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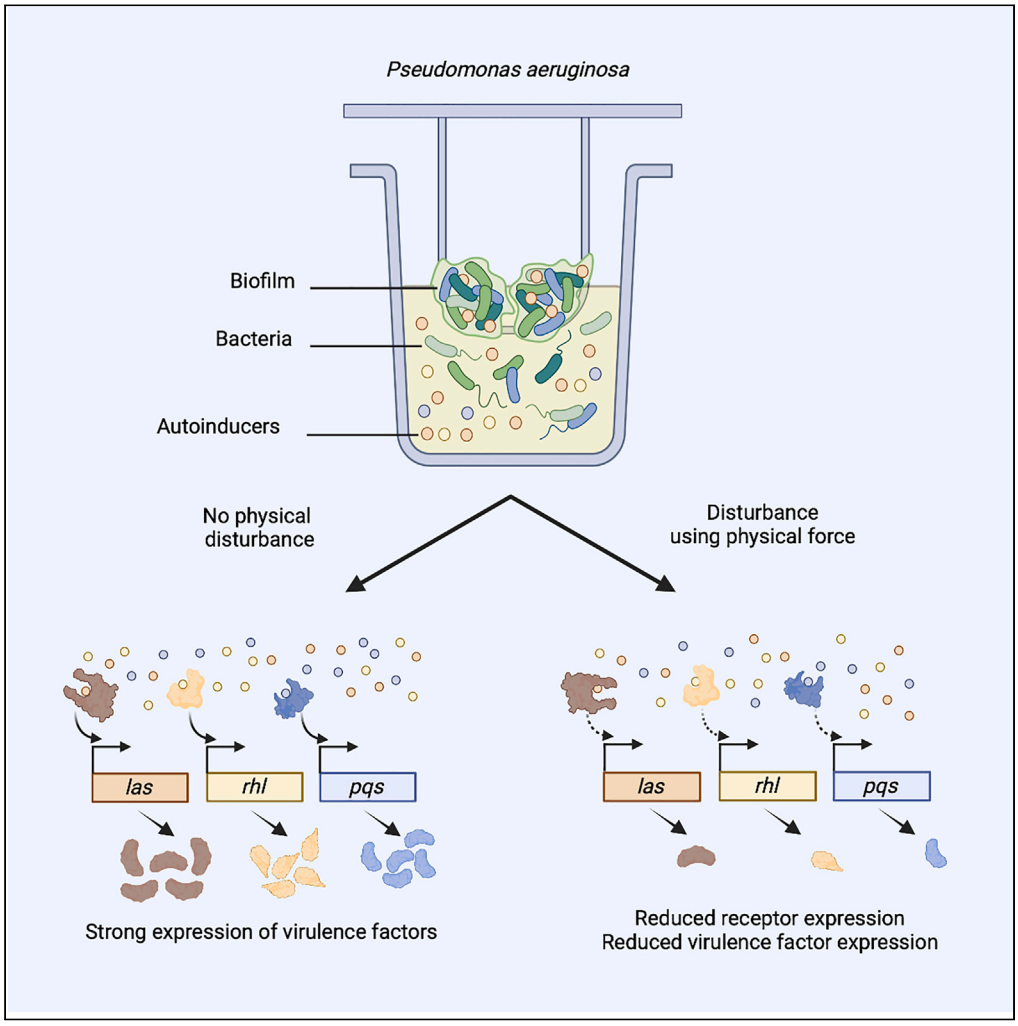

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Highlights

P.aeruginosa uses quorum sensing to regulate expression of virulence factors

Biofilm formation can facilitate expression of these virulence factors

Disturbing biofilms using physical force reduces expression of virulence factors

The LasR receptor buffers the quorum sensing network against disturbance

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Periodically disturbing biofilms reduces expression of quorum sensing-regulated virulence factors in *Pseudomonas aeruginosa*

Laura García-Diéguez,[1] Gabriela Diaz-Tang,[1] Estefania Marin Meneses,[1] Vanessa Cruise,[1] Ivana Barraza,[1] Travis J.A. Craddock,[2,3,4,5] and Robert P. Smith[1,6,7,*]

SUMMARY

***Pseudomonas aeruginosa* uses quorum sensing to regulate the expression of virulence factors. In static environments, spatial structures, such as biofilms, can increase the expression of these virulence factors. However, in natural settings, biofilms are exposed to physical forces that disrupt spatial structure, which may affect the expression of virulence factors regulated by quorum sensing. We show that periodically disturbing biofilms composed of *P. aeruginosa* using a physical force reduces the expression of quorum sensing-regulated virulence factors. At an intermediate disturbance frequency, the expression of virulence factors in the *las*, *rhl*, and *pqs* regulons is reduced. Mathematical modeling suggests that perturbation of the *pqsR* receptor is critical for this reduction. Removing the *lasR* receptor enhances the reduction in the expression of virulence factors as a result of disturbance. Our results allow identification of environments where virulence is reduced and implicate the *lasR* receptor as having a buffering role against disturbance.**

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, opportunistic pathogen that causes approximately 20% of all nosocomial infections, accounting for over 90,000 deaths annually.[1] It can cause a diversity of infections including urinary tract infections, chronic lung infections, and systemic bacteremia.[2,3] Further complicating the treatment of *P. aeruginosa* infections is the rise and spread of antibiotic resistant[4] and hypervirulent strains.[5] Persistent infection with *P. aeruginosa* can lead to additional complications including secondary infections[6] resulting in prolonged treatment and extended hospitalization.[7] There is a need to develop novel tools to disrupt pathogenesis of, and eliminate infections from, *P. aeruginosa*.

One mechanism by which *P. aeruginosa* regulates the expression of virulence factors is through quorum sensing. Quorum sensing involves the production, secretion, and exchange of diffusible small molecules or peptides, called autoinducers, within a population of bacteria. Once the autoinducer reaches a minimal concentration, it binds to its cognate receptor, which facilitates changes in gene expression.[8] The major quorum sensing network in *P. aeruginosa* consists of three interconnected regulons that are controlled by three autoinducers ([2], Figure 1A). In the *las* regulon, *lasI* produces an autoinducer, (N-(3-oxododecanoyl)-homoserine lactone, which binds to the LasR receptor. Once activated, the LasR receptor drives the expression of several virulence factors including *lasA*, *lasB*, and *aprA*.[2,9,10] In the *rhl* regulon, RhlI produces an autoinducer, N-butyryl homoserine lactone, which binds to and activates the RhlR receptor. Activated RhlR will induce the expression of virulence factors *rhlA*, *lasB*, and *hcnA*.[2,9,10] In the *pqs* regulon, the *pqsABCD* operon produces the 2-heptyl-3-hydroxy-4-quinolone (PQS) autoinducer, which binds to the PqsR receptor. Once activated, PqsR drives the expression of multiple virulence factors, including *phzM*.[11] Recent work has also identified a fourth quorum sensing system, the *iqs* regulon, which is composed of the IqsR receptor and the *ambBCDE* operon, which produces the 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) autoinducer.[12] Each quorum sensing regulon is connected to another in a hierarchical fashion.[2] At the top of the network hierarchy is the *las* regulon; activated LasR receptor promotes the expression of *rhlR*, *pqsR*, and *iqsR*. Activated RhlR receptor can repress the expression of *pqsR*, whereas activated PqsR receptor promotes the expression of *rhlR*. Finally, activated IqsR receptor promotes the expression of *pqsR*. Overall, these interconnected quorum sensing regulons control the expression of multiple virulence factors in *P. aeruginosa*.

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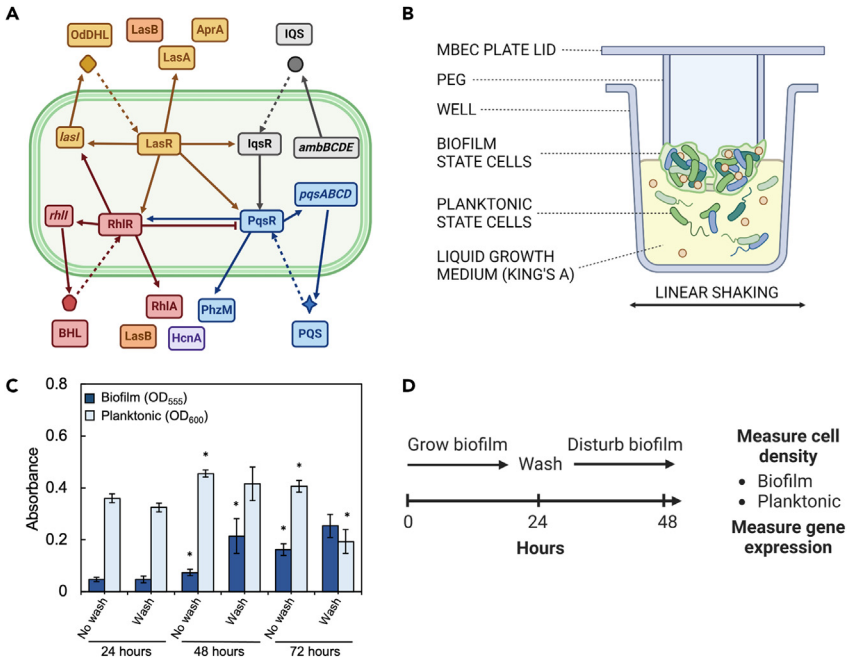

Figure 1. An experimental setup to examine the influence of periodic disturbance on the expression of genes in the central quorum sensing network of *P. aeruginosa*

(A) The central quorum sensing network in *P. aeruginosa*. Each section of the network is color coded: *las* regulon = yellow; *rhl* regulon = red; *pqs* regulon = blue; *iqs* regulon = gray. Each regulon produces its own autoinducer (different shapes outside of the cell) that binds to a receptor on the inside of the cell (denoted with dotted lines). Virulence factors measured in this study and regulated by one or more receptors are shown. Normal arrow indicates activation; blunt end arrow indicates repression. LasB (orange) is regulated by both the *las* and *rhl* regulons. HcnA (purple) is regulated by both the *rhl* and *pqs* regulons.

(B) Experimental approach to growing and disturbing the structure of biofilms. *P. aeruginosa* biofilms were grown in an MBEC Inoculator device. The plate lid contains 96 rounded polystyrene pegs that allow for the attachment and formation of biofilms when immersed in liquid medium; the liquid medium contains a population of bacteria in the planktonic state. Biofilms were disturbed using linear shaking of a microplate reader at defined intervals (called shaking frequency) and amplitudes for 24 h.

(C) Optimizing experimental conditions for growing biofilms. Biofilms were grown for 24 h whereupon the medium surrounding the peg was replaced with fresh medium (wash) or was not replaced (no wash). Biofilm density was quantified using a crystal violet assay (see STAR Methods) and measured at OD_{555}. Density of bacteria in the planktonic state was quantified using OD_{600}. * significant difference in OD measurement. No wash treatment: OD_{600} - 24 h vs. 48 h ($p < 0.001$, two-tailed t-test); 48 h vs. 72 h ($p = 0.026$, two-tailed t-test). OD_{555} - 24 h vs. 48 h ($p = 0.024$, two-tailed t-test); 48 h vs. 72 h ($p = 0.002$, two-tailed t-test). Wash treatment: OD_{600} - 24 h vs. 48 h ($p = 0.089$, two-tailed t-test); 48 h vs. 72 h ($p = 0.004$, two-tailed t-test). OD_{555} - 24 h vs. 48 h ($p = 0.022$, two-tailed t-test); 48 h vs. 72 h ($p = 0.44$, two-tailed t-test). Average and standard deviation from $\geq$4 biological replicates.

(D) Schematic of our experimental approach to disturb the spatial structure of biofilms and measure the impact on the expression of genes regulated by quorum sensing. "Wash" encompasses placing the biofilms affixed to the plate lid in fresh medium for 10 s to remove unadhered bacteria followed by placing the biofilms in additional fresh medium. Thus, disturbance occurs in fresh King's A medium.

Previous work has shown that biofilm formation can alter the expression of virulence factors.[13,14] Multiple bacterial species, including *P. aeruginosa*, form highly structured biofilms, which are largely composed of bacteria encased in extracellular polymeric substances.[15] Biofilms confer multiple properties to a bacterial population including protection from attacks from the immune system[16] and antibiotics.[17,18] In *P. aeruginosa*, expression of virulence factors regulated by quorum sensing is enhanced in biofilms.[19–21] This is likely owing to a local increase in the concentration of autoinducers, as previous research has shown that autoinducers and additional small diffusible molecules can increase in concentration in *P. aeruginosa* aggregates.[22,23] In theory, disrupting the spatial structure of biofilms could disrupt the local accumulation of autoinducers. This would reduce the concentration of autoinducers sensed by bacteria, which could reduce the expression of quorum sensing-regulated virulence factors. Moreover, the majority of studies

that have examined the expression of quorum sensing-regulated virulence factors have occurred in stationary conditions and in the absence of changes in physical force. This is despite the fact that physical forces are known to change periodically in natural environments where *P. aeruginosa* can be found (e.g., soils and[24] tissues[25]). Indeed, previous work has shown that disrupting the spatial structure of bacterial populations can alter quorum sensing[26] and the ability to synthesize virulence factors.[27] Specifically, Wilson et al. used engineered bacteria that required activation of quorum sensing to survive and found that interactions between disturbance-driven access to autoinducer and nutrients would determine population survival.[26] Quinn et al. found that interactions between strain-specific pyoverdine synthesis rates and biofilm formation would determine the impact of disturbance on pyoverdine production.[27] Barraza et al. found that disturbance could alter expression of virulence factors in *Staphylococcus aureus* through changes in access to autoinducer and cellular metabolism.[28] In these studies, it was shown that periodic disturbance of the population could alter the spatial structure of the population. In the case of studies performed with biofilms,[27,28] periodic disturbance at intermediate frequencies could reduce the density of the biofilm. However, owing to an increase in sheer force at high disturbance frequencies, the density of the biofilm could also increase thus resulting in non-linear relationships between disturbance frequency and biofilm density. Currently, it is unclear as to how periodic changes in physical force will impact the expression of virulence factors regulated by quorum sensing in *P. aeruginosa*.

To address this gap in knowledge, we grew biofilms composed of *P. aeruginosa* and disturbed their spatial structure over a variety of disturbance frequencies (the number of disturbance events per hour) and disturbance amplitudes (the distance travelled along one axis during disturbance). We found that periodic disturbance could decrease the expression of virulence factors from multiple quorum sensing regulons in a frequency- and amplitude-dependent manner. A logic model of the *P. aeruginosa* quorum sensing network implicates the PqsR receptor as a key node that is perturbed during disturbance. Moreover, we found that removing *lasR*, but not *rhlR* or *pqsR*, increased the fold reduction in the expression of multiple virulence factors in the quorum sensing network.

RESULTS

Optimizing the growth of biofilms for periodic disturbance assays

We used the MBEC biofilm inoculator device to grow biofilms (Figure 1B). This device consists of a 96-well plate with a lid containing 96 rounded polystyrene pegs; each peg on the lid corresponds to a well in the microplate. Submerging the pegs into medium containing bacteria allows biofilms to form on the peg. The liquid medium surrounding the peg contains a population of bacteria in the planktonic state. The lid containing biofilms can easily be removed and transferred to new microplates where fresh medium can be provided to the bacteria. To optimize the growth of biofilms using the MBEC device, we grew *P. aeruginosa* biofilms for 24, 48, or 72 h in two separate conditions. In the first condition, every 24 h, we removed the lid from the MBEC device and transferred the biofilms into fresh King's A medium (called the "wash condition"). In the second condition, we did not move the lid to a new plate and thus the biofilms existed in the same medium throughout the course of the experiment (called the "no wash condition"). The density of the biofilm was quantified using a crystal violet assay, which was measured using optical density (OD) at 555 nm (OD_{555}). The density of bacteria in the planktonic state was measured using OD_{600}. In the wash condition, biofilms with $OD_{555} \sim 0.2$ were observed after 48 h of growth (Figure 1C). This density was maintained at 72 h and did not show a significant change as compared to 48 h ($p = 0.44$, two-tailed t-test). In the no wash condition, an OD_{555} of ~ 0.2 was only reached after 72 h. Given the shorter time period to achieve a biofilm with an OD_{555} of ~ 0.2, we chose the "wash condition" to perform our experiments. This condition would also allow us to provide fresh medium and disturb the biofilm during development (growth from 24 to 48 h). Thus, for our experimental approach, we chose to first grow biofilms for 24 h, wash the biofilms, place them in fresh medium, and subsequently disturb them for 24 h (Figure 1D). We then measured changes in cell density and gene expression as a result of disturbance.

Periodic disturbance can alter the distribution of bacteria and reduce the expression of genes in the quorum sensing network

We used the linear shaking function of a microplate reader to investigate how the density of bacteria in the biofilm and planktonic states would change as a result of disturbance. We grew and disturbed biofilms as described previously (Figure 1D). After 24 h of disturbance at various frequencies but with a consistent amplitude of 0.3 mm, we quantified the density of bacteria in the biofilm and planktonic states. We observed that the density of the biofilm was reduced at a disturbance frequency of 12 shaking events

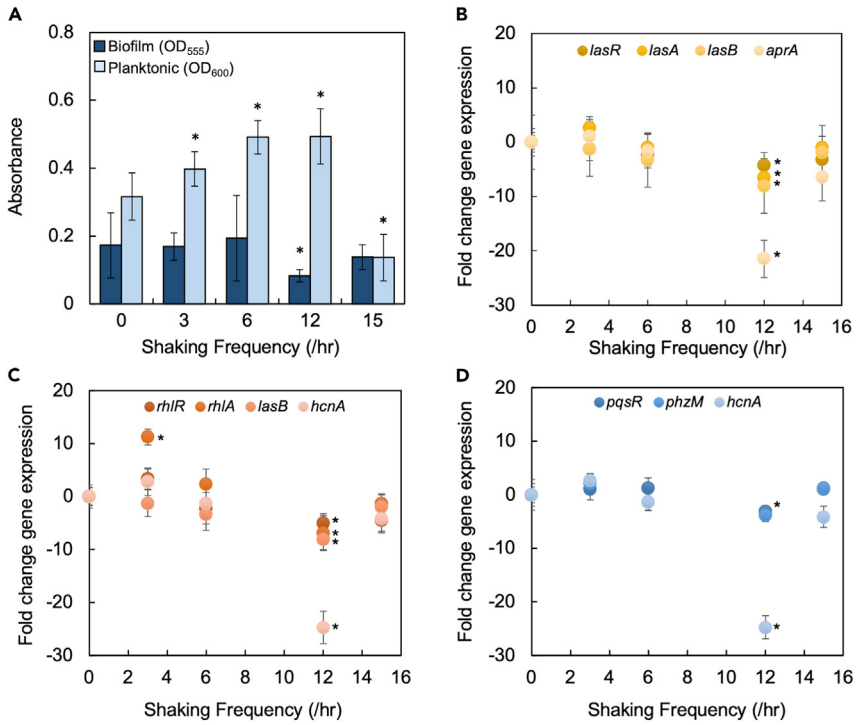

Figure 2. An intermediate disturbance frequency can reduce the expression of virulence factors in the *las*, *rhl*, and *pqs* regulons

(A) Density of bacteria in the biofilm and planktonic states after 24 h of disturbance at the indicated frequency (0.3 mm amplitude). A significant (*) reduction in biofilm density was observed at 12/h ($p = 0.0114$, Mann-Whitney (Shapiro-Wilk; $p = 0.0022$)), as compared to the undisturbed (0/h) control. A significant difference in the density of bacteria in the planktonic state was observed at all disturbance frequencies measured ($p \leq 0.0059$, Mann-Whitney (Shapiro-Wilk; $p < 0.0001$) as compared to 0/h). Average and standard deviation from ≥6 biological replicates.

(B) Effect of periodic disturbance on the expression of genes in the *las* regulon. There was a significant (*) reduction in the expression of *lasR*, *lasA*, *lasB*, and *aprA* at a disturbance frequency of 12/h as compared to 0/h ($p < 0.026$, two-tailed t-test, all p values in Table S2). In panels B–D, average and standard deviation from ≥4 biological replicates. ΔC_t data in Figure S2.

(C) Effect of periodic disturbance on the expression of genes in the *rhl* regulon. There was a significant (*) reduction in the expression of *rhlR*, *rhlA*, *lasB*, and *hcnA* at 12/h as compared to 0/h ($p < 0.041$, two-tailed t-test, all p values in Table S2). A significant increase in the expression of *rhlA* was observed at 3/h ($p = 0.0039$, two-tailed t-test).

(D) Effect of periodic disturbance on the expression of genes in the *pqs* regulon. There was a significant (*) reduction in the expression of *pqsR* and *hcnA* ($p < 0.022$, two-tailed t-test, all p values in Table S2), but not *phzM* ($p = 0.107$), as compared to 0/h.

per hour (12/h) compared to the undisturbed control (0/h, p values reported in the figure legends, Figure 2A). A significant increase in the density of bacteria in the planktonic state was observed at frequencies of 3, 6, and 12/h as compared to 0/h. A significant decrease in the density of bacteria in the planktonic state at 15/h was observed as compared to 0/h. Overall, periodic disturbance could alter the distribution of bacteria in our experimental setup.

We then determined how disturbance affected the expression of genes in the three major interconnected quorum sensing networks; the *las* regulon, the *rhl* regulon, and the *pqs* regulon. As above, we disturbed biofilms at different disturbance frequencies (amplitude = 0.3 mm) for 24 h (Figure 1D). We then extracted RNA from the bacteria in the planktonic state and performed RT-PCR. Fold changes in gene expression were determined relative to 0/h. We found that a disturbance frequency of 12/h reduced the expression of multiple receptors and effectors in all three regulons. In the *las* regulon, a significant decrease in the expression of *lasR*, *lasA*, *lasB*, and *aprA* was observed (Figure 2B). In the *rhl* regulon, there was a significant decrease in the expression of *rhlR*, *rhlA*, *lasB*, and *hcnA* (Figure 2C). Finally, in the *pqs* regulon, there was a significant decrease in the expression of *pqsR* and *aprA*, but not *phzM* (Figure 2D). We note that *lasB* and

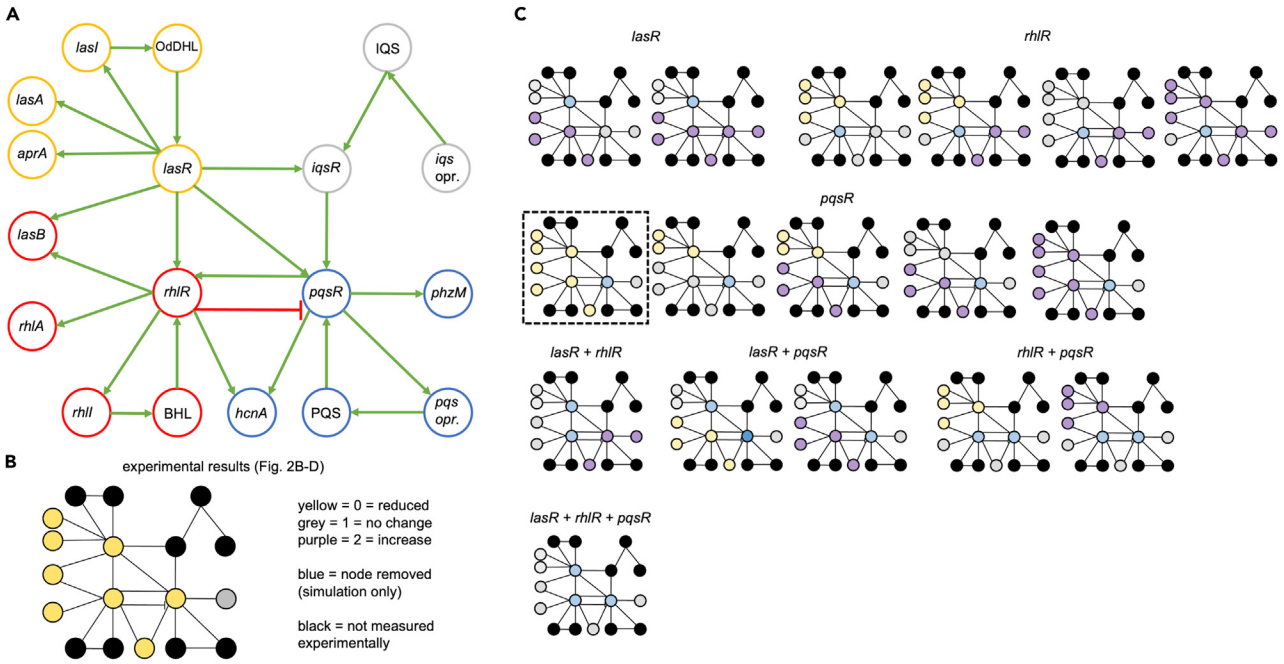

Figure 3. A logic model of the quorum sensing network predicts the effects of disturbance at 12/h by perturbing the *pqsR* node

(A) A logic model of the key nodes and edges in the quorum sensing network in *P. aeruginosa*. Red/blunt end = repressing edges, green/arrow = activating edges. opr = operon.

(B) Left: Experimental results at 12/h (Figures 2B–2D) mapped to our logic model. Right: Legend describing the color coding of each node. Each node in the network was assigned a steady-state value; 0, 1, or 2, representing a decrease, no change, or an increase in node activity. Color scheme applies to panels B and C.

(C) Using our logic model, we systematically removed receptors (*lasR*, *rhlR*, and *pqsR*), both in isolation and in combination, and asked the model to identify possible steady states. One predicated steady state achieved through the removal of *pqsR* resulted in a match to our experimental findings in panel B (highlighted with the black dotted box). Simulation results for the removal of autoinducers and synthases are in Figure S3. Simulation results for nodes that were not measured experimentally are in Table S3. Results from an alternative formulation of the model that includes a repressing edge between *rhlR* and the *pqsR* operon[29] are shown in Figure S4. This model variation continues to implicate *pqsR* as a node that is perturbed during disturbance.

hcnA are regulated by multiple quorum sensing systems (*lasB* = *las* and *rhl* regulons; *hcnA* = *rhl* and *pqs* regulons). Thus, disturbance reduced the expression of effector genes with single (e.g., *lasA* from the *las* regulon) and multiple (*lasB, hcnA*) inputs at a disturbance frequency of 12/h. Significant reductions in the expression of receptors or effectors were not observed at other disturbance frequencies tested (3, 6, and 15/h) and at an amplitude of 0.3 mm.

A network logic model predicts perturbing pqsR can give rise to changes in gene expression observed at 12/h

We created a logic model to identify which nodes in the quorum sensing system were likely being the most impacted by disturbance at 12/h. The model contains the three major quorum sensing regulons, and the *iqs* regulon (Figure 3A), and includes multiple nodes that were measured experimentally. To identify steady states in the network, the model simulation iteratively assigns each node a value of 0, 1, and 2 where 0 represents a decrease in expression, 1 represents no change in expression, and 2 represents an increase in expression relative to 0/h. Each permutation of the assignment was then assessed for stability based on logic rules defined by the connections between nodes. To mimic the impact of disturbance on the functionality of each node, and focusing on the *las*, *rhl*, and *pqs* regulons, we systematically removed autoinducers, synthases, and receptors (both individually and in combination). In the extreme case, removing a node from the network would represent a complete loss of activity of the protein in the network as a result of disturbance. While perturbing some nodes rendered a partial match between our experimental results and simulation predictions, we found that removing *pqsR*, but not autoinducers, synthases, or other receptors, from the network reduced the expression of *lasA*, *lasB*, *aprA*, *lasR*, *rhlR*, *rhlA*, and *hcnA*, but left expression of *phzM* unchanged (Figures 3B and 3C). This pattern in the reduction of gene expression matched that observed in Figures 2B–2D. We also tested an alternative formulation of the logic model that includes a

repressing edge between *rhlR* and the *pqsR* operon[29] (Figure S4). This alternative logic model continues to implicate perturbation of the *pqsR* receptor as key to reproducing the trends in our experimental analysis. Overall, our model predicts that perturbing the functionality of *pqsR* can give rise to the experimentally observed changes in gene expression observed at 12/h.

Amplitude of disturbance events further magnifies the effect of frequency on cell distribution

Previous work has demonstrated that the amplitude of a disturbance event can influence the distribution of bacteria.[21,30,31] Accordingly, we investigated the effects of altering the disturbance amplitude on the expression of quorum sensing-regulated virulence factors. We first determined the impact of disturbance amplitude and frequency on the distribution of bacteria in the biofilm and planktonic states. We focused on disturbance frequencies of 6, 12, and 15/h, which represented frequencies that did (12/h) or did not (6/h, 15/h) reduce expression of genes encoding virulence factors at an amplitude of 0.3 mm. When disturbed at a frequency of 6/h, biofilm density was not significantly different than 0/h at amplitudes of 0.1, 0.2, and 0.3 mm (Figure 4A). The density of bacteria in the planktonic state was significantly higher when the disturbance amplitude was 0.3 mm. Otherwise, significant changes in the density of this population were not observed relative to 0/h. When the disturbance frequency was increased to 12/h, a significant reduction in biofilm density was observed at an amplitude of 0.3 mm, but not 0.1 and 0.2 mm (Figure 4B). A significant increase in the density of bacteria in the planktonic state was observed at amplitudes of 0.1 and 0.3 mm. Finally, when the disturbance frequency was increased to 15/h, a significant reduction in biofilm density was observed at an amplitude of 0.3 mm (Figure 4C). Significant increases in the density of bacteria in the planktonic state were observed at amplitudes of 0.2 and 0.3 mm.

Next, we used RT-PCR to investigate how combinations of disturbance amplitude and frequency impact expression of select virulence factors. Given that both single input and multiple input genes had similar responses to disturbance (Figures 2B–2D), we focused only on the expression of single input genes in the *las*, *rhl*, and *pqs* regulons. In general, a biphasic relationship between the fold change in gene expression (relative to 0/h) and amplitude was observed for each disturbance frequency tested (Figures 4D–4F); gene expression initially decreased with increasing shaking amplitude, but then increased at higher amplitudes. Moreover, we observed that as the disturbance frequency increased, the magnitude of the fold decrease in gene expression was larger. Otherwise put, increasing disturbance frequency increased the average fold reduction in gene expression (Figures 4D–4F, insets). Specifically, at a disturbance frequency of 6/h, significant reductions in the expression of *aprA* and *rhlA* were observed at amplitudes of 0.1 and 0.2 mm relative to 0/h (Figure 4D). Reductions in the expression of *phzM* were not significant. We did not observe a significant change in gene expression at an amplitude of 0.3 mm. When the disturbance frequency was increased to 12/h, significant reductions in the expression of *aprA* and *rhlA* were observed at amplitudes of 0.1, 0.2, and 0.3 mm (Figure 4E). Expression of *phzM* was reduced at an amplitude of 0.2 mm, but not 0.1 or 0.3 mm. Finally, at a disturbance frequency of 15/h, expression of *aprA*, *rhlA*, and *phzM* was reduced at amplitudes of 0.1 and 0.2 mm, but not 0.3 mm (Figure 4F). Overall, we found that while increasing disturbance frequency increased the average fold reduction in gene expression, the largest range of amplitudes over which the expression of at least one virulence factor was reduced occurred at the intermediate disturbance frequency of 12/h.

Removal of the lasR receptor can augment the reduction in gene expression during disturbance

To disentangle how disturbance might impact each of the three quorum sensing regulons, we acquired knockout strains that lack *lasR*, *rhlR*, *pqsR*, or both *lasR* and *rhlR*. Removal of genes encoding these receptors would disrupt the direct input to each regulon but allow the bacteria to continue to produce autoinducers, which have been previously shown to have some crosstalk between regulons.[32] Thus, by removing the receptor, we would preserve minor interactions between the nodes while removing the predominant interaction between receptor and autoinducer in each regulon.

As above, we first quantified the effect of disturbance on the density of bacteria in the biofilm and planktonic states. Consistent with previous literature,[33] and in the 0/h condition, we observed a reduction in density of biofilms produced by the Δ*rhlR* and the Δ*lasR*/Δ*rhlR* strains, although this reduction was not significant (Figure 5A). We also observed a significant increase in the density of bacteria in both the biofilm and planktonic states in the Δ*pqsR* strain. Disturbance at 12/h significantly increased the density of bacteria in the biofilm for the Δ*lasR*, Δ*rhlR*, and the Δ*lasR*/Δ*rhlR* strains. While the biofilm density of the Δ*pqsR* strain was reduced at 12/h, this reduction was not significant, likely owing to a large amount of variability in

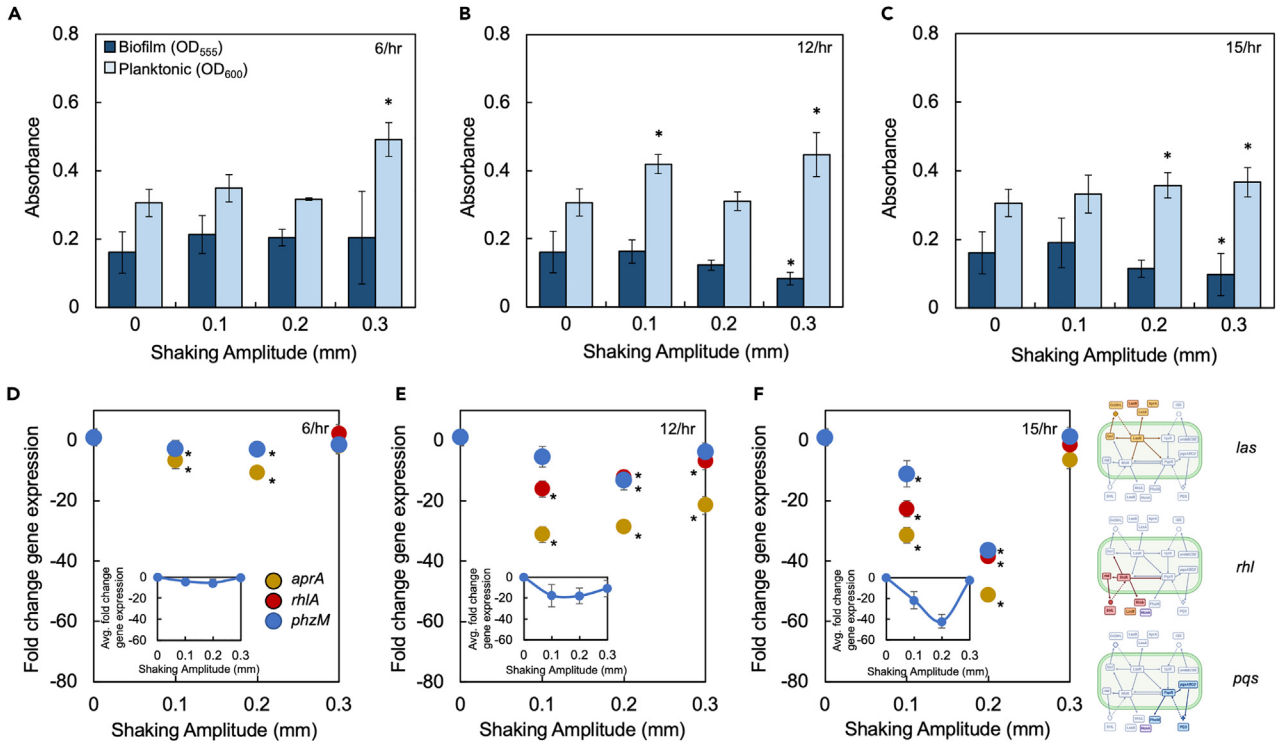

Figure 4. Periodic disturbance can result in a reduction in the expression of virulence factors across multiple disturbance amplitudes

(A) The effect of different disturbance amplitudes on the density of bacteria in the biofilm and planktonic state when disturbed at a frequency of 6/h. A significant (*) increase in the density of bacteria in the planktonic state was observed at an amplitude of 0.3 mm ($p < 0.0001$, Mann-Whitney (Shapiro-Wilk; $p = 0.0166$) compared to 0/h). For panels A–C, biofilm density was measured using a crystal violet assay and OD_{555}; density of bacteria in the planktonic state was measured using OD_{600}. For panels A–C, average and standard deviation from ≥4 biological replicates.

(B) The effect of disturbance amplitude on the density of bacteria in the biofilm and planktonic state when disturbed at a frequency of 12/h. A significant (*) reduction in biofilm density was observed at an amplitude of 0.3 mm ($p = 0.0057$, Mann-Whitney (Shapiro-Wilk; $p = 0.0074$) compared to 0/h). A significant increase in the density of bacteria in the planktonic state was observed at amplitudes of 0.1 mm and 0.3 mm ($p < 0.0001$, two-tailed t-test, compared to 0/h). ΔC_t data in Figure S5.

(C) The effect of disturbance amplitude on the density of bacteria in the biofilm and planktonic state when disturbed at a frequency of 15/h. A significant (*) reduction in biofilm density was observed at an amplitude of 0.3 mm ($p = 0.0095$, Mann-Whitney (Shapiro-Wilk; $p = 0.0125$) compared to 0/h). A significant increase in the density of bacteria in the planktonic state was observed at amplitudes of 0. and 0.3 mm ($p < 0.03$, two-tailed t-test, compared to 0/h).

(D) The effect of disturbance amplitude on the expression of select virulence factors from the *las*, *rhl*, and *pqs* regulons when biofilms were disturbed at 6/h. Expression of *aprA* ($p < 0.035$, two-tailed t-test, compared to 0/h) and *rhlA* ($p < 0.044$, data points behind *phzM* at 0.1 and 0.2 mm) were significantly (*) reduced at amplitudes of 0.1 and 0.2 mm. In panels D–F, average and standard deviation from ≥4 biological replicates; the inset shows the average fold change in expression for all virulence factors measured Data point colors (bottom right) are used consistently for panels D–F. All p values in Table S2; ΔC_t values in Figure S5.

(E) The effect of disturbance amplitude on the expression of select virulence factors from the *las*, *rhl*, and *pqs* regulons when biofilms were disturbed at a frequency of 12/h. Expression of *aprA* ($p < 0.01$, Mann-Whitney (Shapiro-Wilk; $p = 0.0086$), compared to 0/h) and *rhlA* ($p < 0.041$, two-tailed t-test) were significantly (*) reduced at all amplitudes tested. Expression of *phzM* was significantly reduced at an amplitude of 0.2 mm ($p = 0.014$, Mann-Whitney (Shapiro-Wilk; $p = 0.0009$)).

(F) Left: The effect of disturbance amplitude on the expression of select virulence factors from the *las*, *rhl*, and *pqs* regulons when biofilms were disturbed at a frequency of 15/h. Expression of *aprA* ($p < 0.002$, two-tailed t-test, compared to 0/h), *rhlA* ($p < 0.007$, Mann-Whitney (Shapiro-Wilk; $p = 0.0305$), and *phzM* ($p < 0.012$, two-tailed t-test) was significantly (*) reduced at amplitudes of 0.1 and 0.2 mm. Right: The main quorum sensing network in *P. aeruginosa*. Each section of the network is color coded to correspond to the virulence factor gene being measured in panels D–F. *las* regulon = yellow; *rhl* regulon = red; *pqs* regulon = blue.

biofilm density observed. Finally, significant increases in the density of bacteria in the planktonic state were observed in the Δ*rhlR* and the Δ*lasR*/Δ*rhlR* strains.

We then examined expression of both the receptors and single input effector of each regulon using RT-PCR. We performed four different comparisons. First, we compared gene expression observed in the knockout strains relative to the wild-type strain in the 0/h condition (Figure 5B). This allowed us to assess how removing each receptor affected overall gene expression outside of the effects of disturbance. In general, removal of receptors led to a decrease in the expression of both receptors and effectors. In the Δ*lasR*

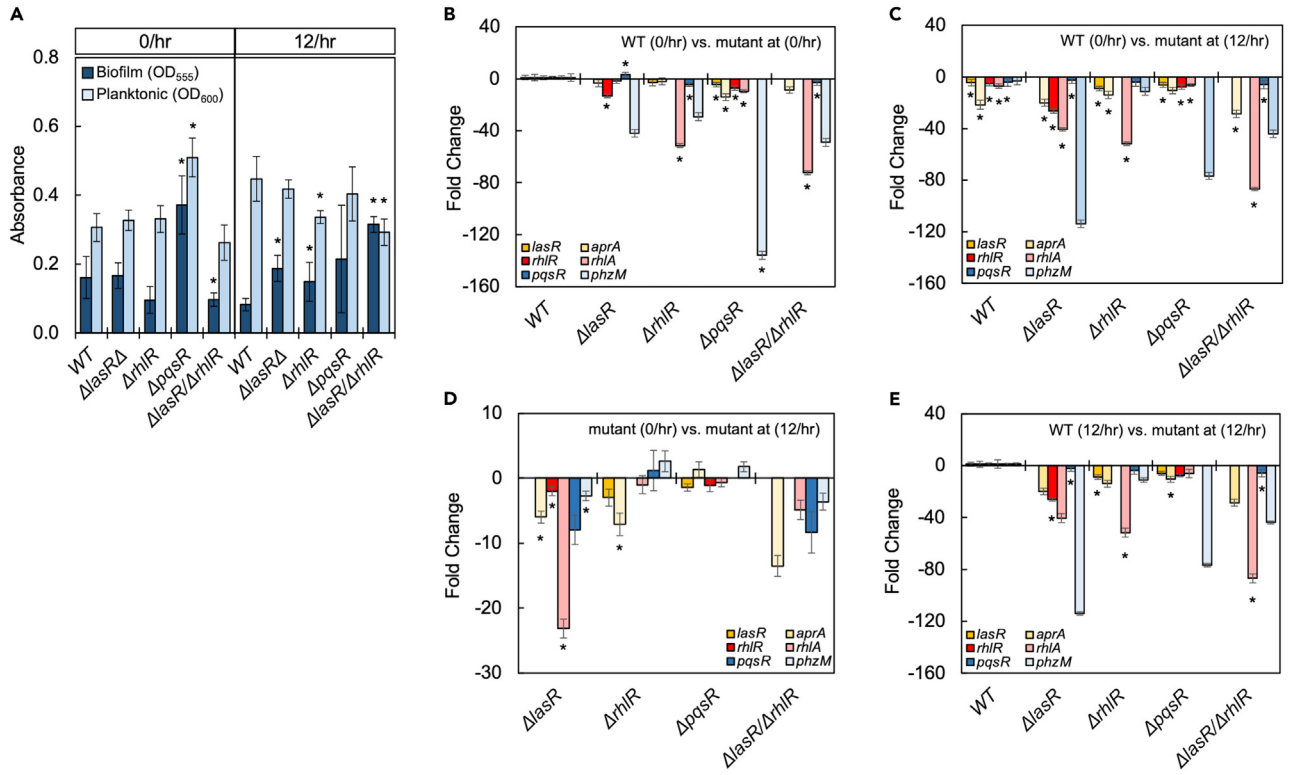

Figure 5. Removal of *lasR* can augment the decrease in expression of select virulence factors at a disturbance frequency of 12/h

(A) Effect of disturbance at 0/h and 12/h (0.3 mm amplitude) on Δ*lasR*, Δ*rhlR*, Δ*pqsR*, and Δ*lasR*/Δ*rhlR* knockout strains. * indicates statistically different from the wild-type strain (WT). At 0/h, there was an increase in the density of both the biofilm and bacteria in planktonic state for the Δ*pqsR* strain ($p \leq 0.0012$, Mann-Whitney ($p \leq 0.0038$, Shapiro-Wilk), and a decrease in the density of the biofilm in the *lasR*/Δ*rhlR* strain ($p = 0.0176$, Mann- Whitney ($p = 0.0003$, Shapiro-Wilk). At 12/h, the density of the biofilm was greater than WT for the Δ*lasR*, Δ*rhlR*, and Δ*lasR*/Δ*rhlR* strains ($p \leq 0.0252$ Mann-Whitney ($p = 0.0231$, Shapiro-Wilk). In addition, the density of bacteria in the planktonic state was greater than WT in the Δ*rhlR* and Δ*lasR*/Δ*rhlR* strains ($p \leq 0.0002$, two-tailed t-test). Average and standard deviation from ≥4 biological replicates.

(B) Effect of removing receptors in the quorum sensing regulons on gene expression of select receptors and effectors. In this panel, gene expression of WT at 0/h is compared to each knockout strain at 0/h * indicates significant difference ($p < 0.02$, each p value in Table S5). For panels B–E, average and standard deviation from ≥4 biological replicates and ΔC_t data in Figure S6.

(C) Effect of disturbance at 12/h on gene expression in knockout strains as compared to the WT strain in the 0/h condition. * indicates significant difference ($p < 0.04$, each p value in Table S5).

(D) Effect of disturbance at 12/h on gene expression in knockout strains as compared to each respective knockout strain at 0/h * indicates significant difference ($p \leq 0.05$, each p value in Table S6).

(E) Effect of disturbance at 12/h on gene expression in knockout strains as compared to the WT strain at 12/h * indicates significant difference ($p < 0.04$, each p value in Table S5).

strain, we found a significant reduction in the expression of *rhlR* concomitant with a small but significant increase in the expression of *pqsR*. Expression of both *rhlA* and *pqsR* was significantly reduced in the Δ*rhlR* strain. In the Δ*pqsR* strain, a significant reduction in all measured receptors and effectors was observed. Finally, in the Δ*lasR*/Δ*rhlR* strain, expression of *rhlA* and *pqsR* was significantly reduced.

Second, we compared the expression of receptors and effectors between the wild-type strain in the 0/h condition and the knockout strains in the 12/h condition (Figure 5C). This allowed us to assess the effect of disturbance on the knockout strains relative to the wild-type strain. In general, there was a reduction in the expression of receptors and downstream effectors for each knockout strain, only some of which were significant. In the Δ*lasR* strain, we observed a significant reduction in the expression of *aprA*, *rhlR*, *rhlA*, and *pqsR*. In the Δ*rhlR* strain, disturbance at 12/h significantly reduced expression of *lasR*, *aprA*, and *rhlA*. In the Δ*pqsR* strain, expression of *aprA*, *rhlR*, and *rhlA* was significantly reduced. Finally, in the Δ*lasR*/Δ*rhlR* strain, expression of *aprA*, *rhlA*, and *pqsR* was significantly reduced.

Third, we compared expression of receptors and effectors within each knockout strain and between the 0 and 12/h conditions (Figure 5D). This would allow us to control for reductions in gene expression owing to removal of each receptor; additional changes in gene expression in this comparison would be owing only to disturbance. We found that removal of *lasR*, but not *rhlR* or *pqsR*, resulted in a significant decrease in the expression of receptors and effectors. Expression of *aprA*, *rhlR*, *rhlA*, and *phzM* was significantly reduced in the Δ*lasR* strain. In the Δ*lasR*/Δ*rhlR* strain, an insignificant decrease in the expression of *aprA*, *rhlA*, and *phzM* was observed. Finally, in both the Δ*lasR* and Δ*lasR*/Δ*rhlR* strains, expression of *pqsR* was insignificantly reduced.

Finally, we compared expression of receptors and effectors between the wild-type strain and each knockout strain both disturbed at 12/h (Figure 5E). In general, we found that expression of all receptors and effectors was reduced, but not all were significant relative to 0/h. Expression of *rhlR* and *pqsR* was significantly reduced in the Δ*lasR* strain. In the Δ*rhlR* strain, expression of *lasR* and *rhlA* was significantly reduced. A significant reduction in the expression of *aprA* was observed in the Δ*pqsR* strain. Finally, in the Δ*lasR*/Δ*rhlR* strain, expression of *rhlA* and *pqsR* was significantly reduced. Overall, we observed that removal of key receptors coupled with disturbance at 12/h could significantly reduce some, but not all, genes in the quorum sensing system in *P. aeruginosa* relative to the wild-type strain.

DISCUSSION

We have found that periodic disturbance can reduce expression of multiple quorum sensing-regulated virulence factors from the three major regulons in *P. aeruginosa*. This is dependent upon both disturbance frequency and amplitude. We also found that removal of *lasR* can further increase the fold reduction in gene expression of single input effectors and receptors owing to disturbance at 12/h. We have previously demonstrated that disturbance can alter the density of *P. aeruginosa* in the biofilm and planktonic states using the linear shaking function of a microplate reader.[27] Indeed, that was the case here as changes in the density of bacteria in both states were observed during disturbance (Figures 2A, 4A–4C, and 5A). Furthermore, we previously showed that intermediate disturbance frequencies can reduce the density of bacteria in the biofilm state.[27] However, further increases in disturbance frequencies can increase the density of the biofilm owing to increased sheer force, which may increase nutrient and oxygen delivery to the bacteria facilitating biofilm formation.[34] This prior observation was confirmed in this study; when using a disturbance amplitude of 0.3 mm, the density of the biofilm was no different than the control at 6 and 15/h, but decreased at the intermediate frequency of 12/h (Figure 2A). In our previous work,[26,27] we were able to use changes in the distribution of bacteria to develop an understanding of how spatial structure was altering the expression of virulence factors and genes regulated by quorum sensing. Despite our best efforts, we were unable to conclusively connect changes in gene expression to changes in the distribution of bacteria in this study (analysis not shown). Here, we looked for relationships between the fold change in gene expression and the density of bacteria in the biofilm, the density of bacteria in planktonic state, the ratio of biofilm density/planktonic density, and the ratio of planktonic density/biofilm density; none of these yielded a consistent trend across conditions. While changes in the distribution of bacteria are likely having a role in this study, the involvement of multiple autoinducers, receptors, and the interconnectedness of the quorum sensing network in *P. aeruginosa* may serve to obscure the impact of changes to the distribution of bacteria. Furthermore, it is unclear how different combinations of disturbance frequency and amplitude affect the change in distribution throughout the experiment; continual measurement of this is unfortunately obscured by the MBEC peg, while crystal violet assays serve as endpoint measures as this assay fixes bacteria. There may also be factors involved that were not measured, including changes to oxygenation of the growth medium owing to disturbance, changes in the expression of other genes that have an indirect impact on the quorum sensing network, and potentially unidentified genes that sense physical forces, which have been previously identified in bacteria.[35–38] Moreover, previous work has found that changes in mechanical force sensed through type IV pili can affect the expression of virulence factors in *P. aeruginosa*[39]; a downstream effector of type IV pili, *vfr*, is thought to promote expression of *lasR*.[40] Accordingly, we cannot conclusively state that the sole reason for reductions in expression of quorum sensing-regulated genes was due to lack of access to autoinducers and/or changes in the distribution of bacteria as a result of disturbance. Nevertheless, it is clear that disturbance had a significant impact on the expression of multiple receptors and effectors in the quorum sensing network.

By testing multiple disturbance amplitudes and frequencies, we were able to observe that gene expression could be reduced over a wide parameter space. We found three prominent trends. First, when the

disturbance amplitude was held constant at 0.3 mm, only a disturbance frequency of 12/h resulted in a decrease in the expression of receptors and effectors (Figures 2B–2D). Second, when varying amplitude, an intermediate disturbance frequency of 12/h had the greatest number of genes whose expression was reduced across the amplitudes measured (Figures 4D–4F). Finally, we found that across three different amplitudes, the average fold change in expression of single input effectors increased with increasing disturbance frequency (Figures 4D–4F). Importantly, these trends may be species and/or strain specific. Our previous work showed that changes in the production of pyoverdine owing to disturbance were strain specific.[27] Moreover, strain-specific resistance to biofilm deformation due to physical forces has been previously noted in *P. aeruginosa*.[41] Nevertheless, our results suggest that combinations of disturbance and frequency may be used as a tool to rationally reduce the expression of genes regulated by quorum sensing.

The *pqsR* receptor is critical in the quorum sensing network and in pathogenesis. Not only does *pqsR* interact with the *lasR* and *rhlR* regulons, it is also responsible for regulating biofilm formation[42] and the synthesis of ~60 compounds,[43] many of which have been implicated in the recalcitrant nature of *P. aeruginosa* infections.[44] Given the central role that *pqsR* plays in the expression of multiple virulence factors and in host inflammation,[45] its inhibition has been central to multiple studies that have developed *pqsR* agonists. These agonists have been shown to reduce quorum sensing,[46] biofilm formation,[47] pathogenicity,[48] and antibiotic resistance.[49] Our network logic model suggests that perturbation of *pqsR* is key to reproducing the majority of the experimental results observed with the wild-type strain (Figure 3). While model predictions matched our experiments when the wild-type strain was perturbed at 12/h and with an amplitude of 0.3 mm, it did not exactly match our findings from when we varied disturbance amplitude (Figures 4D–4F). Chiefly, we found that expression of *phzM* could be significantly reduced by disturbance at various disturbance frequencies and amplitudes (12/h with 0.2 mm, 15/h with 0.1 and 0.2 mm) whereas our model predicts that this will not be impacted by disturbance. Interestingly, when we compared gene expression between the wild type and the Δ*pqsR* strain at 0/h, we found significant reductions in the expression of *lasR*, *rhlR*, *aprA*, *rhlA*, and *phzM* (Figure 5B), which largely matches our modeling predictions (Figure 3C). The discrepancy between our logic model and experimental results as they pertain to the expression of *phzM* could be due to our experimental results not reaching steady state as it pertains to *phzM*, which may be related to differences in disturbance frequency and amplitude. Our model might lack key nodes and edges, such as *vfr*,[40] that can influence activity in the entire quorum sensing pathway. This might also explain why gene expression patterns observed in our experimental analysis of knockouts do not always match our modeling results (Figure 5B). Importantly, we do not understand, nor can our model predict, the mechanism by which *pqsR* is being perturbed by disturbance. Nevertheless, our simulations coupled with our experimental analysis identify *pqsR* as a key receptor whose activity is being perturbed by disturbance. Thus, our work identifies a novel mechanism by which the activity of this key receptor can be reduced.

The analysis of multiple knockout strains provided insight into how removal of key receptors in the quorum sensing network might impact expression of single input virulence factors when coupled with disturbance. In general, we found that disturbance at 12/h of all knockout strains reduced the expression of both receptors and virulence factors relative to the wild-type strain at 0/h (Figure 5C). Moreover, when we compared gene expression of the knockout strains to the wild-type strain disturbed at 12/h, we found that removal of receptors can result in an increased reduction in expression of both receptors and virulence factors (Figure 5E). However, when accounting for reductions in gene expression owing to removal of each receptor in 0/h condition, we found that removal of *lasR*, but not *rhlR* or *pqsR*, resulted in a significant decrease in the expression of *rhlR*, *aprA*, *rhlA*, and *phzM* (Figure 5D). We also noted insignificant reductions in the expression of these genes in the Δ*lasR*/Δ*rhlR* strain. It is interesting to contemplate that either *lasR* itself, or a node leading into *lasR*, may have a role in buffering the network against disturbances. Interestingly, many strains of *P. aeruginosa* isolated from clinical[50] and non-clinical environments[51] lack functional copies of *lasR*. Moreover, these mutants appear to be under positive selection.[52] It would be interesting to study these strains to examine their susceptibility to disturbance. If these strains were more susceptible to disturbance, it may indicate some benefit to *P. aeruginosa* to being more prone to disturbance during the infection process. Overall, our analysis of knockout strains revealed that the *lasR* receptor may have a key role in buffering the quorum sensing network against disturbance.

Multiple methods have been developed to disrupt quorum sensing and/or biofilm formation. These include quorum quenching agents,[53] which have shown promise in the lab and in mouse models as they have effectively reduced the expression of downstream virulence factors, reduced infectivity, and aided in immune system clearance.[54] However, the use of agents that specifically target a single receptor may place a large selective pressure on that target. In contrast, disturbance may not place a selective pressure

directly on any one molecular target. In this scenario, the selective pressure may be spread out more evenly across multiple targets, thus reducing the probability and/or pace at which mutants that can withstand disturbance and return to gene expression levels that are comparable to the wild type arise. Accordingly, disturbance of *P. aeruginosa* has the potential to be used as an approach to reduce quorum sensing and virulence in the clinic without rapidly selecting for disturbance-resistant mutants.

Limitations of the study

We perceive that there are two limitations to our study. First, we were unable to identify the mechanism behind the observed decrease in the expression of virulence factors. While our model strongly suggests that activity of *pqsR* is perturbed, we do not know how. It may be due to changes in the spatial structure of the community, access to or synthesis of autoinducers, changes to the strength of interactions in the quorum sensing network, or a combination of the above. Second, it is unclear as to how disturbance might impact quorum sensing in non-laboratory-based medium. While we used King's A medium for our study as it is considered a standard laboratory growth medium for *P. aeruginosa*, our work is limited in that it does not tell us how disturbance might affect gene expression in more natural environments, such as the human host. These environments will have different nutrient compositions, which may affect multiple aspects of bacterial physiology, including quorum sensing and biofilm formation, outside of any effect of disturbance. Thus, it is unclear as how physical disturbance affects the expression of virulence factors in such environments.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106843.

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AUTHOR CONTRIBUTIONS

L.D.G., G.D.T., E.M.M., V.C., and I.B. performed experiments. L.D.G., G.D.T., E.M.M., and R.P.S. performed analysis. T.J.A.C. performed logic modeling. L.D.G. and R.P.S. wrote the manuscript. R.P.S. conceived of the work.

DECLARATION OF INTERESTS

T.J.A.C. has filed a patent application (US17/537,394) related to the code used in this manuscript. The authors declare no other competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
P. aeruginosa PA14	Lingchong You	N/A
P. aeruginosa PA14 *ΔlasR*	O'Loughlin et al.[55]	N/A
P. aeruginosa PA14 *ΔrhlR*	O'Loughlin et al.[55]	N/A
P. aeruginosa PA14 *ΔlasR/rhlR*	O'Loughlin et al.[55]	N/A
P. aeruginosa PA14 *ΔpqsR*	Scheffler et al.[56]	N/A
Critical commercial assays		
RNeasy Mini Kit	Qiagen	74104
RNase-Free DNase Set	Qiagen	79254
DNase Max Kit	Qiagen	15200-50
iScript Reverse Transcription Supermix	BIO RAD	1708840
iTaq Universal SYBR Green Supermix	BIO RAD	1725121
Deposited data		
Dryad	This study	https://datadryad.org/stash/share/0kpmCqwjPbbdi9Ee69zCkUNJiNmzXfqNYUh6vLXnwOg
Oligonucleotides		
Primers for RT-PCR	Sigma-Aldrich	See Table S1
Software and algorithms		
JMP Pro 16.0.0	SAS Institute Inc.	N/A
Excel (version 16.72)	Microsoft	N/A
Network Logic Model	This study	Protected by patent application US17/537,394
Other		
MBEC plates	Innovotech	19113
Individual PCR tubes 8-tube strip, clear	BIO RAD	TLS0801
Optical Flat 8-cap Strips	BIO RAD	TCS0803

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Robert P Smith (rsmith@nova.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All raw data have been deposited at the Dryad Digital Repository and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- All original code is integral to patent application (US17/537,394) and cannot be released to the public.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Strains and general growth conditions

Pseudomonas aeruginosa strain PA14 was used throughout this study. Knockout strains (Δ*lasR*, Δ*rhlR*, and Δ*lasR*/Δ*rhlR*) were obtained from.[55] The Δ*pqsR* strain was obtained from.[56] Overnight cultures were initiated from single colonies isolated from Luria Bertani (MP Biomedical, Solon, OH) agar plates containing 1% agar (Alfa Aesar, Ward Hill, MA). Overnight cultures were created from single colonies inoculated in 3 mL of liquid LB medium placed in 16 mL culture tubes (Genesee Scientific, Morrisville, NC). Bacteria in culture tubes were shaken at 250 revolutions per minute (RPM) at 37°C for 24 hours.

Growing biofilms

Biofilms were grown in MBEC biofilm inoculator plates (Innovotech, Edmonton, AB, Canada). Overnight cultures were washed once with fresh King's A medium (2% peptone (Fisher Scientific, Hampton, NH), 1% potassium sulphate (Thermo Scientific, Waltham, MA), 0.164% magnesium sulphate (BDH, VWR, Radnor, PA), 1% glycerol (Acros Organics, Geel, Belgium)[57] and subsequently diluted 1000X into fresh King's A medium. 175 μL of diluted culture was added to the wells of the MBEC plate. The wells surrounding the culture were filled with 200 μL of autoclaved dH_2O to reduce evaporation. The MBEC lid was then placed on the wells, the plate was sealed with two layers of parafilm, and was subsequently shaken at 110 RPM at 37°C for 24 hours. During shaking, the incubator contained a beaker filled with ~100 mL of dH_2O.

METHOD DETAILS

Measuring the density of bacteria

Biofilm density

Biofilms were grown as described in 'Growing biofilms'. After 24 hours of growth, the lid of the MBEC plate was removed and biofilms were washed by placing the pegs in 200 μL of fresh King's A medium for 10 seconds that was housed in a new 96-well plate. The lid with pegs was then placed for 10 minutes at room temperature in 125 μL of 0.1% m/w crystal violet (Acros Organics, NJ, USA) in 95% ethanol (Fisher Bioreagents). Excess crystal violet was removed from the biofilms by washing the pegs four times in 200 μL of dH_2O for 10 seconds that was housed in new 96 well plates. Finally, the pegs were incubated for 10 minutes in 200 μL of 30% acetic acid housed in a new 96 well plate (Fisher Chemical). Absorbance was measured using optical density (OD) at 555 nm (OD_{555}) in a Victor X4 plate reader (Perkin Elmer, Waltham, MA). Background absorbance of 30% acetic acid was subtracted from all measurements.

Planktonic state

The density of bacteria in the planktonic state was measured using OD_{600} in a Victor X4 plate reader. Background absorbance of bacteria-free King's A medium was subtracted from all measurements.

Disturbance of biofilms

Biofilms were grown as described in 'Growing biofilms'. After 24 hours, the pegs were washed for 10 seconds in 200 μL of fresh King's A medium (that was housed in a new 96-well plate). The washed biofilms were placed in 180 μL fresh King's A medium. The plate was then placed in a Victor X4 Perkin Elmer microplate reader set to 37°C. Biofilms were disturbed at the frequency and amplitude indicated in the text using the linear shaking function (shaking along the x-axis, fast setting = frequency of 4800/mm/min) for 10 seconds per shaking event for a total of 24 hours.

RNA extraction

Biofilms were grown as described in 'Growing biofilms' and disturbed as described in 'Disturbance of biofilms.' 175 μL of media was removed from the wells of the MBEC plate and placed into 1.5 mL microcentrifuge tubes. Bacteria were pelleted via centrifugation (2 minutes at 12,000 RPM) and were washed twice in fresh King's A medium. The cell pellet was subsequently resuspended in 30 μL of 10 mg/mL lysozyme solution (MP Biomedicals) in 1X Tris-EDTA (Thermofisher). This mixture was vortexed every 2 minutes at room temperature for a total of 20 minutes. Total RNA was then isolated using an RNeasy MiniKit (Qiagen) following the manufacturer's recommended protocol including the in-column DNase digestion. After RNA was isolated, the Qiagen DNase Max Kit (Qiagen) was used to remove any residual DNA by following the manufacturer's QuickStart protocol.

cDNA synthesis

cDNA synthesis was performed in a C1000 Touch Thermalcycler using the iScript Reverse Transcription Supermix from BIO RAD (Hercules, CA). The following protocol was used: 5 minutes at 25°C, 20 minutes at 46°C and 1 minute at 95°C. Volumes for the reaction were: 4 μL of iScript RT Supermix, 10 μL of RNA template, and 6 μL of nuclease free water.

Real-time PCR

Real-time (RT) PCR was performed using the iTaq Universal SYBR Green Supermix (BIORAD) kit following the manufacturer's instructions. Each reaction contained 5 μL iTaq SYBR green, 3.5 μL RNA/DNAse-free water, 0.5 μL (10 μM) forward primer, 0.5 μL (10 μM) reverse primer, and 0.5 μL of template (cDNA, RNA, or dH_2O). RT-PCR was performed in a Bio-Rad CFX96 Touch Real-Time PCR Detection System using the following protocol: 95°C for 3 minutes, 95°C for 10 seconds, 63°C for 30 seconds, (repeat 39X), 95°C for 5 seconds. Melt curve analysis was performed directly after the 40th cycle. Fold change in gene expression was determined using normalized average threshold cycle (C_q) with the comparative C_t method.[58] All *Ct* values were normalized to *rpoD*. Primers used in RT-PCR are listed in Table S1. Representative gel image confirming PCR product amplification and functioning of controls in Figure S1.

Discrete ternary logic analysis of regulatory network

Using a similar approach reported in,[59–62] the *P. aeruginosa* quorum sensing system was captured as a logic connectivity model consisting of interconnected nodes with three discrete states: 0 (inhibited), 1 (nominal) and 2 (activated). In brief, the state of the system at a point in time was described by an assignment of discrete states to all nodes. The state that each node in the system transitions to in the next time step was determined from a set of balanced ternary logic statements (see[63]), the node's current state, and the defined interactions (i.e. activate or inhibit) of the neighboring input nodes. The logic is such that an increase in activators raises the node value, while a decrease in inhibitors decreases the value. In cases where both activators and inhibitors were increased, the node value remained unchanged. The system was updated asynchronously (allowing only one variable to change at a time), such that for each current state there are potentially several subsequent states towards which it may evolve. The number of states, and the values they can be assigned, determine the total number of states available to the model system. With the ternary logic used here, a model of N nodes has 3^N states. As such, our model possesses 3^{18} = 387,420,489 possible states. By analyzing all possible states of the system, a temporal sequence of states was discerned. Steady states were defined as those states for which the current system state did not evolve in time.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All statistical tests were performed in JMP Pro 16 (SAS Institute Inc., Cary, NC) and Microsoft Excel (Redmond, WA). A Shapiro-Wilk test was used to determine the distribution of the data. In non-normally distributed data sets, a Mann–Whitney U test was performed. In normally distributed data sets, a two-tailed t-test was performed (unpaired, unequal variation). ΔC_t values were used to assess significant differences in gene expression data. All P values for gene expression analysis are listed in Tables S2, S5 and S6. Significant differences ($P \leq 0.05$) are indicated with * throughout the manuscript. The number of biological replicates (*n*) can be found in the figure legends. Averages and standard deviation (error bars) as determined in Microsoft excel are used as throughout the manuscript. * is used to indicate statistical significance on all figures in the manuscript. The specific statistical test used is noted in the figure legend. In the case where multiple statistical tests are used for the same figure panel(s), the statistical test and all related P values can be found in the associated table in the supplemental information. Each table is noted in the figure legend.