). There were no significant differences between 48 hours and 72 hours in nutrient-rich groups (t-test, p-value = 0.05714). Biofilms were greater at 72 hours for the nutrient-depleted group (t-test, p-value = 0.04456). As such, we established that replenishing liquid media in 48 hours old biofilms led to significantly denser biofilms.

Differences In Biofilm and Planktonic State Populations

Next, we wanted to determine how periodic disturbances to *P. aeruginosa* structures alter the distribution of biofilm and planktonic state cells (Fig. 4). Previous work by Quinn et al. demonstrated that the linear shaking operation on a microplate reader can be used to perturb the spatial arrangement of bacterial populations [54]. A single shaking event displaces bacteria in the biofilm into the planktonic state, as such, increasing the frequency of these events thus decreases the organization of the bacterial populations [41, 54]. Using this approach, we hypothesized that as shaking frequency increases so would the distribution of cells from the biofilm to the planktonic state. In order to test this, we grew mature *P. aeruginosa* biofilms for 24 hours, replenished the KA growth medium, and then perturbed them for another 24 hours at increasing shaking frequencies. We then measured the cell density of planktonic state cells using optical density at 600nm (OD_{600}), and biofilm state cells using crystal violet at (OD_{555}). We observed a nonlinear relationship between increasing shaking frequency and planktonic cell density (Fig. 4). As shaking frequency increases, so did the planktonic state cell density, with 6, 12 and 15 shakes/hour being significantly higher than the undisturbed control (0/hr) (6/hr (t-test, p-value = 3.933e-08), 12/hr (t-test, p-value = 7.717e-06), 15/hr (t-test, p-value = 0.002094). Planktonic cell density of 3/hr was not significantly different than 0/hr (t-test, p-value = 0.2775). We saw the inverse relationship in the biofilm state cells; the nonlinear relationship showed biofilm cell density decreasing as shaking frequency increases, with 12/hr and 15/hr being significantly lower than 0/hr (12/hr (t-test, p-value = 0.009162), 15/hr (t-test, p-value =0.0008128). Biofilm cell density of 3/hr and 6/hr were not significantly different than 0/hr (Wilcoxon, p-value = 0.05032; Wilcoxon, p-value = 0.203). Thus, we hypothesized that after the bacterial structures reach a threshold of disturbances in their environment (> 12/hr) shear forces promote aggregation back to the biofilm, thus limiting the effect of the linear shaking on cell dispersal.

Changes in the Expression of Genes Involved in QS Occur at 12/hr

Our results showed that there were changes in *P. aeruginosa* cell density in both planktonic and biofilm populations owing to physical perturbations in their environment. As such, we then sought to determine how these changes in cell density influenced the expression of major QS-regulated virulence factors. Given the complex hierarchal structure of the QS pathway/system, we

grouped the results according to the major QS suite into three main groups: *las, rhl,* and *pqs.* Similar to the nonlinear relation between cell density and shaking frequency, expression of QS-regulated genes also followed a similar trend, with most genes from all suites being significantly downregulated at 12 /hr (Fig. 5).

Expression of the *las* suite of genes followed the same trend, with regulator *lasR*, and effector genes *lasA, lasB,* and *aprA* being significantly downregulated at 12/hr (Fig. 5A) (Wilcoxon, p-value = 0.02443, p-value = 0.0007816, p-value = 0.0007816, p-value = 0.002468). Similarly, all genes in *rhl* suite were significantly downregulated at 12/hr, including transcription regulator *RhlR*, and effector genes *rhlA, lasB,* and *hcnA* (Fig. 5B) (Wilcoxon, p-value = 0.002953, p-value = 0.01061, p-value = 0.0007816, p-value = 0.0004936). Interestingly, *rhlA* was upregulated at 3/hr (Fig. 7A) (Wilcoxon, p-value = 0.00279). Lastly, the genes of the *pqs* suite were also downregulated at 12/hr, with *pqsR* and *hcnA* being significantly downregulated at 12/hr (Fig. 5C) (Wilcoxon, p-value = 0.007898, p-value = 0.0004936). While our cell distribution results suggested that the frequency of periodic disturbance influenced the distribution of bacteria in the system, our gene expression analysis demonstrated that virulence factors were only significantly downregulated when disturbed at 12/hr and with a shaking amplitude of 0.3mm. Overall, we observed major decreases in the expression of most QS-virulence genes accounting for exotoxins, proteases, and rhamnolipids at 12/hr.

Importantly, we performed an agarose gel-electrophoresis to confirm the amplicon specificity of the qRT-PCR products (Fig. 6). We saw clear bands at around 300bps in lanes 1, 4, 7, and 10 corresponding to cDNA/primer amplicons *rpoD, aprA, rhlA,* and *phzM* from planktonic *P. aeruginosa* undisturbed condition (0/hr) (Fig.5 A). As such, we concluded and confirmed that there were no primer-dimers or genomic DNA contamination in RNA and water controls.

Changes in Cell Density at Varying Shaking Frequencies and Amplitudes

Prior work established that amplitude, *i.e.*, the distance of an isolated linear shaking event, also influences the distribution of cells [41]. Thus, we wanted to investigate whether a combination of physical forces of increasing shaking frequency and amplitude were responsible for the decrease in cell distribution. We selected the frequencies of 6/hr, 12/hr, and 15/hr as these resulted in the most drastic increase in planktonic cell density when disrupted at 0.3mm (Fig. 4). As such we sought to assess the combined effect of select shaking frequencies 6/hr, 12/hr, and 15/hr over a

range of shaking amplitudes 0.1mm, 0.2mm, 0.3mm, and 0.4mm on the distribution of *P. aeruginosa* cell density (Fig. 7).

Disturbing biofilms at 0.1 and 0.2mm at 6/hr alters the cell distribution in the biofilm state (Fig. 7A) (t-test, p-value = 0.0301; p-value = 0.009744). While there were no significant differences in planktonic cell density at 0.1 and 0.2mm (t-test, p-value = 0.1102; p-value = 0.4261), or in biofilm at 0.3mm (Wilcoxon, p-value = 0.203), there was a significant increase distribution of planktonic state cells at 0.3mm (t-test, p-value = 3.933e-08).

At 12/hr, biofilm density was significantly decreased when perturbed at 0.2, 0.3, and 0.4mm (Fig. 7B) (t-test, p-value = 0.02563; p-value = 0.009162; p-value = 0.006725). Planktonic cell density was significantly higher at 0.3 and 0.4mm (t-test, p-value = 7.717e-06; p-value = 0.0004025). Lastly, planktonic cell density was significantly higher at 0.3mm at 15/hr (Fig. 7C) (t-test, p-value = 0.002094). Biofilm density was statistically reduced at 0.3 mm compared to the control (t-test, p-value = 0.0008128).

These results suggest that across a wide range of shaking frequencies, disturbing the biofilm structure at 0.3 mm consistently results in the most significant change in cell distribution. While lower amplitudes had a stronger effect on biofilm density at lower shaking frequencies (6/hr), higher amplitudes reduced biofilm cell density at higher shaking frequencies (15/hr). However, our findings show that disturbing *P. aeruginosa* at 12/hr across several shaking amplitudes resulted in significant differences in cell distribution at 0.2, 0.3, and 0.4. This suggests that perhaps a combination of both frequency and amplitude at this range is sufficient to alter and dysregulate cooperation and formation of biofilm structures.

Lower Shaking Amplitudes Lead to Significant Decreases in QS Gene Expression

Next, we examined the effect of both shaking amplitude and shaking frequency on the expression of QS-virulence factors of *P. aeruginosa*. We selected single-input effector genes, *i.e.*, genes that are predominantly dependent on a single transcription node, from each major QS suite to establish direct effect from their respective receptor systems. As such, we determined the expression of effector genes *aprA*, *rhlA*, and *phzM* in *P. aeruginosa* when perturbed at a range of 0.1-0.4mm and 6/hr, 12/hr, and 15/hr (Fig. 8).

Disturbing biofilms at 0.1, 0.2, and 0.3mm at a frequency of 6/hr caused a significant decrease in the expression of *aprA* at 0.1mm and 0.2mm (Wilcoxon, p-value = 0.04848; p-value =

0.01616) (Fig. 8A). Expression of all other genes (*rhlA*, *phzM)* were not significantly different than in the undisturbed control (Wilcoxon, p-value = 0.6042, p-value = 0.4606, p-value = 1, p-value = 0.5697).

Disturbing biofilms at 0.1, 0.2, 0.3 and 0.4mm at a frequency of 12/hr caused the strongest significant decrease in the expression of QS-effector genes (Fig. 8B). At 0.1mm, effector genes *aprA* and *rhlA* were significantly downregulated (Wilcoxon, p-value = 0.00836; p-value = 0.01119). At 0.2mm and 12/hr, *aprA*, *rhlA* and *phzM* were significantly downregulated (t-test, p-value = 0.002604; p-value = 0.002604; p-value = 0.008481). At 0.3mm, only *aprA* and *rhlA* were significantly downregulated (Wilcoxon, p-value = 0.002468; p-value = 0.002953). Lastly, all effector genes (*aprA*, *rhlA* and *phzM*) were significantly downregulated when disturbed at an amplitude of 0.4mm (Wilcoxon, p-value = 0.0003108; p-value = 0.02551; p-value = 0.04009). Expression of *phzM* was not statistically different than 0/hr at 0.1mm and 0.3 mm (t-test, p-value = 0.05191; Wilcoxon, p-value = 0.2359).

Finally, perturbing biofilms at 0.1, 0.2, and 0.3mm at a frequency of 15/hr resulted in a significant decrease in the expression of all three effector genes at 0.1 and 0.2 mm (Fig. 8C). At 0.1mm, QS-effector genes *aprA*, *rhlA* and *phzM* were significantly downregulated (Wilcoxon, p-value = 0.00404; p-value = 0.01119; p-value = 0.02828). At 0.2 mm and 15/hr, all three single-node QS effector genes *aprA*, *rhlA* and *phzM* were significantly downregulated (t-test, p-value = 0.0001245; p-value = 0.000397; p-value = 0.001463). Effector genes were not significantly different from the undisturbed (0/hr) at 0.3mm (t-test, p-value = 0.1804; p-value = 0.6634; p-value = 0.8122).

Overall, we found that disturbing *P. aeruginosa* at lower amplitudes causes significant decreases in gene expression of QS-virulence effector genes over a wide range of shaking frequencies.

Using Knockout Strains to Ascertain Mechanisms During Shaking Events

Lastly, we examined the effect of periodic disturbances on QS-deficient strains of *P. aeruginosa* PA14 lacking *las* and *rhl* receptors (ΔLasR, ΔRhlR, and ΔLasRΔRhlR) to ascertain which QS-pathway was most affected during shaking events. These knockout strains were grown as previously described and disturbed at 12/hr at an amplitude of 0.3mm for 24 hours. The cell

density of planktonic cells was measured with OD_{600}, and biofilm cell density was quantified using crystal violet and OD_{555} at 0/hr and 12/hr (Fig. 9).

There were no significant differences in planktonic cell density between WT PA14 and ΔLasR at 0/hr (t-test, p-value = 0.1871). However, planktonic cell density of ΔLasR PA14 was significantly higher after 12/hr compared to the undisturbed (0/hr) ΔLasR PA14 control (t-test, p-value = 0.003937). Interestingly, we found no significant differences in biofilm cell density between 0/hr ΔLasR and 0/hr WT PA14 (t-test, p-value = 0.3051) as well as 12/hr ΔLasR and 12/hr WT (Wilcoxon, p-value = 0.2977), which could indicate that in WT, shaking events disrupt the *las* system most significantly.

Additionally, there were no significant changes in planktonic cell density between WT and ΔRhlR PA14 at 0/hr (t-test, p-value = 0.305), nor after disturbance at 12/hr (t-test, p-value = 0.8218). Biofilm cell density of the ΔRhlR KO was also not significantly different than the WT at 12/hr (t-test, p-value = 0.2211). Planktonic cell density of ΔLasRΔRhlR PA14 was significantly different from the WT PA14 at 0/hr (t-test, p-value = 0.01472), but not from ΔLasRΔRhlR 12/hr (t-test, p-value = 0.3792), indicating that disturbances did not affect planktonic cell density of double mutant. Biofilm cell density of ΔLasRΔRhlR PA14 was significantly higher than the WT at 12/hr (Wilcoxon, p-value = 0.007937).

Gene Expression of QS Virulence Factors Decrease in Knockout Strains

Initial findings show a grossly exaggerated decrease in the expression of QS receptor and effector genes in the mutant strains ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR using the gene expression of WT PA14 at 0/hr as the comparative control group (Fig. 10). Baseline expression of QS receptors *lasR*, *rhlR*, and *pqsR* were significantly downregulated at 0/hr for knockout strains ΔLasR (t-test, p-value = 0.001232; p-value = 0.0002712; p-value = 0.0008902) and ΔLasR/ΔRhlR (t-test, p-value = 0.04329; p-value = 0.005316; p-value = 0.0008333) compared to WT at 0/hr (Fig. 10A). Baseline expression of QS receptor *rhlR* and *pqsR* was also significantly downregulated at 0/hr compared to WT for knockout strain ΔRhlR (t-test, p-value = 0.04444; p-value = 2.188e-05). Expression of *lasR* was not downregulated for ΔRhlR at 0/hr (t-test, p-value = 0.1381) (Fig.10A).

Periodically disturbing at 12/hr caused a further decrease in the expression of all QS-receptor genes in WT and KO strains. More concretely, when compared to WT at 0/hr, ΔLasR showed a decrease in *lasR*, *rhlR*, and *pqsR* expression at 12/hr (t-test, p-value = 0.0001456; p-value = 4.316e-05; p-value = 0.002886). Compared to WT at 0/hr, ΔRhlR also showed a decrease in *lasR*, *rhlR*, and *pqsR* expression at 12/hr (t-test, p-value = 0.001021; p-value = 0.002293; p-value = 0.007273). Lastly, ΔLasR/ΔRhlR also had a decrease in QS-receptor expression at 12/hr (t-test, p-value = 0.02072; p-value = 0.01304; p-value = 0.03944).

We then measured the baseline expression of QS-effector genes at 0/hr compared to WT at 0/hr (Fig. 10B). Effector genes *aprA*, *rhlA* and *phzM* were not significantly downregulated in ΔLasR at 0/hr (t-test, p-value = 0.2532; p-value = 0.3007; p-value = 0.2062). Baseline expression of effector gene *rhlA* was significantly downregulated in ΔRhlR at 0/hr compared to WT (t-test, p-value = 0.0004085). Effector genes *aprA* and *phzM* were not significantly different from undisturbed WT in ΔRhlR at 0/hr (t-test, p-value = 0.6388; p-value = 0.09744). Effector genes *aprA* and *rhlA* were significantly downregulated in ΔLasR/ΔRhlR at 0/hr compared to the undisturbed WT (t-test, p-value = 0.03142; p-value = 0.0002353), but not *phzM* (t-test, p-value = 0.2322).

After periodic disturbance events at 12/hr, expression of effector genes was significantly reduced across all mutant strains compared to WT at 0/hr (Fig. 10B). Interestingly, gene fold change of effectors *aprA* and *rhlA* were significantly reduced in both WT (Wilcoxon, p-value = 0.01061; p-value = 0.002468) and ΔLasR (t-test, p-value = 0.004487; p-value = 0.0005081) at 12/hr compared to WT at 0/hr. Expression of effector gene *phzM* was not significantly reduced in WT or ΔLasR at 12/hr (Wilcoxon, p-value = 0.2359; p-value = 0.8081). Expression of all effector genes was significantly downregulated in KO strain ΔRhlR at 12/hr (t-test, p-value = 0.02828; p-value = 0.002293; p-value = 0.01443). Expression of effector genes *aprA*, *rhlA*, and *phzM* were significantly reduced in ΔLasR/ΔRhlR strain at 12/hr compared to WT (t-test, p-value = 0.006721; p-value = 0.0002461; p-value = 0.04137).

These initial findings establish that mutant strains indeed do not express QS-receptors LasR and RhlR, as there is an over 1000 gene fold reduction in expression compared to the WT. Furthermore, these findings demonstrate that before periodic disturbance, there is already a

decrease in expression of some receptors (*pqsR*) and effectors (*rhlA*) in some of the mutants. Lastly, these findings demonstrate that in both WT and KO strains, periodic disturbances cause a downregulation in the expression of QS genes.

Periodic Disturbances Alter Expression of QS Genes Regardless of KO

The expression of QS genes *aprA, rhlA, pqsR* and *phzM* in KO strains were quantified after periodic disturbance at 12/hr. Gene fold change was normalized to housekeeping gene *rpoD*, and compared to gene expression of WT at 12/hr (Fig. 12). Compared to WT at 0/hr, WT at 12/hr shows significant decrease in expression of *pqsR, aprA,* and *rhlA* (Wilcoxon, p= 0.007898, p=0.002468, p=0.002953). Significant differences in QS gene expression indicate that disturbing at 12/hr siginificantly downregualtes expression of QS genes compared to WT at 12/hr.

After disturbance at 12/hr, ΔLasR showed a significant decrease in QS receptor *pqsR* compared to WT at 12/hr (Wicoxon, p-value = 0.002797), but not in effector genes *aprA, rhlA*, or *phzM* (Wicoxon, p-value = 0.503; p-value = 0.05035; p-value = 0.3301). Compared to WT at 12/hr, KO ΔRhlR showed a decrease in expression of *rhlA* (t-test, p-value = 0.03908), but not in *aprA, pqsR,* or *phzM* (t-test, p-value = 0.6105; p-value = 0.3743; p-value = 0.9844). Lastly, expression of *rhlA* and *pqsR* were significantly reduced in ΔLasR/ΔRhlR compared to WT at 12/hr (t-test, p-value = 0.01557; p-value = 0.01557). The expression of QS effector genes *aprA* and *phzM* was not statistically different from the gene expression of WT at 12/hr (t-test, p-value = 0.7278; p-value = 0.232). These results illustrate that, comparatively, periodically disturbing WT and KO PA14 biofilms alter the expression of QS-virulence genes *aprA, rhlA, pqsR* and *phzM* in a similar manner. This suggests that periodic disturbances alone can disrupt the QS and transcription ability of WT *P. aeruginosa* to mimic or resemble that of a strain with non-functional QS system (i.e., ΔLasR).

Finally, we performed agarose gel-electrophoresis to confirm the amplicon specificity of the qRT-PCR products on three biological replicates of the QS-deficient strain ΔRhlR (Fig. 12). There were clear bands near 200bp corresponding to cDNA/primer amplicons *aprA* and *phzM* from planktonic ΔRhlR controls 0/hr. On the other hand, there were very faint bands in wells 1-3 corresponding to gene expression of *rhlA* (0/hr) (Fig. 10B). As these bands were barely visible, it

is likely that the deletion of the *rhl* receptor in this knockout strain entirely modulated the expression of *rhlA.*

Discussion

Previous studies have shown that periodically disturbing spatially organized bacterial communities using physical linear force decouples the effective concentration of bacteria and autoinducers in their environment [41, 54]. Quinn *et al* further demonstrated that, under certain environmental and growth conditions, periodic disturbances hinder the production of the fluorescent siderophore pyoverdine in *P. aeruginosa* [54]. However, the extent to which QS-mediated gene expression is affected by physical forces had yet to be investigated in *P. aeruginosa.*

Our initial findings suggest that the specific shaking frequency of periodic disturbances aof 12 shakes per hour (12/hr) strongly attenuates the expression of QS-virulence genes in *P. aeruginosa.* Our initial results suggested that the uncoupling of bacteria from the biofilm into the planktonic state would dictate the extent to which QS genes were affected. Literature states that frequent periodic disturbances to a biofilm lead to a higher cell density of free cells, which, unlike biofilm state cells, do not have proper access to AIs needed for QS, as they diffuse rapidly in the media [41]. However, this was not the case for a great part of our data. The changes reported in gene expression could not be solely attributed to the significant changes seen in cell density distribution, since there was a greater distribution of planktonic cells at 6/hr and 15/hr (Fig.4), but no changes in QS gene expression at those specific shaking frequencies (Fig. 5). Instead, we found that QS-regulated genes were most persistently downregulated at a specific shaking frequency of 12/hr compared to the undisturbed control 0/hr (Fig. 5). All major components of the QS-system were affected at this 12/hr: *las, rhl*, and *pqs.* Additionally, we saw a dramatic downregulation of almost all downstream effector genes, accounting for exotoxins, proteases, and rhamnolipids, with the notable exception of *phzM* (Fig. 5C). This implies that at a sufficiently high enough disturbance frequency, *P. aeruginosa* halts a majority of QS-mediated transcription, and instead depends on *pqs*-mediated QS to promote behaviors that ensure survival, colonization, and immune evasion when *las* and *rhl* QS is indisposed [34, 46].

Additionally, we found that a combination of physical forces of increasing shaking frequency and amplitude disturbed the structural integrity of *P. aeruginosa* biofilms, as this

combination of forces caused an increase in cell distribution from biofilm to planktonic states. While our working hypothesis is that linear shaking will disrupt the structural organization of biofilms and uncouple AI concentration, it is well established that biofilms can withstand significant mechanical, biological, and chemical stress [38, 55]. However, this does not mean biofilms are entirely resistant to physical pressures. Non-motile bacteria, such as *S. aureus,* benefit from shear forces in their environment as it enables shear-mediated rolling migration. This mechanism allows *S. aureus* to transport part or most of the aggregated biofilm community to a new environment when fluid shear forces surpass the viscoelasticity of the biofilm [56]. In contrast, motile organisms, such as *P. aeruginosa*, are more likely to resist normal and shear forces and are less susceptible to detachment as their biofilms are viscoelastic. In fact, biofilms exposed to high shear forces have a greater and more strongly attached EPS matrix than biofilms grown with lower shear [27, 39, 55]. Interestingly, we found that perturbations at higher shaking amplitudes significantly reduced biofilm cell density across all shaking frequencies (Fig. 7), while lower amplitudes seem to promote larger biofilms (Fig. 7A). A single shaking event in the microplate reader assay lasts 10s, in which the plate reader displaces the MBEC biofilm plate by 0.1, 0.2, 0.3, or 0.4mm for any given shaking frequency (/hr). As such, it is likely that displacing the biofilms on the pegs at lower amplitudes (smaller distance) for the same amount of time as their high amplitude counterparts (larger distance) exposes the biofilms to higher liquid shear forces. As such, our findings suggest that disturbing biofilms at 12/hr results in a wider range of effective shaking amplitudes that lead to a more significant proportion of cell displacement (Fig. 7B). This could mean that more frequent exposure to low shear forces strongly displaces bacteria from the biofilm as seen in 12/hr and 15/hr.

Interestingly, perturbing *P. aeruginosa* biofilms at lower amplitudes led to significant decreases in the expression of virulence factor effector genes over a wide range of shaking frequencies. While receptor expression provides an insight into the total possible network activity in the cell, virulence factor effector expression instead directly mirrors what is happening in that particular node. As such, we selected single-input effector genes, *i.e.*, genes that are predominantly dependent on a single transcription node, from each major QS suite to establish direct effect from their respective receptor systems. Expression of alkaline protease *aprA* was significantly reduced in both 6/hr and 15/hr at lower shaking amplitudes (Fig. 8A, Fig. 8C), but was not significantly

different from the undisturbed control (0/hr) at 0.3mm. The expression of *aprA* is directly regulated by *lasR* but is generally not considered a necessary metabolite for aggregation and biofilm formation [45]. This could indicate that at lower amplitudes the pathogenic behavior regulated by the *las* system is downregulated. On the other hand, expression of all effector genes, *aprA, rhlA,* and *phzM,* were significantly downregulated at any given amplitude of 12/hr (Fig. 8B). This discrepancy in gene expression reduction is likely attributed to the diminished distribution of biofilm and planktonic cells.

Bacteria embedded in biofilms accumulate AI more readily and perform QS at higher rates than those dispersed in media [34, 38, 57]. However, both 6/hr and 15/hr had limited cell dispersal from the biofilm at lower amplitudes, and as such, it is likely that a majority of QS-performing colonies are encased in the biofilm instead. Furthermore, since QS is both population and AI-dependent, poor availability of AI access in planktonic state cells at 6/hr and 15/hr likely limits QS. On the other hand, the drastic distribution of biofilm to planktonic cells at 12/hr is indicative that there is a sufficiently high population to perform QS. While the transcription of pyocyanin (*phzM*) at 12/hr + 0.3mm was not significantly different from the undisturbed control (0/hr), several other key QS-receptors and effectors were significantly downregulated (Fig. 5). This suggests that combining low shear forces and high-frequency disturbances (such as 0.3mm and 12/hr) can be used to modulate and attenuate key virulence factors in *P. aeruginosa.*

Owing to the highly interconnected pathways in *P. aeruginosa*, it is difficult to establish the exact mechanism or QS-pathway activated during periodic disturbance events. Examining the response to physical perturbations in QS-deficient knockout strains can provide valuable insights into the mechanisms employed by *P. aeruginosa.* Our findings illustrate key differences in cell dispersal and biofilm formation in mutant strains perturbed at 12/hr + 0.3mm (Fig. 9). Planktonic cell density was highest in WT and ΔLasR, and lowest in ΔRhlR and ΔLasR/ΔRhlR. Inversely, biofilm density was the lowest in WT and ΔLasR and the highest in ΔRhlR and ΔLasR/ΔRhlR. Interestingly, while there were significant differences in planktonic cell density between WT and ΔLasR/ΔRhlR, there were no significant differences between WT and ΔLasR (Fig. 9). Considering the roles of *las, rhl,* and *pqs* in biofilm formation, it is interesting to note that ΔLasR did not have the least biofilm density. In fact, *las* effectors must be transcribed in tandem with *rhl* and *pqs*

effectors to modulate the production of elastase, pyocyanin, and rhamnolipids necessary for attachment [8]. Elastase B (*lasB*) is promoted by both *lasR* and *rhlR*, and as such can still be transcribed in a ΔLasR strain, accounting for biofilm formation [58]. Similarly, under loss of function of *rhlR*, *lasR* continues to promote transcription of *lasB* and *pqsR* effectors, such as pyocyanin [38, 49], still forming biofilms to a lesser extent. Interestingly, complete inhibition of *lasR* and *rhlR* does not damper virulence, as ΔLasR/ΔRhlR knockouts display uncontrolled and unmitigated expression of virulence factors and are associated with higher pathogenicity *in vivo* [49, 59, 60]. These findings concur that WT phenotype behaviors most resemble those of ΔLasR under physical stress, further implying that periodic disturbances effectively disrupt the functionality of the *las* QS suite.

Gene expression analysis revealed that the baseline gene fold change of QS receptors and effectors was reduced by several orders of magnitude in the ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR strains compared to WT at 0/hr (Fig. 10). The initial gene expression of QS receptors *lasR*, *rhlR*, and *pqsR* was already significantly reduced for the knockout strains compared to WT, and periodic disturbances further amplified this lack of expression particularly in *lasR* for ΔRhlR. The most notable difference between the plots at 0/hr and 12/hr is the significant decrease in the expression of *pqsR* across all knockout strains after periodic disturbance at 12/hr (Fig. 10A). A possible explanation behind this finding could be related to the gene excision using λ-red recombinase by Bassler *et. al.* before we acquired the mutant strain [49, 61]. However, considering the magnitude of downregulation of gene expression of the receptor genes in the knockout strains (Fig. 10), it is more likely that the perceived difference in our comparative gene expression analysis is an artifact from amplification of some trace expression of the receptor [61] which would account for such an extreme decrease in gene expression compared to WT. While the decrease in *lasR* and *rhlR* also seem extreme, the subsequent downstream effect on *pqsR* expression demonstrates that as the system loses integrity and robustness, PA14 becomes more sensitive to changes in environment.

Additionally, we observed significant changes in the expression of virulence genes *aprA*, *rhlA*, and *phzM* in WT and knockout strains at 12/hr (Fig. 10B). The baseline expression of single node effectors was not significantly different from WT at 0/hr in ΔLasR strain. However, the baseline expression of *rhlA* at 0/hr was significantly lower than WT in ΔRhlR and ΔLasR/ΔRhlR

strains owing to the limited functionality of the *rhl* system in these knockout strains. Periodic disturbance at 12/hr caused a significant decrease in effector gene expression across all major QS suites in ΔRhlR and ΔLasR/ΔRhlR compared to the WT, accounting for significant reduction in the transcription of virulence factors related to toxicity and colonization. Interestingly, WT and ΔLasR showed similar significant reductions in the expression of *aprA* and *rhlA*, an expected result from a system lacking *las* and therefore limited transcription to downstream *las* effectors such as alkaline protease and rhamnolipids [45, 62]. WT and ΔLasR did not show any significant changes or reduction in the expression of *pqs* effector *phzM*, in turn owing to the robustness of the *pqs* system when *las* is disrupted or non-functional under environmental stress [34, 50]. As such, our findings concur that disrupting the spatial structures with an already unbalanced QS system further interferes with the organism's ability to display pathogenic behaviors related to toxicity, biofilm formation, and colonization.

Finally, we noted that the periodic disturbances can hinder QS stability. Comparative gene expression analysis of single node effectors in WT and KO strains showed some similarities and differences in fold change when disturbed at 12/hr (Fig. 11). Comparing the effect of periodic disturbances at 12/hr on WT and KO strains,

The expression of QS receptor *pqsR* in ΔLasR strains was significantly reduced compared to WT when disrupted at 12/hr, a reduction that could be highly attributed to the overtaking of the *rhl* suite in a *las* deficient system, as *rhlR* inhibits transcription of *pqsR* (Fig. 1).

Notably, our findings shows that there were no significant differences between WT and KO strains at 12/hr for QS effector genes *aprA, phzM,* and *rhlA* (Fig. 11). This strongly suggests that disturbances at 12/hr alter the behavior of WT PA14 to resemble that of strains with faulty QS systems, most similarly to that of ΔLasR, perhaps making it more susceptible to changes in its environment at a transcriptional level. As such, our findings illustrate that QS system stability determines susceptibility to environmental disturbances.

In absence of LasR, clinical strains of *P. aeruginosa* display a diminished expression of *las* and *rhl* genes and thus depend on the *rhl* and *pqs* systems to regulate pathogenicity [32, 49, 62]. Moreover, *in vivo* studies have shown that double null strains of ΔLasR/ΔRhlR *P. aeruginosa* display enhanced virulence as a result of an uninhibited and poorly controlled upregulation of *pqs*

and limited *rhl* genes (Fig. 1) [34, 49]. However, none of our findings display any significant increase or upregulation of QS virulence genes in WT or knockout genes that would suggest higher virulence and pathogenic behaviors. In fact, our findings continuously demonstrate a reduction in transcription and expression of QS virulence genes in knockout and WT strains of *P. aeruginosa* alongside an increase in cell dispersion from periodic disturbances. While higher cell density and distribution in the planktonic media might suggest higher rates of QS and virulence factor transcription, our results show that QS is consistently and significantly reduced in a disturbed environment. As such, while there are actively more detached cells in the planktonic state, they are unable to effectively detect AI, communicate, and coordinate the expression of virulence genes, and as such, display significantly limited virulent and pathogenic behaviors. Moreover, we found that a combination of both the amplitude and frequency of spatial disturbances can disrupt the effectiveness and function of the quorum sensing system in WT *P. aeruginosa*, contributing to higher cell dispersion and lower gene expression of virulence factors as the *las* system is most readily affected and causes a downstream disruption on the other QS suites systems. We believe this demonstrates that spatial arrangement and access to AI in *P. aeruginosa* biofilm environment plays an equally as important role in the attenuation of virulence as the presence and functionality of transcription regulators in the QS system itself.

These findings may have implications for possible future intervention protocols in clinical settings that aim to reduce the spread and virulence of pathogenic *P. aeruginosa* on sensitive medical equipment, such as ventilators and catheters [13, 40, 63]. Most medical equipment is sterilized using chemical germicides (soap, chemical disinfectants) or physical methods (pasteurization, autoclaving, plasma gas sterilization) [64]. However, despite current practices and best efforts to maintain sterile conditions, many drug-resistant bacteria, such as *Staphylococcus epidermidis, S. aureus,* and *P. aeruginosa* will rapidly and easily contaminate and colonize prosthetic, implantable, and life-support medical devices [11, 65]. As such, applying periodic disturbances to these devices could provide an additional method or step in the sterilization process to prevent formation of resistant biofilm colonies, and to disarm *P. aeruginosa* from its QS system. Moreover, these findings demonstrate the effects of a successful non-antibiotic approach used to mitigate virulence and spread of a notorious nosocomial pathogen. These methods could be used

in tandem or as an adjuvant in more biofilm studies, in non-synergistic bacterial infections, or as a preventative method in clinical settings [66].

However, this study presents two major limitations that should be addressed in future research. First, we only examined the effect of periodic disturbances on gene expression from planktonic state cells. While this approach greatly illustrated the dynamics of cell dispersion and QS in detached *P. aeruginosa* cells, there is much we do not yet know about the dynamics and behaviors of the cells in the biofilm. Performing qRT-PCR on biofilm state cells using the MBEC growth methodology proved arduous and resulted in inconsistent RNA/ cDNA extractions (data not shown) given the amount of extracellular genomic material naturally found in EPS biofilms [50, 66, 67]. As such, future prospects should therefore implement more suitable RNA extraction techniques in order to thoroughly investigate the effects of periodic disturbances on gene expression of QS-mediated virulence genes of adhered *P. aeruginosa* biofilm state cells as well.

Another limitation this study presents pertains to the sterile nature of *in vitro* experimentation. While there are a great number of advantages to performing *in vitro* studies, such as being able to extrapolate direct causation in complex systems, *in vitro* growing conditions rarely mimic the natural environments pathogens such as *P. aeruginosa* or other QS-bacteria usually colonize. As such, future research must indeed apply these findings to more representative growing conditions to account for naturally occurring events, such as polymicrobial co-cultures commonly found in infectious sites (i.e., *S. aureus* and *P. aeruginosa*) [37, 68-70] and exposure to higher environmental stress [57, 70-73] in addition to physical periodic disturbances.

Additionally, we propose to further examine the effect of periodic disturbances on the QS pathway of *P. aeruginosa* using a mathematical model to predict expected bacterial phenotypes and behaviors based on specific input conditions (AI detection, ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR experiments, amplification and frequency tests, and baseline QS network schematics). Delineating *P. aeruginosa* QS dynamics would provide valuable insight on the mechanics of virulence and AI detection in disturbed environments [39, 74].

Lastly, we propose to examine the effect of periodic disturbance on QS stability. QS mediates a plethora of both essential and pathogenic behaviors, including motility, AI production, and transcription of antimicrobial resistance genes [37, 46, 75]. As select physical forces disrupt

QS transcription and function in *P. aeruginosa*, future research should therefore examine the effect of periodic disturbances under different environmental conditions (such as high AI environment, antiseptic environment, or with antibiotic treatment) to determine extent of QS system function and colony susceptibility under environmental stress.

Conclusion

For the past 100 years, we have been in an antibiotic arms race with infectious agents, slowly losing the battle against infection and antimicrobial resistance. Pathogenic bacteria, such as *P. aeruginosa* and *S. aureus*, confer AMR is through QS, a population density-dependent communication mechanism that relies on the secretion and detection of AIs to propagate infection and coordinate expression of virulence factors.

There is a growing interest in the development of non-antibiotic-based techniques to attenuate pathogenicity and virulence of infectious agents in clinical settings. As such, the main goal of this study was to examine the effect of spatial periodic disturbances on the expression of QS-regulated virulence genes in *P. aeruginosa.* Our central hypothesis was that spatial disturbances (i.e., linear shaking) uncouple the effective concentration of cells in biofilm and AI in the environment, causing a decrease in detection and expression of virulence genes.

First, we determined that a combination of frequency and amplitude of shaking produced the greatest increase in cell dispersion from the biofilm, with the frequency of 12/hr resulting in the most dispersed biofilm and planktonic cell populations at any given amplitude (0.2, 0.3, 0.4mm) (Figs.4 & 7). Next, we found that most QS receptors and effector genes from *las, rhl,* and *pqs* QS system were greatly and significantly downregulated when perturbed at 12/hr compared to the 0/hr control (Fig. 5). We then determined that disturbing *P. aeruginosa* at lower amplitudes causes significant decreases in gene expression of QS-virulence effector genes over a wide range of shaking frequencies due to the possible combined effects of shear force and frequent disturbances (Fig. 8). Lastly, we determined that periodic disturbances affect WT PA14 in a similar manner as ΔLasR, causing an overall decrease in expression of *las*-mediated effector genes for alkaline protease and rhamnolipid (Figs. 10 & 11). Overall, we were able to localize a range of

physical disturbances that limit and dysregulate QS-mediated transcription of virulence genes in pathogenic strains of *P. aeruginosa*.

These results may have implications for possible intervention methods in clinical settings that aim to reduce virulence of *P. aeruginosa* on medical equipment. Moreover, these findings demonstrate the effects of a non-antibiotic approach to mitigate virulence and spread of a notorious nosocomial pathogen.

To address the major limitations of this study, we propose to further investigate the effect of periodic disturbances on QS-virulence gene expression of biofilm states cells in *P. aeruginosa.* Understanding this could provide major insight into the dynamics of biofilm formation and dispersal, AI synthesis, and nutrient scavenging at a transcriptional and molecular level. We further propose to investigate the effect of periodic disturbances on polymicrobial conditions, as well as under different environmental conditions, to further examine the effect of spatial structure in QS stability. Finally, we propose to delineate *P. aeruginosa* QS system dynamics during disturbance using a mathematical model to better understand the mechanics of virulence and AI detection, and to be able to predict expected bacterial phenotypes and behaviors based on specific input conditions.

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Appendix

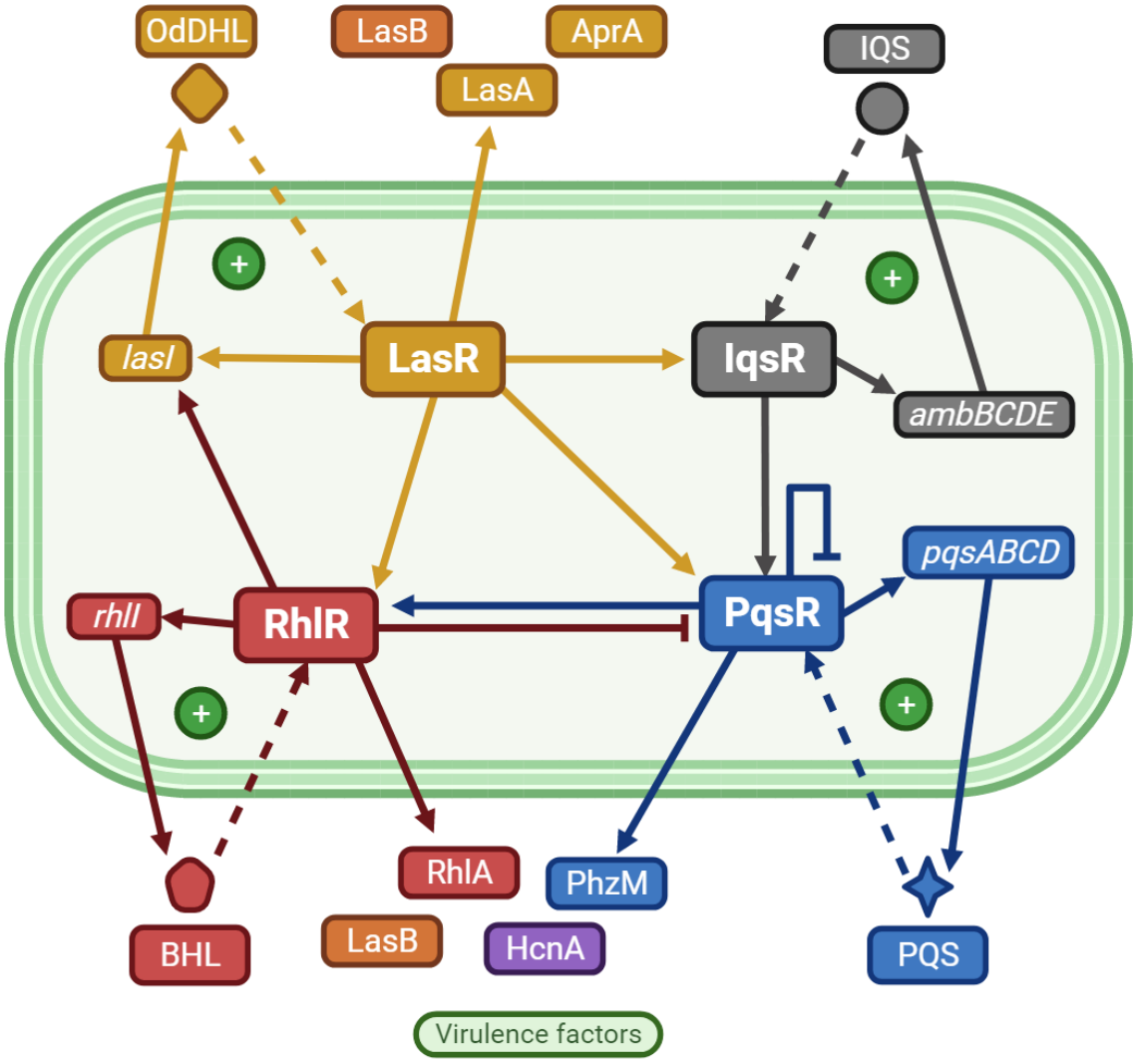

Figure 1: The *P. aeruginosa* quorum sensing system is a highly interconnected hierarchy of autoinducing receptors and transcription regulators that modulate the expression of broad class exotoxins, metalloenzymes, and biosurfactants. The expression of these virulence factors is highly regulated by the concentration of AI detected in the surrounding environment, the interplay between the receptors of each suit of QS genes, and the activation of positive AI-QS-Receptor feedback loops. At a sufficiently high concentration of OdDHL, the cytoplasmic Las Receptor dimerizes and initiates a signal transduction cascade, promoting the transcription of *lasI* and other LasR-dependent QS-regulated products. The dimerization of LasR also promotes the activation of RhlR, IQS, and PQS, as well as *lasI*, further amplifying the QS signal, and respective QS-regulated products. Graphic made using BioRender 2022 (Toronto, Canada).

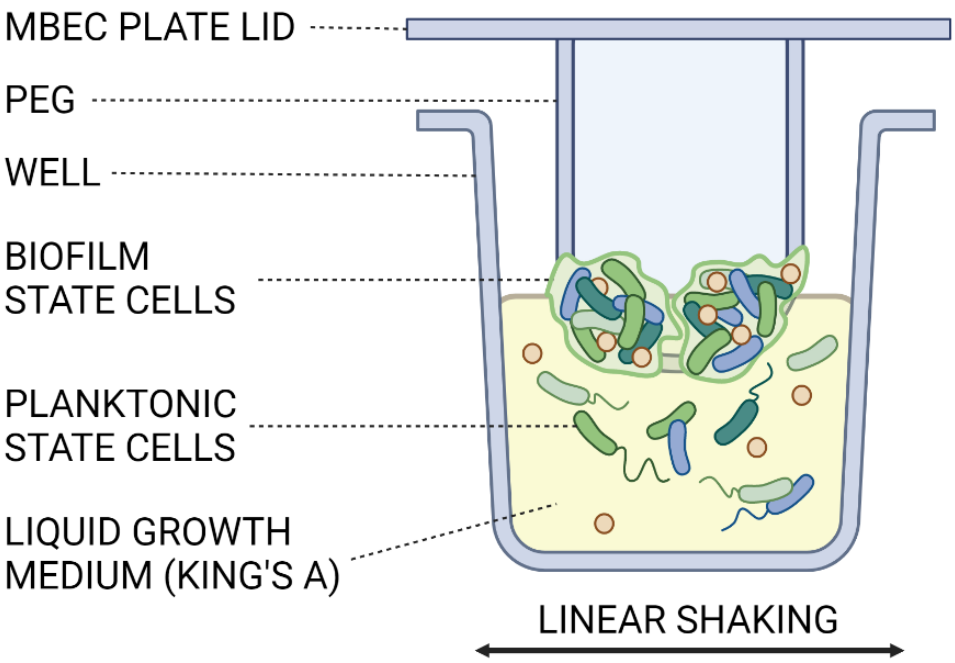

Figure 2: Experimental set-up using MBEC inoculator plates. *P. aeruginosa* biofilms were grown in Innovotech MBEC Innoculator 96-well plates. The MBEC plate lid contains 96 rounded polystyrene pegs that allow for the attachment and formation of biofilms when immersed in liquid media bacterial cultures. Once established, biofilm structures were then perturbed using linear shaking at defined hourly intervals in a microplate reader for 24 hours. Perturbations uncouple bacteria and autoinducers from the biofilm as they enter the planktonic state in the liquid media. Graphic made using BioRender 2022 (Toronto, Canada).

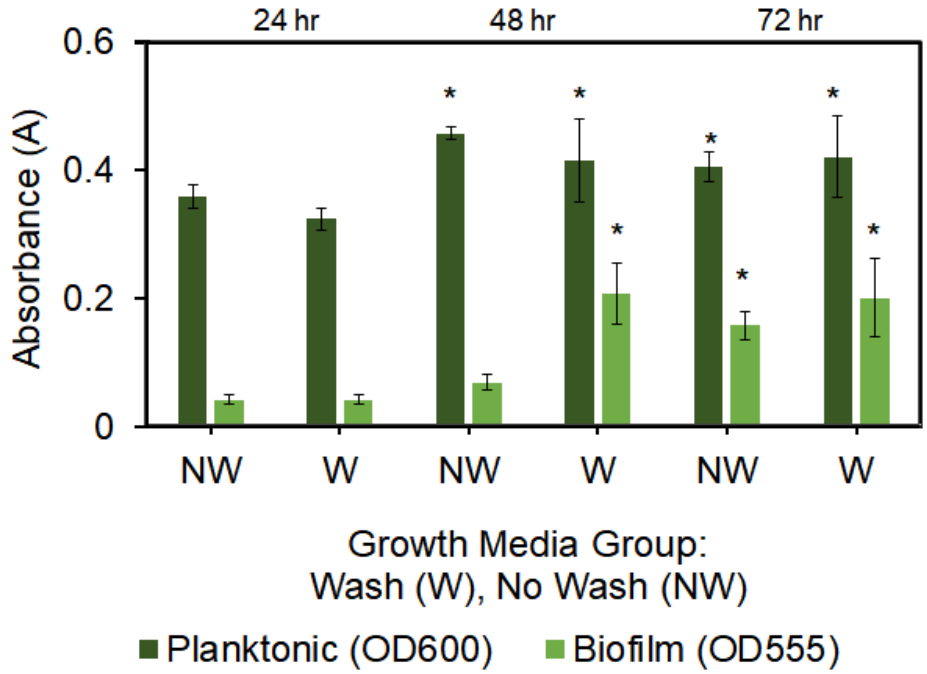

Figure 3: Determining growing conditions for robust biofilm formation. Growing conditions lead to robust mature biofilms at 48 hours when provided nutrients every 24 hours. Under nutrient-deprived conditions, biofilms took up to 72 hours to achieve mature cell density (t-test, p-value = 0.04456), while in nutrient-rich conditions, biofilms were adequately populated at 48 hours (t-test, p-value = 0.04456). Significant differences in planktonic cell populations can be attributed to bacterial migration and starvation in nutrient-deprived conditions (ANOVA, nutrient group p-value = 0.000411, time p-value = 2.23e-05, group and time p-value = 0.001084). Planktonic cell differences in a nutrient-rich environment are attributed to the availability of carbon sources and amino acids necessary for proliferation. There were no significant differences between 48 hours and 72 hours in nutrient-rich groups (t-test, p-value = 0.05714). Planktonic cell density was measured using OD_{600}, and biofilm cell density was quantified using crystal violet assay at OD_{555}. The standard deviation for > 4 biological replicates, * indicates significance.

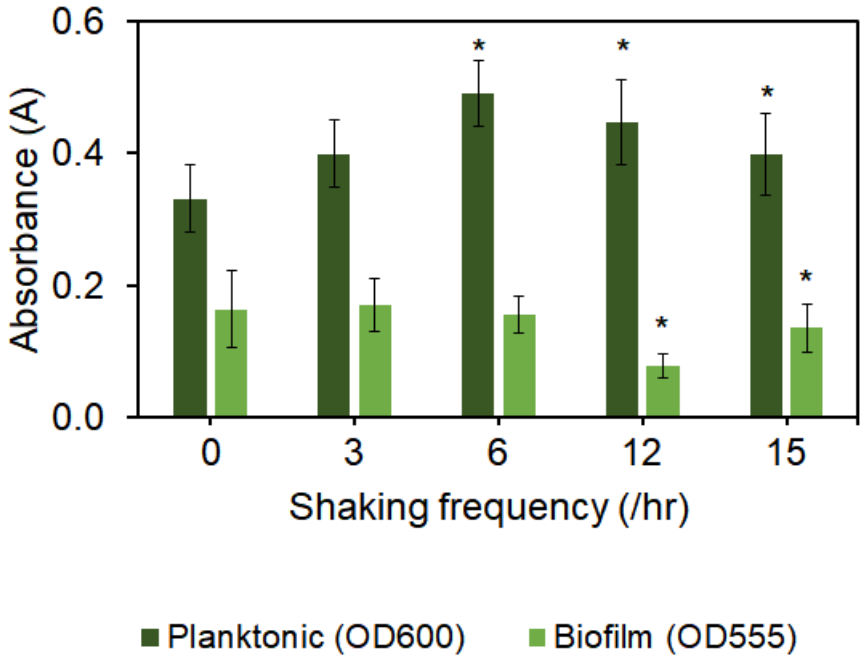

Figure 4: Periodic disturbances at increasing shaking amplitudes alter cell distribution in biofilm and planktonic state cells. Cell density in planktonic state was measured using OD_{600}, while biofilm cell density was measured using crystal violet at OD_{555}. Linear shaking causes a proportional shift from bacteria in the biofilm to disperse to the planktonic state in the surrounding liquid media. Periodic disturbances at 3/hr did not result in significant difference in planktonic cell distribution compared to control 0/hr (t-test, p-value = 0.2775). Planktonic cell density was statistically different from the undisturbed control at 6/hr, 12/hr and 15/hr (t-test, p-value = 3.933e-08; p-value = 7.717e-06; p-value = 0.002094). Biofilm density was significantly lower than the control at 12/hr and 15/hr (t-test, p-value = 0.009162; p-value =0.0008128). Biofilm density at 3/hr and 6/hr were not significantly different than 0/hr (Wilcoxon, p-value = 0.05032; Wilcoxon, p-value = 0.203). Standard deviation for > 6 biological replicates, * indicates significance.

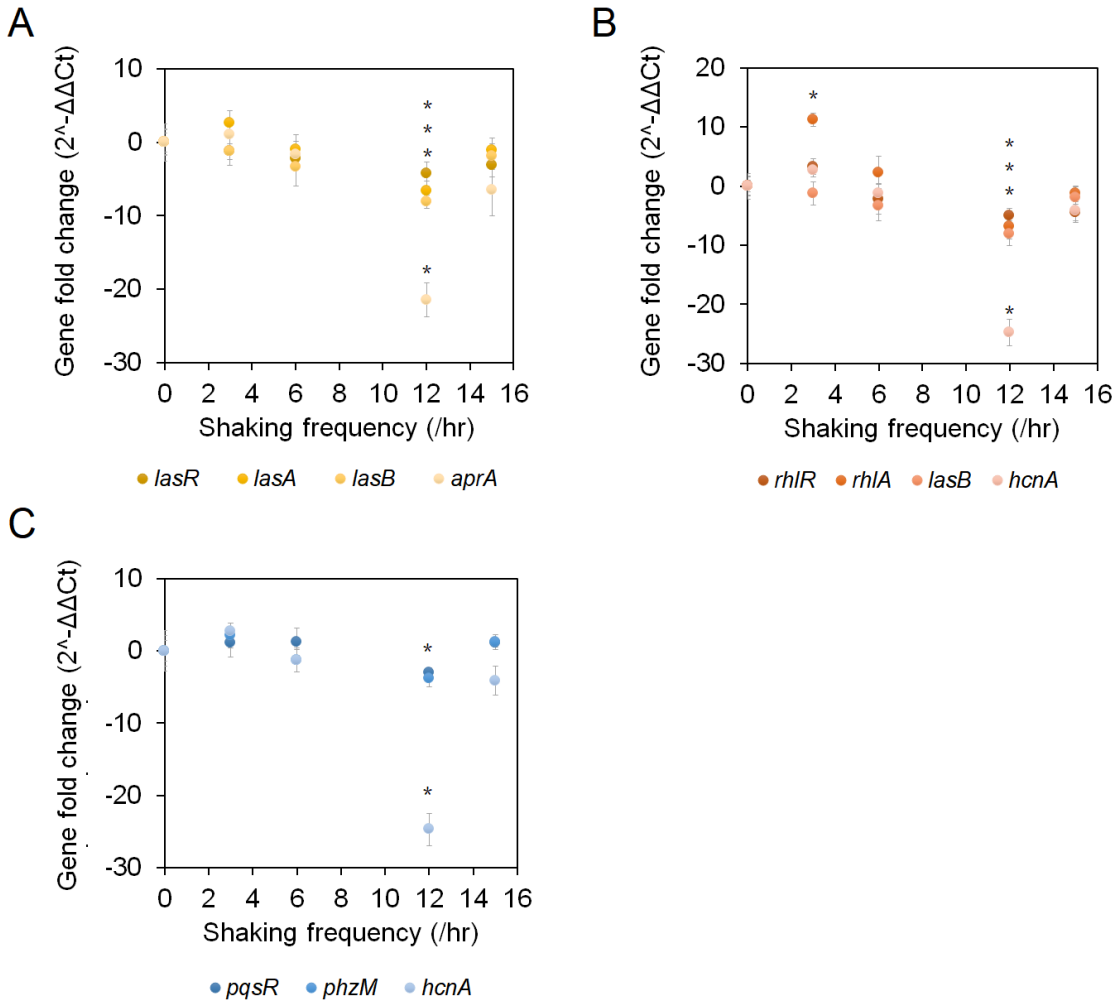

Figure 5: The effect of periodic disturbance on QS-regulated genes at increasing shaking frequencies. qRT-PCR analysis shows that increasing shaking frequencies (/hr) significantly decreases the gene fold change ($2^{-\Delta\Delta Ct}$) of QS-virulence genes of *P. aeruginosa* at 12/hr. **A)** All genes in *las suite* were were significantly downregulated at 12/hr, including transcription regulator *LasR*, and effector genes *lasA*, *lasB*, and *aprA* (Wilcoxon, p-value = 0.02443, p-value = 0.0007816, p-value = 0.0007816, p-value = 0.002468). **B)** All genes in *rhl* suite were significantly downregulated at 12/hr, including transcription regulator *RhlR*, and effector genes *rhlA*, *lasB*, and *hcnA* (Wilcoxon, p-value = 0.002953, p-value = 0.01061, p-value = 0.0007816, p-value = 0.0004936). Effector gene *rhlA* was upregulated at 3/hr (Wilcoxon, p=0.002797). **C)** Most of the genes in the *pqs* suite were significantly downregulated at 12/hr, including transcription regulator *pqsR*, and effector gene *hcnA* (Wilcoxon, p-value = 0.007898, p-value = 0.0004936). Standard deviation for > 6 biological replicates, * indicates significance.

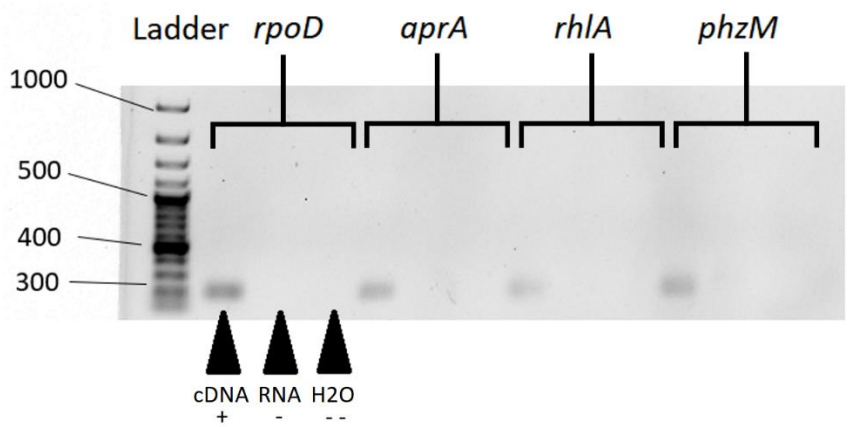

Figure 6: Gel electrophoresis of RTq-PCR products confirming amplicon specificity for *P. aeruginosa* undisturbed control (0/hr). Lane L contains Gene Ruler 100bp Ladder (ThermoFisher). qRT-PCR templates loaded on gel lanes are (1) experimental/ positve cDNA control for select gene, (2) negative control/ no-RT RNA control for select gene, and (3) negative control no-RT nuclease free water. Lanes 1-3 are amplicons for housekeeping gene *rpoD*, lanes 4-6 are *aprA* amplicons, lanes 7-9 are *rhlA* amplicons, and lanes 10-12 are *phzM* amplicons. Bands were detected around 370bp.

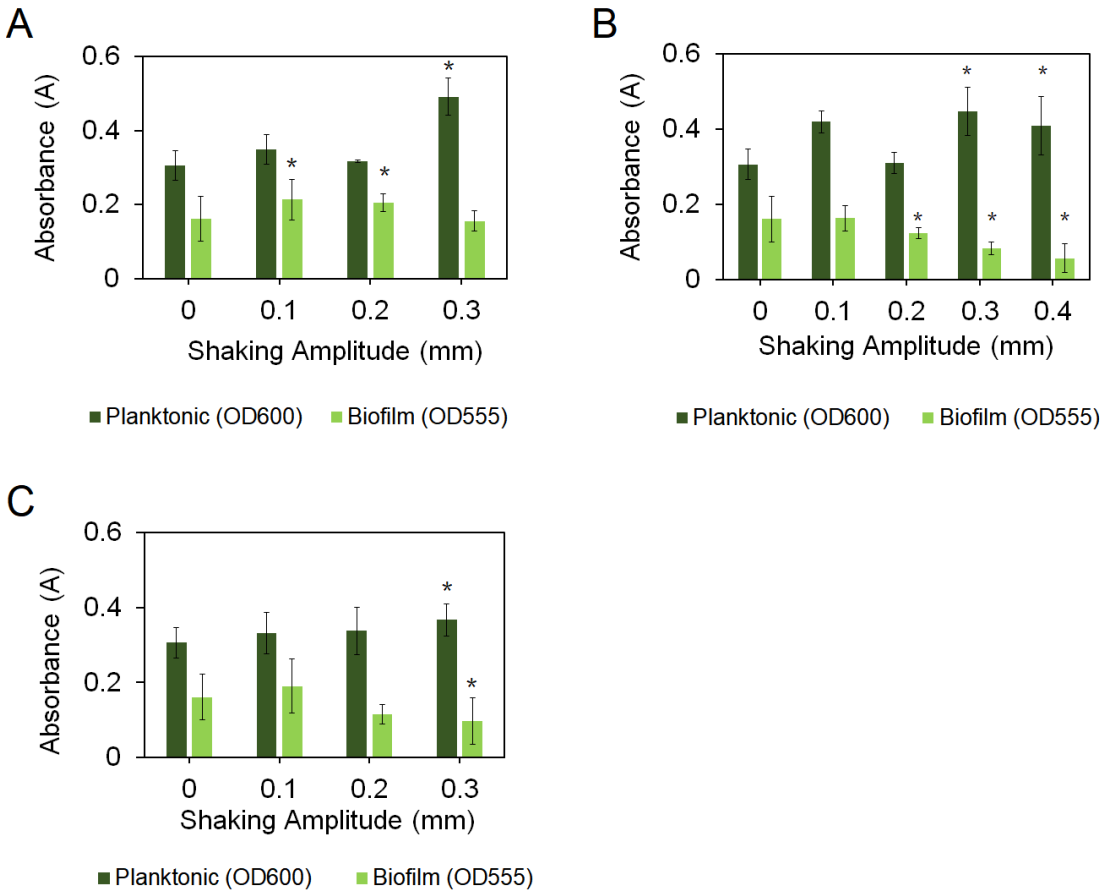

Figure 7: Combining shaking amplitude and frequency alters the distribution of cells in biofilm and planktonic states of *P. aeruginosa* compared to an undisturbed control (0/hr). Cell density in planktonic state was measured using OD_{600}, and biofilm cell density was measured using crystal violet at OD_{555}. **A)** Disturbing structure of PA14 at 0.1 and 0.2mm at 6/hr alters the cell distribution towards the biofilm state (t-test, p-value = 0.0301; p-value = 0.009744). The distribution of planktonic state cells also significantly increases at 0.3mm (t-test, p-value = 3.933e-08). There are no changes in planktonic at 0.1 and 0.2mm (t-test, p-value = 0.1102; p-value = 0.4261) nor in biofilm at 0.3mm (Wilcoxon, p-value = 0.203). **B)** At 12/hr, biofilm density was significantly decreased when perturbed at 0.2, 0.3, and 0.4mm (t-test, p-value = 0.02563; p-value = 0.009162; p-value = 0.006725). Planktonic cell density was significantly higher at 0.3 and 0.4 mm (t-test, p-value = 7.717e-06; p-value = 0.0004025). **C)** at 15/hr, planktonic cell density was significantly higher at 0.3 mm (t-test, p-value = 0.002094). Biofilm density was significantly reduced at 0.3 mm compared to the control (t-test, p-value = 0.0008128). Standard deviation for 4 biological replicates, * indicates significance.

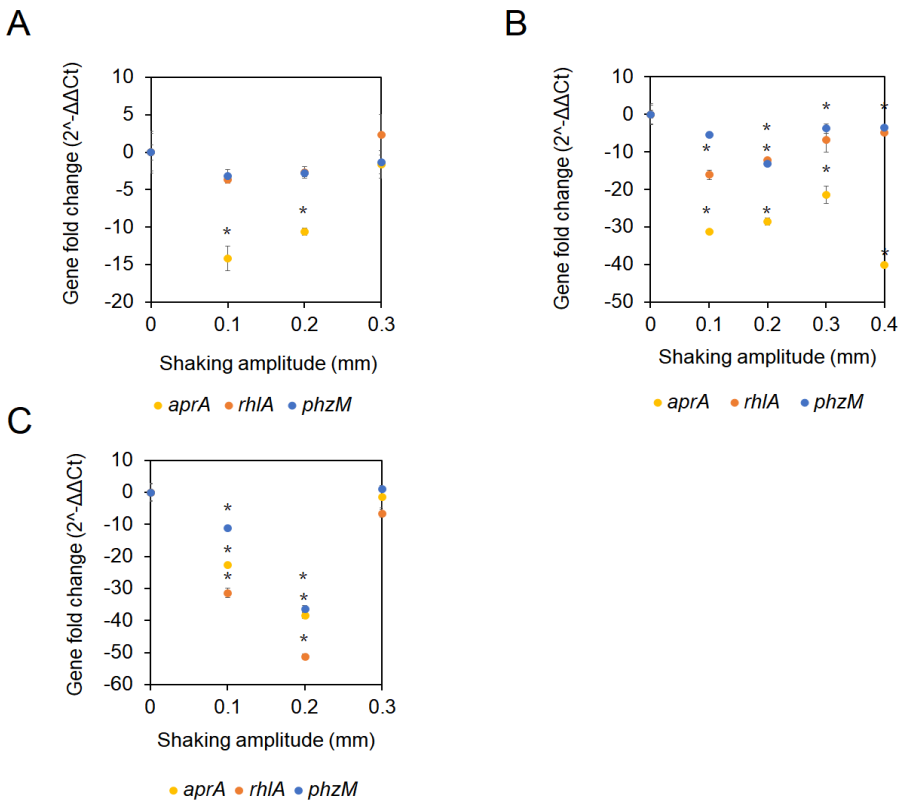

Figure 8: Disturbing *P. aeruginosa* at lower amplitudes causes significant decreases in gene expression of QS-virulence effector genes over a wide range of shaking frequencies. Gene fold change ($2^{-\Delta\Delta Ct}$) of QS-effectors *aprA*, *rhlA* and *phzM* was plotted across inreasing shaking amplitudes (mm) per select shaking frequencies (6, 12, 15/hr). **A)** Disturbing PA14 biofilms at 0.1, 0.2, and 0.3mm, at a frequency of 6/hr caused a significant decrease in the expression of *aprA* at 0.1 and 0.2mm (Wilcoxon, p-value = 0.04848; p-value = 0.01616). **B)** Disturbing PA14 biofilms at 0.1, 0.2, 0.3 and 0.4mm at a frequency of 12/hr caused a significant decrease in the expression of QS-effector genes. QS effectors *aprA* and *rhlA* were significantly downregulated at 0.1mm (Wilcoxon, p-value = 0.00836; p-value = 0.01119). All three single-node effector genes *aprA*, *rhlA* and *phzM* were significantly downregulated at 0.2mm (t-test, p-value = 0.002604; p-value = 0.002604; p-value = 0.008481). Only effector genes *aprA* and *rhlA* were downregulated at 0.3mm (Wilcoxon, p-value = 0.002468; p-value = 0.002953). Lastly, *aprA*, *rhlA* and *phzM* were significantly downregulated at 0.4mm (Wilcoxon, p-value = 0.0003108; p-value = 0.02551; p-value = 0.04009). Expression of *phzM* was not statistically different than 0/hr at 0.1mm and 0.3 mm (t-test, p-value = 0.05191; Wilcoxon, p-value = 0.2359). **C)** Disturbing PA14 biofilms at 0.1, 0.2, and 0.3mm at a frequency of 15/hr caused a significant decrease in the expression of all three effector genes at 0.1 and 0.2 mm. At 0.1mm QS-effector genes *aprA*, *rhlA* and *phzM* were significantly downregulated (Wilcoxon, p-value = 0.00404; p-value = 0.01119; p-value = 0.02828). At 0.2 mm and 15/hr, QS effectors *aprA*, *rhlA* and *phzM* were significantly downregulated (t-test, p-value = 0.0001245; p-value = 0.000397; p-value = 0.001463). Effector genes were not significantly different from the undisturbed at 0.3mm (t-test, p-value = 0.1804; p-value = 0.6634; p-value = 0.8122). Standard deviation for 4 biological replicates, * indicates significance.

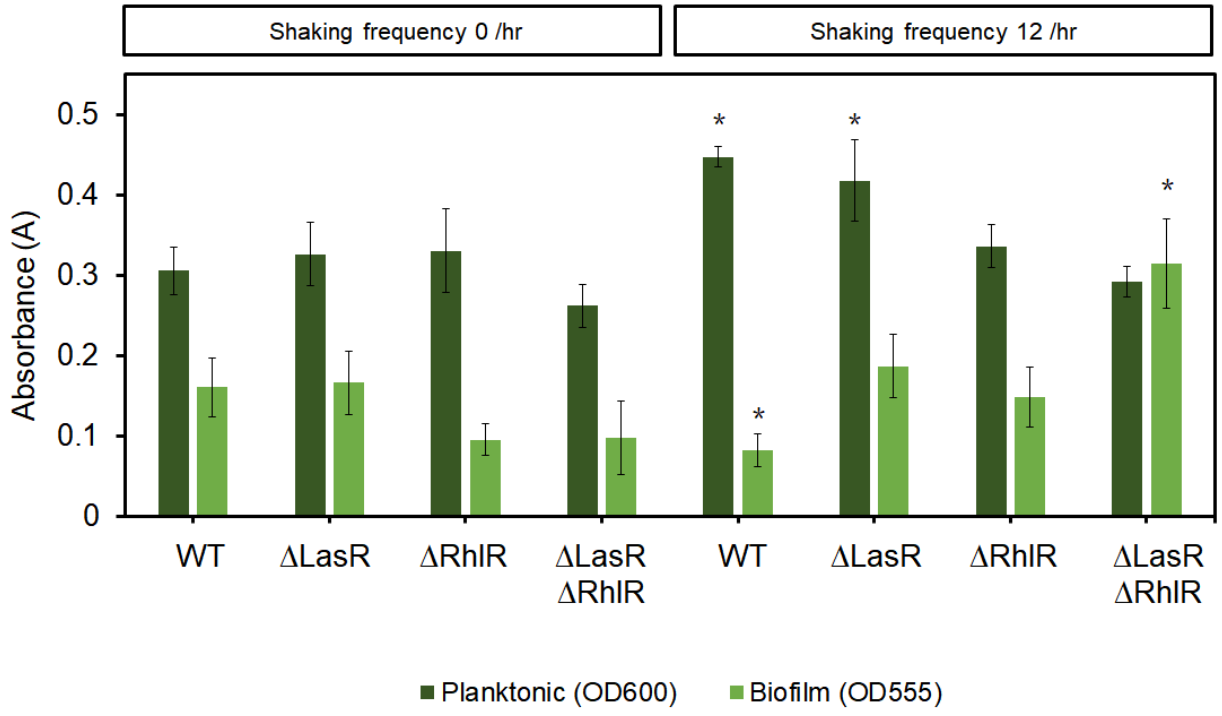

Figure 9: QS stability mediates biofilm structure and cell dispersal under physical perturbations. Cell density in planktonic state was measured using OD_{600}.Biofilm cell density was measured using crystal violet at OD_{555}. Baseline cell density at 0/hr shows no differences in planktonic and biofilm cell distribution among WT and KO strains. Periodic disturbances at 12/hr led to singificanlty higher plankonic cell density in ΔLasR compared to ΔLasR at 0/hr (t-test, p-value = 0.003937), and was not different from WT at 12/hr (Wilcoxon, p-value = 0.2977). Cell density of both planktonic and biofilm ΔRhlR were not statistically different after shaking event (t-test, p-value = 0.305, p-value = 0.8218). At 12/hr, biofilm cell density of ΔLasR/ΔRhlR was significantly higher than undisturbed control (Wilcoxon, p-value = 0.007937). Planktonic cell density of ΔLasR/ΔRhlR was not singificantly different form KO at 0/hr (t-test, p-value = 0.3792). Standard deviation for > 4 biological replicates, * indicates significance.

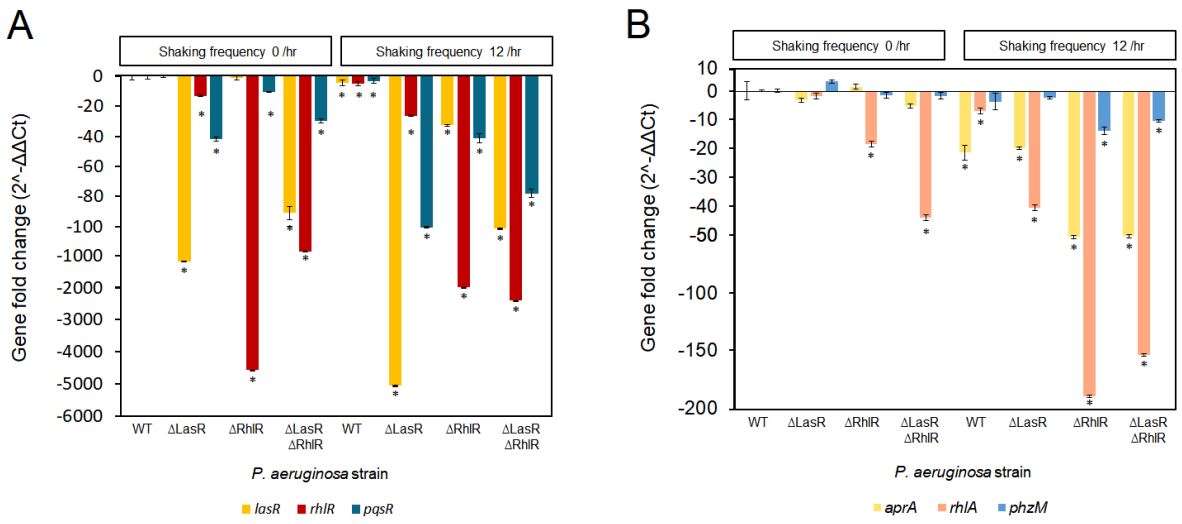

Figure 10: The expression of QS receptor and effector genes is significantly reduced in knockout strains of *P. aeruginosa.* **A)** Comparative gene expression analysis shows a massive reduction in the expression of QS receptor genes in ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR, compared to WT at 0/hr confirming lack of QS receptors in KO strains. QS receptors *lasR*, *rhlR*, and *pqsR* were significantly downregulated at 12/hr for all knockout strains compared to WT at 0/hr: ΔLasR (t-test, p-value = 0.001232; p-value = 0.0002712; p-value = 0.0008902) and ΔLasR/ΔRhlR (t-test, p-value = 0.04329; p-value = 0.005316; p-value = 0.0008333). Baseline expression of QS receptor *rhlR* and *pqsR* was also significantly downregulated at 0/hr compared to WT for knockout strain ΔRhlR (t-test, p-value = 0.04444; p-value = 2.188e-05). **B)** Baseline differences in gene expression of QS effectors *aprA*, *rhlA* and *phzM* vary among KO, and are significantly reduced with periodic shaking at 12/hr. Gene fold change of effectors *aprA* and *rhlA* were significantly reduced in the WT (Wilcoxon, p-value = 0.01061; p-value = 0.002468) and ΔLasR (t-test, p-value = 0.004487; p-value = 0.0005081) at 12/hr compared to WT at 0/hr. Expression of all effector genes was significantly downregulated in KO strain ΔRhlR at 12/hr (t-test, p-value = 0.02828; p-value = 0.002293; p-value = 0.01443). Expression of effector genes *aprA*, *rhlA*, and *phzM* were significantly reduced in ΔLasR/ΔRhlR strain at 12/hr compared to WT (t-test, p-value = 0.006721; p-value = 0.0002461; p-value = 0.04137). Standard deviation for > 4 biological replicates, * indicates significance.

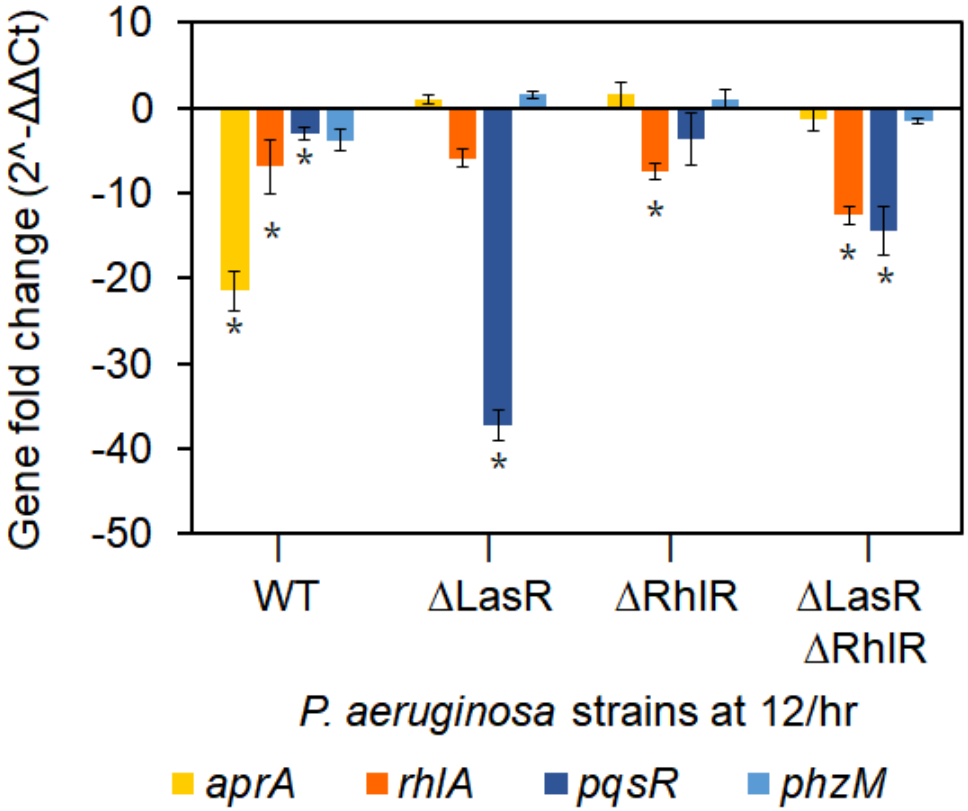

Figure 11: Comparative gene expression analysis between WT and ΔLasR, ΔRhlR, and ΔLasR/ΔRhlR KO strains at 12/hr indicates similarities in QS receptor expression in disrupted systems. The expression of QS effectors *aprA*, *rhlA*, and *phzM*, and QS receptor *pqsR* were normalized to housekeeping gene *rpoD* and WT gene expression at 0/hr. WT at 12/hr shows significant decrease in expression of *pqsR*, *aprA*, and *rhlA* (Wilcoxon, p= 0.007898, p=0.002468, p=0.002953). Expression of QS effectors and *pqsR* was normalized to KO housekeeping gene *rpoD* and comapred to WT at 12/hr. Significant differences in QS gene expression indicate that disturbing at 12/hr siginificantly downregualtes expression of QS genes compared to WT at 12/hr (ΔLasR *pqsR* W = 0, p-value = 0.002797, ΔRhlR *rhlA* W = 4, p-value = 0.03357, and ΔLasR/ΔRhlR *rhlA* and *pqsR* W = 4, p-value = 0.03357, W = 0, p-value = 0.002797). QS effector genes *aprA* and *phzM* were not significantly different between WT and KO at 12/hr (ΔLasR *aprA* and *phzM* W = 23, p-value = 0.5035, W = 25, p-value = 0.3301; ΔRhlR *aprA* and *phzM* W = 23, p-value = 0.5035, W = 16, p-value = 0.8252; ΔLasR/ΔRhlR *aprA* and *phzM* W = 17, p-value = 0.9399 W = 15, p-value = 0.7105) Standard deviation for >4 biological replicates, * indicates significance.

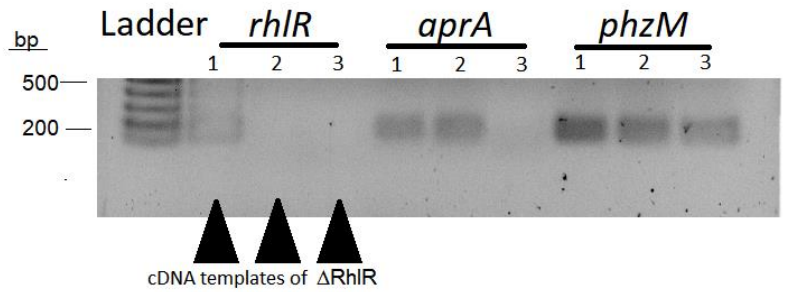

Figure 12: Gel electrophoresis of qRT-PCR products confirming amplicon specificity for ΔRhlR PA14 in the undisturbed control (0/hr) in 3 biological replicates. Lane L corresponds to Gene Ruler 100bp Ladder (ThermoFisher); Lanes sequentially labeled 1, 2, and 3, correspond to positive cDNA from qRT-PCR products from biological replicates 1, 2, and 3 of ΔRhlR PA14 strain. Lanes 1-3 are *rhlR* amplicons per biological replicate, lanes 4-6 are *aprA* amplicons per biological replicate, and lanes 7-9 are *phzM* amplicons per biological replicate. There was no band for *rhlR* in the mutant strains. Bands were detected at around 250bp.

Table 1: Bacterial strains used

Strain	Origin
***Pseudomonas aeruginosa* UCBPP- PA14**	American Type Culture Collection (ATCC) Lingchong You [28]
ΔLasR *P. aeruginosa* PA14	Bonnie Bassler, Julie Valastyan [49]
ΔLasRΔRhlR *P. aeruginosa* PA14	Bonnie Bassler, Julie Valastyan
ΔRhlR *P. aeruginosa* PA14	Bonnie Bassler, Julie Valastyan

Table 2: Growth media components

Growth Media	Quantity	Components	Manufacturer
Luria Broth (LB) Media (1L)	20 g	Luria Broth Powder	MP Biomedical, Solon, OH
	1000 mL	DI H_2O	
King's A Media (KA) (1L) [51]	20 g	Protease Peptone	Fisher Bioreagent, Janssen
	10 g	Magnesium Chloride	Alfa Aesar
	1.64 g	Potassium Sulfate	Acros Organics
	10 mL	Glycerol 99%	Acros Organics
	990 mL	DI H_2O	

Table 3: *P. aeruginosa* PA14 QS gene primers used in qRT-PCR (Sigma-Aldrich, Saint Louis, MO)

QS gene & Product	Effect to host & benefit to *P. aeruginosa* [8, 34, 38]	Direction	Primer Sequence (5'-3')
rpoD **RNA polymerase sigma factor**	'Housekeeping gene' promotes the attachment of RNA polymerase	Forward	GGGCGAAGAAGGAAATGGTC
		Reverse	CAGGTGGCGTAGGTGGAGAA
lasR **transcriptional regulator LasR**	OdDHL Receptor; Major transcriptional activator of *lasI* and *las* suite, promotes transcription of *rhl* and *pqs.* Necessary for biofilm formation and toxicity.	Forward	GCGCCATCGGCAAGACCAG
		Reverse	GCGGGAGGTCACACCGAAC
lasA **protease LasA**	Disrupts epithelium; staphylolytic, colonization, immune evasion	Forward	TGTCGCGCTGCCAGGTAC
		Reverse	CTGCTGGCCGTTGCTCAC
lasB **Elastase LasB**	Breaks down matrix proteins (elastin), iron acquisition	Forward	AGACCGAGAATGACAAAGTGGGAA
		Reverse	GTAGGAGACGTTGTAGACCAGTTG
aprA **Alkaline metalloproteinase**	Degrades cytokines and host complement system; immune evasion aids in colonizing CF lungs	Forward	ACCCTGTCCTATTCGTTCC
		Reverse	GATTGCAGCGACAACTTGG
rhlR **Acyl homoserine lactone-dependent transcriptional regulator**	BHL receptor; promotes transcription of *rhlI* and *rhl* genes, inhibits activation of *pqs.*	Forward	GCGTGTTCGCCGTCCTGGA
		Reverse	GGCCGGGTGAAGGGAATCG

Table 3: (Continued) *P. aeruginosa* PA14 QS gene primers used in qRT-PCR (Sigma-Aldrich)

QS gene & Product	Effect to host & benefit to *P. aeruginosa* [8, 34, 38]	Direction	Primer Sequence (5'-3')
rhlA **Rhamnosyl-transferase chain A**	Macrophage and lymphocyte necrosis; biofilm formation, immune evasion	Forward	TGGCCGAACATTTCAACGT
		Reverse	GATTTCCACCTCGTCGTCCTT
hcnA **Hydrogen cyanide synthase**	Arrests cellular respiration in host; airway and lung damage; promotes colonization	Forward	CTCAATGGCCAGCCCGTCG
		Reverse	GCATACGCCCATGCCGCAG
phzM **Phenazine-specific methyltransferase**	Forms pyocyanin; oxidative effects to cellular respiration, airway paralysis, inhibit inflammation, neutrophil damage. Infection, colonization, immune evasion,	Forward	CGGGATCGACAGCGACGAG
		Reverse	TCGCGGGTATCGCCCTGG
mvfR/ pqsR **Transcriptional regulator**	PQS Receptor; promotes *pqsI* and *pqs* genes	Forward	CCATCCGCAGCACCCGTTG
		Reverse	TCGAATGCTGCCCGGAGC