Quorum-sensing regulation of antibiotic resistance and antibiotic-dependent effects on social cheating in the bacterial pathogen *Pseudomonas aeruginosa*

By

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Quorum-sensing regulation of antibiotic resistance and antibiotic-dependent effects on social cheating in the bacterial pathogen *Pseudomonas aeruginosa*

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Abstract

The *Pseudomonas aeruginosa* LasR-I quorum sensing system regulates secreted proteases that can be exploited by cheaters, such as quorum sensing receptor-defective (*lasR*) mutants. The LasR-I system is essential for virulence and increases resistance to aminoglycoside antibiotics. However, *lasR* is a mutation hotspot in clinical *P. aeruginosa* isolates. Here, we explore how adaptation to aminoglycosides tobramycin and gentamicin influences the evolutionary trajectory of quorum sensing. We show that both antibiotics suppressed the emergence of *lasR* mutants in casein-passaged populations. Several mutations accumulated in those populations indicating evidence of antibiotic adaptation. We found that inactivation of the nitrogen phosphotransferase gene, *ptsP,* suppressed cheaters through the policing toxin pyocyanin. Cheater suppression through *ptsP*-independent mechanisms were also evident in gentamicin-adapted variants. Although *ptsP* inactivation suppresses cheating, Δ*ptsP* populations are prone to collapse. Population collapse was also observed in Δ*ygdP* and Δ*mdpA*. The mechanistic pathway behind the population collapse remains unknown. We also show that a point mutation in the elongation factor, *fusA1* G61A (FusA1^{A21T}) reverses the role of LasR in tobramycin resistance. This mutation increases the antibiotic resistance of the *lasR* mutants through an ArmZ-dependent upregulation of the MexXY efflux pump. Inactivation of *lasR* has an additive effect on ribosome stalling which induces ArmZ expression. This illustrates how losing LasR function may be advantageous in antibiotic-treated environments and may provide a potential explanation for the high frequency of *lasR* mutants in clinical samples. Overall, this work provides insights on how antibiotic selection alters quorum sensing and quorum sensing-regulated functions. These pleiotropic effects of antibiotics on quorum sensing might be important for populations adapting to antibiotics during interspecies competition or infections.

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CHAPTER I: Introduction

Two sections (*Overview of quorum sensing* and *Cooperation and social cheating in Pseudomonas aeruginosa*) of this chapter were written as per the Review Article:

Abisado RG*, Benomar S*, Klaus JR*, Dandekar AA, Chandler JR. 2018. Bacterial Quorum Sensing and Microbial Community Interactions. mBio. 9(3):e02331-17. doi: 10.1128/mBio.02331-17 (*equal contributions by **RGA**, SB, JRK).

Cooperation is a widespread social behavior in bacteria. Cooperative behaviors in the opportunistic pathogen *Pseudomonas aeruginosa* are carried out using quorum sensing, a type of cell-cell signaling. Studies on quorum sensing systems in *P. aeruginosa* have contributed in the better understanding of the biology of cooperation in this pathogen. *P. aeruginosa* is also notable for its innate drug resistance and ability to acquire drug resistance factors. Antibiotics can have pleiotropic effects on bacterial cells. Understanding how quorum sensing and cooperation are influenced by antibiotic adaptations may provide insights on how *P. aeruginosa* behave in natural habitats and infection environments. In this chapter, a background on the biology, quorum sensing, cooperation, social cheating, and antibiotic resistance in *P. aeruginosa* is discussed.

Overview of quorum sensing

Quorum sensing is a type of cell-to-cell signaling that regulates gene expression in a population density-dependent manner (1-4). When the population reaches a critical density, quorum sensing triggers behavioral changes through production and sensing of extracellular signals. At low population cell density, the signal is produced at a low concentration and accumulates in the local environment as the population density increases. Once a threshold signal concentration is reached, the signal interacts with a receptor protein which causes a coordinated change in gene expression in the population (1, 2). In many Proteobacteria, the quorum-sensing signal is in the form of acyl-homoserine lactone (AHL) (4, 5). AHL quorum sensing systems are composed of LuxR family signal receptors and LuxI family signal synthases (**Fig. 1**).

AHLs are typically produced by LuxI synthases from *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) from the fatty acid biosynthesis pathway. The length and

substitution of the acyl side chain determine the specificity of the AHL quorum sensing systems (6-8). The LuxR proteins have a conserved N-terminal AHL-binding domain and a C-terminal helix-turn-helix DNA-binding domain (9, 10). The interaction of the LuxR N-terminal domain with AHLs induces homodimerization of the LuxR homolog and subsequent binding to a specific DNA sequence called the *lux* box in the target promoter (11, 12). The AHL-bound LuxR often induces transcription of its cognate signal synthase *luxI* signal synthase gene, resulting in a positive feedback loop that further increases the concentration of AHLs (**Fig. 1**) (13).

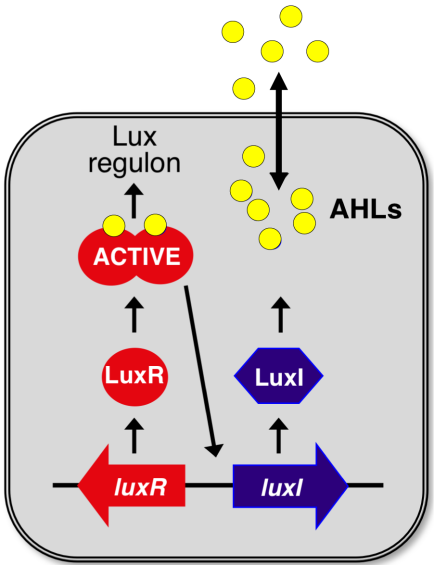

Fig. 1. AHL quorum sensing system architecture. AHL signals (yellow circles) are synthesized by LuxI family signal synthases. At threshold concentration, AHLs interact with LuxR family transcription factors. Interaction between AHL and LuxR causes LuxR to dimerize and become active, resulting in the production of more AHLs and induction of target gene regulation. Figure adapted from (14).

Most LuxR-family proteins act as transcriptional activators, although a subset of them are repressors, which can be identified by sequence relatedness (for a review, see (15)). Cognate LuxI- and LuxR-family proteins are often encoded adjacent to one another. However, some

species have orphan LuxR receptors which encode LuxR-type proteins with no cognate LuxI-family AHL synthase (16). LuxR- and LuxI-type proteins are widely distributed in Proteobacteria, and many quorum sensing architectures involve more than one signal-receptor combination (2, 5, 15). The selective pressures that result in multiple AHL signaling circuits are not clear, although it has been proposed that the different properties of AHL signals might provide specific benefits in different environments (17).

AHL quorum sensing systems control diverse behaviors such as production of secreted toxins and virulence factors, biofilm matrix components, and DNA conjugation (for reviews, see (1-3, 18)). The secreted or excreted goods can be shared by the entire group and are important for cooperation (4). Some of the cooperative activities, such as secretion of toxins, might also be important for competition with other species of bacteria or other organisms (19, 20).

Pseudomonas aeruginosa and its quorum sensing systems

One of the model bacteria for understanding quorum sensing is the Gram-negative *Pseudomonas aeruginosa* (21, 22). *P. aeruginosa* is a motile non-spore forming rod biofilm-forming bacterium that was first discovered from green pus-contaminated bandages (21, 23, 24). The production of pyocyanin gives *P. aeruginosa* the blue-green color (21, 24). *P. aeruginosa* is ubiquitous, thriving both in soil and water habitats and with a broad host range including animals and plants (21, 25). It is considered an opportunistic pathogen, hardly infecting healthy people but frequently associated with nosocomial infections and poses risk to immunocompromised individuals such as AIDS, burn, cancer, cystic fibrosis, and solid-organ transplant patients (26-30). *P. aeruginosa* is also notable for its multidrug resistance nature, making it one of the superbugs (28, 31-33). About 10% of the genes in this pathogen is regulated by quorum sensing.

Some of the quorum sensing-regulated genes are genes coding for proteases, pyocyanin, rhamnolipid, and hydrogen cyanide (21, 34-38).

P. aeruginosa has four known overlapping hierarchically organized quorum sensing networks (**Fig. 2**) (3, 22, 39-45). The first two are AHL-based quorum sensing systems, the LasR-I and RhlR-I systems. The other two systems are *Pseudomonas* quinolone signal (PQS) system and the integrated quorum sensing system (IQS) (39, 40). The LasR-I quorum sensing system is composed of the LasR signal receptor and LasI signal synthase and responds to 3-oxododecanoyl-homoserine lactone (3OC12-HSL) signals. The LasR-I system, which is the focus of this work, is required to activate the other three quorum sensing systems. Thus, LasR is considered the master quorum sensing regulator (3, 4, 22).

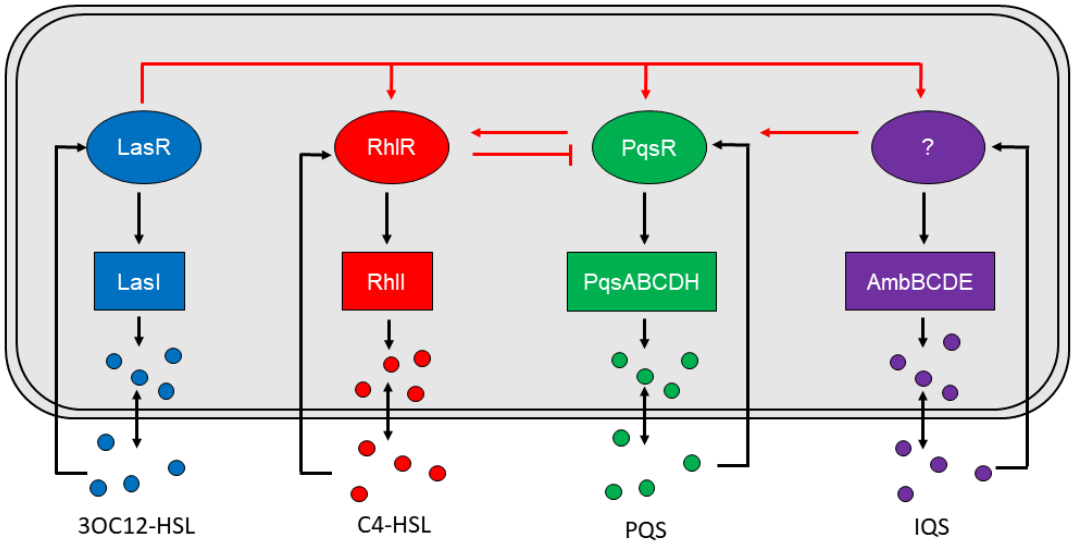

Fig. 2. *Pseudomonas aeruginosa* quorum sensing systems. Each quorum sensing circuit is composed of transcription factor or signal receptor (LasR, RhlR, PqsR, unknown for IQS), signal synthase (LasI, RhlI, PqsABCDH, AmbBCDE), and signal (3OC12-HSL, C4-HSL, PQS, IQS). LasR-I and RhlR-I are AHL-based quorum sensing systems. These four quorum sensing systems are interrelated as depicted by the red lines. Black arrows indicate autoinduction; red arrows indicate stimulatory effect; and short vertical lines indicate inhibitory effect. Figure modified from (3).

The RhlR-I quorum sensing system, which responds to butanoyl-homoserine lactone (C4-HSL) signals, is composed of the RhlR signal receptor and RhlI signal synthase. Activation of the RhlR-I system also requires PQS, a quorum sensing system that operates using PqsR and quinolone signals (2-heptyl-3-hydroxy-4-quinolone) synthesized by PqsABCDH. Inhibition of the PQS system by RhlR is thought to ensure achieving a correct 3OC12-HSL to C4-HSL ratio, which dictates PQS system activation (4, 5, 30). The fourth known quorum sensing system in *P. aeruginosa* is IQS which responds to IQS signals (2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde) synthesized by AmbBCDE (39). PQS activation also requires IQS (22, 39). Under some conditions (*e.g.* limited phosphate) and certain genetic background (*e.g.* presence of other mutation), activation of the RhlR-I, PQS, or IQS is observed even in the absence of LasR, indicative of a multifaceted quorum sensing cascade in this pathogen (22, 39, 40, 43-46).

Loss of LasR function is a common adaptation for cystic fibrosis *P. aeruginosa* isolates (47). These *lasR* mutants have reduced virulence in acute infection models (48-50). Many *lasR* mutants form flattened colonies (due to lysed surface) with metallic iridescent sheen (51). *lasR* mutants undergo autolysis and produce a metallic iridescent sheen due to the accumulation of 4-hydroxy-2-heptylquinoline (HHQ), the precursor for the synthesis of PQS (51). Autolysis can also be observed in *lasR*-intact strains when PQS accumulates (52). HHQ accumulates in *lasR* mutants because PqsH, which catalyzed the conversion of HHQ into PQS, is LasR-controlled (35, 53).

Cooperation and social cheating in *Pseudomonas aeruginosa*

Laboratory studies have demonstrated that quorum sensing systems can be cooperative (54, 55). Many quorum sensing-regulated products are shared “public goods” that can be used by

any member of the community (56). Typically these are secreted or excreted products, such as secreted proteases (37, 57). Synthesis of public goods imparts a metabolic cost for an individual cell, but is beneficial for all the other cells within the population (56).

The cost involved in the production of quorum sensing-dependent public goods makes it prone to exploitation, or social cheating. Social cheating can offer growth or survival advantages to individual bacteria (58-60). Since cheaters thrive at the expense of cooperators, their presence may destabilize cooperation: if the proportion of social cheaters becomes too high, the population will no longer produce sufficient public goods. If public goods are needed for growth, the entire population stops growing and, ultimately, collapses (56, 60-62).

In situations where quorum sensing controls the production of public goods, the social cheaters that arise are quorum sensing receptor mutants (55). Laboratory studies have shown that (a) cooperators have a growth advantage over cheaters when they are grown separately in these conditions and (b) cheaters exploit the cooperators and proliferate when they are grown as a mixed culture.

Quorum sensing regulation of cooperation and cheating was first demonstrated using *P. aeruginosa*. *P. aeruginosa* uses quorum sensing to control production of a protease called elastase (38). Elastase production is required for growth when populations are grown on casein as a sole source of carbon and energy (54, 55). When *P. aeruginosa* populations are passaged in casein broth, mutations in the gene encoding the quorum sensing receptor LasR emerge within <100 generations. The mutations are typically single nucleotide changes that abolish or significantly reduce LasR function (55). These mutants are cheaters, as they are unable to grow by themselves in casein broth, however, they proliferate when grown in a mixed culture with the wild type.

Other systems for studying cooperating and cheating in *P. aeruginosa* have since been developed. A protease-dependent laboratory model has been used to demonstrate quorum sensing-dependent cooperation in biofilm conditions (63). Quorum sensing and sociality were also studied in the context of swarming, another social trait due to cooperative production of secreted surfactants (64, 65). Quorum sensing exploitation by cheaters has also been demonstrated using *in vivo* models (66-68). Rumbaugh and colleagues (68) demonstrated that *P. aeruginosa* LasR mutants act as cheaters during wound infections in mice. In the study, LasR mutants were attenuated compared to wild type in single-strain infections. However, when a mixture of both strains was used as an infection inoculum, the LasR mutants outgrew the wild type and ultimately dominated the population (68).

It is possible that quorum sensing-null bacteria have nonsocial advantages in some settings (51, 69, 70). Surveys of some communities from infections and other environments indicate quorum sensing mutants (signal receptor-deficient) can readily be isolated from *P. aeruginosa* populations (47, 71-79). However, it remains unclear whether these mutants function as cheaters in these natural communities. In one study of *P. aeruginosa* infections of ventilator-intubated patients, LasR mutants were shown to proliferate only when quorum sensing-intact cells were present (80), supporting the idea that the LasR mutants are social cheaters. However, in other studies, LasR mutants appear to have an intrinsic growth advantage and be better adapted to certain growth environments than the wild type, suggesting there is a selective advantage of mutating LasR (51, 69). These social cheaters may also have evolved for resource optimization, population stability, and stress protection (70, 81-83).

Because the rise of cheaters can threaten cooperation in a population, a recurring question in evolutionary biology is: how do cooperative systems persist, despite the ongoing threat of

cheating (84)? Microbial systems are emerging as an excellent tool for studying cheater control because microbes have the advantage of rapid growth, high population yields, and reproducible growth in the laboratory. Studies in these systems suggest cheater control is widespread in bacteria (56).

Quorum sensing can stabilize cooperation by decreasing the incentives to cheat or by sanctioning cheaters. One such mechanism of cheater control is through pleiotropy, where quorum sensing co-regulates public goods with goods that provide an individual benefit (private goods) (62, 85). In *P. aeruginosa*, linking public (*e.g.* elastase) and private (*e.g.* nucleoside hydrolase) goods through pleiotropy causes a disincentive to cheat due to loss of the private good (62). Quorum sensing can also stabilize cooperation through a mechanism involving selective harm of cheaters, a type of policing or enforcement mechanism similar to that described in animals (86). Policing toxins in *P. aeruginosa* include hydrogen cyanide, pyocyanin, and rhamnolipid (87-90). The cooperators are not harmed by the policing toxins because the resistance factors against the policing toxins are also quorum sensing-controlled (87-90).

Cheating can also be deterred through "metabolic prudence", *i.e.* delaying production of costly products until nutrients required for growth are exhausted (64). For example, in swarming *P. aeruginosa* colonies, cheaters exploit cooperating cells that secrete rhamnolipid biosurfactants, which are needed to swarm (64). Quorum sensing-control of rhamnolipid production results in a delay in the production of this metabolically costly biosurfactant, minimizing the benefit of cheating (64). Evolution theory predicts that limited dispersal through spatial structuring or high viscosity is also protective against cheating (91). Conditions of limited dispersal increase the probability that interacting individuals are close relatives (92), such that cooperative public goods are shared only among related cooperator cells. Indeed, quorum

sensing is protected from cheaters in *P. aeruginosa* populations grown on casein in conditions of high relatedness (54). Quorum sensing-dependent biofilm formation and spatial structuring might also increase cooperation, which could be important for stabilizing quorum sensing in natural environments (93, 94).

Interplay of quorum sensing and antibiotic resistance in *Pseudomonas aeruginosa*

The rampant use of antibiotics resulted in the evolution of superbugs that are resistant to different classes of antibiotics, leading us on the edge of post-antibiotic era (95-97). If the antimicrobial resistance dilemma is not addressed, the world is predicted to face about 10 million deaths yearly by 2050 (98). One of the multidrug-resistant pathogens posing public health risks worldwide is *P. aeruginosa* (99, 100).

P. aeruginosa is known for its intrinsic resistance to many antibiotics as well as its natural ability to acquire antibiotic resistance machineries and enhance its antibiotic resistance arsenals (99, 101, 102). Intrinsic resistance mechanisms in *P. aeruginosa* arises from the natural presence of antibiotic resistance genes and lack of genes coding for antibiotic target sites in its genome (101, 103, 104). Mutations in the intrinsic antibiotic resistance resulting from antibiotic selection and horizontal gene transfers both contribute in the development of acquired antibiotic resistance in *P. aeruginosa* (99, 105-108). Under stressful conditions, *P. aeruginosa* also exhibits transient, unstable, and inducible adaptive resistance mechanisms which are mediated by changes in gene expression triggered by external factors (109, 110). The major antibiotic resistance mechanisms in *P. aeruginosa* are well-characterized, but there are still more uncharacterized molecular pathways that contribute in *P. aeruginosa* antibiotic resistance (99, 103, 109, 111, 112).

One of the poorly understood regulatory pathways with regards to antibiotic resistance is quorum sensing. Although the link between antibiotic resistance mechanisms and quorum sensing in *P. aeruginosa* remains murky, there is a growing body of research showing an interplay between antibiotic resistance and quorum sensing (99, 111, 113, 114). Several studies showed evidences of quorum sensing-dependent low-level antibiotic resistance (63, 115-117). LasR mutants were found to be more susceptible to the aminoglycosides under biofilm conditions (63, 115-117). Several studies showed how quorum sensing inhibitors could potentiate the effects of antibiotics (117-119). Clinical isolates of *P. aeruginosa* with elevated expression of *mexY*, one of the genes in the operon coding for the MexXY multidrug efflux pump were also observed to have enhanced expression of the LasR-I system (120). Moreover, expression of some efflux pumps in *P. aeruginosa* is transcriptionally induced by quorum sensing or enhanced by quorum sensing signals (34, 121). Quorum sensing may also be contributing to antibiotic resistance through biofilms, formation of persister cells, and regulation of bacterial products that confer antibiotic tolerance (122-124). At the other end of the spectrum, some studies showed a correlation between antibiotic resistance and loss of quorum sensing functions. LasR mutants ability to enhance nitrite-based metabolism has been shown to confer tolerance to tobramycin and ciprofloxacin suggesting that susceptibility of LasR mutants to antibiotics could be highly conditional (125). Increased β-lactamase activity in LasR mutants enhanced resistance to β-lactam antibiotics, but these phenotypes could be a result of adaptation (51, 126). Evolution experiments also revealed that *lasR*-intact and *lasR*-null mutants could undergo different antibiotic adaptations, *i.e.* selection of *lasR* mutation first could restrain evolution of antibiotic resistance while selection of mutations conferring antibiotic resistance first could restrain emergence of *lasR* mutants (127).

Overall, the connection between quorum sensing and antibiotic resistance remains a critical gap in knowledge of quorum sensing biology in *P. aeruginosa*. Understanding how quorum sensing influences antibiotic resistance and how quorum sensing systems adapt to antibiotic selection during therapy would be a substantial step towards filling this gap in knowledge. This will provide valuable insights in the development of effective treatments for *P. aeruginosa* infections, particularly those that focus on therapies that function by blocking quorum sensing.

Dissertation Overview

This work explores the impact of aminoglycoside-induced adaptations on the evolutionary trajectory of quorum sensing. Antibiotics are known to have a wide spectrum of effects on bacteria other than killing. Here, the changes induced by antibiotics on *P. aeruginosa* quorum sensing systems and quorum-sensing regulated phenotypes such as cooperation and antibiotic resistance are described. Understanding the pleiotropic effects of antibiotics on quorum sensing contributes to a better understanding of quorum sensing biology. This study focuses on the effect of the aminoglycosides tobramycin and gentamicin, which are both clinically relevant antibiotics, and on the LasR-I quorum sensing system of *P. aeruginosa*.

Chapter II describes the effect of tobramycin adaptation on quorum sensing activity and the dynamics of cooperator-cheater relationships. This work demonstrates that tobramycin can suppress the emergence of *lasR* mutants in casein-passaged populations. Suppression can occur directly because of the higher tobramycin susceptibility of the *lasR* mutants, or indirectly, through the pleiotropic effects of tobramycin-induced adaptations. In this chapter, the mechanism by which tobramycin can indirectly control emergence of cheaters is further described.

Chapter III describes how tobramycin-induced adaptation can alter the antibiotic resistance of *lasR* mutants. LasR is known to contribute to tobramycin resistance, virulence, and pathogenesis. However, *lasR* mutants are surprisingly common in clinical samples. This chapter describes a potential explanation for the abundance of *lasR* mutants in tobramycin-treated infection environments. The mechanism of tobramycin resistance in the tobramycin-adapted *lasR* mutants is further explored in this chapter.

Chapter IV describes the adaptations of *P. aeruginosa* in the aminoglycoside gentamicin. This work reveals similarities and differences in the genetic adaptations acquired by *P. aeruginosa* in different types of aminoglycosides. This chapter further illustrates other genetic basis of enhanced antibiotic resistance in LasR-intact and LasR-deficient *P. aeruginosa* as well as enhanced quorum sensing system activity, other potential mechanisms of cheater suppression induced by antibiotic selection, and how antibiotic selection could lead to accelerated population collapse.

Chapter V further illustrates the effect of tobramycin adaptations in the dynamics of cooperator-cheater relationships. Here we show that tobramycin adaptation does not always lead to stability of quorum-sensing regulated cooperation. Some tobramycin adaptive mutations can also induce population collapse. We discuss in this section the adaptive mutations that trigger population collapse and the conditions by which population collapse occurs.

Overall, this work provides insights on how antibiotic selection shapes the evolutionary trajectory of quorum sensing in the multidrug resistant opportunistic pathogen *P. aeruginosa*. Aminoglycoside-induced adaptive mutations alter quorum sensing and quorum sensing-regulated functions such as antibiotic resistance and cooperation. These pleiotropic effects of antibiotics might be important for populations adapting to antibiotics during interspecies competition or

infections. Moreover, this work contributes to a deeper understanding of quorum sensing biology.

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CHAPTER II: Tobramycin adaptation enhances policing of social cheaters in *Pseudomonas aeruginosa*

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Abstract

The *Pseudomonas aeruginosa* LasR-I quorum-sensing system regulates secreted proteases that can be exploited by cheaters, such as quorum sensing receptor-defective (*lasR*) mutants. *lasR* mutants emerge in populations growing on casein as a sole source of carbon and energy. These mutants are exploitative cheaters because they avoid the substantial cost of engaging in quorum sensing. Previous studies showed that quorum sensing increases resistance to some antibiotics, such as tobramycin. Here, we show that tobramycin suppressed the emergence of *lasR* mutants in casein-passaged populations. Several mutations accumulated in those populations indicating evidence of antibiotic adaptation. We found that mutations in one gene, *ptsP,* increased antibiotic resistance and also pleiotropically increased production of a quorum sensing-controlled phenazine, pyocyanin. When passaged on casein, *ptsP* mutants suppressed cheaters in a manner that was tobramycin independent. We found the mechanism of cheater suppression in *ptsP* mutants relied on pyocyanin, which acts as a policing toxin by selectively blocking growth of cheaters. Thus, tobramycin suppresses *lasR* mutants through two mechanisms: first, through direct effects on cheaters and second, by selecting mutations in *ptsP* that suppressed cheating in a tobramycin-independent manner. This work demonstrates how adaptive mutations can alter the dynamics of cooperator-cheater relationships, which might be important for populations adapting to antibiotics during interspecies competition or infections.

Importance

The opportunistic pathogen *Pseudomonas aeruginosa* is a model for understanding quorum sensing, a type of cell-cell signaling important for cooperation. Quorum sensing controls production of cooperative goods, such as exoenzymes, which are vulnerable to cheating by quorum sensing-defective mutants. Because uncontrolled cheating can ultimately cause a population to collapse, much focus has been on understanding how *P. aeruginosa* can control cheaters. We show that an antibiotic, tobramycin, can suppress cheaters in cooperating *P. aeruginosa* populations. Tobramycin suppresses cheaters directly because the cheaters are more susceptible to tobramycin than cooperators. Tobramycin also selects for mutations in a gene, *ptsP,* that suppresses cheaters independent of tobramycin through pleiotropic regulation of a policing toxin, pyocyanin. This work supports the idea that adaptation to antibiotics can have unexpected effects on the evolution of quorum sensing and has implications for understanding how cooperation evolves in dynamic bacterial communities.

Introduction

Many Proteobacteria have quorum-sensing systems that sense and respond to *N*-acyl-homoserine lactones (AHLs) to cause population density-dependent changes in gene expression (1-3). AHL systems involve a LuxR-family signal receptor and a LuxI-family signal synthase (1, 4, 5). LuxR-I systems in many Proteobacteria control production of exoproducts such as proteases and toxins, which can be considered public goods (3, 6, 7). Public goods can be used by any member of the population; however, some population members do not contribute to public good production and are called freeloaders or cheaters (7-9). An example of a cheater is individuals with null mutations in the gene coding for the LuxR-family signal receptor; these mutants do not produce quorum sensing-dependent public goods but can still exploit public goods produced by quorum sensing cooperators in the population (8-10).

Cheater proliferation presents a serious threat to the stability of cooperating populations. Because public goods are metabolically costly, cheaters can overrun cooperators (8, 9, 11). In conditions where the public goods are required for growth, uncontrolled cheating can lead to population collapse (7, 12-14). Mechanisms must exist to control cheating in order for cooperative phenotypes to be maintained. One such mechanism is policing (15, 16). In bacteria, cooperators police cheaters by linking toxin and toxin-resistance factor production, such as through co-regulation of each of these by quorum sensing (16-18). Quorum sensing can also control cheating through co-regulation of public goods with private goods (13) that benefit only producing cells. Previously, we showed that the soil bacterium *Chromobacterium subtsugae* (formerly *Chromobacterium violaceum*) uses quorum sensing to co-regulate a publicly available secreted protease with a privately available cell-associated tetracycline-specific efflux pump (10). When *C. subtsugae* is grown on casein as a sole carbon and energy source, quorum

sensing-dependent protease production can be exploited by quorum sensing-defective cheaters. However, these cheaters are suppressed when tetracycline is included in the casein medium, because they do not express the efflux pump conferring resistance (10). This type of cheater suppression likely requires some other selective force ensuring that public and private goods are maintained under co-regulation (19).

In this study, we sought to determine if antibiotics can restrain the emergence of quorum sensing-defective cheaters in bacteria other than *C. subtsugae.* We were also interested in understanding how adaptation under antibiotic selection can alter the dynamics of cooperation and cheating. Previous reports show that AHL quorum sensing regulates antibiotic resistance in *Pseudomonas aeruginosa* (20-23). There are two AHL quorum-sensing systems in *P. aeruginosa,* the LasR-I and RhlR-I systems. These two systems produce and respond to the signals 3-oxododecanoyl-homoserine lactone (3OC12-HSL) and butanoyl-homoserine lactone (C4-HSL), respectively (3, 24, 25). The systems are hierarchical with LasR-I controlling activation of RhlR-I (25, 26). In biofilm conditions, deletions in LasR were previously shown to cause sensitivity to tobramycin antibiotic in at least one strain of *P. aeruginosa,* PAO1(20-22).

Here, we show the LasR-I system increases tobramycin resistance in planktonic conditions in the *P. aeruginosa* strain PA14. We also show that tobramycin can suppress the emergence of *lasR*-mutant cheaters in cooperating PA14 populations grown on casein, similar to previous observations with *C. subtsugae* (10). We sequenced the genomes of isolates from tobramycin-evolved populations. All of the isolates had mutations in or upstream of *ptsP*, a gene coding for phosphoenolpyruvate-protein phosphotransferase (EI^{Ntr}). This enzyme is the first in a global regulatory system known as the nitrogen phosphotransferase system (PTS^{Ntr}) (27). Mutations in *ptsP* are known to increase tobramycin resistance (28, 29). Interestingly, we

observed that *ptsP* mutations can also lead to suppression of cheaters, even in populations passaged with no antibiotic. We demonstrate cheater suppression is due to increased production of the toxin pyocyanin in the *ptsP* mutants (30). Our results show that selection by tobramycin can lead to both direct and pleiotropic effects on cheating. These results provide new information on policing mechanisms in *P. aeruginosa* and demonstrate how antibiotic selection can lead to changes in cooperative activity.

Results

LasR promotes tobramycin resistance in *P. aeruginosa* PA14 planktonic cultures

As an initial test that LasR contributes to tobramycin resistance in planktonic conditions, we determined the minimum inhibitory concentration (MIC) of tobramycin against the laboratory strain PA14, or PA14 with a deletion of *lasR* or *lasI*. To increase the MIC detection sensitivity, we generated two tobramycin dilution series that were staggered by using two different starting concentrations (31). We observed a small but reproducible 1.7-fold decrease in the Δ*lasR* mutant MIC relative to PA14 (**Fig. 1A**). The difference was observed when tobramycin was used to treat cultures grown to an optical density at 600 nm of 4 (OD_{600} 4), but not cultures at lower cell densities (**Fig. S1**). After 24 h treatment with 1.1 μg ml^{-1} tobramycin, about 200-fold fewer Δ*lasR* mutant cells were recovered than wild type (**Fig. 1B**). We also observed a decrease in the MIC of the Δ*lasI* mutant similar to that of the Δ*lasR* mutant (**Fig. 1A**). We could restore resistance to the Δ*lasI* mutant by adding synthetic 3OC12-HSL (**Fig. 1A**). There was no significant difference between the wild type and the Δ*rhlR* mutant, supporting the hypothesis that the RhlR-I system is not important for the resistance phenotype. Further, the MIC of the Δ*lasR,* Δ*rhlR* double mutant was similar to that of the Δ*lasR* single mutant. Together, these results show that the LasR-I system, but not the RhlR-I system, contributes to tobramycin resistance in planktonically-grown *P. aeruginosa* strain PA14.

Tobramycin suppresses cheating in *P. aeruginosa*

Our results support the idea that tobramycin could limit the emergence of *lasR*-mutated cheaters in cooperating *P. aeruginosa* populations. *P. aeruginosa* requires a LasR-controlled protease to grow in minimal medium with casein as the sole source of carbon and energy (8, 9).

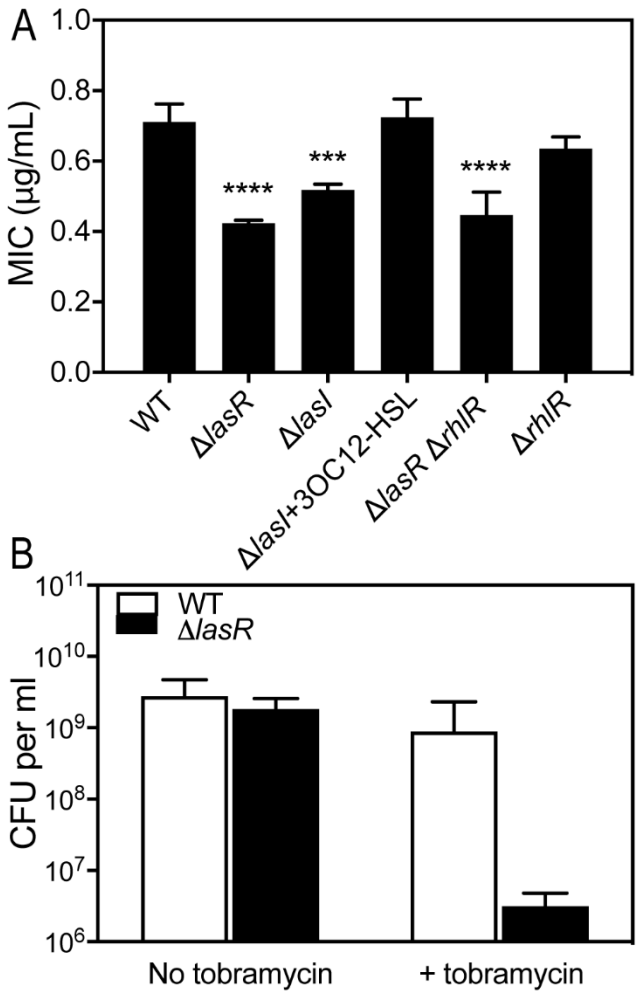

Fig. 1. *lasR* contributes to tobramycin resistance in planktonically-grown *P. aeruginosa* PA14. **A**. The minimum inhibitory concentration (MIC) of tobramycin was determined for each of the strains indicated as described in Materials and Methods. 3OC12-HSL was added before inoculation where indicated (10 μM final). Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type: *** $p<0.001$, **** $p<0.0001$. **B.** Cells recovered following tobramycin treatment with 1.1 μg ml^{-1}. Cells were treated as in part A and the surviving cells were enumerated by serial dilution and plating following treatment. The difference in effective concentrations of tobramycin in part A and B is likely due to the varying potencies of different tobramycin stocks used for each set of experiments. For both A and B, the values shown represent the average of three independent experiments and the error bars represent the standard deviation.

We initially verified that our strain shows similar dependence on LasR for growth on casein (**Fig. S2A**). Next, we serially passaged PA14 in casein broth and monitored the emergence of *lasR*-mutant cheaters. We distinguished *lasR* mutants by colony phenotypes as described in the Materials and Methods (32, 33), and for a subset of the identified *lasR* mutants we confirmed the location of the mutation using Sanger sequencing (**Table S1**). In populations with no antibiotic, *lasR* mutants emerged between 5–8 days and increased to 98-99% of the population (**Fig. 2**). The rapid increase in frequency of *lasR* mutants in the population shows mutating *lasR* provides a fitness advantage to these individuals and supports that they are acting as cheaters, as has been demonstrated by prior work in other *P. aeruginosa* strains (8, 9). We also observed that none of

our populations showed evidence of a collapse, which is also consistent with prior work (8, 9). It has been proposed that cooperation is maintained so long as cheaters remain below a certain percentage in the population, such as by hydrogen cyanide-dependent policing (16). To test the role of tobramycin in cheater emergence, we carried out our passaging experiment in casein broth with added tobramycin at a sublethal concentration (0.6 μg ml^{-1} tobramycin). In the populations with tobramycin, *lasR* mutant frequencies remained low or below the detection level throughout the experiment (**Fig. 2**). These results show that tobramycin can suppress the emergence of LasR-mutated cheaters in casein-grown *P. aeruginosa* populations.

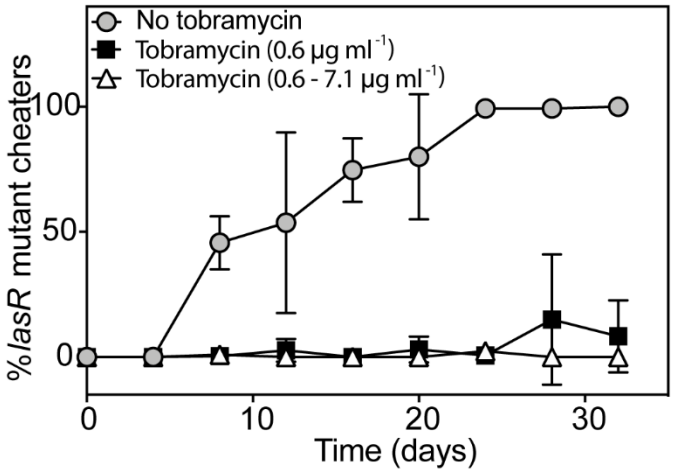

Fig. 2. Tobramycin suppresses the emergence of *lasR*-mutant cheaters. *P. aeruginosa* populations were transferred daily in 1% casein broth for 32 days, and cheaters were enumerated every 4 days by patching as described in Materials and Methods. For each experiment, one culture was initially started with no tobramycin, and after 72 h split into three cultures propagated in one of three conditions; i) with no tobramycin (○), ii) with tobramycin added at 0.6 μg ml^{-1} (■), iii) with tobramycin added at 0.6 μg ml^{-1} initially, and increased by 50% every 4 days to a final concentration of 7.1 μg ml^{-1} (△). For conditions ii and iii, tobramycin was added every other day just after transfer to fresh medium. The detection level of cheaters was 1%. The values shown represent the average of three independent experiments and the error bars represent the standard deviation.

Variants from tobramycin-treated populations undergo genetic adaptation

To assess how antibiotic adaptation influences cheater emergence and suppression, we carried out a second experiment using higher tobramycin concentrations for stronger selection. We used an initial concentration of 0.6 μg ml^{-1} tobramycin and increased the concentration by 50% every 4 days to reach a final concentration of 7.1 μg ml^{-1}. In three independent cultures, the *lasR* mutant population remained at less than 5% of the total population (**Fig. 2**). We did not observe any significant growth inhibition at any stage of the experiment, even at the highest tobramycin concentration (**Fig. S2B**), suggesting genetic adaptation occurred during passage. To test this hypothesis, we isolated one representative variant from each of the passaged populations (variants T1, T2, and T3 from the 0.6–7.1 μg ml^{-1} tobramycin-passaged populations and variants T4, T5, and T6 from the 0.6 μg ml^{-1} tobramycin-passaged populations), and determined the MIC. All six variants showed a higher tobramycin MIC than the ancestor strain (**Fig. S3A**) or variants from identically treated populations with no added antibiotic (clones N1–N3, **Fig. S3B**).

To identify the mutations that accumulated in tobramycin-evolved variants, we sequenced the genomes of our six tobramycin-evolved variants. For comparison, we also sequenced the parent PA14 strain and clone N2 described above, an isolate from a population passaged with no antibiotic. We identified 3–6 mutations in each of the tobramycin-evolved variants that were not in either the parent PA14 or isolate N2 (**Table 1**). Most of the tobramycin-evolved variants had mutations in two genes: *ptsP,* which codes for phosphoenolpyruvate protein phosphotransferase and *fusA1*, which codes for translation elongation factor EF-G1A and is considered essential (34). To verify the role of *ptsP* and *fusA1* mutations in tobramycin resistance, we introduced mutations of each to the PA14 genome. We used Δ*ptsP* or the *fusA1* G1643A mutation from isolate T5, because *fusA1* deletions are thought to be nonviable (34). We

also constructed a Δ*ptsP, fusA1* G1634A double mutant. We compared the MIC of the mutated strains with that of the PA14 parent (**Fig. S3C, S3D**). Each of the individual mutations increased PA14 resistance by about 2-fold and combining mutations increased the MIC by about 3-fold (**Fig. S3E**).

Table 1. Mutations in variants from tobramycin-passaged populations.

Variant[a]	Gene mutation[b]		
	ptsP	*fusA1*	others
T1	1547T	G61A	*pmrB, mdpA*
T2	841ΔC	ND[c]	*mexZ*, *rrf2*, *psdR,* PA14_RS15595
T3	1547T	G61A	*dppA3*, *ostA*
T4	**G392228A[d]**	A1655G	*psdR*
T5	**G392228A[d]**	G1634A	*mdpA*
T6	**A392231G[d]**	ND	*mdpA, pstS*, *ftsH*, *nuoM*, **Δ4064707-4092544**

[a]Variants isolated from populations passaged with tobramycin at 0.6-7.1 μg ml^{-1} (T1-T3) or 0.6 μg ml^{-1} (T4-T6).
[b]Bolding indicates promoter mutations and large chromosomal deletions that are given by genomic location; all other gene mutations are given by nucleotide location.
[c]ND: not detected
[d]Indicates DNA sequence in the predicted promoter of *ygdP,* which is immediately upstream of *ptsP*. *ygdP* and *ptsP* are predicted to be co-transcribed using the DOOR database for prokaryotic operons (35), accessed through the website pseudomonas.com (36).

ptsP-mutated strains have enhanced LasR activity and pyocyanin production

We focused our attention on the *ptsP* mutation because disrupting *ptsP* was previously shown to increase LasR activity (30), and we were interested in understanding how these effects could alter the cooperator-cheating dynamic. First, we tested the hypothesis that the LasR-LasI

system is elevated at least in the T1, T2, and T3 mutants. To do so, we used a P*lasI-gfp* plasmid reporter (37), which showed ~2–3-fold higher fluorescence in the Δ*ptsP* mutant than that of the wild-type PA14 strain (**Fig. S4A**), similar to previous results (30). The T1, T2, and T3 variants carrying this reporter also showed ~3-fold higher fluorescence than that of PA14. The elevated fluorescence levels in these variants could be restored to wild-type levels by introducing *ptsP* to the neutral *attB* site in the genome (**Fig. 3A**). The reporter activities of isolates N1, N2, and N3 were similar to that of PA14 (**Fig. S4B**). The difference in reporter activity in the PA14 Δ*ptsP* mutant or the T1–T3 strains could not be explained by differences in growth (**Table S2**).

Disruptions in *ptsP* were previously shown to increase pyocyanin production by about 3-fold (30). To test whether pyocyanin production was similarly elevated in our tobramycin-evolved variants, we compared pyocyanin produced by the T1, T2, and T3 variants with that of PA14. Our results showed the variants have ~3-fold higher pyocyanin production than the PA14 parent, and that pyocyanin production can be reduced to wild-type levels in the variants by introducing *ptsP* to the *attB* site in the genome (**Fig. 3B**). This increase was not observed in isolates N1–N3 (**Fig. S4C**). Thus, *ptsP* mutations also increase pyocyanin production in strains passaged with tobramycin.

lasR-mutant cheaters are suppressed in *ptsP* mutant populations

Because *ptsP* mutations alter quorum sensing regulation, we predicted that these mutations might influence cheating dynamics even in the absence of tobramycin. Specifically, we predicted *lasR* mutant cheaters might emerge at a faster rate in *ptsP* mutant populations due to the metabolic burden associated with higher LasR activity. To test this hypothesis, we

passaged the T1, T2, and T3 variants in casein broth for 32 days in the absence of antibiotic and monitored the emergence of *lasR*-mutant cheaters (**Fig. 4A, S5**). Surprisingly, cheaters emerged

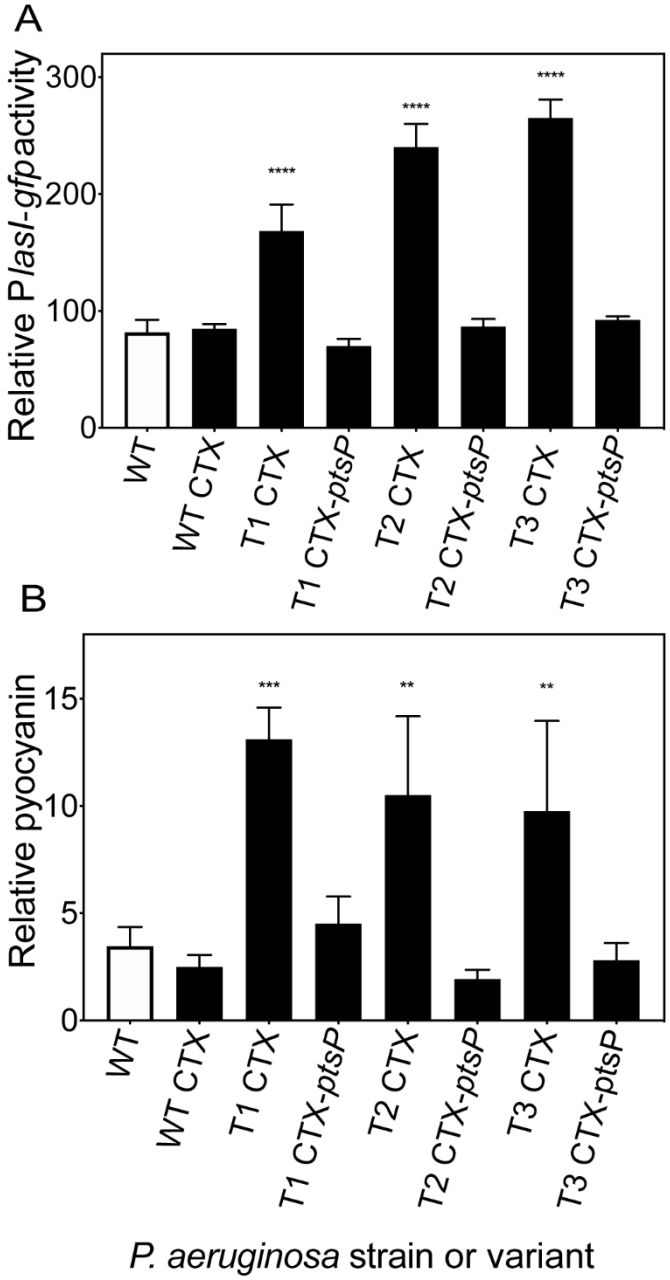

Fig. 3. Effects of *ptsP* inactivation on LasR activity and pyocyanin production. **A.** Activity of LasR. *P. aeruginosa* strains were electroporated with a plasmid containing a LasR-responsive GFP reporter (pBS351). Reported values are fluorescence normalized to culture density at 18 h of growth. **B.** Pyocyanin production. Cultures were inoculated into pyocyanin-producing media, grown for 18 h, and extracted before quantifying pyocyanin as described in Materials and Methods. In all cases, reported values are µg/ml pyocyanin normalized to culture density at the time of measurement. Strains carried either the empty CTX-1 cassette (CTX), or the CTX-1-*ptsP* cassette, inserted at the neutral *attB* site in the genome. The values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type: ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

later in these populations than did those of the PA14 ancestor (23 to >32 days for T1–T3 vs. 6–8 days for PA14). For T2, cheaters were never observed even by 32 days. Further, the total cheater frequency in the T1 and T3 populations did not reach the same levels as that of PA14 (33–67%

for T1 and T3 vs. >98% for PA14) (**Fig. S5A**). There were no differences in cheating observed with variants N1–N3 passaged with no tobramycin, compared with PA14 (**Fig. S5C**). The total cell density remained similar for all evolution experiments, suggesting the differences in cheating were not due to differences in total population of the experiments (**Fig. S5B, S5D**).

We hypothesized that the difference in cheating observed with the tobramycin-evolved variants was due to the disrupted *ptsP* gene in these strains. To test this hypothesis, we tested cheating in a Δ*ptsP* mutant. We passaged the Δ*ptsP* mutant carrying either CTX-1 or the CTX-1-*ptsP* cassette in 1% casein broth for 32 days and monitored cheater frequency over time. We also monitored cheating in PA14 and PA14 CTX-1. We observed no differences in cheating of PA14 CTX-1 compared with PA14, verifying that the CTX-1 cassette had no effect on cheating. However, cheaters emerged later and did not reach the same frequency in the Δ*ptsP*-CTX-1 populations compared with either PA14 strain (**Fig. 4B, S5E**). Cheating was indistinguishable from PA14 in the Δ*ptsP*-CTX-1-*ptsP* populations (**Fig. 4B, S5E**). There were no notable differences in total growth of any of these populations (**Fig. S5F**). Together, our results show that cheating is suppressed in *ptsP* mutant populations compared with that of wild type PA14. The observation that the *lasR* mutants are not completely suppressed in *ptsP* mutant populations suggests there is still some fitness benefit associated with mutating *lasR*, though that benefit is reduced.

Pyocyanin produced by Δ*ptsP* mutants is active against *lasR*-mutated cheaters

A potential explanation for delayed cheating in the Δ*ptsP* mutant populations is that the Δ*ptsP* mutant has a growth advantage compared with the PA14 ancestral strain. However, PA14 and Δ*ptsP* showed identical growth rates (**Table S2**), suggesting there is another explanation. We

hypothesized that the Δ*ptsP* mutant produces a toxin that inhibits growth of *lasR* mutant cheaters. To test this hypothesis, we filtered culture fluid from a Δ*ptsP* mutant and tested its

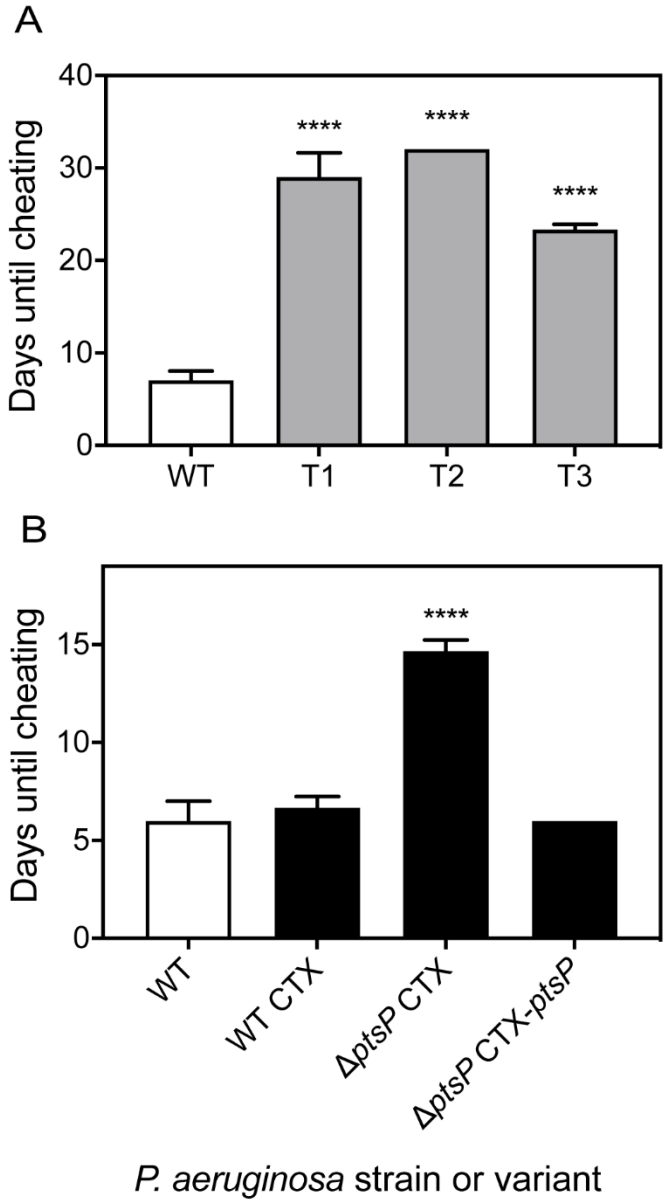

Fig. 4. *ptsP* inactivation suppresses cheaters. P. *aeruginosa* populations were transferred daily in 1% casein broth for 32 days, and cheaters were enumerated every 4 days by patching as described in Materials and Methods. Values shown indicate the number of days until *lasR*-mutated cheaters emerge in **A**) populations of wild type PA14 (WT) and PA14 variants (T1, T2, and T3) from tobramycin-evolved populations and **B**) WT or the Δ*ptsP* mutant carrying either the empty CTX-1 cassette (CTX), or the CTX-1-*ptsP* (CTX-*ptsP*) cassette, inserted at the neutral *attB* site in the genome. Cheater emergence is defined as when *lasR* mutants reach a frequency in the population of 8%, at which point it was generally observed that cheater frequencies continued increasing rather than decreasing or remaining constant. Full data sets of cheater frequency and total populations are shown in Fig. S5. Values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type for A; and by one-way ANOVA and Tukey's multiple comparisons test for B: **** $p<0.0001$.

ability to inhibit growth of logarithmically growing PA14 or PA14 with deletions of *ptsP*, *lasR,* or both *ptsP* and *lasR*. We performed identical treatments with filtered fluid from PA14 or with

unspent culture media (untreated control) and enumerated cell growth after 24 h. Treatment with PA14 culture fluid had no effects on growth, and results were similar to that of the untreated control (**Fig. 5**). However, treatment with the Δ*ptsP* mutant filtrate reduced growth of the Δ*lasR* mutant by 83-fold and Δ*ptsP* Δ*lasR* mutants by 13,000-fold, compared with no treatment. Thus, the Δ*ptsP* mutant secretes a substance that is growth inhibitive to *lasR*-mutated strains and particularly to Δ*ptsP* Δ*lasR* mutants.

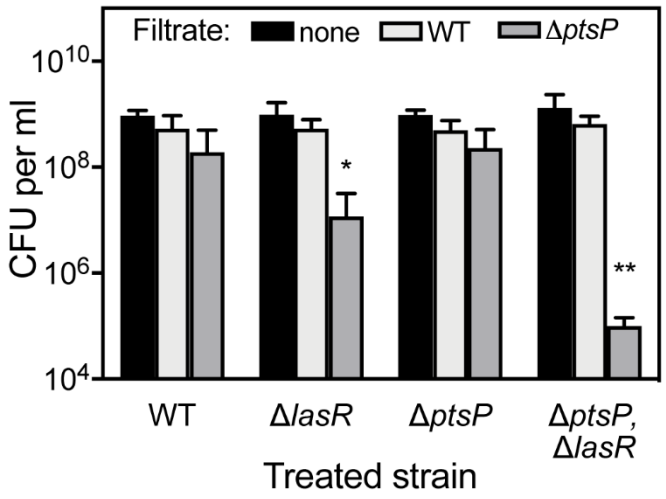

Fig. 5. Δ*ptsP* mutant cultures has antimicrobial activity against *lasR* mutants. Final cell densities of *P. aeruginosa* strains treated for 24 h with filtered fluid from 1% casein-grown cultures of untreated (black), wild-type PA14 (light grey), or the *ptsP* mutant (dark grey). The initial cell density of the treated cultures was 5–7 X 10^5 cells ml^{-1}. The values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by two-way ANOVA and Dunnett's multiple comparison test with untreated for each strain. * $p<0.05$, ** $p<0.01$.

We hypothesized that the activity in the Δ*ptsP* mutant culture fluid was due to pyocyanin. This hypothesis was based on our result that *ptsP* mutations increase pyocyanin production (**Fig. 3**) and prior work demonstrating pyocyanin can be toxic to *lasR* mutants under certain conditions (17, 38). Pyocyanin is produced from the *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*) gene clusters and the *phzM* and *phzS* genes (**Fig. 6A**) (39, 40). Several of the pyocyanin intermediates are toxic, such as 5-methylphenazine 1-carboxylic acid (5-Me-PCA) (41-43) . We tested several gene mutations in this pathway to determine whether any of the products have activity against *lasR* mutants. We deleted *phzM,* which is needed to convert the precursor phenazine-1-

carboxylic acid (PCA) to 5-Me-PCA, *phzS* which is needed to convert 5-Me-PCA to pyocyanin (39, 44), and *phzH* which is not needed for pyocyanin production but involved in production of another product of this pathway, phenazine-1-carboxamide (PCN) (39).

We initially tested whether pyocyanin biosynthesis contributes to the antimicrobial activity of a *ptsP* mutant by making a Δ*ptsP* Δ*phz1* Δ*phz2* triple mutant. We compared antimicrobial activity in culture fluid of this strain with that of the single Δ*ptsP* mutant, and tested against the Δ*ptsP,* Δ*lasR* mutant. Deleting both *phz1* and *phz2* abolished the antimicrobial activity observed with the Δ*ptsP* single mutant (**Fig. 6B**). Mutations in *phzM* or *phzS* similarly abolished this activity (**Fig. 6B**). However, no effects were observed by deleting *phzH* (**Fig. 6B**), which is important for production of the pyocyanin bi-product PCN but not pyocyanin itself. These results support that the antimicrobial activity in Δ*ptsP* mutant culture fluid is due to pyocyanin. We also tested whether commercially supplied pyocyanin could restore antimicrobial activity of the Δ*ptsP* Δ*phzS* mutant by adding 10 μg ml^{-1} pyocyanin to Δ*ptsP* Δ*phzS* culture filtrates prior to treating Δ*ptsP* Δ*lasR* cells. Adding pyocyanin reduced Δ*ptsP,* Δ*lasR* mutant growth by ~10^7-fold, whereas PA14 growth was only reduced by ~10-fold (**Fig. 6C**). Together, these results support the conclusion that pyocyanin can selectively limit growth of Δ*ptsP,* Δ*lasR* mutants.

Pyocyanin production leads to enhanced policing by Δ*ptsP* mutants

We also tested the role of pyocyanin in policing cheaters using competition experiments. We mixed the Δ*lasR* mutant with a cooperator starting at a 1:99 (cheater:cooperator) ratio in 1% casein media and transferred the population daily for 3 days. The results are shown in **Fig. 6D**. In cocultures with PA14 as the cooperator, Δ*lasR* mutants increased from an initial frequency of 1%

to a final frequency of 56%. However, in identical competition experiments with the Δ*ptsP* mutant, the Δ*lasR* mutants increased from 1% to only 16% final frequency, consistent with limited cheater proliferation observed with the Δ*ptsP* mutant (**Fig. 4B**). Deleting *phzM* in PA14 did not alter the proliferation of Δ*lasR* mutants from that of PA14, demonstrating pyocyanin is not important for policing Δ*lasR* mutant cheaters in PA14 in the conditions of our experiment. However, deleting *phzM* in the Δ*ptsP* mutant caused the final frequency of Δ*lasR* mutants to increase to 88%. Thus, pyocyanin is responsible for cheater suppression in the Δ*ptsP* mutant. We find it interesting that Δ*lasR* cheaters increase to a higher frequency in competition experiments with a Δ*ptsP,* Δ*phzM* mutant as compared with the Δ*phzM* mutant or the PA14 parent (88% vs. ~50% final Δ*lasR* cheater frequency). It's possible deleting *phzM* in a Δ*ptsP* mutant causes some deleterious effect on fitness that gives the Δ*lasR* mutants an additional advantage. Altogether, our results support that the cheater suppression observed in the Δ*ptsP* mutant is dependent on pyocyanin.

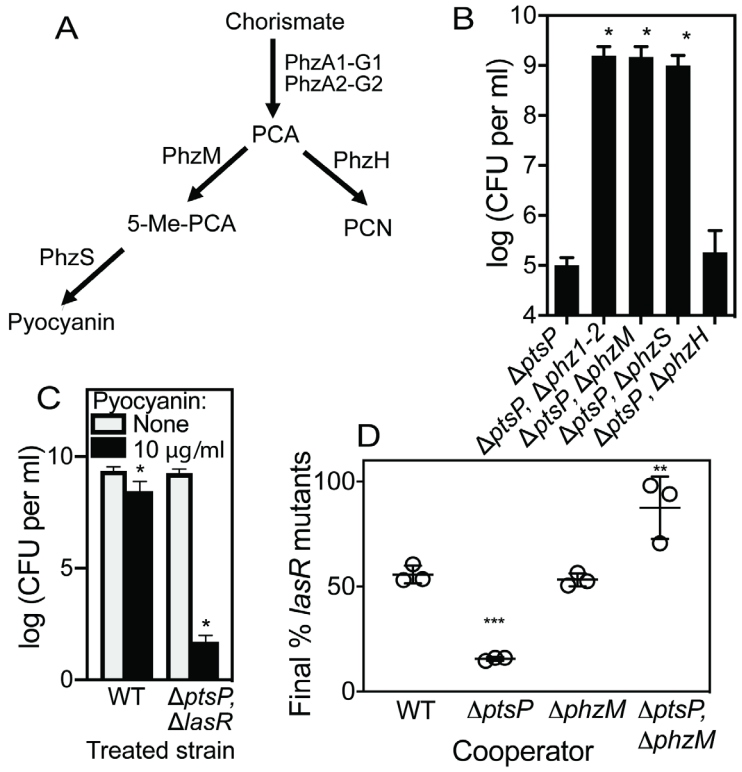

Fig. 6. Δ*ptsP* mutants police cheaters using pyocyanin. **A.** Pyocyanin biosynthesis steps. The products of the *phzA1-G1* (*phz*1) and *phzA2-G2* (*phz*2) gene clusters synthesize phenazine-1-carboxylic acid (PCA) from chorismate. PhzM and PhzH convert PCA into 5-methylphenazine 1-carboxylic acid (5-Me-PCA) or phenazine-1-carboxamide (PCN), respectively. PhzS converts PCA into pyocyanin. **B.** Final cell densities of Δ*ptsP*, Δ*lasR* cells treated with filtered fluid from 1% casein-grown cultures of a Δ*ptsP* mutant or Δ*ptsP* with the entire *phz1* and *phz2* operons deleted (Δ*phz*1-2) or with *phzM, phzS,* or *phzH* deleted. The initial cell density of the treated cultures was 5–7 X 10^5 cells ml^{-1}. Data are the average of three independent experiments with standard deviation. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons test with Δ*ptsP,* *p<0.05. **C.** Final cell densities of wild-type PA14 (WT) or the Δ*ptsP*, Δ*lasR* mutant treated with 0 or 10 µg ml^{-1} pyocyanin and filtered fluid from 1% casein-grown Δ*ptsP* Δ*phzS* cultures. The initial cell density of treated cultures was 4–6X10^5 CFU ml^{-1}. Data are the average of four independent experiments with standard deviations. Statistical analysis by two-way ANOVA and multiple comparisons test with no-pyocyanin control, * p<0.05. **D.** Cheater suppression during competition. Competition cultures were inoculated with the *lasR* mutant and each cooperator strain at an initial ratio of 1:99 (cheater:cooperator) in 1% casein broth and transferred to fresh medium daily for 3 days. On day 3, cheaters were enumerated by patching as described in Materials and Methods. Each data point represents an independent experiment. The horizontal line represents the mean and the vertical line represents the standard deviation of all the experiments in each set. Statistical analysis by one-way ANOVA with Tukey's multiple comparisons test of each set with the wild-type cooperator experiments: ** p<0.01, *** p<0.001. Total population densities of each experiment are shown in **Fig. S6**.

Discussion

Previous studies have demonstrated that *P. aeruginosa* quorum sensing plays a small but appreciable role in antibiotic resistance (20-23). Here, we confirm the role of quorum sensing in antibiotic resistance and also show that antibiotics can stabilize quorum sensing by directly suppressing cheaters and also by selecting mutations in *ptsP* that have pleiotropic effects on cheating. The *ptsP* mutations cause antibiotic-independent cheater suppression by enhancing production of the policing toxin, pyocyanin. Importantly, the results demonstrate how tobramycin adaptation can have pleiotropic effects on cheating. Together, these results support the idea that cheater control mechanisms can change under selection by environmental pressures such as antibiotics.

Mutations in *ptsP* were genetic adaptations to the antibiotic tobramycin. Adaptive mutations have been shown to influence cooperation and cheating in other studies (45-47). For example, adaptive mutations can improve the fitness of cooperators so they can outcompete cheaters (19, 46) or cause re-wiring of cooperative traits and allow the cheaters to cooperate again (45, 48). In this study, adaptive mutations in *ptsP* were selected by tobramycin. The mutations in *ptsP* had pleiotropic effects on pyocyanin production that led to policing of cheaters. Our results show how adaptation to antibiotics could have important effects on cooperation and cheating. In natural settings, adaptation could be much more complex. For example, populations may encounter multiple antibiotics, other stressors, or face competition from other species for nutrients. In addition, the adaptive mutations might not be homogeneous throughout the population, for example if there is spatial structure created by the formation of biofilms.

In our study, cheater suppression was caused by increased production of the toxin pyocyanin. Pyocyanin was previously shown to be involved in policing *lasR* mutant cheaters (17, 38). Pyocyanin leads to the generation of highly toxic hydroxyl radicals (49-51), which can cause oxidative stress and cell death (52, 53). LasR mutant cheaters have been shown to be more susceptible to pyocyanin (54, 55), possibly because they fail to upregulate enzymes involved in relieving pyocyanin-induced oxidative stress such as catalase and superoxide dismutase (SOD) (17, 50, 52, 54). Detoxifying enzymes produced by cooperators can also have some benefits to the cheaters (19), which may explain how *lasR* cheaters were still able to increase in frequency in *ptsP* mutant populations (**Fig. 6**). It will be interesting to determine whether pyocyanin production could have similar effects on stabilizing quorum sensing in natural communities, such as infections.

Mutations in *ptsP* have been previously shown to increase pyocyanin production (30). PtsP, along with two other enzymes PtsN and PtsO, make up a poorly understood system called the nitrogen phosphotransferase system (PTS^{Ntr}). In *Escherichia coli,* PTS^{Ntr} is involved in regulating diverse physiological changes in response to nitrogen starvation (56). It is unclear if PTS^{Ntr} plays a similar role in *P. aeruginosa*. In *P. aeruginosa,* PtsP has been shown to contribute to pathogenesis (57), biofilm formation (27), and quorum sensing regulation (30). Mutations in *ptsP* have also been shown to contribute to tobramycin resistance (28, 29), consistent with our results. The mechanistic pathway by which PtsP contributes to pathogenesis, biofilm formation, and tobramycin resistance are as-yet unknown. In the case of quorum sensing, it is thought that PtsP somehow represses LasR through the antiactivator QscR (30), although it is not clear if PtsP acts entirely through QscR or modifies quorum sensing through other pathways. The important

effects of mutating PtsP on *P. aeruginosa* virulence and virulence-associated behaviors suggest PtsP and the PTS^{Ntr} system might have potential as a new target for therapeutic development.

Our results also show that quorum sensing has a small but appreciable contribution to antibiotic resistance in *P. aeruginosa* strain PA14 under planktonic conditions (**Fig. 1**). Similar results have been reported for other strains and species, for example in *P. aeruginosa* PAO1 in biofilms (20-23), and *C. subtsugae* (10). In *C. subtsugae,* resistance is attributed to an efflux pump, CdeAB-OprA, which is transcriptionally activated by the CviR quorum-sensing receptor in response to the cognate quorum-sensing signal *N*-hexanoyl-homoserine lactone (10, 31). In *P. aeruginosa,* there may be multiple factors contributing to antibiotic resistance. There are at least three efflux pumps known to have overlapping specificity for aminoglycosides; MexAB (58, 59), MexXY (60, 61), and PA1874-1877 (62); there are also aminoglycoside-inactivating enzymes (58). Quorum-control of antibiotic resistance may provide an important evolutionary benefit. For example, quorum sensing may increase resistance to protect against self-produced toxins, or synchronize resistance factor expression across members of the population (63) to protect neighboring cells from exported antibiotic (64). Understanding how and why quorum sensing contributes to antibiotic resistance will provide important new information about the biology of quorum sensing and will be relevant to designing new therapies that function by blocking quorum-sensing systems.

Materials and Methods

Culture conditions and reagents

Bacteria were routinely grown in Luria-Bertani broth (LB) or LB buffered to pH 7 with 50 mM 3-(morpholino)-propanesulfonic acid (MOPS), or on LB agar (LBA; 1.5% (weight per volume) Bacto-Agar). Growth media for specific experiments were M9-caseinate (casein broth; 6 g l^{-1} Na_2HPO_4, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl; pH 7.4, 1% sodium caseinate); MOPS minimal medium (25 mM D-glucose, freshly prepared 5 µM $FeSO_4$, 15 mM NH_4Cl, and 2 mM K_2HPO_4 added to a 1X MOPS base buffer consisting of 50 mM MOPS, 4 mM tricine, 50 mM NaCl, 1 mM K_2SO_4, 50 µM $MgCl_2$, 10 µM $CaCl_2$, 0.3 µM $(NH_4)_6Mo_7O_{24}$, 40 µM H_3BO_3, 3 µM Cobalt(II) acetate ($Co(OAc)_2$) , 1 µM $CuSO_4$, 8 µM $MnSO_4$, and 1 µM $ZnSO_4$) (65); a modified M9-casamino acid broth (200 ml l^{-1} 10X M9, 0.6 g l^{-1} thiamine hydrochloride, 1 ml l^{-1} 1 M $MgSO_4$, 1 ml l^{-1} 0.2 M $CaCl_2$, 10 g l^{-1} casamino acid; freshly prepared; filter sterilized), Pyocyanin Producing Media (PPM) (66), and 4% skim milk agar (SMA) (9). All *P. aeruginosa* broth cultures were grown at 37ºC with shaking at 250 rpm, 18 mm test tubes (for 2 ml cultures), 125 ml baffled flasks (10 ml cultures), or 250 ml baffled flasks (60 ml cultures), unless otherwise specified. For *E. coli*, 100 µg ml^{-1} carbenicillin, 15 µg ml^{-1} gentamicin, and 10 µg ml^{-1} tetracycline were used. For *P. aeruginosa,* 300 µg ml^{-1} carbenicillin, 50–200 µg ml^{-1} gentamicin, and 200 µg ml^{-1} tetracycline were used. AHLs were purchased from Cayman Chemicals (MI, USA) and handled as described elsewhere (10). Genomic or plasmid DNA was extracted using Qiagen Puregene Core A kit (Hilden, Germany) or IBI Scientific plasmid purification mini-prep kit (IA, USA) while PCR products were purified using IBI Scientific PCR clean-up/gel extraction kits, according to the manufacturer's protocol. All antibiotics were purchased from GoldBio (MO, USA) except for tetracycline which is from Fisher Scientific (PA, USA).

Pyocyanin and DMSO (solvent for pyocyanin) were purchased from Sigma Aldrich (MO, USA) and Acros Organics (WI, USA), respectively.

Bacterial strains and strain construction

All bacterial strains, plasmids, and primers used in this study are listed in **Tables 2–5**. *P. aeruginosa* strain UCBPP-PA14 ('PA14') (67) and PA14 derivatives were used for these studies. Markerless deletions in specific loci of *P. aeruginosa* PA14 were generated using allelic exchange as described previously (68). To generate plasmids for allelic exchange, DNA fragments with the mutated or deleted gene allele plus 500 bp flanking DNA were generated by PCR using primer-incorporated restriction enzyme sites. The PCR product was digested and ligated to pEXG2 (*fusA1* G1634A) or pEX18Ap (Δ*lasR* and Δ*rhlR*) and transformed into the appropriate *P. aeruginosa* strain. The plasmids for Δ*lasI* and Δ*ptsP* were described elsewhere (27, 69). Merodiploids were selected on Pseudomonas Isolation Agar (PIA)-carbenicillin (300 μg ml^{-1}) for Δ*lasR* and Δ*rhlR*, PIA-gentamicin (200 μg ml^{-1}) for Δ*lasI* and Δ*ptsP*, and LBA-gentamicin (50 μg ml^{-1}) for *fusA1* G1634A. Deletion mutants were counterselected using NaCl-free 15% sucrose. Putative mutants were verified through antibiotic sensitivity tests and gene-targeted Sanger sequencing. Plasmid transformations were described previously (27, 37, 70, 71). Complementation strains were constructed by integrating the mini-CTX-1 vector at the neutral chromosomal *attB* locus (27, 71).

Table 2. *P. aeruginosa* and *E. coli* strains used in this study.

Strain	Relevant properties	Reference or source
P. aeruginosa		
UCBPP-PA14	Ancestral wild type	(67)
PA14 Δ*lasR*	PA14 with a deletion of *lasR*	This study
PA14 Δ*rhlR*	PA14 with a deletion of *rhlR*	This study
PA14 Δ*lasR* Δ*rhlR*	PA14 Δ*lasR* with a deletion of *rhlR*	This study
PA14 Δ*lasI*	PA14 with a deletion of *lasI*	This study
PA14 Δ*ptsP*	PA14 with a deletion of *ptsP*	This study
PA14 Δ*ptsP* Δ*lasR*	PA14 Δ*ptsP* with a deletion of *lasR*	This study
PA14 Δ*phzM*	PA14 with a deletion of *phzM*	(41)
PA14 Δ*phzS*	PA14 with a deletion of *phzS*	(41)
PA14 Δ*phz1* Δ*phz2*	PA14 with a deletion of *phz1 and phz2*	(72)
PA14 Δ*phzH*	PA14 with a deletion of *phzH*	(73)
PA14 Δ*ptsP* Δ*phzM*	PA14 Δ*ptsP* with a deletion of *phzM*	This study
PA14 Δ*ptsP* Δ*phzS*	PA14 Δ*ptsP* with a deletion of *phzS*	This study
PA14 Δ*ptsP* Δ*phz1* Δ*phz2*	PA14 Δ*ptsP* with a deletion of *phz1* and *phz2*	This study
PA14 Δ*ptsP* Δ*phzH*	PA14 Δ*ptsP* with a deletion of *phzH*	This study
PA14 *fusA1* G1634A	PA14 with the *fusA1* G1634A mutation	This study
PA14 Δ*ptsP fusA1* G1634A	PA14 Δ*ptsP* with the *fusA1* G1634A mutation	This study
PA14 Δ*ptsP* CTX-1-*ptsP*	PA14 Δ*ptsP* with *attB*::CTX-1-*ptsP* insertion	This study
PA14 Δ*ptsP* CTX-1	PA14 Δ*ptsP* with *attB*::CTX-1 insertion	This study
PA14 CTX-1	PA14 with *attB*::CTX-1 insertion	This study
Escherichia coli		
DH5α	F^- Φ80*lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17*(r_K^- m_K^+) *recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen

S17-1	*recA pro hsdR RP4-2-Tc::Mu-km::Tn7*	(74)
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pi*r	(74)
Rho3	*thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA*, integrated RP4-2 Tcr::Mu (λ*pir* $^{+}$)Δ*asd*::FRT Δ*aphA*::FRT	(75)

Table 3. *P. aeruginosa* PA14 variants.

Strain	Relevant properties	Reference or source
Variants isolated after daily transfer for 32 days in 1% casein		
T1	Isolate from experiment with tobramycin added at 0.6-7.1μg ml^{-1}	This study
T2	Isolate from experiment with tobramycin added at 0.6-7.1μg ml^{-1}	This study
T3	Isolate from experiment with tobramycin added at 0.6-7.1μg ml^{-1}	This study
T4	Isolate from experiment with tobramycin added at 0.6 μg ml^{-1}	This study
T5	Isolate from experiment with tobramycin added at 0.6 μg ml^{-1}	This study
T6	Isolate from experiment with tobramycin added at 0.6 μg ml^{-1}	This study
N1	Isolate from experiment with no added tobramycin	This study
N2	Isolate from experiment with no added tobramycin	This study
N3	Isolate from experiment with no added tobramycin	This study
Modified variants		
T1 CTX-1	T1 Δ*ptsP* with *attB*::CTX-1 insertion	This study
T1 CTX-1-*ptsP*	T1 Δ*ptsP* with *attB*::CTX-1-*ptsP* insertion	This study
T2 CTX-1	T2 Δ*ptsP* with *attB*::CTX-1 insertion	This study
T2 CTX-1-*ptsP*	T2 Δ*ptsP* with *attB*::CTX-1-*ptsP* insertion	This study

T3 CTX-1	T3 Δ*ptsP* with *attB*::CTX-1 insertion	This study
T3 CTX-1-*ptsP*	T3 Δ*ptsP* with *attB*::CTX-1-*ptsP* insertion	This study

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Reference or source
pEX18Ap	Suicide vector, Ap^r	(76)
pDA8	pEX18Ap containing flanking sequences of *lasR,* Ap^r	An and Parsek, unpublished
pDA9	pEX18Ap containing flanking sequences of *rhlR*, Ap^r	An and Parsek, unpublished
pEXG2	Suicide vector, Gm^r	(77)
pEXG2-Δ*lasI*	pEXG2 containing Δ*lasI* with flanking sequences, Gm^r	(69)
pEXG2-*ptsP*	pEXG2 containing Δ*ptsP* with flanking sequences, Gm^r	(27)
pEXG2-*fusA1* G1634A	pEXG2 containing G1634A mutation in *fusA1,* Gm^r	This study
pPROBE-GT	Broad-host-range pVS1/p15a GFP reporter, Gm^r	(78)
pBS351	pPROBE-GT with -1 through-501 5' region of *lasI*, Gm^r	(37)
CTX-1	mini-CTX-1, *P. aeruginosa* integrative plasmid, Tet^r	(71)
CTX-1-*ptsP*	CTX-1 containing the *ptsP* gene, Tet^r	(27)

Table 5. Primers used in this study.

Primer	Sequence
lasR-A	GCGCGC**GAATTC**AACATGGTCACCTCCAGCA
lasR-B	GCTGAGAGGCAAGATCAGAG**CTGCAG**CATAGCGCTACGTTCTTCTT
lasR-C	GCAAGAAGAACGTAGCGCTATG **CTGCAG**CTCTGATCTTGCCTCTCA
lasR-D	GCGCGC**AAGCTT**GTTACCGTCACCAGCGTCT
rhlR-A	GCGCGC**GAATTC**GCTGTTCGACGGCAGTAT
rhlR-B	GCGCGTCGAACTTCTTCTGGAT**CTGCAG**CATTGCAGTAAGCCCTGA
rhlR-C	GCTCAGGGCTTACTGCAATG**CTGCAG**ATCCAGAAGAAGTTCGACGC
rhlR-D	GCGCGC**AAGCTT**CGGACCGCAGAGAGACTA
rgaoligo74	TAATAA**AAGCTT**CTCGGTGAAAGGCAAGAAAGA
rgaoligo75	TAATAA**TCTAGA**GCTTCGGCGTATTTGGAGAA

[a]Bolded text indicates restriction sequences
[b]rgaoligo74 and 75 were used to incorporate the *fusA1* G1634A mutation. Other oligos were used for *lasR* or *rhlR* deletions as indicated by their name.

Evolution experiments

To prepare the inoculum for evolution experiments, overnight (18 h) pure cultures were grown in LB-MOPS, diluted 1:50 into 2 ml LB-MOPS, and grown to an OD_{600} of ~3.5. To start the experiment, 50 µl from this starter culture was transferred to 2 ml fresh casein broth in an 18 mm tube. At 24 h intervals, cultures were diluted 1:50 into fresh casein broth in a new tube. Tobramycin was added every other day where indicated, similar to previous experiments with *C. subtsugae* (10). CFU ml^{-1} for each lineage was determined by viable plate counts every 96 h. The % *lasR* mutant cheater (*lasR* cheater) was determined by patching 100 colonies, unless otherwise

specified, on SMA. LasR cheaters form flattened colonies with iridescent, metallic sheen surface, and decreased skim milk proteolysis in SMA (9, 32, 33). Sixteen *lasR* mutants were sequence-verified by Sanger sequencing (**Table S1**).

Whole genome sequencing

Genomic DNA was extracted using the Qiagen Puregene yeast/bacteria kit and a sequencing library was constructed with 350-bp inserts (strain T2) or 200-bp inserts (all other strains). Sequencing was performed using Illumina HiSeq 4000 (for strain T2) or Illumina MiSeq with ~25X coverage (all other strains). The raw reads were aligned to the *P. aeruginosa* UCBPP-PA14 reference genome (UCBPP-PA14 Accession NC_008463) using Strand NGS (Bangalore, India) software v 3.1.1, using a pipeline described previously (79). Mutations of interest were verified by gene-targeted Sanger sequencing. Sequence reads for the ancestral PA14 (SAMN16823471) and tobramycin-evolved isolates (SAMN16823472 - SAMN16823478) can be found at the NCBI SRA under BioProject PRJNA678537.

Antimicrobial susceptibility assays

Tobramycin susceptibility was determined by MIC according to the 2020 guidelines of the Clinical and Laboratory Standards Institute (CSLI), using a modified dilution method. Briefly, tobramycin was added to MOPS minimal medium and successively diluted 2-fold in a 200 µl volume in 2 ml tubes. For each experiment, two dilution series were staggered by starting them at different tobramycin concentrations to cover a broader range of concentrations. The MICs were determined as followed. We prepared *P. aeruginosa* starter cultures by growing in LB to an optical density at 600 nm (OD_{600}) of 3.2-4 (**Fig. 1**) or as indicated (**Fig. S1**). The starter

cultures were then diluted 1:40 into each tube containing tobramycin to start the MIC experiment. The inoculated tubes were incubated with shaking for 20 h. After incubation, turbidity was measured using a BioTek Synergy 2 plate reader. The MIC was defined as the lowest concentration of tobramycin (μg ml^{-1}) in which bacterial growth was not measurable. In some cases, CFU ml^{-1} was also determined by viable plate counts.

To determine susceptibility to *P. aeruginosa* culture fluid, we prepared culture fluid by inoculating overnight cultures to an optical density at 600 nm (OD_{600}) of 0.1 into 60 ml casein broth, grew these cultures for 20 h, then passed cultures through a 0.22 μm filter to remove cells. The filtered fluid was mixed with 100 μl M9-casamino acid broth to a final volume of 2 ml and this was inoculated at an initial OD_{600} of 0.001 with either wild type, Δ*lasR*, Δ*ptsP*, or Δ*ptsP* Δ*lasR* from logarithmic-stage LB-MOPS cultures at an OD_{600} 0.2–0.6. The M9-casamino-filtrate mixture was incubated for 24 h and the initial and final population counts (CFU ml^{-1}) were enumerated by colony counting on plates.

Measurements of LasR activity and pyocyanin production

To measure LasR activity, we first introduced the LasR-responsive plasmid pBS351 to *P. aeruginosa* mutants or wild-type PA14 strains by electroporation (37). Electrocompetent cells were prepared from overnight cultures using 300 mM sucrose (80). Transformants were selected on LB agar using gentamicin at 50–200 μg ml^{-1} and routinely grown with gentamicin (50 μg ml^{-1} for agar and 15 μg ml^{-1} for broth) for plasmid maintenance. Transformants were grown in LB-MOPS with 15 μg ml^{-1} gentamicin for 18 h, washed with phosphate buffered saline (PBS), and fluorescence was measured using a BioTek Synergy 2 plate reader. For pyocyanin measurements, we extracted pyocyanin as described previously (81-83). Briefly, cells were

grown for 18 h in Pyocyanin-producing media (66), and 5 ml whole culture was extracted with 2 ml chloroform. The organic layer was separated and extracted a second time with 0.2 N HCl. The absorbance of the aqueous layer was measured at 520 nm and multiplied by 17.072 to calculate the pyocyanin concentration (μg ml^{-1}) (82). LasR activity and pyocyanin measurements were normalized to culture density (optical density at 600 nm) for reporting data.

Coculture assays

Coculture experiments were conducted in 2 ml casein broth in 18 mm test tubes. To prepare the inoculum, overnight (18 h) pure cultures were grown in LB-MOPS, diluted to an optical density at 600 nm (OD_{600}) of 0.025 for cheaters or 0.05 for cooperators into LB-MOPS, and grown to an OD_{600} of ~3.5 before combining at a 99:1 (cooperator:cheater) ratio. This mixture was then diluted 1:40 into casein broth to start the coculture. Cocultures were diluted 1:40 into fresh casein broth in a new tube every 24 h until the end of the experiment at 72 h. The initial and final total population counts (CFU ml^{-1}) were determined by viable plate counts. The % *lasR* cheater was determined by patching 200 colonies on SMA.

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Supplementary Information

Table S1. *lasR* mutants isolated from casein evolution experiments.

Isolate[a]	*lasR* mutation(s)[b]
NT1D1	425T, C692T
NT1D2	C692T
NT1D3	G72A, G99A, G144A, T215G
NT1D4	C692T
NT1D5	C649T
NT1D6	521C
NT1D7	C692T
NT1D8	G142A, G193C, G287A, C692T
NT2D1	G676A
NT2D2	G676A
NT2D3	G676A
NT2D4	G676A
NT2D5	G676A
NT2D6	C350T
NT2D7	G217T
NT2D8	G676A

[a]Isolates were from cultures passaged with no antibiotic.
[b]Gene mutations are given by nucleotide location.

Table S2. Doubling time of some strains used in this study[a]

Strain	Doubling Time (min^{-1})		
	LB-MOPS	Casein broth	LB-MOPS-15 µg ml^{-1} gentamicin
WT	39.35 ± 1.7	78.77 ± 15	
Δ*ptsP*	39.23 ± 0.56	62.16 ± 8	
WT P*lasI-gfp*			35.98 ± 0.57
Δ*ptsP* P*lasI-gfp*			35.87 ± 1.12
T1 P*lasI-gfp*			39.74 ± 1.94
T2 P*lasI-gfp*			37.54 ± 1.02
T3 P*lasI-gfp*			42.84 ± 3[b]

[a]Doubling times were calculated from hourly cell density measurements using optical density at 600 nm of cultures grown in LB-MOPS or CFU ml^{-1} of cultures grown in casein broth. The values shown are average of three independent experiments with the standard deviation.
[b]Significantly different from wild type P*lasI-gfp* by one-way ANOVA and Dunnett's multiple comparisons test ($p < 0.05$).

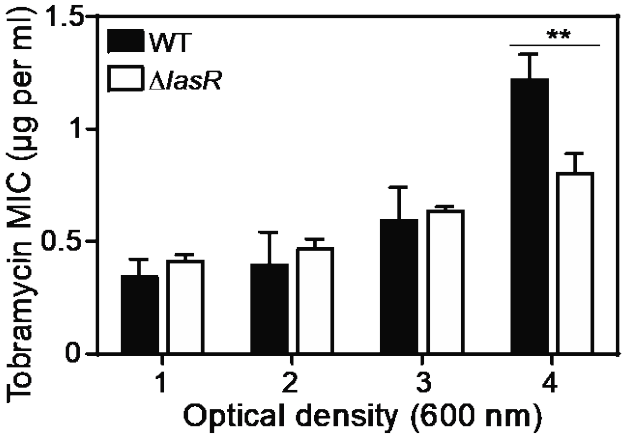

Fig. S1. Tobramycin resistance is LasR dependent at high cell density. The minimum inhibitory concentration (MIC) of tobramycin for *P. aeruginosa* PA14 (WT) or the PA14 Δ*lasR* mutant was determined as described in the Materials and Methods. Cultures were grown to the density indicated prior to diluting and adding tobramycin. The values represent the average of two independent experiments and the error bars represent the standard deviation. Statistical analysis by two-way ANOVA and Sidak's multiple comparisons test with wild type: ** $p<0.01$.

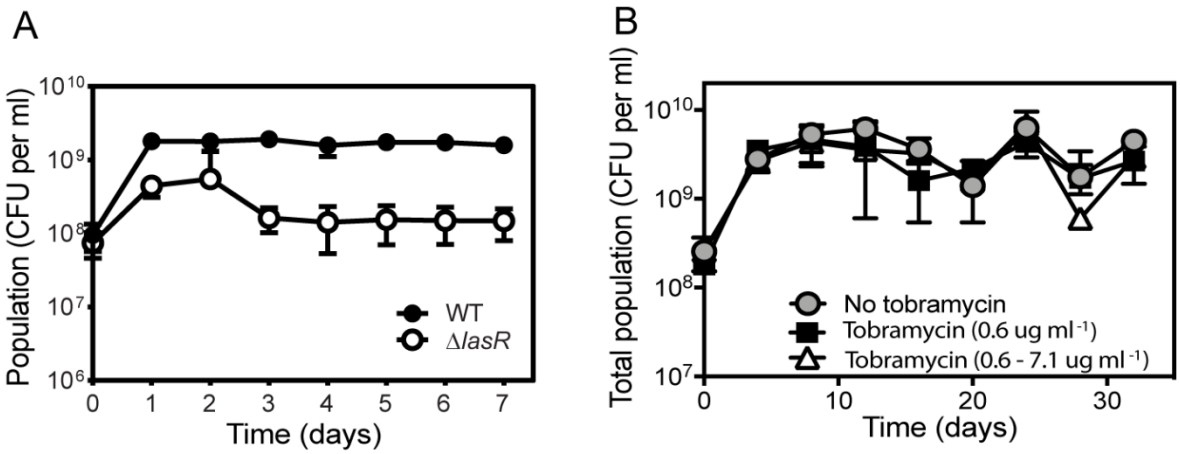

Fig. S2. Cell densities of of *P. aeruginosa* passaged in casein broth. **A.** Cell density of *P. aeruginosa* wild type and Δ*lasR* mutant in casein broth. Cell densities were enumerated daily by spread plating serially diluted bacteria on LB agar. *P. aeruginosa* strains were transferred daily in 1% casein broth for 7 days. **B.** Total cell densities of *P. aeruginosa* populations passaged in casein broth. Cell densities were enumerated every 4 days by spot plating serially diluted bacteria on LB agar. *P. aeruginosa* populations were transferred daily in 1% casein broth for 32 days, and cheaters were enumerated every 4 days by patching as described in Materials and Methods. For each experiment, one culture was initially started with no tobramycin, and after 72 h split into three cultures propagated in one of three conditions; i) continuing with no tobramycin (○), ii) with tobramycin added at 0.6 µg ml^{-1} (■), iii) with tobramycin added at 0.6 µg ml^{-1} initially, and increasing by 50% every 4 days to a final concentration of 7.1 µg ml^{-1} (△). For conditions ii and iii, tobramycin was added every other day just after transfer to fresh medium. The values (A and B) represent the average of three independent experiments and the error bars represent the standard deviation.

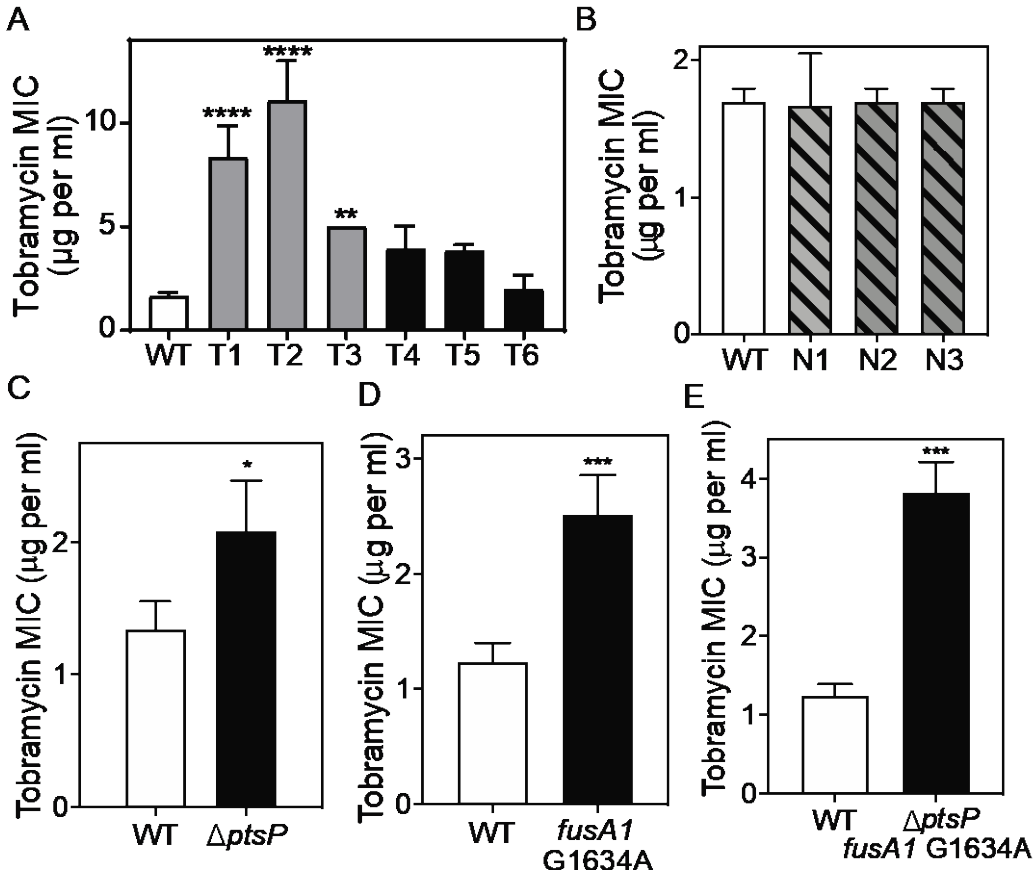

Fig. S3. Tobramycin susceptibility of *P. aeruginosa* mutants. **A.** The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. T1–3 are isolates from populations passaged in 0.6–7.1 μg ml^{-1} tobramycin while T4–6 are isolates from populations passaged in 0.6 μg ml^{-1} tobramycin. **B.** MIC of tobramycin for strains passaged in casein with no tobramycin (N1, N2, N3). **C.** MIC of tobramycin for the PA14 Δ*ptsP* mutant. **D.** MIC of tobramycin for the PA14 *fusA1* G1634A mutant. **E.** MIC of tobramycin for the PA14 Δ*ptsP*, *fusA1* G1634A mutant. The values represent the average of three (A, B, C, and E) or four (D) independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type (A or B) or unpaired t-test (C, D, and E): * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

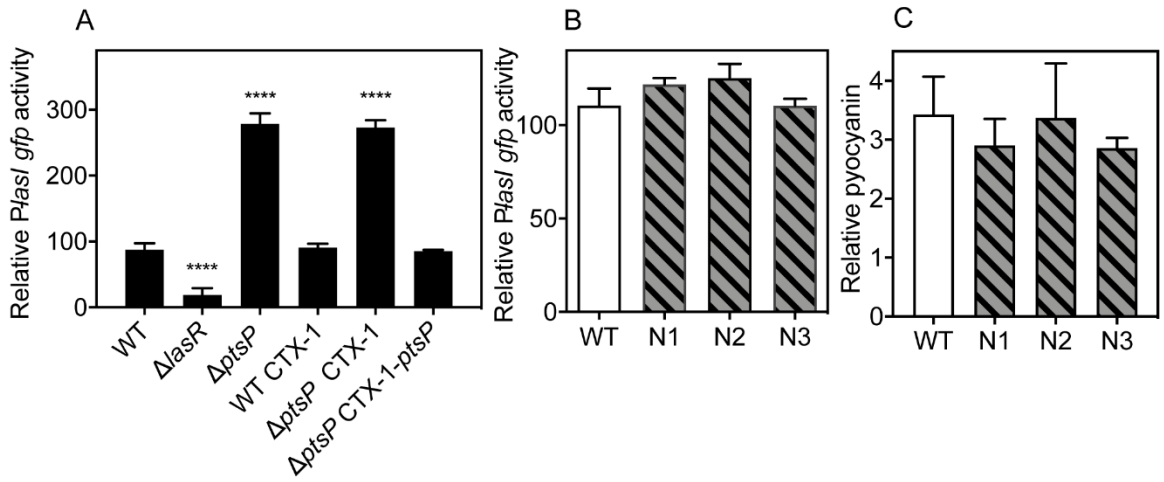

Fig. S4. LasR activity and pyocyanin production in *P. aeruginosa* mutants. **A** and **B.** LasR activity. *P. aeruginosa* strains were electroporated with a plasmid containing a LasR-responsive GFP reporter (pBS351). Reported values are fluorescence normalized to culture density at 18 h of growth. **C.** Pyocyanin production. Cultures were inoculated into pyocyanin-producing media, grown for 18 h, and extracted before quantifying pyocyanin as described in Materials and Methods. Reported values are μg ml^{-1} pyocyanin normalized to culture density at the time of measurement. The values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type, **** $p<0.0001$. For B and C, there was no significant difference ($p>0.05$).

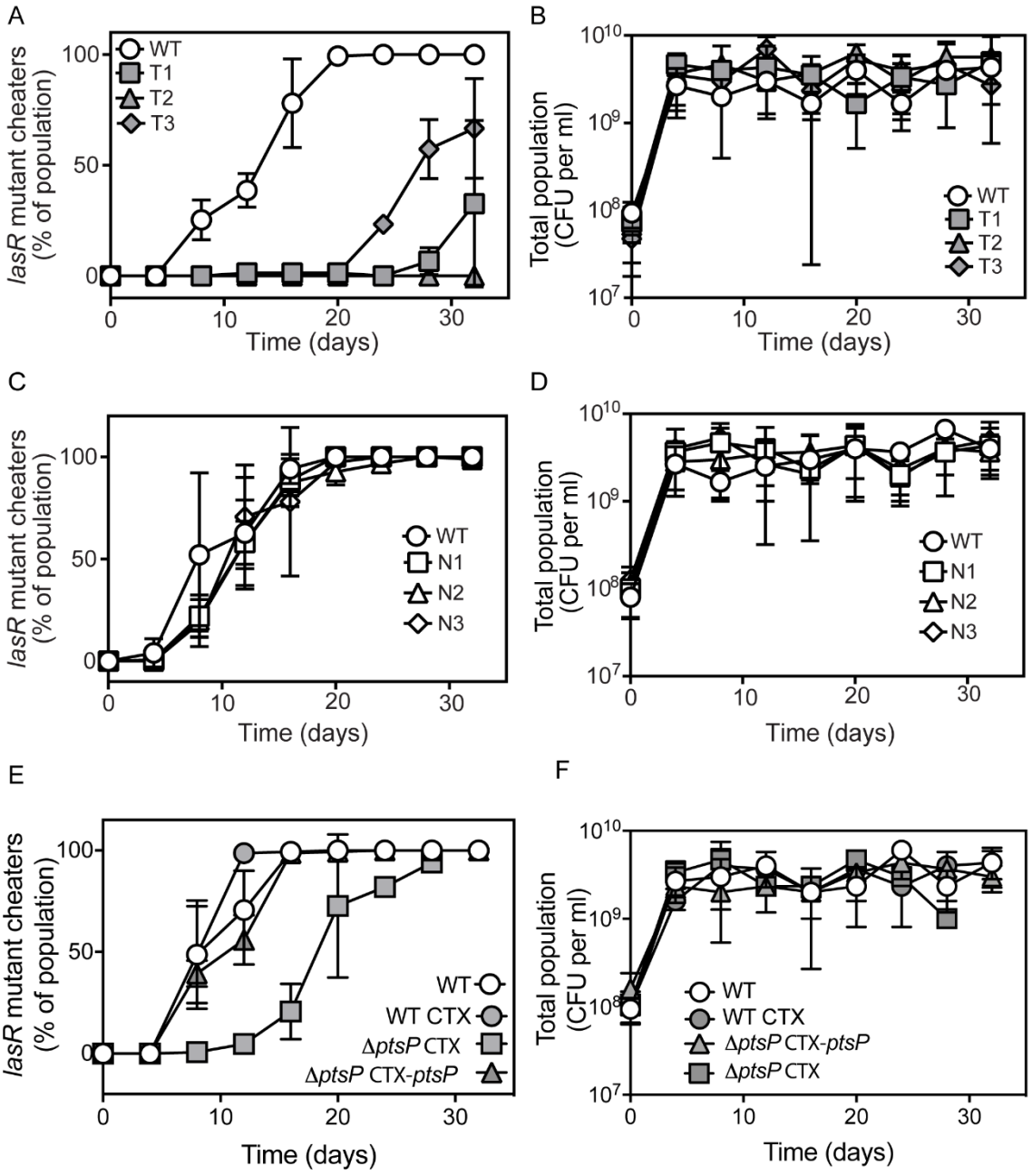

Fig. S5. Frequency of *lasR* mutant cheaters (A, C, and E) and total populations (B, D, and F) for *P. aeruginosa* strains undergoing daily passage in 1% casein for 32 days. A and B show results for strains transferred with tobramycin and corresponds with data shown in Fig. 4A. C and D show results of strains passaged with no tobramycin. E and F show results of the Δ*ptsP* mutant and Δ*ptsP* CTX-*ptsP* and corresponds with data shown in Fig. 4B. The limit of detection of cheaters was 2%. The values for all graphs represent the average of three independent experiments and the error bars represent the standard deviation.

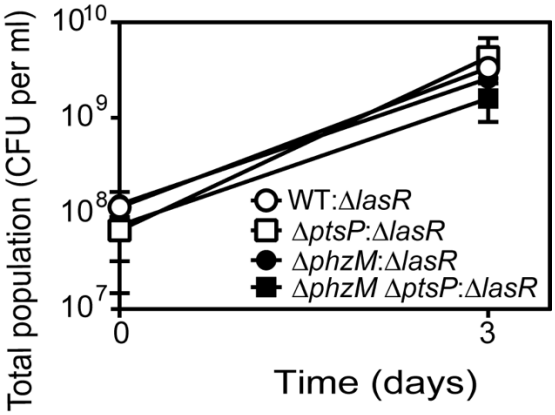

Fig. S6. Initial and final populations in competition experiments. Cultures were initiated at a ratio of 99:1 (cooperator:cheater) and passaged in 1% casein broth daily for 3 days. Initial and final populations were enumerated by serial dilution plating. The values represent the average of three independent experiments and the error bars represent the standard deviation. These data correspond with the data in **Fig. 6D**.

CHAPTER III: Tobramycin adaptation alters the antibiotic susceptibility of *Pseudomonas aeruginosa* quorum sensing-null mutants

Workload allocations:

Rhea G. Abisado contributed in conceptualization, investigation (coculture, MIC, growth curve, RNA extraction, ddPCR, mutant construction), data analysis, supervision, and writing of the chapter; John H. Kimbrough contributed in investigation (antibiotic-adapted strain isolation and purification); Kade A. Townsend (coculture, MIC, growth curve, RNA extraction, mutant construction), Kathryn E. Woods (coculture, MIC, growth curve, RNA extraction, mutant construction), Brielle M. McKee (coculture), Vaughn D. Craddock (coculture), and Alexandra J. Holder (mutant construction, growth curve) contributed in investigation; Josephine R. Chandler contributed in funding acquisition, conceptualization, supervision, data analysis, and editing of the chapter.

Abstract

The LasR-I quorum sensing system is essential in virulence and pathogenesis of the opportunistic pathogen *Pseudomonas aeruginosa*. LasR also contributes in the resistance to the antibiotic tobramycin, a clinically relevant antibiotic that can suppress the emergence of *lasR* mutants *in vitro*. Ironically, *lasR* mutations are a common genetic adaptation of *P. aeruginosa* in cystic fibrosis lung infections which are frequently treated with tobramycin. Here, we explore the effect of tobramycin adaptive mutations on *lasR*-null mutants. We show that *P. aeruginosa* lineages can acquire a point mutation in the elongation factor, *fusA1* G61A (FusA1^{A21T}) under selection by tobramycin, which alters the antibiotic sensitivity of *lasR* mutants. Thus, *fusA1* G61A mutations reverse the role of LasR in tobramycin resistance. The interaction between *fusA1 G61A* and *lasR* inactivation has a sign epistatic effect on *lasR* inactivation. LasR inactivation is beneficial in *fusA1* G61A mutant but deleterious in the wild type in the presence of tobramycin. The *fusA1* G61A mutation increases tobramycin resistance in *lasR* mutants through the MexXY efflux pump in a manner that is dependent on the repressor MexZ and the MexZ anti-repressor ArmZ. Results suggest that activation of the MexXY pump through ArmZ/MexZ involves enhanced ribosome stalling, consistent with previous evidence of how this pathway is activated. Overall, our results show how antibiotics can alter the role of quorum sensing. These results might provide an explanation of how quorum sensing systems might be lost under antibiotic selection and have important implications for understanding the evolution of regulatory networks in changing conditions.

Introduction

About 10% of the genes in the opportunistic multidrug resistant pathogen *Pseudomonas aeruginosa* are regulated by quorum sensing, a type of bacterial communication that regulates gene expression in a population density-dependent manner (1-4). One of the two complete *N*-acyl-homoserine lactone (AHL) quorum-sensing systems in this pathogen is the LasR-I system, which produces and responds to the signal 3-oxododecanoyl-homoserine lactone (3OC12-HSL) (2-5) . This system is composed of a LuxR-family signal receptor LasR and a LuxI-family signal synthase LasI (2, 6, 7). The LasR-I system is on the top of the hierarchy in the quorum sensing regulatory pathways of *P. aeruginosa*, making LasR a master regulator (5, 6, 8).

The LasR-I system is essential for pathogenesis in several acute infection animal models (9-11). It also regulates the expression of some virulence factors (10). Moreover, this system contributes in *P. aeruginosa* resistance to the antibiotic tobramycin (12-15). Tobramycin is a clinically relevant antibiotic that is commonly used to treat chronic *P. aeruginosa* lung infections that are prevalent in individuals with the genetic disease cystic fibrosis (16-19). Clinical isolates of *P. aeruginosa* evolve as a consequence of adaptations to the cystic fibrosis lung environment (20-22). One of the most commonly observed adaptations in clinical infections are LasR mutations, resulting in the loss of quorum-sensing function (22-26). This is quite a surprising finding considering the importance of LasR-I system for virulence, pathogenesis, and tobramycin resistance.

One potential explanation for the emergence of LasR-null mutants in clinical infections is that they are social cheaters. LasR-null mutants can act as exploitative cheaters in laboratory conditions where a quorum sensing-dependent protease is required for growth (27-29). Secreted or excreted proteases can be used by any member of the population and are therefore considered

exploitable public goods (4, 27-30). In natural environments such as infections, the possibility that LasR-null mutants are acting as cheaters could not be disregarded. However, there is also evidence that the LasR-null mutants might emerge for other reasons. For example, in some cases the quorum sensing system hierarchy in *P. aeruginosa* is actually rewired and not lost in clinical isolates with LasR mutations (22, 31-34). In laboratory strains, LasR activates the RhlR-I system, the second AHL quorum sensing system in *P. aeruginosa*, and many cystic fibrosis isolates can retain RhlR activity even in the absence of the master quorum sensing regulator LasR (22). It is also possible that LasR mutants are selected in the conditions of the infection environment (23, 24, 35, 36). There is also the possibility that other mutations, such as those selected by antibiotics, lead to changes that promote the emergence of *lasR.* A number of adaptive mutations emerge in the clinical setting (25), and the effects of these mutations on quorum sensing evolution has not been explored.

One such mutation commonly found in clinical isolates is in *fusA1* coding for elongation factor G1A (EF-G1A) (37-41). *fusA1* is an essential gene, catalyzing the translocation of the ribosome along the mRNA during translation and the recycling of the ribosome (42, 43). Mutation in this gene confers resistance to several antibiotics such as tobramycin (15, 40, 44, 45). In some cases the mechanism of resistance appears to be through the MexXY efflux pump (37, 46). MexXY is one of the Resistance-Nodulation-Division (RND)-type multidrug efflux pumps, and is comprised of the membrane fusion protein MexX and the transporter MexY (47-51). Lacking its own outer membrane protein, MexXY uses the OprM of another RND pump, MexAB (47, 49, 51). The MexXY pump is regulated by the repressor MexZ and the anti-repressor ArmZ (52, 53). MexXY expression is known to be induced by ribosome stalling caused by ribosome disruption, oxidative stress, and membrane damage (54-57). It is thought that

mutations in *fusA1* induce the MexXY pump through ribosome stalling possibly by directly disrupting the elongation step of translation. It is also possible ribosome stalling is induced through indirect mechanisms involving the generation of reactive oxygen species or induction of stress-responsive pathways caused by disrupting this essential function.

Here, we show that a point mutation in *fusA1* gene, *fusA1* G61A ($FusA1^{A21T}$), increases tobramycin resistance in *lasR*-null mutants by increasing expression of the MexXY pump in an ArmZ-dependent manner. We also show that inactivation of *lasR* increases MexXY expression in a *fusA1* G61A mutant through enhanced ribosome stalling. The *fusA1* G61A mutation may make losing LasR a favorable strategy in a population under selection by tobramycin. The interaction between *fusA1 G61A* and *lasR* inactivation has a sign epistatic effect on *lasR* inactivation. In other words, LasR inactivation is beneficial in *fusA1* G61A mutant but deleterious in the wild type in the presence of tobramycin. Sign epistasis is a phenomenon where a trait is beneficial in certain genetic backgrounds and harmful in others (58-60). Epistatic interactions have implications on antimicrobial resistance (60). Overall, our results provide new insight into how LasR-null mutations could arise in a clinical setting and broaden our understanding of how antibiotic selection can contribute to the evolution of quorum sensing.

Results

fusA1 G61A mutations have epistatic effects on *lasR*-null mutant antibiotic resistance

Previously, we isolated and characterized variants of *P. aeruginosa* PA14 passaged in increasing concentrations of tobramycin (15). Variants T1 and T3 were isolated from PA14 populations passaged for 32 days in increasing concentrations of tobramycin (0.6–7.1 μg ml^{-1}), where antibiotic concentration was increased by 50% every 4 days. Both T1 and T3 showed elevated tobramycin resistance relative to the ancestral wild-type PA14 (15). Unexpectedly, deleting *lasR* in either T1 or T3 further increased resistance by about 1.4- to 2-fold (**Fig. 1A, S1A**), whereas deleting *lasR* in the ancestral PA14 strain decreases resistance (12-15) (**Fig. 1B**). We also observed an increase in the MIC of the Δ*lasI* mutant similar to that of the Δ*lasR* mutant, which could be restored to that of the parent by adding 10 μM synthetic 3OC12-HSL (**Fig. 1A, S1A**).

Variants T1 and T3 have two shared mutations, *fusA1* G61A ($FusA1^{A21T}$) and *ptsP* 1547T (a frameshift mutation which is predicted to result in an inactivated protein) (15). To identify the genetic mutation that increased tobramycin resistance in Δ*lasR* mutants of T1 and T3 variants, we introduced the *fusA1* G61A or *ptsP* 1547T mutations to the genome of PA14 or PA14 Δ*lasR* using allelic exchange, and we determined the MIC of each of the mutated strains. We found that deleting *lasR* in the *ptsP* 1547T decreased the MIC by 1.4-fold, which was similar to that observed with the PA14 parent (**Fig. 1B**). However, deleting *lasR* in the *fusA1* G61A mutant increased the MIC by about 2-fold (**Fig. 1B**). Hence, the *fusA1* G61A mutation was responsible for the altered susceptibility of the adapted *lasR* mutants. We could also decrease the tobramycin resistance in variants T1 and T3 by 2.4-fold and 1.4-fold, respectively, through introducing wild type *fusA1* to the neutral *att*Tn7 site in the genome (**Fig. S1B**). Single-copy complementation of

fusA1 G61A mutation with wild type *fusA1* did not decrease the tobramycin resistance of variants T1 and T3 to PA14 ancestral strain levels because of the presence of other mutations (15) that confer antibiotic resistance such as the *ptsP* 1547T mutation (**Fig. S1B**). Our attempts to introduce the wild type *fusA1* gene into the genome of *fusA1* G61A, and corresponding Δ*lasR* mutants of T1 and T3 were unsuccessful. Together, these results show that tobramycin-selected *fusA1* G61A mutations alter the antibiotic resistance phenotype of Δ*lasR* mutants, making *lasR* inactivation sign epistatic.

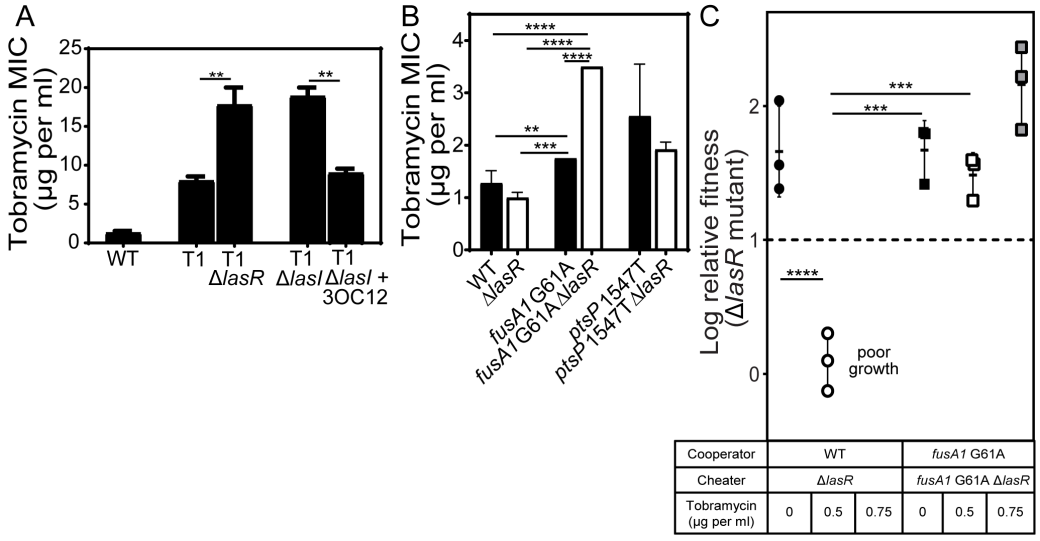

Fig. 1. Tobramycin susceptibility and fitness of *P. aeruginosa fusA1* G61A mutants **A.** MIC of tobramycin for quorum-sensing mutants of tobramycin-adapted variant T1. **B.** MIC of tobramycin for genetically engineered PA14 *fusA1* G61A and *ptsP* 1547T mutants. The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. The values represent the average of three independent experiments and the error bars represent the standard deviation. **C.** Fitness of *fusA1* G61A Δ*lasR* cheater during competition in tobramycin. Competition cultures were inoculated with the Δ*lasR* mutant and each cooperator strain at an initial ratio of 1:99 (cheater:cooperator) in 1% casein broth with different concentrations of tobramycin and transferred to fresh medium daily for 2 days. The antibiotic concentrations used were optimized for each experiment. Cheaters were enumerated by patching as described in Materials and Methods on day 2. Each data point represents an independent experiment. The horizontal line represents the mean and the vertical line represents the standard deviation of all the experiments in each set. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons test (A) or two-way ANOVA and Tukey's multiple comparisons test (B and C): ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

The *fusA1* G61A mutation alters fitness of *P. aeruginosa lasR*-mutated cheaters in competition experiments in the presence of tobramycin

Previously, we showed that tobramycin suppressed the emergence of exploitative *lasR* mutants in cooperating populations grown on casein (15). We hypothesized that tobramycin would not be able to suppress *lasR* mutants in populations with the *fusA1* G61A mutation. We tested this hypothesis using competition experiments. We mixed the Δ*lasR* mutant with a cooperator starting at a 1:99 (cheater: cooperator) ratio in 1% casein media with different concentrations of antibiotics and transferred the population daily for 2 days. We distinguished *lasR* mutants by colony phenotypes as described in the Materials and Methods (23, 24, 28). Consistent with previous studies (15), the Δ*lasR* increased to 37% of the population in cocultures with no tobramycin, but remained at 1% of the population with tobramycin at sublethal concentration (0.5 ug/ml) (**Fig. 1C**). In the cocultures with *fusA1* G61A and *fusA1* G61A Δ*lasR,* the Δ*lasR* mutant increased in frequency in the no-tobramycin cocultures, similar to that of PA14. However, in cocultures with tobramycin at 0.5 μg ml^{-1} or at an even higher concentration of 0.75 μg ml^{-1}, we observed the *lasR* mutants increased to a similar frequency as the cocultures with no tobramycin. *lasR* mutants of the T1 and T3 variant were similarly not suppressed by tobramycin (**Fig. S2A, S2C**). At the highest tobramycin concentration, the *lasR* mutants reached an even higher frequency than that of the no-tobramycin cocultures, with T1 and T3 showing statistically significant differences while G61A showed a similar, not significantly different frequency. There were no significant differences in the total population densities across each coculture experiment (**Fig. S2B, S2D, S2E)**. Overall, our results support that *fusA1* G61A alters the fitness of *lasR*-mutated cheaters in tobramycin-treated conditions.

Other mutations in *fusA1* do not cause sign epistasis of Δ*lasR* mutations

fusA1 is frequently mutated in *P. aeruginosa* exposed to tobramycin as well as in clinical isolates (40, 44, 45). We sought to test whether other *fusA1* mutations can alter the antibiotic resistance phenotype of Δ*lasR* mutants similar to the G61A mutation. We introduced the mutations *fusA1* T119A (FusA1^{L40Q}), G352A (FusA1^{G118S}), A1366G (FusA1^{T456A}), C1664A (FusA1^{A555E}), or A2011G (FusA1^{T671A}) to the genome of PA14, deleted *lasR* in the resulting *fusA1* mutant strains, and determined the MIC of each strain pair. In each case, the *lasR* mutant was either more susceptible than the corresponding *lasR*-intact strain or has the same susceptibility as the corresponding *lasR*-intact strain (**Fig. 2**). Thus, there is something specific to the *fusA1* G61A mutation that causes sign epistasis of Δ*lasR* mutants.

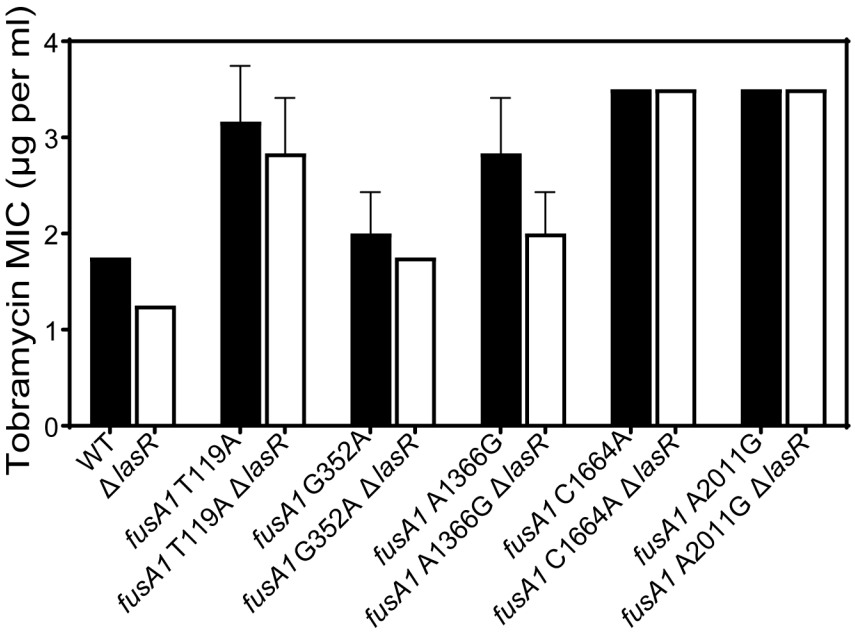

Fig. 2. Tobramycin susceptibility of *P. aeruginosa fusA1* mutants. The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. The values represent the average of one independent experiment with three technical replicates and the error bars represent the standard deviation.

MexXY is needed for *fusA1* G61A-dependent epistatic effects on Δ*lasR* mutations

Next, we sought to determine the mechanism of Δ*lasR* sign epistasis on tobramycin resistance. FusA1 mutations have been shown to enhance aminoglycoside resistance in *P. aeruginosa* through elevated expression of the RND multidrug efflux pump, MexXY (37, 46). We tested whether the MexXY efflux pump is also responsible for enhanced antibiotic resistance in our *fusA1* G61A Δ*lasR* mutants. As a first step to test this hypothesis, we quantified transcripts of *mexX,* which codes for the efflux membrane fusion protein in PA14 and PA14 Δ*lasR* and the corresponding strains of each of the *fusA1* G61A mutated strains (*fusA1* G61A, T1, and T3) (**Fig. 3A**). Our results showed *mexX* transcripts were 5-fold higher in the *fusA1* G61A, Δ*lasR* double mutant than in the *fusA1* G61A single mutant. The T1 and T3 variants showed a similar pattern with 7- and 2-fold differences, respectively. *mexX* transcripts were nearly identical in PA14 and PA14 Δ*lasR*. These results suggest the MexXY pump is activated by deleting *lasR* in a *fusA1* G61A mutant. These results also suggest MexXY is not LasR-controlled in the ancestral PA14 parent.

Our *mexX* transcription results are consistent with the idea that *fusA1* G61A-dependent sign epistasis of Δ*lasR* mutants requires the MexXY pump. To test this idea, we deleted *mexY* in T1 and T1 Δ*lasR*, and we compared the MIC of each of the *mexY*-null strains with that of the parent. We also deleted *mexY* in PA14 and PA14 Δ*lasR*. Our attempts to delete *mexY* in *fusA1* G61A, T3, and T3 Δ*lasR* were unsuccessful. Deleting *mexY* in PA14 or PA14 Δ*lasR* did not significantly affect the tobramycin MIC (**Fig. 3B**). However, deleting *mexY* in IT1 and IT1 Δ*lasR* decreased the MIC to nearly that of PA14 and PA14 Δ*lasR,* supporting the idea that MexY is important for tobramycin resistance in IT1. Importantly, the MIC of IT1 Δ*mexY* and IT1 Δ*lasR* Δ*mexY* were nearly identical. Together, these results are consistent with the idea that MexXY is

needed for sign epistasis of Δ*lasR* in a *fusA1* G61A mutant. Our results also support the idea that MexXY is not important for LasR-dependent tobramycin resistance in the PA14 ancestor.

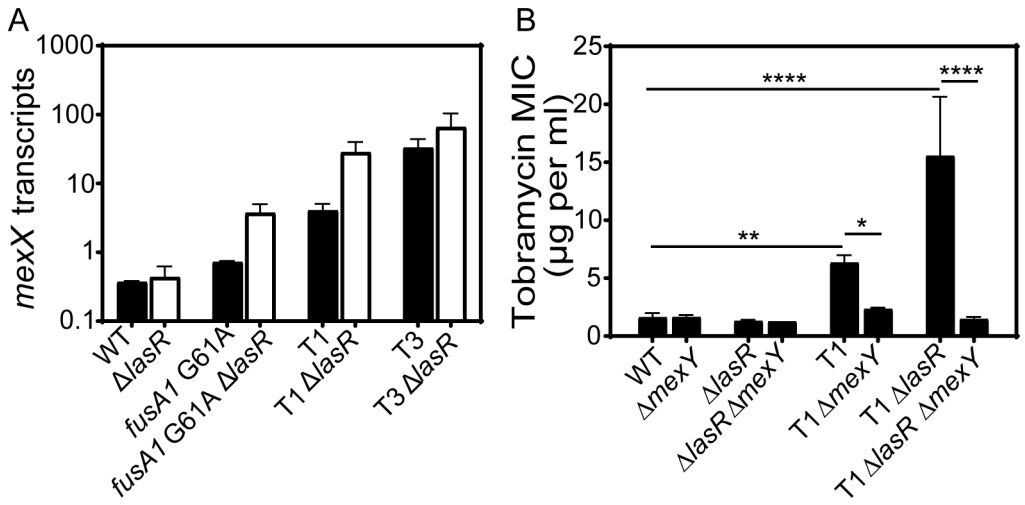

Fig. 3. *fusA1* G61A mutation increases antibiotic resistance in Δ*lasR* mutants through the MexXY efflux pump. **A** *mexX* expression. Results are shown as *groEL*-adjusted transcript values. **B** Tobramycin MIC for *mexY* mutants of T1 and the T1 Δ*lasR* variant. The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. The values represent the average of two (A) or three (B) independent experiments and the error bars represent the standard deviation. Statistical analysis by two-way ANOVA and Tukey's multiple comparisons test: * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

fusA1 G61A mutation upregulates MexXY efflux pump expression in *P. aeruginosa lasR*-null mutants in ArmZ-dependent manner

The MexXY operon is repressed by an adjacently encoded regulator, MexZ. MexZ, in turn, is repressed by the ArmZ antirepressor (52, 53). ArmZ is induced upon exposure to ribosome targeting antibiotics through a mechanism involving transcription attenuation (52). Antibiotic exposure causes ribosome stalling, which causes alternate mRNA folding of an ArmZ leader peptide called PA5471.1 and this in turn allows transcription progression into the *armZ* gene and ultimately translation of ArmZ (52, 54, 61). We investigated whether ArmZ is needed

for sign epistasis of Δ*lasR* in a *fusA1* G61A mutant. First, we measured the expression of *armZ* in the *fusA1* G61A mutated strains (**Fig. 4A**). Our results showed *armZ* transcripts were 1.9-fold higher in the *fusA1* G61A, Δ*lasR* double mutant than in the *fusA1* G61A single mutant. The T1 and T3 variants also showed a similar pattern with ~5- and 1.4-fold differences, respectively. The *armZ* transcripts were nearly identical in PA14 and PA14 Δ*lasR*. These results are consistent with the idea that the *fusA1* G61A mutation causes epistatic interactions with *lasR* inactivation that induce production of ArmZ in a Δ*lasR* mutant.

We also deleted *armZ* in T1 and T1 Δ*lasR* mutants, and we compared the MIC of the mutated strains with that of T1 and T1 Δ*lasR*. Whereas deleting *armZ* had no significant effects in PA14 or PA14 Δ*lasR*, deleting *armZ* in the T1 strains caused a significant reduction of the MIC to nearly identical levels (**Fig. 4B**). We observed a similar pattern when we deleted *armZ* in T3 and T3 Δ*lasR* (**Fig. 4C**). Our attempts to delete *armZ* in *fusA1* G61A were also unsuccessful. Altogether, our results show that *fusA1* G61A-dependent sign epistasis of Δ*lasR* is through upregulation of ArmZ expression.

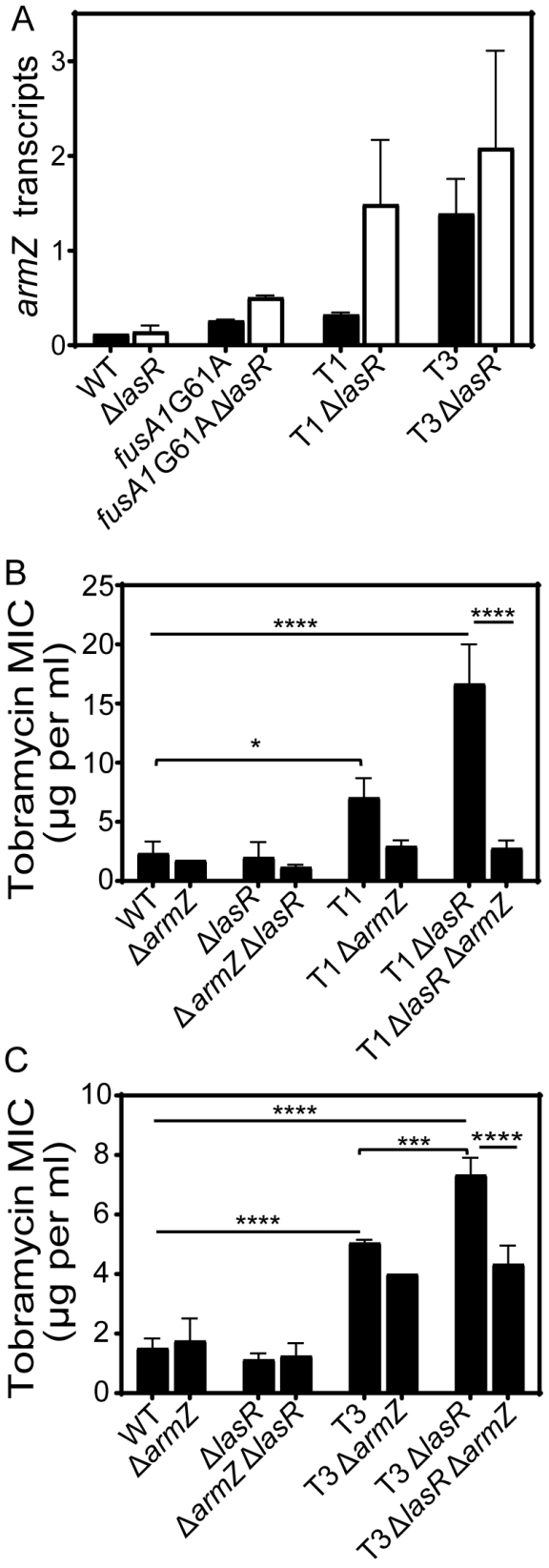

Fig. 4. A *armZ* expression. Results are shown as *groEL*-adjusted transcript values. **B** Tobramycin MIC for *armZ* mutants of T1 and the T1 Δ*lasR* variant. **C** Tobramycin MIC for *armZ* mutants of T3 and the T3 Δ*lasR* variant. The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. The values represent the average of two (A) or three (B and C) independent experiments and the error bars represent the standard deviation. Statistical analysis by two-way ANOVA and Tukey's multiple comparisons test: * $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Inactivation of *lasR* in *a fusA1* G61A mutant increases ribosome stalling

Because stalling of the ribosome is one of the known mechanisms that induce expression of the MexXY efflux pump (54, 57, 61), and *fusA1* mutations are associated with slower growth rates and increased ribosome stalling (37, 46, 62), we hypothesized that *fusA1* G61A mutations caused enhanced ribosome stalling that somehow leads to LasR-dependent MexXY activation. Growth rate is a proxy for ribosome stalling. Thus, we measured doubling time during growth in MOPS minimal media as an initial test for ribosome stalling (**Table 1**). The doubling time of PA14 and PA14 Δ*lasR* were similar. Doubling times in strains with the *fusA1* G61A mutant were all higher (*i.e.* slower growth rate) than PA14, supporting the idea that this mutation causes ribosome stalling. In each case, deleting *lasR* further slowed growth, supporting the hypothesis that deleting *lasR* in the *fusA1* G61A mutant somehow increases ribosome stalling leading to activation of MexXY.

Table 1. Growth rates in MOPS minimal media[a].

Strain/Variant	Doubling time (per min)
WT	62.5±1.98
Δ*lasR*	61.4±2.9
fusA1 G61A	66.3±2.9
fusA1 G61A Δ*lasR*	78.2±2.2***
T1	67.7±4.0
T1 Δ*lasR*	79.7±2.5***
T3	74.88±3.35
T3 Δ*lasR*	82.93±3.5*

[a]The values represent the average of three independent experiments and standard deviation. Statistical analysis by one-way ANOVA and Sidak's multiple comparisons test: * $p<0.05$, *** $p<0.001$.

Discussion

Quorum sensing has a small but appreciable contribution to tobramycin resistance in *P. aeruginosa* both under biofilm and planktonic conditions (12-15). Here, we show that one mutation, *fusA1* G61A, can reverse the role of quorum sensing on antibiotic resistance. The *fusA1* G61A mutation arose under selection by tobramycin. Our results showed that inactivation of the quorum-sensing receptor LasR was beneficial in conditions of antibiotic selection, whereas *lasR*-null mutants are detrimental in the PA14 parent. Thus, we have demonstrated sign epistasis of *lasR*-null mutations caused by a particular type of mutation, a *fusA1* G61A mutation. We have also demonstrated that sign epistasis of *lasR*-null mutations can occur as *P. aeruginosa* adapts to selective pressures in its environment, which could potentially explain how *lasR*-null mutations survive and even proliferate in antibiotic-treated infections.

LasR is important for pathogenesis and virulence (9-11), and also contributes to antibiotic resistance (12-15, 63). Paradoxically, it is common for *P. aeruginosa* infection isolates to have mutations that abolish function of the LasR-I quorum-sensing system, which sits at the top of the quorum-sensing circuitry and is needed to activate the other quorum-sensing systems (22-26). A potential explanation for the abundance of *lasR* mutants in the clinical isolates include the rewiring of the quorum sensing hierarchy. Rewiring can occur through inactivation of the efflux pump regulator MexT, which enables activation of the RhlR-I system as well as the non-AHL Pseudomonas quinolone signal (PQS) in *lasR*-null mutants (31, 32, 34). Another potential explanation is that increased tolerance of *lasR*-null mutants to antibiotics could be conditional. The ability of *lasR*-null mutants to enhance nitrite-based metabolism has been shown to confer tolerance to tobramycin and ciprofloxacin (64). Here, we provide another potential explanation: adaptive mutations that accumulate during infection could reverse the fitness benefits of a *lasR*-

null mutation and cause selection of these mutants by antibiotics. It is also possible that *lasR*-null mutants accumulate antibiotic resistance mutations after they emerge, as has been reported for β-lactam antibiotic resistance in laboratory evolution experiments and clinical *lasR* mutant isolates (24, 65).

There is a growing body of research on the impact of epistasis on antibiotic resistance (60, 66-68). For example, bacterial fitness is altered by epistatic interactions between conjugative resistance plasmids and resistance mutations conferring resistance to nalidixic acid, rifampicin, or streptomycin (67). The mutational trajectories conferring high level cefotaxime resistance in *E. coli* are constrained by sign epistasis (66). Evolution experiments also revealed that *lasR*-intact and *lasR*-null mutants could undergo different antibiotic adaptations due to negative sign epistasis (68). When *lasR* mutation is selected first, evolution of antibiotic resistance could be restrained. However, when mutations conferring antibiotic resistance are selected first, emergence of *lasR* mutants could be restrained in the presence of antibiotics (68). Epistasis may have implications on how *P. aeruginosa* and other pathogens evolved and how they affect their hosts especially during chronic infections.

The *fusA1* G61A mutation increases tobramycin resistance in *lasR*-null mutants through an ArmZ-dependent MexXY efflux pump (**Fig. 3, 4**). The MexXY pump is known to be involved in the active efflux of aminoglycosides, fluoroquinolones, tetracycline, and erythromycin (47, 49). This implies that the *lasR*-null mutants may also have developed resistance to other drugs which have significant consequences on the treatment of clinical infections. Growth rates (**Table 1**) suggest that there is more ribosome stalling occurring in strains with both *fusA1* G61A and *lasR*-null mutations, and this could possibly explain upregulation of MexXY in those strains. The mechanism behind the increased ribosome stalling

in *lasR*-null mutants with *fusA1* G61A mutation needs further investigation. A potential explanation is that *fusA1* G61A mutation may increase production of reactive oxygen species (ROS) that could contribute to ribosome stalling. Quorum sensing is known to activate ROS detoxification systems such as the enzyme superoxide dismutase (69). The *lasR* mutant, which does not activate ROS detoxifiers, may have higher levels of ROS and more stalling. These are testable hypotheses that will be pursued in the near future. There could be other types of mutations that have effects similar to *fusA1* G61A, such as mutations in oxidative stress systems or membrane damage repair (55-57). A recent study showed that a *fusA1* mutation can alter expression of virulence genes in *P. aeruginosa* (46). Whether *fusA1* G61A mutation has the same effect on the virulence of *lasR*-null mutants is yet to be explored.

Overall, our work provides additional evidence on how antibiotic selection can contribute to the evolution of quorum sensing. Previously, we reported how LasR activity and cooperation-cheater dynamics were influenced by tobramycin adaptation (15). Here, we illustrate how tobramycin adaptation could cause a reversal of some quorum sensing-dependent phenotypes such as antibiotic resistance. Understanding the evolution of *P. aeruginosa* quorum sensing under antibiotic selection will contribute in a better understanding of quorum sensing biology and provide insights to ongoing studies on alternative therapies involving novel therapeutics that target or block quorum sensing.

Materials and methods

Culture conditions and reagents

Bacteria were routinely grown in Luria-Bertani broth (LB), 50 mM 3-(morpholino)-propanesulfonic acid (MOPS)-buffered LB (pH 7), or on LB agar (LBA) with 1.5% weight per volume Bacto-Agar. For specific experiments, M9-caseinate (15), MOPS minimal medium (15, 70), and 4% skim milk agar (SMA) were used (28). All *P. aeruginosa* broth cultures were grown at 37ºC with shaking at 250 rpm using 18 mm test tubes (for 2 ml cultures) or 125 ml baffled flasks (10 ml cultures). For *E. coli*, 100 μg ml^{-1} carbenicillin, 15 μg ml^{-1} gentamicin, 50 μg ml^{-1} streptomycin, and 2.5–10 μg ml^{-1} tetracycline were used while 150–300 μg ml^{-1} carbenicillin, 50–200 μg ml^{-1} gentamicin, 4000 μg ml^{-1} streptomycin, and 15–100 μg ml^{-1} tetracycline were used for *P. aeruginosa*. AHLs were purchased from Cayman Chemicals (MI, USA) and handled as described elsewhere (71). Genomic or plasmid DNA was extracted using Qiagen Puregene Core A kit (Hilden, Germany) or IBI Scientific plasmid purification mini-prep kit (IA, USA) while PCR products were purified using IBI Scientific PCR clean-up/gel extraction kits, according to the manufacturer's protocol. All antibiotics were purchased from GoldBio (MO, USA) except for tetracycline which is from Fisher Scientific (PA, USA).

Bacterial strains and strain construction

All bacterial strains, plasmids, and primers used in this study are listed in **Tables 2–4**. *P. aeruginosa* strain UCBPP-PA14 ('PA14') and PA14 derivatives were used for these studies. Markerless deletions in specific loci of *P. aeruginosa* PA14 were generated using allelic exchange as described previously (72). To generate plasmids for allelic exchange, DNA fragments with the mutated or deleted gene allele plus 500 bp flanking DNA were generated by

PCR using primer-incorporated restriction enzyme sites (*fusA1* G61A and *ptsP* 1547T) or by stitch PCR (Δ*lasI*). The PCR product was digested and ligated to pEXG2 (*fusA1* G61A and *ptsP* 1547T) or by isothermal assembly with pEXG2 (Δ*lasI*) and transformed into the appropriate *P. aeruginosa* strain. The plasmids for Δ*lasR* (15), Δ*mexY* (73), Δ*armZ* (52), *fusA1* T119A (37), *fusA1* G352A (37) , *fusA1* A1366G (37), *fusA1* C1664A (37), and *fusA1* A2011G (37) were described elsewhere. Merodiploids were selected on Pseudomonas Isolation Agar (PIA)-carbenicillin (150–300 μg ml^{-1}) for Δ*lasR*; PIA-gentamicin (50–200 μg ml^{-1}) for *fusA1* G61A, Δ*lasI*, and *ptsP* 1547T; PIA-streptomycin (4000 μg ml^{-1}) for *fusA1* T119A, *fusA1* G352A, *fusA1* A1366G, *fusA1* C1664A, and *fusA1* A2011G; and PIA-tetracycline (15–100 μg ml^{-1}) for Δ*mexY* and Δ*armZ*. Deletion mutants were counterselected using NaCl-free 15% sucrose. Putative mutants were verified through antibiotic sensitivity tests and gene-targeted Sanger sequencing. To make the P*lac-fusA1* expression cassette, we cloned *fusA1* into pUC18T miniTn7 *lac*-Gm and introduced the cassette into *P. aeruginosa* by electroporation as described previously (74). Transformants were selected on LBA with 50–200 μg ml^{-1} gentamicin and verified insertion of the cassette in the *att*Tn7 site by PCR as described previously (74).

Antimicrobial susceptibility assay

Tobramycin susceptibility was determined by MIC according to the 2020 guidelines of the Clinical and Laboratory Standards Institute (CSLI), using a modified dilution method described elsewhere (15). Briefly, two dilution series were made from staggered starting tobramycin concentrations to cover a broader range of concentrations in MOPS minimal medium, and successively diluted 2-fold in a 200 μl volume in 2 ml tubes. The starter cultures were prepared by growing *P. aeruginosa* in LB-MOPS to OD_{600} of 4. Then, the starter cultures

were diluted 1:40 into each tube containing tobramycin to start the MIC experiment. After 20 h of incubation with shaking, turbidity was measured using a Biotek Synergy 2 plate reader. The MIC was defined as the lowest concentration of tobramycin (μg ml^{-1}) in which bacterial growth was not measurable.

Table 2. Bacterial strains used in this study.

Strain	Relevant properties	Reference or source
P. aeruginosa		
UCBPP-PA14	Ancestral wild type	(75)
PA14 Δ*lasR*	PA14 with a deletion of *lasR*	(15)
PA14 *fusA1* G61A	PA14 with the *fusA1* G61A mutation	This study
PA14 *fusA1* G61A Δ*lasR*	PA14 *fusA1* G61A with a deletion of *lasR*	This study
PA14 *ptsP* 1547T	PA14 with the *ptsP* 1547T mutation	This study
PA14 *ptsP* 1547T Δ*lasR*	PA14 *ptsP* 1547T with a deletion of *lasR*	This study
PA14 Δ*mexY*	PA14 with a deletion of *mexY*	This study
PA14 Δ*lasR* Δ*mexY*	PA14 Δ*lasR* with a deletion of *mexY*	This study
PA14 Δ*armZ*	PA14 with a deletion of *armZ*	This study
PA14 Δ*lasR* Δ*armZ*	PA14 Δ*lasR* with a deletion of *armZ*	This study
PA14 *fusA1* T119A	PA14 with the *fusA1* T119A mutation	This study
PA14 *fusA1* G352A	PA14 with the *fusA1* G352A mutation	This study
PA14 *fusA1* A1366G	PA14 with the *fusA1* A1366G mutation	This study
PA14 *fusA1* C1664A	PA14 with the *fusA1* C1664A mutation	This study
PA14 *fusA1* A2011G	PA14 with the *fusA1* A2011G mutation	This study

PA14 *fusA1* T119A Δ*lasR*	PA14 *fusA1* T119A with a deletion of *lasR*	This study
PA14 *fusA1* G352A Δ*lasR*	PA14 *fusA1* G352A with a deletion of *lasR*	This study
PA14 *fusA1* A1366G Δ*lasR*	PA14 *fusA1* A1366G with a deletion of *lasR*	This study
PA14 *fusA1* C1664A Δ*lasR*	PA14 *fusA1* C1664A with a deletion of *lasR*	This study
PA14 *fusA1* A2011G Δ*lasR*	PA14 *fusA1* A2011G with a deletion of *lasR*	This study

P. aeruginosa PA14 variants

Variants isolated after daily transfer for 32 days in 1% casein

T1	Isolate from experiment with tobramycin added at 0.6–7.1µg ml^{-1}	(15)
T3	Isolate from experiment with tobramycin added at 0.6–7.1µg ml^{-1}	(15)

Modified variants

T1 Δ*lasR*	T1 with a deletion of *lasR*	This study
T1 Δ*lasI*	T1 with a deletion of *lasI*	This study
T1 Δ*mexY*	T1 with a deletion of *mexY*	This study
T1 Δ*lasR* Δ*mexY*	T1 Δ*lasR* with a deletion of *mexY*	This study
T1 Δ*armZ*	T1 with a deletion of *armZ*	This study
T1 Δ*lasR* Δ*armZ*	T1 Δ*lasR* with a deletion of *armZ*	This study
T3 Δ*lasR*	T3 with a deletion of *lasR*	This study
T3 Δ*lasI*	T3 with a deletion of *lasI*	This study
T3 Δ*armZ*	T3 with a deletion of *armZ*	This study
T3 Δ*lasR* Δ*armZ*	T3 Δ*lasR* with a deletion of *armZ*	This study

Escherichia coli

DH5α	F$^-$ Φ80*lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17*(r_K^- m_K^+) *recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
S17-1	*recA pro hsdR RP4-2-Tc::Mu-km::Tn7*	(76)
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pi*r	(76)
Rho3	*thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA*, integrated RP4-2 Tcr::Mu (λ*pir* $^+$)Δ*asd*::FRT Δ*aphA*::FRT	(77)
CC118 λ*pir*	*Δ(ara-leu) ara D ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recAl lysogenized with λpir phage*	(78)
HB101	*supE44 hsdS20(*r_B*-* m_B*-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	(79)

Coculture assays

Overnight (18 h) pure cultures were grown in LB-MOPS, diluted to OD_{600} of 0.025 for cheaters or 0.05–0.15 for cooperators into LB-MOPS, and grown to an OD_{600} of ~3.5 before combining at a 99:1 (cooperator:cheater) ratio. This mixture was then diluted 1:40 into casein broth with different antibiotic concentrations to start the coculture in 18 mm test tubes. Cocultures were diluted 1:40 into fresh casein broth in a new tube every 24 h until the end of the experiment at 48 h. The initial and final total population counts (CFU ml^{-1}) were determined by viable plate counts. The % *lasR* cheater was determined by patching 200 colonies on SMA where *lasR* cheaters form distinct colony phenotypes (15, 23, 24, 28).

Table 3. Plasmids used in this study.

Plasmid	Relevant properties	Reference or source
pEX18Ap	Suicide vector, Ap^r	(80)
pDA8	pEX18Ap containing flanking sequences of *lasR,* Ap^r	(15)
pEXG2	Suicide vector, Gm^r	(81)
pEXG2-Δ*lasI*	pEXG2 containing Δ*lasI* with flanking sequences, Gm^r	Matthew Cabeen
pEXG2-*fusA1* G61A	pEXG2 containing G61A mutation in *fusA1,* Gm^r	This study
pEXG2-*ptsP* 1547T	pEXG2 containing 1547T mutation in *ptsP,* Gm^r	This study
pUC18T-miniTn7-lac-*fusA1*	pUC18T-miniTn7-lac containing *fusA1,* Gm^r	This study
pEX18Tc	Suicide vector, Tc^r	(80)
pCSV05	pEX18Tc containing Δ*mexY* with flanking sequences, Tc^r	(73)
pYM008	pEX18Tc containing Δ*armZ* with flanking sequences, Tc^r	(52)
pKNG101	Marker exchange suicide vector in *P. aeruginosa*; sacBR mobRK2 oriR6K; Str^r	(82)
pKNG101-*fusA1*$_{T119A}$	pKNG101 containing *fusA1* T119A, Str^r	(37)
pKNG101-*fusA1*$_{G352A}$	pKNG101 containing *fusA1* G352A, Str^r	(37)
pKNG101-*fusA1*$_{A1366G}$	pKNG101 containing *fusA1* A1366G, Str^r	(37)
pKNG101-*fusA1*$_{C1664A}$	pKNG101 containing *fusA1* C1664A, Str^r	(37)
pKNG101-*fusA1*$_{A2011G}$	pKNG101 containing *fusA1* A2011G, St^r	(37)
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids; ColE1 Tra^+ Mob^+ Kan^r	(83)
pTNS3	Helper plasmid encoding transposase gene necessary for the chromosomal integration of mini-Tn7	(84)

Table 4. Primers used in this study.

Primer	Sequence
rgaoligo72	TAATAA**AAGCTT**GTGCTGGCTGATCCGAAATA
rgaoligo73	TAATAA**TCTAGA**GACCGACGAAGTTCTCTTCAG
Droplet Digital PCR	
mexXF	CGAAGAAGCAGCGGACAC
mexXR	GCAGCTCGCTGGTGATG
armZF	CGATCCAGCAACTGCAAAC
armZR	CGATCCAGCAACTGCAAAC
groELF[c]	GTACCGTGATCAGCGAAGAA
groELR[c]	CATCGATGATGGTGGTGTTT

[a]Bolded text indicates restriction sequences
[b]rgaoligo72 and 73 were used to incorporate the *fusA1* G61A mutation
[c]Primer sequence provided by Nicole Smalley (University of Washington)

Droplet digital PCR

Overnight (18 h) pure cultures were grown in LB-MOPS, subcultured in LB-MOPS, and grown to an OD_{600} of ~4. Stationary-phase *P. aeruginosa* (OD_{600} of 4) was diluted to OD_{600} 0.1 in MOPS minimal medium and grown to OD_{600} 0.5. RNA was harvested using the RNeasy minikit (Qiagen), following the manufacturer's instructions. Droplet digital PCR (ddPCR) was performed on a Bio-Rad QX200 ddPCR system using Eva Green supermix. Each reaction mixture contained 1.8 ng μl^{-1} cDNA template, 0.8 µM of each primer, and 10 µl Eva Green supermix in a 20-µl final volume. After generating 40 µl of oil droplets, 40 rounds of PCR were conducted using the following cycling conditions: 95°C for 30s, 62°C for 30s, and 68°C for 30s.

Absolute transcript levels were determined using Bio-Rad QuantaSoft software. In all cases, a no-template control was run with no detectable transcripts. The reference gene used was *groEL*, and the results are reported as the calculated transcript amount of a given gene per calculated *groEL* transcript.

Growth Curve

Overnight (18 h) pure cultures were grown in LB-MOPS, subcultured in LB-MOPS, and grown to an OD_{600} of ~4. Stationary-phase *P. aeruginosa* (OD_{600} of 4) was diluted to OD_{600} 0.1 in MOPS minimal medium, and OD_{600} was measured in a Jenway spectrophotometer every hour min for 8 h. Doubling time was calculated for each strain.

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Supplementary Information

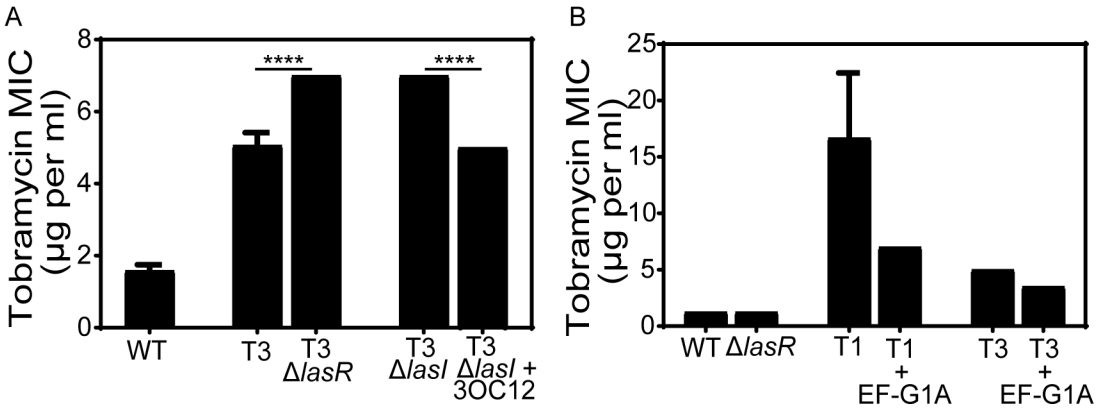

Fig. S1. Tobramycin MIC for tobramycin-adapted variants. **A.** Quorum-sensing mutants of tobramycin-adapted variant T3. **B.** Variants T1 and T3 with wild type *fusA1* gene (EF-G1A). Strains carried either the P*lac* or the P*lac-fusA1* cassette, inserted at neutral *att*Tn7 site in the genome. The minimum inhibitory concentration (MIC) of tobramycin was determined as described in Materials and Methods. The values represent the average of three independent experiments (A) or one independent experiment with three technical replicates (B). The error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons test: **** $p<0.0001$.

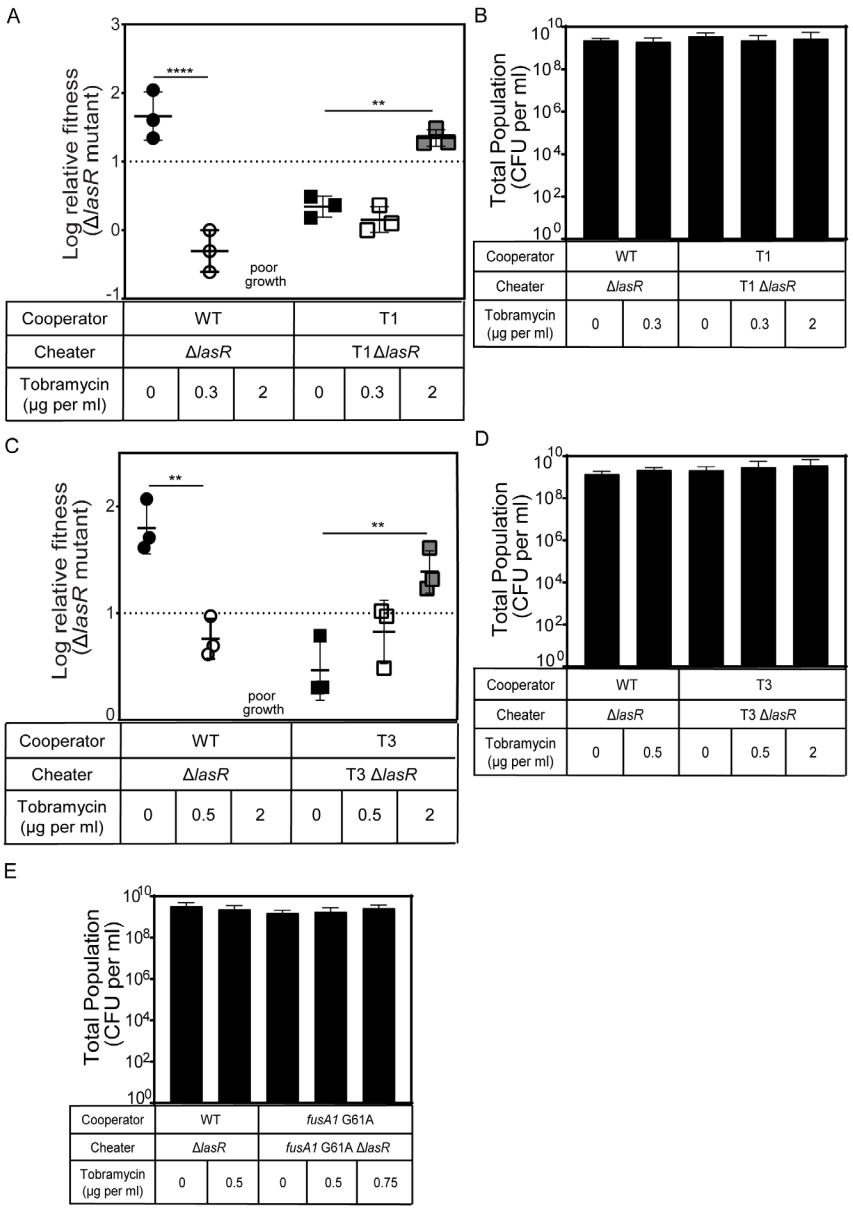

Fig. S2. Fitness of tobramycin-adapted Δ*lasR* mutant cheater during competition in tobramycin. **A.** Competition between variants T1 and T1 Δ*lasR*. **B.** Population counts for competition between variants T1 and T1 Δ*lasR* **C.** Competition between variants T3 and T3 Δ*lasR*. **D.** Population counts for competition between variants T3 and T3 Δ*lasR*. **E.** Population counts for competition between variants *fusA1* G61A and *fusA1* G61A Δ*lasR*. Competition cultures were inoculated with the Δ*lasR* mutant and each cooperator strain at an initial ratio of 1:99 (cheater:cooperator) in 1% casein broth with different concentrations of tobramycin and transferred to fresh medium daily for 2 days. The antibiotic concentrations used were optimized for each experiment. Cheaters were enumerated by patching as described in Materials and Methods on day 2. Each data point represents an independent experiment. The horizontal line represents the mean and the vertical line represents the standard deviation of all the experiments in each set. Statistical analysis by one-way ANOVA and Sidak's multiple comparison test: ** $p<0.01$, **** $p<0.0001$.

CHAPTER IV: Gentamicin-induced alterations on LasR-I quorum sensing system of *Pseudomonas aeruginosa*

Workload allocations:

Rhea G. Abisado contributed in conceptualization, investigation (evolution, MIC, *gfp* reporter assays, whole genome sequencing, mutant construction), supervision, data analysis, and writing of the chapter; John H. Kimbrough contributed in conceptualization, investigation (evolution, antibiotic-adapted strain isolation and purification), and data analysis; Vaughn D. Craddock (whole genome sequencing, evolution, *gfp* reporter assays), and Brielle M. McKee (evolution, mutant construction) contributed in investigation; Nicole E. Smalley contributed in conceptualization and bioinformatics analysis; Ajai A. Dandekar contributed in funding acquisition, supervision, and conceptualization; Josephine R. Chandler contributed in funding acquisition, supervision, conceptualization, data analysis, and editing of the chapter.

Abstract

In the opportunistic pathogen *Pseudomonas aeruginosa,* the quorum-sensing regulator LasR is important for virulence and pathogenesis. LasR also contributes to aminoglycoside resistance. Paradoxically, *lasR* mutants are common in clinical samples from infections of patients often treated with antibiotics. It is unclear if these mutants are somehow selected by the infection environment or if they can act as social cheaters, which can exploit cooperator-produced public goods without contributing any costs. Another possibility is that the entire population, or the *lasR* mutants in particular, acquire mutations during adaptation that alter the susceptibility of the *lasR* mutants to antibiotics. To understand the influence of antibiotic adaptation on the LasR-I system, we previously generated and characterized *P. aeruginosa* populations passaged on tobramycin and showed that mutations accumulate in these populations that inactivate *ptsP* (phosphoenolpyruvate-protein phosphotransferase gene). Inactivating *ptsP* increases antibiotic resistance, quorum-sensing activity, pyocyanin production, and policing of *lasR* cheaters. Here we show that gentamicin triggers a different evolutionary trajectory. Gentamicin selection leads to accumulation of mutations that enhance LasR activity and cheater suppression through *ptsP*-dependent and independent mechanisms. Some of the gentamicin-adapted variants show enhanced antibiotic resistance when LasR is inactivated. In addition, *lasR* variants that emerge naturally in these populations also show enhanced antibiotic resistance. Both of these findings offer potential alternative explanations for the high frequency of *lasR* mutants in clinical environments. This work demonstrates how adaptive mutations can influence quorum sensing and its regulated functions and provides new insight into understanding quorum sensing evolution in infections.

Introduction

The LasR-I quorum sensing system of the opportunistic pathogen *Pseudomonas aeruginosa* senses and responds to the N-acyl homoserine lactone (AHL) signal, 3-oxododecanoyl-homoserine lactone (3OC12-HSL) to cause changes in gene expression based on population density (1-5). The LasR-I system is composed of the signal receptor LasR and the signal synthase LasI which synthesizes 3OC12-HSL (1, 5-9). Exoproducts such as proteases and toxins, which can be considered public goods, are regulated by the LasR-I system (3, 10, 11). Any member of the population can use the metabolically costly public goods whether they contribute to public good production or not. Those freeloaders that do not share the metabolic cost for public good production are called social cheaters (11-14). An example of a cheater are *lasR*-null mutants which do not produce quorum sensing-dependent public goods but can still benefit from public goods produced by quorum sensing cooperators in the population (12, 13).

In conditions where the public goods are required for growth, the *lasR* cheaters may overgrow the cooperators and cause a population collapse (3, 15, 16). However, proliferation of cheaters are regulated by mechanisms of cheater control (3, 10, 17, 18). One of the known mechanisms in *P. aeruginosa* is a punishment-based strategy called policing. In policing, the cooperators produce a toxin (*e.g.* pyocyanin, hydrogen cyanide, rhamnolipid) that restricts cheaters and a toxin-resistance factor that prevents auto-poisoning (15, 19, 20). Our group recently reported that policing of cheaters through pyocyanin can be enhanced by inactivation of *ptsP*, a gene coding for phosphoenolpyruvate-protein phosphotransferase (EI^{Ntr}) which is the first enzyme in the global regulatory system, nitrogen phosphotransferase system (PTS^{Ntr}) (21-25). Null mutations in *ptsP* attenuate biofilm formation and c-di-GMP production (24). Inactivation of *ptsP* also upregulates quorum sensing activity and pyocyanin production in *P. aeruginosa*

(22, 25). PtsP is also a mutation hotspot for *P. aeruginosa* during tobramycin adaptation *in vitro* (25-28).

Although *P. aeruginosa* has mechanisms in place to regulate cheaters, *lasR* mutants remain common in certain human infection environments including cystic fibrosis lung infections (29-36). The propagation of *lasR* mutants in infection environments was surprising because the importance of the LasR-I system in the virulence of *P. aeruginosa* is well established (37-41). There is also a mounting evidence on the importance of quorum sensing in antibiotic resistance in *P. aeruginosa* and other Proteobacteria (25, 42-48). In fact, the AHL-based quorum sensing systems also adapt to antibiotic selection as we have observed with the aminoglycoside tobramycin (25). The selection of these mutants in the clinical environments and their roles in infection are not well understood primarily due to the high diversity of clinical strains and complexity of infection environments, but there is already a growing body of research exploring why and how such *lasR* mutants emerge in clinical environments (30, 49-52).

In this study, we show that LasR is important for gentamicin resistance and gentamicin can suppress the emergence of cheaters in cooperating *P. aeruginosa* populations grown on casein, similar to our findings with tobramycin (25). Isolates from the antibiotic-exposed populations showed evidence of adaptation to antibiotics including mutations in *ptsP* and in *mexZ*, a gene coding for a MexXY efflux pump repressor (53, 54). We confirm that our gentamicin-evolved isolates have enhanced LasR-I activity and demonstrate delays in cheater emergence even when passaged in the absence of antibiotic in a PtsP-dependent and independent mechanisms. We also show that *lasR* mutants can acquire mutations in genes associated with antibiotic resistance that may allow survival in clinical environments rich in antibiotics. Overall, these results provide more evidence of how antibiotic selection can influence cooperative activity

and offer a potential alternative explanation for why *lasR* mutants are commonly observed in *P. aeruginosa* infections.

Results

LasR promotes gentamicin resistance in *P. aeruginosa* PA14 planktonic cultures

We have previously shown that LasR contributes to tobramycin resistance in planktonic conditions (25). We hypothesized that LasR also contributes to resistance to other aminoglycosides. We determined the minimum inhibitory concentration (MIC) of gentamicin against the laboratory strain PA14, or PA14 with a deletion of *lasR*. We observed about 1.5-fold decrease in the Δ*lasR* mutant MIC relative to PA14 (**Fig. 1**). This result shows that the LasR-I system also has a small contribution to gentamicin resistance in planktonically-grown *P. aeruginosa* strain PA14.

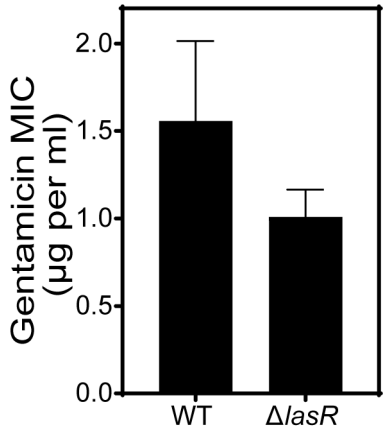

Fig. 1. *lasR* contributes to gentamicin resistance in planktonically-grown *P. aeruginosa* PA14. The minimum inhibitory concentration (MIC) of gentamicin was determined for each of the strains indicated as described in Materials and Methods. The values represent the average of two independent experiments, and the error bars represent the standard deviation.

Gentamicin suppresses emergence of *lasR* mutant cheaters in *P. aeruginosa* populations

The aminoglycoside tobramycin suppresses emergence of *lasR* mutant cheaters in *P. aeruginosa* populations passaged in casein (25). With the higher susceptibility of *lasR* mutants to gentamicin, we hypothesized that gentamicin also suppresses emergence of *lasR* mutant cheaters.

To test this hypothesis, we passaged *P. aeruginosa* daily in casein broth with gentamicin and enumerate the frequency of *lasR* cheaters. We identified the *lasR* mutants by phenotype as described in the Materials and Methods. We also verified a subset of the *lasR* mutants by sequencing the *lasR* gene. The *lasR* cheaters emerged by 5–8 days and increased in frequency (>99%) in all the lineages passaged in the absence of gentamicin as we have previously observed with tobramycin (**Fig. 2**) (25).

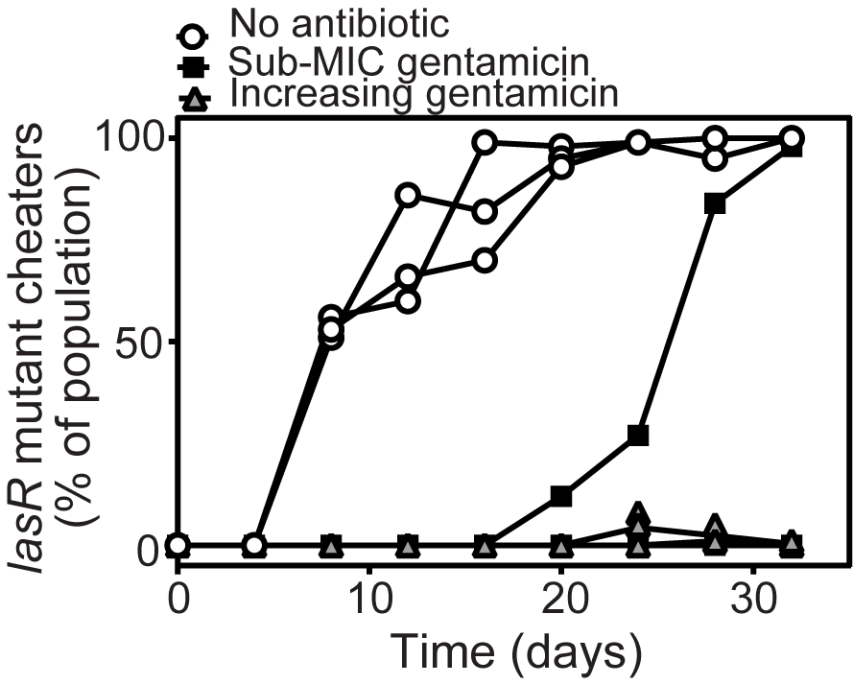

Fig. 2. Gentamicin suppresses cheating in casein-passaged *P. aeruginosa* populations. Cultures were transferred daily in 1% casein broth for 32 days, and cheater abundance was determined every 4 days by patching 100 colonies onto 4% skim milk agar. Populations passaged in antibiotics were exposed to either sub-MIC level of gentamicin (0.5 μg ml^{-1}) or an initial concentration of 0.5 μg ml^{-1} gentamicin with repeated 50% increases in gentamicin every 4 days (maximum of 5.7 μg ml^{-1}). A 100% cheater level means the wild type is below the detection level (1%). One out of three lineages exposed to sub-MIC gentamicin showed high frequency of *lasR* cheaters. Each line represents an independent lineage (three lineages for each antibiotic concentration).

In populations consistently passaged with 0.5 μg ml^{-1} gentamicin (sub-MIC gentamicin), we observed a delayed emergence of cheaters (17–24 days). Two of the population lineages showed a lower frequency of cheaters at the 30-day endpoint of the experiment (at most 8%). In the third lineage, the cheaters increased in frequency comparable to the lineages evolved in the absence of antibiotics (98%), although cheaters were also delayed in this population (17 days vs. 5–8 days). For a stronger selection, we also passaged *P. aeruginosa* populations in 0.5–5.7 μg ml^{-1} gentamicin (increasing gentamicin concentrations). In these populations, cheaters did not increase above 8%. Overall, our results showed cheater suppression by gentamicin.

Genetic adaptation occurred in *P. aeruginosa* passaged in gentamicin

We did not observe differences in the total population densities (**Fig. S1**), even at the highest gentamicin concentration, in our gentamicin-passaged *P. aeruginosa* populations so we predicted that genetic adaptations happened during passage. To test this hypothesis, we isolated one representative variant from each of the passaged populations (variants G1, G2, and G3 from the 0.5–5.7 μg ml^{-1} gentamicin-passaged populations and variants G4, G5, and G6 from the 0.5 μg ml^{-1} gentamicin-passaged populations), and determined the MIC. All variants except G5 and G6 showed a higher gentamicin MIC than the ancestor strain (**Fig. 3**). We also investigated the effect of adaptation on antibiotic resistance when *lasR* is inactivated in the gentamicin-adapted variants. We deleted *lasR* in each of the gentamicin-adapted strains, and determined the MIC. We did not observe a change in MICs in all variants when *lasR* is inactivated except for two variants (**Fig. 3**). Interestingly, inactivation of *lasR* increased gentamicin resistance in both G4 and G6 by about 2-fold (**Fig. 3**). These results showed that adaptations have occurred as a result of gentamicin selection.

To understand the genetic adaptations that occurred in gentamicin-adapted variants, we used whole genome and gene-targeted sequencing to identify the mutations that were acquired by each gentamicin-adapted variant (**Table 1**). We focused on the mutations that were observed in the gentamicin-adapted variants and absent both in the PA14 wild type ancestral strain and a variant from a population passaged in the absence of gentamicin.

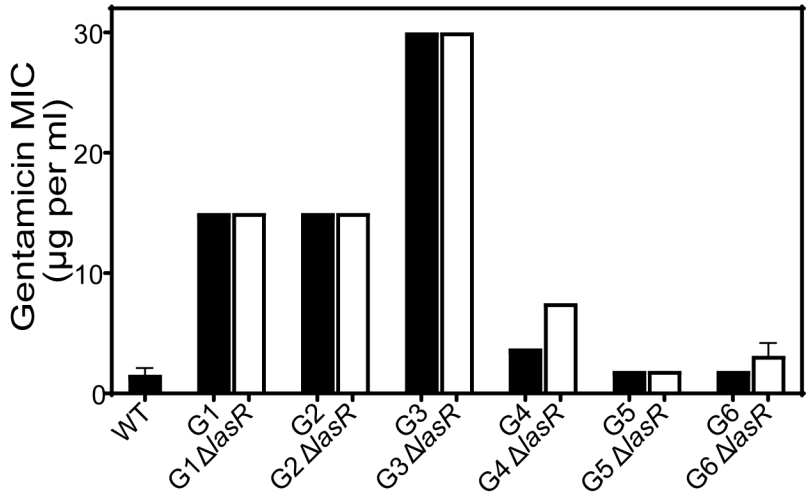

Fig. 3. Gentamicin susceptibility of *P. aeruginosa* mutants. The minimum inhibitory concentration (MIC) of gentamicin was determined as described in Materials and Methods. G1, G2, and G3 are isolates from populations passaged in 0.5–5.7 µg ml^{-1} gentamicin (increasing gentamicin concentrations) while G4, G5, and G6 are isolates from populations passaged in 0.5 µg ml^{-1} gentamicin. The values represent one independent experiment, and the error bars represent the standard deviation of the technical replicates.

All the gentamicin-adapted variants had mutations in *psdR*, which is coding for a transcriptional repressor that regulates genes involved in transport and processing of dipeptides (55). Mutations in *mexZ,* encoding a transcriptional repressor for MexXY efflux pump, were also common (53, 54). All the gentamicin-adapted variants from populations passaged in increasing gentamicin concentrations showed mutations in *ptsP* which is coding for phosphoenolpyruvate protein phosphotransferase (21). These three genes were also mutated among tobramycin-

adapted *P. aeruginosa* variants, but *psdR* and *mexZ* were not mutation hotspots in tobramycin-adapted variants (25). The other genetic mutations shared with some tobramycin-evolved strains were mutations in *fusA1* (translation elongation factor EF-G1A) and *pmrB* (sensor kinase of the two-component regulatory system PmrAB) (25). Both *mexZ* and *ptsP*-null mutations contribute to antibiotic resistance (25-28, 53, 56, 57). We confirmed the role of *mexZ* in increasing gentamicin resistance in PA14 (**Fig. S2**). We also tested whether inactivation of *psdR* could increase gentamicin resistance. Our results showed that null mutations in *psdR* do not contribute to gentamicin resistance, suggesting that *psdR* mutations are not selected for gentamicin adaptations.

Gentamicin-adapted variants have increased LasR activity and delayed emergence of cheaters

Inactivation of the gene *ptsP* increases LasR activity (25, 58). Since *ptsP* mutations were found in isolates G1, G2, and G3 (**Table 1**), we hypothesized that these evolved strains have increased LasR activity. We introduced pBS351 (P*lasI-gfp* reporter) in the gentamicin-adapted variants and compared GFP activity with that of wild type (**Fig. 4**). We observed a ~3-fold increase in GFP activity in strains G1, G2 and G3, like the previously reported results (25). We also observed a significant increase in LasR activity in isolate G5, even though this isolate lacked a *ptsP* mutation, suggesting that there are other genetic mutations that could result in increased LasR activity (**Fig. 4, Table 1**).

Table 1. Genetic mutations acquired by the gentamicin-evolved strains.

Gentamicin-passaged isolates[a]	Gene[b]			
	psdR	*mexZ*	*ptsP*	Others
G1	A491G	156CAAGAA	1317–1325$_{\Delta}$	*pmrA*, *pelG*
G2	A491G	A464G	T1502C	*pmrA*, *pelG*, putative transcriptional regulator
G3	435ΔC	535–545$_{\Delta}$	G1663T	*pmrB*, *pelG*
G4	146–156$_{\Delta}$	43–59$_{\Delta}$	ND	*phoQ, fusA1*
G5	G397A	ND[c]	ND	*lptA*, acyl-coA dehydrogenase, filamentous hemagglutinin N-terminal domain-containing protein
G6	G119A	A464G	ND	*phoQ*

[a]G1, G2, and G3 are variants from 0.5–5.7 μg ml^{-1} gentamicin-passaged populations; G4, G5, and G6 are variants from 0.5 μg ml^{-1} gentamicin-passaged populations; All were isolated at day 32 of the evolution experiments in **Fig. 2**.
[b]Gene mutations are given by nucleotide location
[c]ND: not detected

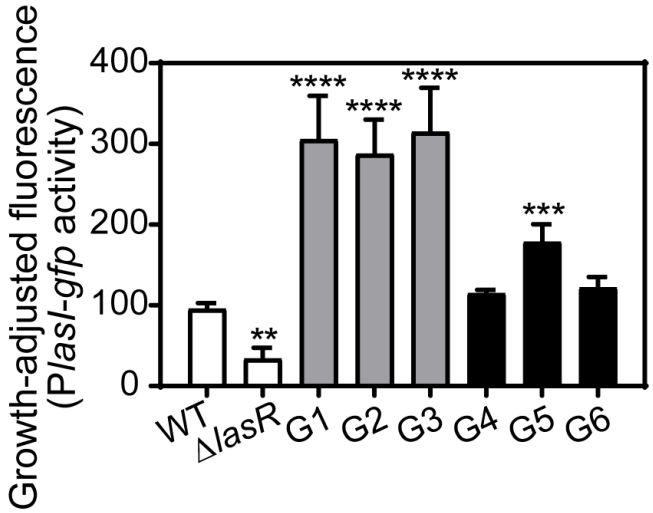

Fig. 4. Gentamicin-adapted *P. aeruginosa* variants have increased LasR activity. LasR activity was measured as GFP fluorescence normalized with OD_{600}. All strains/variants contained pBS351. The values represent the average of six independent experiments. The error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type ** $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

Inactivation of *ptsP* also delays the emergence of cheaters by increasing the production of the policing phenazine, pyocyanin (19, 25, 58). Hence, we hypothesized that cheaters might be similarly delayed in passaged populations of G1, G2, and G3. To test this hypothesis, we passaged these strains in casein broth for 32 days in the absence of antibiotic and monitored the emergence of *lasR* mutant cheaters (**Fig. 5A, S3A**). We observed that cheaters emerged later in G1, G2, and G3 populations compared with that of the PA14 population (13 to 15 days for G1, G2, and G3 vs. 6–7 days for PA14). However, unlike the lower total cheater frequency observed with passaged populations of the tobramycin-adapted isolates (25), the total cheater frequency in passaged G1, G2, and G3 populations reached the same levels as that of PA14 (85–97% for G1, G2, and G3 vs. >98% for PA14) **(Fig. S3A**). We also observed population collapse for G2 and G3, which was not observed for PA14 or the other passaged populations in these conditions. Collapse of G2 and G3 could not be explained by differences in growth yield early in the experiment, as the total cell density remained similar for all evolution experiments before the collapse was observed (**Fig. S3A, S3B**).

We also observed delayed cheating in variants G4, G5, and G6, which cannot be explained by *ptsP* inactivation, as these variants have an intact *ptsP* gene (**Fig. 5B, Table 1**). At least 8% *lasR* cheaters emerged in G4, G5, and G6 populations after 11–15 days of passaging in casein (**Fig. 5B, S3C**). The total cheater frequency (91–>98%) in G4, G5, and G6 populations were also high as observed in G1, G2, and G3 populations (**Fig. S3A, S3C**). The differences in total cell density for G4, G5, and G6 populations were not significantly different (**Fig. S3D**). Thus, variants G4, G5, and G6 showed a *ptsP*-independent delay in cheater emergence. We sought to determine what other mutations might delay cheating. All of these isolates have mutations in *psdR,* and variants G4 and G6 have mutations in *mexZ* (**Table 1**). Mutations in *psdR*

stabilize cooperation in *P. aeruginosa* PAO1 (59), and mutations in *mexZ* increase aminoglycoside resistance by increasing production of the MexXY efflux pump (53, 56, 60). We generated PA14 strains with either a Δ*psdR* or a Δ*mexZ* mutation and determined the cheater emergence of PA14 or each of the mutants passaged on casein using an evolution experiment. However, compared with the PA14 parent, we did not see a delay in cheating in passaged populations of the Δ*psdR* mutant (**Fig. 6A, S4A**) or the Δ*mexZ* mutant (**Fig. 6B, S4C**).

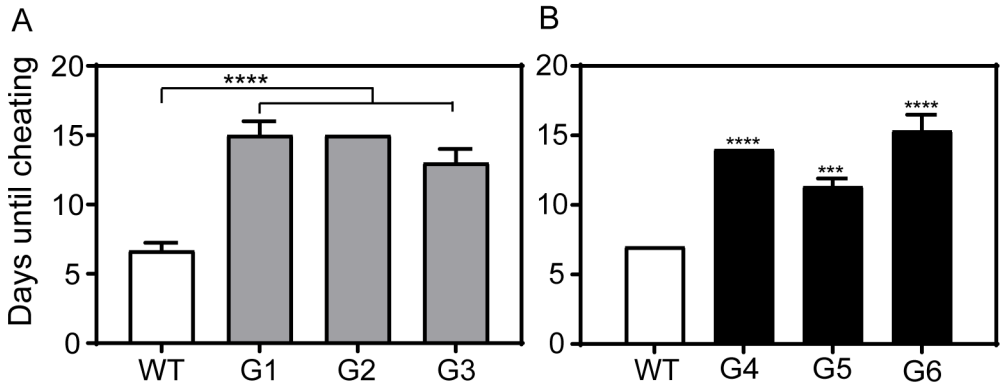

Fig. 5. Gentamicin-adapted *P. aeruginosa* variants have delayed cheating. **A.** Variants from 0.5–5.7 μg ml^{-1} gentamicin-passaged populations. **B.** Variants from 0.5 μg ml^{-1} gentamicin-passaged populations. Strains were passaged in 1% casein broth daily for 32 days, and cheater abundance was determined by patching 50 colonies onto 4% skim milk agar. Screening for cheaters was done daily until 8% cheaters was observed, then every 4 days after observing 8% cheaters in the population. Bars represent number of days it takes to observe 8% cheaters in the population. The values in A and B represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Dunnett's multiple comparisons test with wild type: *** $p<0.01$, **** $p<0.0001$.

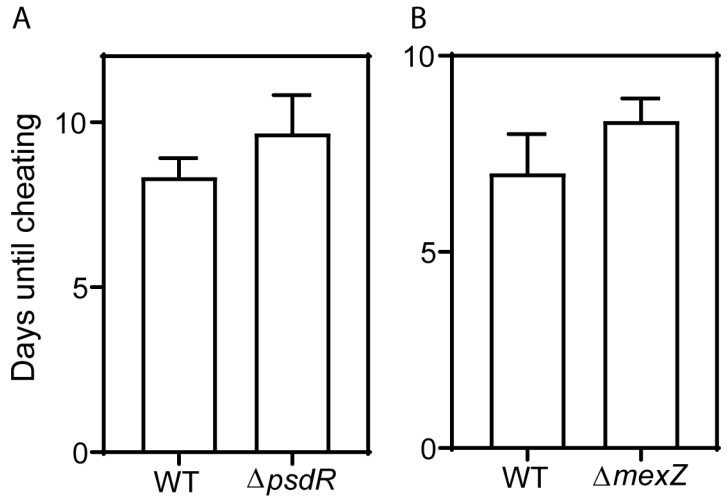

Fig. 6. *psdR* and *mexZ* mutations do not delay cheating. **A.** *psdR* mutant **B.** *mexZ* mutant. Method was as described in **Fig. 5**. Bars represent number of days it takes to observe 8% cheaters in the population. The values in A and B represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by t-test (p=0.05) showed no significant difference. **Fig. S4B** and **S4D** show the total population density in the evolution experiments.

The *lasR* cheaters can acquire genetic mutations conferring antibiotic resistance

In one of the lineages passaged in sub-MIC gentamicin (G5), the cheaters were able to increase in frequency similar to the lineages evolved in the absence of antibiotics (**Fig. 2**). We hypothesize the *lasR* mutants that emerge in this population acquired genetic mutations that conferred resistance, allowing them to break-free from suppression by gentamicin. To test this hypothesis, we isolated spontaneous *lasR* mutants (G5C1, G5C2, and G5C3) from G5 population and determine the MIC. We observed about 2-fold increase in MIC relative to the ancestral PA14 strain and less than 2-fold increase in MIC relative to the LasR-intact G5 variant (**Fig. 7**), suggesting that *lasR* mutants also undergo adaptation.

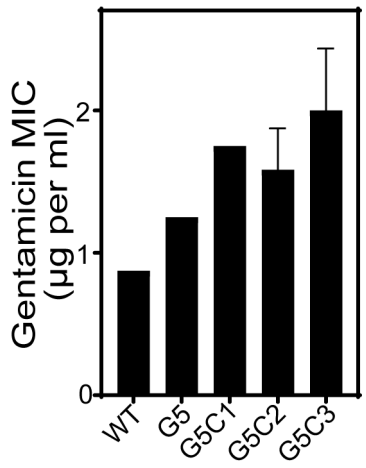

Fig. 7. Gentamicin susceptibility of spontaneous *lasR* mutants. The minimum inhibitory concentration (MIC) of gentamicin was determined as described in Materials and Methods. G1, G2, and G3 are isolates from populations passaged in 0.5–5.7 µg ml^{-1} gentamicin while G4, G5, and G6 are isolates from populations passaged in 0.5 µg ml^{-1} gentamicin. The values represent one independent experiment, and the error bars represent the standard deviation of the technical replicates.

We also use whole genome sequencing to identify the genetic mutations that the spontaneous *lasR* mutants have accumulated (**Table 2**). These *lasR* isolates had acquired mutations not observed in G5 isolate which may confer antibiotic resistance. All *lasR* mutants had a mutated CusA/CzcA family heavy metal efflux (HME) resistance nodulation division (RND) gene. This HME-RND efflux pump is predicted to be important for antimicrobial resistance in Gram-negative bacteria (61). G5C1 and G5C2 also had mutations in *mexZ* and *phoQ* (two-component sensor). Null mutations in both genes have been reported to increase aminoglycoside resistance (60, 62). G5C3 also had a mutation in *pmrB* (two-component regulator system signal sensor kinase), and mutations in *pmrB* are associated with aminoglycoside and polymyxin resistance development (63, 64). The only mutation shared by all these *lasR* mutants with G5 is a deletion in the 314–324 loci of the acyl-coA dehydrogenase gene (**Table 1, 2**). A mutation (T3720C) in the filamentous hemagglutinin N-terminal domain-containing protein is shared by all except for G5C2. The gene *psdR* is also mutated in G5, G5C1, and G5C2, but they all differ in terms of the mutated loci.

Table 2. Genetic mutations acquired by spontaneous *lasR* mutant cheaters isolated from G5 population.

Gene[a]	G5 spontaneous *lasR* mutant cheaters		
	G5C1	G5C2	G5C3
lasR	T599G	T599G	122–156$_{\Delta}$
CusA/CzcA family heavy metal efflux RND	963A	963A	963A
mexZ	340ΔG	340ΔG	ND
phoQ	T779G	T779G	ND
acyl-coA dehydrogenase	314–324$_{\Delta}$	314–324$_{\Delta}$	314–324$_{\Delta}$
psdR	G115A	G19A	ND
filamentous hemagglutinin N-terminal domain-containing protein	T3720C	ND	T3720C
others	ND	ND	*lptD*, *pmrB*, aminopeptidase P family protein

[a]Gene mutations are given by nucleotide location; These mutations were not confirmed by gene-targeted sequencing.
[b]ND: not detected

Discussion

Antibiotic selection has pleiotropic effects on bacteria that are not well understood (65). We recently reported some of these pleiotropic effects in *P. aeruginosa* (25). Adaptation to the antibiotic tobramycin resulted in a more active LasR-I quorum sensing system and a change in the dynamics of social cheating, resulting in a more stabilized cooperative behavior (25). In this study, we explore changes in quorum sensing of *P. aeruginosa* when adapting to the aminoglycoside gentamicin.

Previously, we demonstrated that LasR has a small role in tobramycin resistance and that tobramycin can suppress the emergence of cheaters (25). Our other work shows that in another Proteobacteria, *Chromobacterium subtsugae* (formerly *C. violaceum*), quorum sensing increases resistance to tetracycline and bactobolin (42), and that these antibiotics can suppress cheater emergence similar to tobramycin in *P. aeruginosa*. Here, we show that LasR is also important for gentamicin resistance and that gentamicin suppresses cheater emergence in *P. aeruginosa* (**Fig. 1, 2**). We also observed development of antibiotic resistance (**Fig. 3**), increased LasR activity (**Fig. 4**), and delayed cheater emergence (**Fig. 5, S3**), even in the absence of antibiotics, confirming our results with tobramycin (25).

Our sequencing results (**Table 1**) showed a different set of genetic mutations selected during gentamicin adaptation as compared to that of tobramycin adaptation (25). This result suggests there is a difference in the evolutionary trajectory toward resistance that is selected for by each antibiotic although they both belong to the aminoglycoside group. This difference is probably due to differences in the specificity of these antibiotics to different regions of the 16S rRNA A-site of the 30S ribosome (66, 67). However, a common genetic adaptation shared by gentamicin- and tobramycin-evolved strains are mutations in *ptsP* (**Table 1**, (25)). As we have

demonstrated previously and reported by other groups, inactivation of *ptsP* is an adaptation to tobramycin exposure and accounts for increased LasR activity, delayed cheating emergence through a pyocyanin policing mechanism, and small increases in antibiotic resistance (22, 25, 27, 28). Knowledge on the role of *ptsP* in *P. aeruginosa*, how it regulates QS systems, and its relevance in the clinical settings remains sparse.

Variant G5 showed increased LasR activity and delayed cheating emergence (**Fig. 4, 5B, S3C**) while G4 and G6 showed delayed cheating emergence despite having an intact *ptsP* gene (**Fig. 5B, S3C, Table 1**), suggesting that there are other genetic determinants for both phenotypes. We determined that either *psdR* or *mexZ* inactivation does not cause a significant delay in cheating emergence (**Fig. 6, S4**). Our results with *psdR* showed disparity with a previous report that inactivation of *psdR* results in a lower cheater load (59), although the previous study was in a different strain (PAO1), which could explain this disparity. We also did not further investigate the genetic basis of either the increased LasR activity in G5 nor delayed cheating in G4, G5, and G6 (**Fig. 4, 5B, S3C**). G5 carries a mutation in *lptA,* which in PAO1 increases transcription of *lasI* (68), providing a potential mechanism of increased LasR activity and/or delayed cheating in this variant. The other variants have a mutation in *phoQ*, which can upregulate pyocyanin biosynthesis (69), which can in turn suppress cheating by policing (25). PhoQ mutations are also clinically relevant because they are one of the common resistance-associated determinants in *P. aeruginosa* cystic fibrosis isolates (70).

Although there are a lot of limitations associated with investigating infection environments, several empirical evidences have been reported on what enables the *lasR* mutants to proliferate in host environments. Some of the factors that could be contributing to the selection of *lasR* mutants in the cystic fibrosis lung environment are a growth advantage on certain amino

acids, which could be serving as carbon and nitrogen sources in the cystic fibrosis lung, ability of clinical isolates to modify the quorum sensing hierarchy through acquiring genetic mutations, and potential to act as social cheaters (32, 37, 49, 52). Here, we show an additional contributing factor for the survival of *lasR* mutants in the clinical environments which is their ability to become antibiotic resistant by acquiring mutations in genes that confer antibiotic resistance (**Table 2**) or acquisition of genetic mutations that reverses the role of LasR in antibiotic resistance which is a form of epistatic interaction (**Fig. 3**) (71-74) . Investigating the role of each mutation in antibiotic resistance and the order of appearance of the genetic changes in the evolution of these *lasR*-mutated strains could contribute in a better understanding of the evolutionary pathway for *lasR* mutants in antibiotic-exposed environments.

Understanding the consequences of antibiotics on the sociality and quorum sensing system of pathogenic bacteria is important for a more thorough understanding of pathogenesis. Social cheaters may have impact on the development and success of *P. aeruginosa* infections as they can destroy cooperation eventually leading to reduced virulence (37, 51, 75). However, the ability of these cheaters to adapt should not be underrated. Quorum sensing inhibitors are also currently being explored as a potential alternative treatment against quorum sensing pathogens (10, 18, 44, 45). These alternative drug search efforts would definitely benefit from insights gained from the evolutionary trajectory of quorum sensing and the evolutionary dynamics of cooperator-cheater relationships.

Materials and Methods

Bacterial culture conditions and reagents

Bacteria were routinely grown in Luria-Bertani broth (LB) or in LB with 1.5% (wt per vol) Bacto-Agar (LBA). LB was buffered to pH 7 with 50 mM 3-(morpholino)-propanesulfonic acid (MOPS) for *P. aeruginosa* strains. All broth cultures were incubated at 37 ºC with shaking at 250 rpm for 18 h, unless specified otherwise. For 2 ml and 10 ml cultures, 18 mm test tubes and 125 ml baffled flask were used, respectively. Growth media for specific experiments include Pseudomonas Isolation Agar (PIA), 1% (wt per vol) sodium caseinate (casein broth) (25), 4% (wt per vol) skim milk agar (SMA) (13), and modified MOPS minimal medium (25, 76). Genomic DNA extraction, PCR product purification, and plasmid DNA extraction were done using a Puregene Core A kit, plasmid purification mini-prep kit, and PCR clean-up/gel extraction kits (Qiagen (Hilden, Germany) or IBI Scientific (IA, USA)), respectively, according to the manufacturer's protocol. Gentamicin and tetracycline were purchased from GoldBio (MO, USA) and Fisher Scientific (PA, USA), respectively.

Bacterial strains and strain construction

All bacterial strains and plasmids used in this study are listed in **Table 3–4** . *P. aeruginosa* strain UCBPP-PA14 ('PA14') and PA14 derivatives were used for this study. Markerless deletions in specific loci of PA14 were generated using allelic exchange as described previously (77). Briefly, for Δ*psdR*, DNA fragments were generated by DNA synthesis (Genscript, NJ, USA) containing ~500 bp DNA flanking the *psdR* gene and fused together creating an unmarked, non-polar deletion of the gene with incorporated restriction sites. The DNA fragments were ligated into pEXG2 and delivered into PA14 by mating. Deletion

constructs for Δ*lasR* (25) and Δ*mexZ* (78) were described elsewhere. *E. coli* containing pEXG2-Δ*psdR* or pEX18Tc-Δ*mexZ* was grown in 15 μg ml^{-1} gentamicin or 10 μg ml^{-1} tetracycline, respectively. Merodiploids were selected on PIA-gentamicin (200 μg ml^{-1}) for Δ*psdR* or PIA-tetracycline (100 μg ml^{-1}) for Δ*mexZ*. Deletion mutants were counterselected in NaCl-free 15% (wt per vol) sucrose. Putative mutants were verified by testing for antibiotic sensitivity, PCR-amplifying the deletion region, and sequencing the PCR product. Strains with pBS351 (30) were introduced into sucrose-electrocompetent cells as described previously (79) and selected on LBA-gentamicin (50 μg ml^{-1}).

Evolution experiments

Evolution experiments were carried out as described previously (25). Briefly, 40 μl from OD_{600} 3.5 culture was transferred to 2 ml fresh casein broth in an 18 mm tube. At 24 h intervals, cultures were diluted 1:40 into fresh casein broth in a new tube. In the case of antibiotic-exposed lineages, antibiotics were added every other day (42). CFU ml^{-1} for each lineage was determined by viable plate counts every 96 h. The % *lasR* mutant cheater (*lasR* cheater) was determined by patching 100 colonies, unless otherwise specified, on SMA. LasR cheaters form flattened colonies with iridescent, metallic sheen surface, and decreased skim milk proteolysis in SMA (13, 31, 32).

Strains that were the subject of sequence and phenotypic analyses were selected from evolution experiments described previously (25). Briefly, after the initial three dilutions (72 h), cultures were split into three lineages with continued passaging every 24 h but each with separate antibiotic treatment conditions. One of the lineages had no antibiotic as in the first three days. One of the other lineages was treated with gentamicin at a sub-MIC dose (0.5 μg ml^{-1}). The third

lineage was treated initially with gentamicin at 0.5 μg ml^{-1} and then the gentamicin concentration was increased by 50% every 4 days (0.5–5.7 μg ml^{-1}).

Table 3. *P. aeruginosa* and *E. coli* strains used in this study.

Strain	Relevant properties	Reference or source
P. aeruginosa		
UCBPP-PA14	Ancestral wild type	(80)
PA14 Δ*lasR*	PA14 with a deletion of *lasR*	(25)
PA14 Δ*mexZ*	PA14 with a deletion of *mexZ*	This study
PA14 Δ*psdR*	PA14 with a deletion of *psdR*	This study
Variants isolated after daily transfer for 32 days in 1% casein		
G1	Isolate from experiment with gentamicin added at 0.5–5.7 μg ml^{-1}	This study
G2	Isolate from experiment with gentamicin added at 0.5–5.7 μg ml^{-1}	This study
G3	Isolate from experiment with gentamicin added at 0.5–5.7 μg ml^{-1}	This study
G4	Isolate from experiment with gentamicin added at 0.5 μg ml^{-1}	This study
G5	Isolate from experiment with gentamicin added at 0.5 μg ml^{-1}	This study
G6	Isolate from experiment with gentamicin added at 0.5 μg ml^{-1}	This study
G1 Δ*lasR*	Isolate G1 with a deletion of *lasR*	This study
G2 Δ*lasR*	Isolate G2 with a deletion of *lasR*	This study
G3 Δ*lasR*	Isolate G3 with a deletion of *lasR*	This study
G4 Δ*lasR*	Isolate G4 with a deletion of *lasR*	This study
G5 Δ*lasR*	Isolate G5 with a deletion of *lasR*	This study
G6 Δ*lasR*	Isolate G6 with a deletion of *lasR*	This study

G5C1	Spontaneous *lasR* mutant isolated from G5 population	This study
G5C2	Spontaneous *lasR* mutant isolated from G5 population	This study
G5C3	Spontaneous *lasR* mutant isolated from G5 population	This study
Escherichia coli		
DH5α	F^- Φ80*lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17*(r_K^- m_K^+) *recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
S17-1	*recA pro hsdR RP4-2-Tc::Mu-km::Tn7*	(81)
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pi*r	(81)
Rho3	*thi-1 thr-1 leuB26 tonA21 lacY1 supE44 recA*, integrated RP4-2 Tcr::Mu (λ*pir* $^+$)Δ*asd*::FRT Δ*aphA*::FRT	(82)

Gentamicin susceptibility assay

Gentamicin susceptibility was determined by MIC according to the 2020 guidelines of the Clinical and Laboratory Standards Institute (CSLI), using a modified dilution method described previously (25). Briefly, gentamicin was added to MOPS minimal medium, successively diluted 2-fold to create a dilution series, and 200 µl of each dilution was dispensed in 2 ml tubes. *P. aeruginosa* inocula (OD_{600} of 4) from LB cultures were diluted 1:40 into each tube and the tubes were incubated with shaking for 20 h. After incubation, turbidity was measured using a Biotek Synergy 2 plate reader. The MIC was defined as the lowest concentration of gentamicin (µg ml^{-1}) in which bacterial growth was not measurable.

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Reference or source
pEXG2	Suicide vector, Gm^r	(83)
pEXG2-Δ*psdR*	pEXG2 containing Δ*psdR* with flanking sequences, Gm^r	This study
pPROBE-GT	Broad-host-range pVS1/p15a GFP reporter, Gm^r	(84)
pBS351	pPROBE-GT with -1 through-501 5' region of *lasI*, Gm^r	(30)
pEX18Tc	Suicide vector, Tc^r	(85)
pYM021	pEX18T containing Δ*mexZ* with flanking sequences, Tc^r	(78)

Whole genome sequencing

Genomic DNA was extracted using the Qiagen Puregene yeast/bacteria kit and a sequencing library was constructed with 200-bp inserts (G1, G2, G3, G4, G5, and G6). Sequencing was performed using Illumina MiSeq with ~25X coverage. For G5C1, G5C2, and G5C3, sequencing library was constructed with 350-bp inserts and sequencing was performed using Illumina HiSeq 4000. The raw reads were aligned to the *P. aeruginosa* UCBPP-PA14 reference genome (UCBPP-PA14 Accession NC_008463) using Strand NGS (Bangalore, India) software v 3.1.1, using a pipeline described previously (86). Mutations of interest were verified by gene-targeted Sanger sequencing.

Transcription reporter assay

Strains with pBS351 were grown in 2 ml LB-MOPS- gentamicin (15 μg ml^{-1}). Cultures were centrifuged, cell pellets were resuspended in PBS, and diluted 1:10. 200 μl of the diluted

sample was aliquoted into clear-bottomed, black-walled, 96-well plates, where fluorescence and OD_{600} were measured using a BioTek Synergy 2 plate reader. Fluorescence was normalized to OD_{600}.

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Supplementary Information

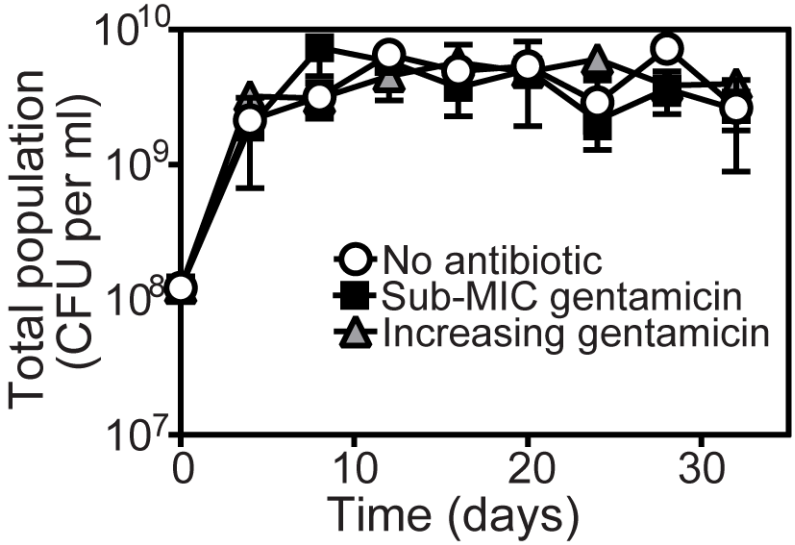

Fig. S1. Total population count of *P. aeruginosa* passaged in casein with different concentrations of antibiotics. Cultures were transferred daily, and population count was determined every 4 days by viable plate counts. Populations passaged in sub-MIC gentamicin was exposed to 0.5 μg ml^{-1} while populations passaged in increasing gentamicin was exposed to an initial concentration of 0.5 μg ml^{-1} with repeated 50% increases in gentamicin every 4 days (maximum of 5.7 μg ml^{-1}). The values represent the average of three independent experiments and the error bars represent the standard deviation.

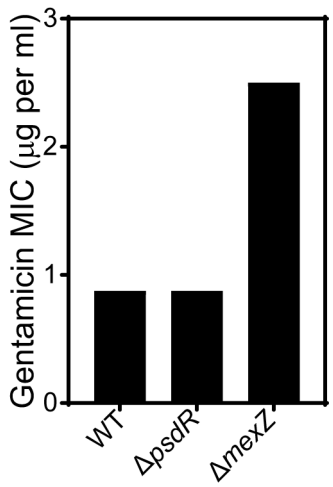

Fig. S2. Gentamicin susceptibility of *psdR* and *mexZ* mutants. The minimum inhibitory concentration (MIC) of gentamicin was determined as described in Materials and Methods. The values represent one independent experiment with three technical replicates.

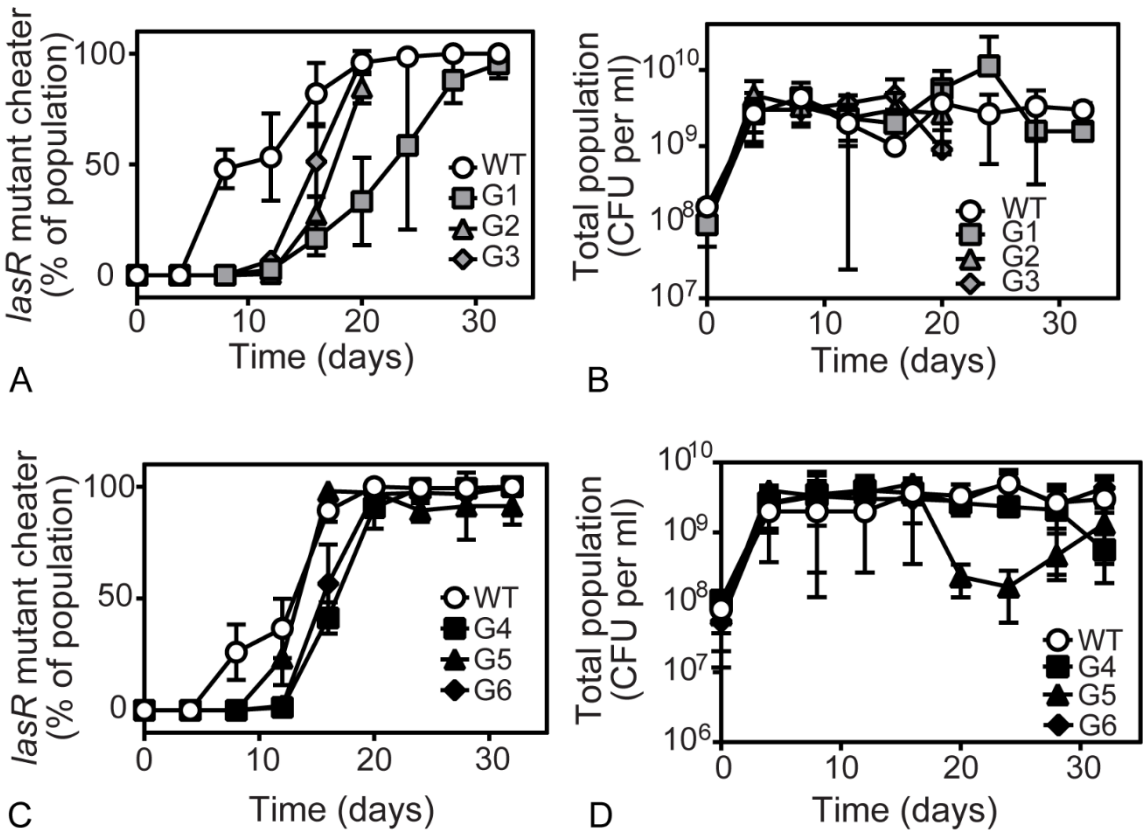

Fig. S3. Percent *lasR* mutant cheaters and total population in *P. aeruginosa* strains passaged in casein daily for 32 days. **A.** *lasR* mutant cheaters in the evolution experiment for isolates from populations passaged in increasing gentamicin. **B.** Total population in the evolution experiment for isolates from populations passaged in increasing gentamicin. **C.** *lasR* mutant cheaters in the evolution experiment for isolates from populations passaged in sub-MIC gentamicin. **D.** Total population in the evolution experiment for isolates from populations passaged in sub-MIC gentamicin. Cheaters were enumerated by patching 50 colonies in 4% skim milk agar while total population was determined by viable plate counts. A 100% cheater level means the wild type cells are below the detection level (2%). Experiments were terminated when population collapsed was observed (G2 and G3). The values for all graphs represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons test for S3D showed no significant differences at p=0.05.

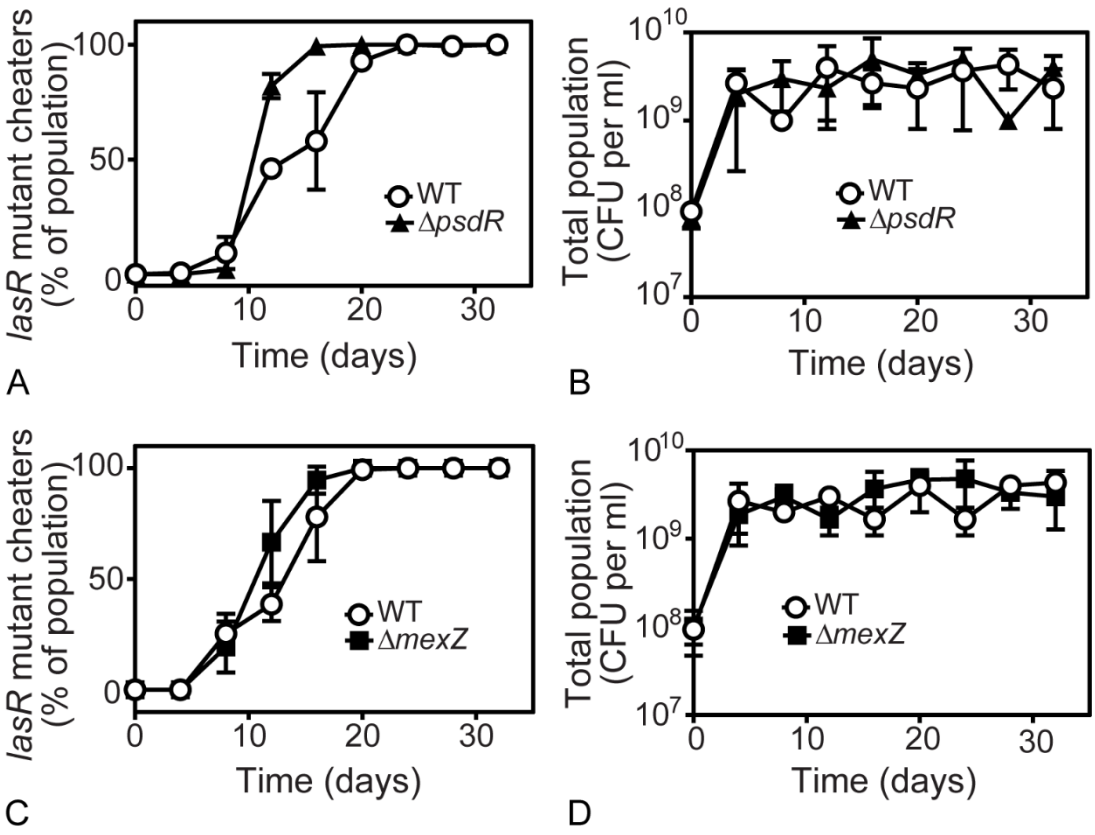

Fig. S4. Percent *lasR* mutant cheaters and total population in *psdR* and *mexZ* mutants passaged in casein daily for 32 days. **A.** *lasR* mutant cheaters in the evolution experiment for *psdR* mutant. **B.** Total population in the evolution experiment for *psdR* mutant. **C.** *lasR* mutant cheaters in the evolution experiment for *mexZ* mutant. **D.** Total population in the evolution experiment for *mexZ* mutant. Methods are the same as in **Fig. S3**. One replicate for *psdR* evolution was terminated early because of population collapse. The values for all graphs represent the average of three independent experiments and the error bars represent the standard deviation.

CHAPTER V: Tobramycin adaptations destabilize cooperation in *Pseudomonas aeruginosa*

Workload allocations:

Rhea G. Abisado contributed in conceptualization, investigation (coculture, evolution, mutant construction, protease assay), data analysis, supervision, and writing of the chapter; Brielle M. McKee (coculture, evolution, mutant construction), and Kade A. Townsend (coculture, mutant construction) contributed in investigation; Josephine R. Chandler contributed in funding acquisition, conceptualization, supervision, data analysis, and editing of the chapter.

Abstract

The LasR-I quorum-sensing system mediates cooperation in the opportunistic pathogen *Pseudomonas aeruginosa*. It regulates production of costly extracellular public goods, such as elastase, which are prone to exploitation by freeloading social cheaters. A commonly observed cheater in conditions requiring quorum sensing for growth are the *lasR*-null mutants. The proliferation of the LasR cheaters may drive the population to a collapse if the public good is an essential growth factor and the cooperators or public good production becomes insufficient to support the entire population. We recently reported a tobramycin adaptation that stabilizes cooperation in *P. aeruginosa* through a quorum sensing-controlled policing toxin pyocyanin, which is enhanced by PtsP inactivation. Mutations in the gene *ptsP,* coding for the first enzyme in the nitrogen phosphotransferase (PTS^{Ntr}) system, are common adaptation to tobramycin. Here we show that *ptsP* inactivation triggers a population collapse despite its suppressing effect on the emergence of *lasR* cheaters. We also show that tobramycin adaptations, *mdpA*- and *ygdP*-null mutations can also trigger a population collapse. The gene *mdpA* codes for a metallopeptidase while *ygdP* codes for dinucleoside polyphosphate hydrolase. The mechanistic pathway by which *ptsP*, *mdpA*, or *ygdP* induces population collapse still needs to be investigated. Identifying the factors contributing to collapse of cooperating populations will provide valuable insights for efforts to find alternative treatment strategies for *P. aeruginosa* infections.

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is a well-studied model organism for understanding quorum sensing, a type of bacterial communication that regulates gene expression in a population density-dependent manner (1-4). *P. aeruginosa* has hierarchically organized, overlapping, multifaceted quorum-sensing networks (2, 5). On top of the hierarchy, is the LasR-I system, which produces and responds to the signal 3-oxododecanoyl-homoserine lactone (3OC12-HSL) (2, 6, 7). In this system, LasR is the signal receptor while LasI is the signal synthase (1, 6, 8). One of the quorum sensing-regulated social behaviors in *P. aeruginosa* is public good cooperation (3, 9-12).

Public goods are extracellular products which can be used freely by any member of the local bacterial population (9, 10, 13). Because public good synthesis is metabolically costly, the freeloaders that do not pay any cost in producing the public goods are considered social cheaters (9-11, 14, 15). In conditions where quorum sensing is required for growth, quorum sensing-defective social cheaters with null mutations in LasR spontaneously emerge (11, 14, 16). LasR mutants are also frequently isolated in environmental and clinical samples (3, 17-19), although it is unclear if they are cheaters in this context or emerge for some other reasons. The cheaters may have growth advantage over the cooperators because of their lower metabolic cost and may outnumber the cooperators. When the cooperators and public good production become insufficient to support the growth of the entire population, collapse may occur (10, 20-22). Collapsing populations may start with a population decline and crash over time. Both population collapse and population decline could lead to extinction (23, 24). In this study, we define population collapse as having a population count less than 10^9 CFU ml-1.

Given the challenge of social cheating, how cooperation is sustained in nature continues to puzzle evolutionary biologists (25). In bacterial systems, there are mechanisms in place to control cheating (3, 12, 26). Some of the known mechanisms are policing, pleiotropy, and metabolic prudence (16, 22, 27-31). We recently reported that cheating control mechanisms in *P. aeruginosa* can be enhanced by tobramycin adaptation (16). Inactivation of *ptsP,* coding for the first enzyme in the nitrogen phosphotransferase (PTS^{Ntr}) system, enhances restriction of cheaters in *P. aeruginosa* through upregulation of the policing toxin, pyocyanin (16). PtsP inactivation also confers low-level antibiotic resistance, increases LasR activity and pyocyanin production as well as attenuates biofilm formation, c-di-GMP production, and pathogenesis in *P. aeruginosa* (16, 32-37).

Interestingly, *ptsP* inactivation can trigger a population collapse, despite its role in suppression of *lasR* cheaters. Collapse in Δ*ptsP* population occurs only when *lasR* cheater increased to a high frequency (>80%) before 32 days. At the same or even higher cheater frequency, the wild type PA14 population does not collapse. We also identified two more tobramycin adaptations (16) that can trigger population collapse, null mutations in *mdpA* and *ygdP*. The gene *mdpA* codes for metallopeptidase while *ygdP* codes for dinucleoside polyphosphate hydrolase. The mechanistic pathway by which *ptsP*, *mdpA*, or *ygdP* induces population collapse remains unknown. Identifying the factors contributing to collapse of cooperating populations will provide valuable insights in developing alternative treatment strategies for *P. aeruginosa*.

Results

Occasional population collapse occurs in Δ*ptsP* mutant populations passaged on casein

We recently reported that inactivation of *ptsP* delays cheating in *P. aeruginosa* (16). We passaged the Δ*ptsP* mutant or the wild-type PA14 (WT) in 1% casein broth for 32 days and monitored cheater frequency over time. We observed the *lasR* cheaters emerged at about 15 days vs. 6–8 days for the wild type (16). In some cases (4/15), the cheaters eventually reached high levels in the *ptsP* mutant population (>80%) (**Table S1**). In all the lineages where there were >80% cheaters before 32 days, the cultures ceased production of the blue-green pyocyanin and eventually become colorless similar to uninoculated casein broth (**Fig. S1A**), suggesting the culture was no longer supporting growth and a population collapse has occurred. Prior to population collapse, the cell density between growing and collapsed cultures were similar (**Table S2**). The collapse was not observed in any of the PA14 wild-type populations at least in the 32 days of passaging in casein broth, similar to that of previous studies (**Table S1**) (11, 14, 16). In fact, no collapse of the PA14 populations was observed even when the *lasR* cheater load was very high (>98%), and we could observe >98% *lasR* cheaters as early as 8 days of passaging the wild type in casein broth (16) (**Table S1**). Our evolution experiments suggest that Δ*ptsP* population can collapse occasionally.

To determine if Δ*ptsP* has growth defects in casein that could possibly explain the population collapse, we monitored growth of Δ*ptsP* as a monoculture in casein broth. We did not observe a difference in the population counts between the wild type and Δ*ptsP* in the 7 days of passaging in casein broth (**Fig. S1B**). We also previously reported that Δ*ptsP* has similar doubling time as the wild-type PA14 (16). Growth in casein as a sole source of carbon and energy requires production of exoprotease, a public good supplied by the cooperator (14). Thus,

we determined whether Δ*ptsP* has defects in protease production that may have caused the population collapse (**Fig. S1C**). However, we did not observe significant differences in protease production between Δ*ptsP* and the wild type, suggesting that the population collapse observed in Δ*ptsP* population is not due to a defect in protease production. Overall, these experiments showed that the population collapse in Δ*ptsP* populations passaged in casein is not due to growth defects of Δ*ptsP* in casein.

Δ*ptsP* Δ*lasR* mutant triggers the population collapse

We used coculture experiments to determine whether the Δ*ptsP* cooperator or the Δ*ptsP* Δ*lasR* cheaters are the cause of population collapse. As an initial experiment to determine conditions where collapse is observed in coculture, we mixed the Δ*ptsP* cooperator with either Δ*lasR* or Δ*ptsP* Δ*lasR* at different starting ratios (1:1, 9:1, 99:1 cooperator:cheater), transferred to fresh 1% casein every day and monitored the total population density in the initial culture and at 3 days. With the exception of the coculture started at a 1:1 ratio of Δ*ptsP:*Δ*ptsP* Δ*lasR*, the coculture densities increased from ~10^8 initially to ~3.4x10^9 at 3 days. However, the Δ*ptsP:*Δ*ptsP* Δ*lasR* (1:1) coculture stayed near ~10^8 at 3 days, suggesting this coculture may have collapsed (**Fig. S2C, S2D**).

To examine this pattern more closely, we repeated the experiment using this ratio of 1:1 (cooperator:cheater) and either wild type or Δ*ptsP* as a cooperator and Δ*lasR* or Δ*ptsP* Δ*lasR* as the cheater, and determined the population density after each daily transfer for 7 days. In both of the cocultures with Δ*lasR* as the cheater, the total population counts increased from 10^8 initially to about 1.4x10^9 CFU ml^{-1} after 24 h and remained stable throughout the 7 days of passaging in casein broth (**Fig. 1**). In identical coculture experiments with the Δ*ptsP* Δ*lasR* cheater, the total

population increased to about $1.6x10^9$ CFU ml^{-1} by 3 days but then decreased by ~10-fold after that, suggesting evidence of collapse. From days 5 to 7, we observed that the total population counts were still slightly higher than the starting inoculum, suggesting that either the cocultures did not completely collapse in at least 7 days that we passaged the cocultures or there could be readily available nutrients in the casein broth that sustain the cocultures at that population count.

There were no significant differences in the total Δ*ptsP* Δ*lasR* cheater counts in the cocultures that could possibly explain the population collapse (**Fig. S2A**). As a control, we monitored the spontaneous emergence of *lasR*-null cheaters in a Δ*ptsP* population transferred daily for 7 days, and there were no cheaters for all 7 days (**Fig. S2B**), consistent with the conclusion that naturally emerging cheaters did not emerge in the time-frame of our coculture experiments similar to previous studies (16). These results suggest Δ*ptsP* population collapse is due to the emergence of Δ*ptsP* Δ*lasR* cheaters and not due to the Δ*ptsP* cooperator.

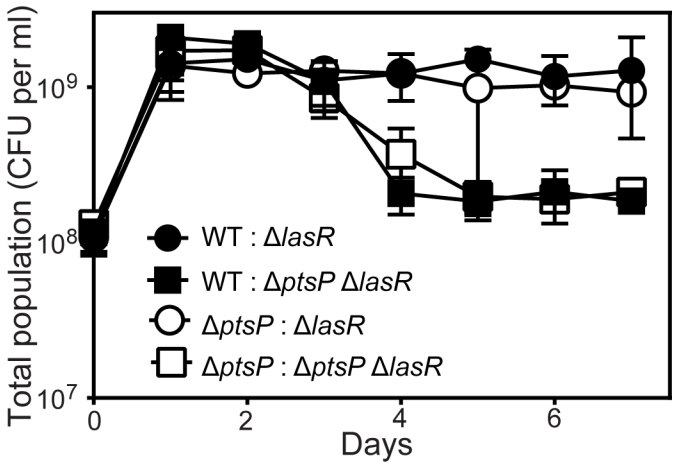

Fig. 1. Population collapse in coculture experiments. Cultures were grown to OD 5 and mixed together at 1:1 (cooperator:cheater) ratio. The cocultures were passaged in 1% casein broth daily for 7 days. Total population was determined daily by viable plate counts. The values represent the average of three independent experiments. The error bars represent the standard deviation.

We hypothesized that inactivating Δ*ptsP* in a Δ*lasR* background could result in a growth advantage in casein, which could explain the collapse. However, Δ*lasR* and Δ*ptsP* Δ*lasR* both had similarly poor growth in pure culture casein passaging experiments suggesting that Δ*ptsP* Δ*lasR* did not have any growth advantage in casein broth (**Fig. S1B**). Although Δ*ptsP* Δ*lasR* monoculture did not have growth advantage in casein, it can compete with either the wild type or Δ*ptsP* better than a Δ*lasR* mutant (**Fig. S2E**). Altogether, these results suggest that it is the Δ*ptsP* Δ*lasR* mutant that is triggering population collapse.

Δ*ptsP* Δ*lasR*-dependent population collapse is not due to pyocyanin, hydrogen cyanide, or pseudomonas quinolone signal (PQS)

Inactivation of *ptsP* enhances production of the phenazine, pyocyanin (16, 38). We also observed that the Δ*ptsP* Δ*lasR* has elevated pyocyanin production relative to the wild type or the Δ*lasR* mutant (**Fig. S3**). Thus, we hypothesized that Δ*ptsP* Δ*lasR* is causing collapse by contributing to production of pyocyanin and phenazine intermediates, killing both itself and the cooperator. Pyocyanin is produced from the *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*) gene clusters and the *phzM* and *phzS* genes. The products of *phz1* and *phz2* convert the precursor chorismate to phenazine-1-carboxylic acid (PCA); PhzM converts PCA to 5-Methyl-PCA; PhzS converts 5-Methyl-PCA to pyocyanin; while PhzH is involved in the production of phenazine-1-carboxamide (PCN) from PCA (39-41). We deleted the genes coding for pyocyanin and phenazine intermediates in the Δ*ptsP* Δ*lasR* double mutant and determine the effect of deletion in the population collapse through coculture experiments. Our results showed that deleting the genes involved in the production of these toxins did not prevent population collapse (**Table S3**).

We also tested other quorum sensing-regulated toxins such as Pseudomonas quinolone signal (PQS) and hydrogen cyanide (HCN). PQS production involves the genes *pqsA*, *pqsH*, and

pqsR. PqsR is the transcriptional regulator for the genes involved in PQS synthesis (*pqsA–D* and *phnAB*). The products of *pqsA–D* and *phnAB* are involved in the production of 4-hydroxy-2-heptylquinoline (HHQ), which is the precursor for PQS production; PqsH catalyzes the conversion of HHQ into PQS (42). Our results also showed that deleting the genes (*pqsA*, *pqsH*, *pqsR* for PQS and *hcnC* for HCN) involved in the production of these toxins did not prevent population collapse (**Table S3**). Overall, these results suggest that pyocyanin, phenazine intermediates, hydrogen cyanide, or PQS in Δ*ptsP* Δ*lasR* was not causing the population collapse.

Population collapse also occurs in Δ*ygdP* and Δ*mdpA* mutant populations passaged on casein

PstP mutations are adaptive mutations for tobramycin (16, 33, 35, 36). To determine whether there are other tobramycin adaptive mutations that cause population collapse, we screened genetic mutations identified from tobramycin-adapted variants (16). Two of the mutations we have identified in the tobramycin-adapted variants were promoter mutations in *ygdP* and point mutations in *mdpA* (16). The gene *ygdP* codes for dinucleoside polyphosphate hydrolase while *mdpA* codes for a metallopeptidase. We deleted either *ygdP* or *mdpA* in PA14 and passaged Δ*ygdP or* Δ*mdpA* mutant or the wild type in 1% casein broth for 32 days and monitored cheater frequency and total populations over time. We observed the *lasR* cheaters emerged at about 7 and 11 days in Δ*ygdP* and Δ*mdpA* populations, respectively vs. 8 days for the wild type (**Fig. 2**). The Δ*ygdP* and Δ*mdpA* populations collapsed after 20 and 16 days of passaging in 1% casein broth, respectively (**Fig. 2, S4**). These results showed that *mdpA* and *ygdP* inactivation can also trigger population collapse.

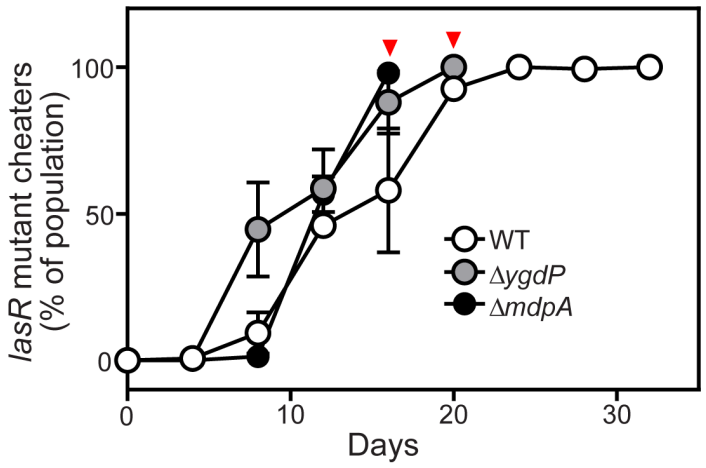

Fig. 2. Inactivation of *ygdP* and *mdpA* triggers population collapse. Collapsed cultures are marked by red inverted triangles. *P. aeruginosa* populations were transferred daily in 1% casein broth for 32 days, and cheaters were enumerated every 4 days as described in Materials and Methods. Values represent the average of three independent experiments and the error bars represent the standard deviation.

Inactivation of *mdpA* increases tobramycin resistance and *lasR* activity in casein

To determine whether *mdpA* mutations were selected for adaptation to tobramycin, we determined the effect of this mutation in tobramycin resistance. We determined the minimum inhibitory concentration (MIC) to tobramycin of the *mdpA*-null mutation and the point mutation (*mdpA* A646T) that we identified from a previously characterized tobramycin adapted variant, T1 (16). *mdpA* A646T is also predicted to be a loss-of-function mutation. We found that null-mutations in *mdpA* result in a 2-fold increase in tobramycin resistance of *P. aeruginosa* (**Fig. S5A**), suggesting that *mdpA* mutations are adaptations to tobramycin just like *ptsP* mutations (16, 33, 35, 36). Deletion of *lasR* in *mdpA* A646T mutant resulted in a 2.8-fold decrease in tobramycin resistance, suggesting that this mutation increases antibiotic resistance in a LasR-dependent manner (**Fig. S5A**).

We also determined whether *mdpA* inactivation has effect on LasR activity. We used a *lasR*-responsive P*lasI-gfp* plasmid reporter to measure the LasR activity (43). Our results showed that inactivation of *mdpA* also increases LasR activity in casein, and this could be contributing

both to the LasR-dependent increase in tobramycin resistance and population collapse (**Fig. S5B**).

Discussion

Microbial systems display the tell-tale signs of sociality (44). Individuals in a microbial population also have the natural tendency to cheat because freeloading is favorable at the individual level (3, 9-11, 14, 15, 20). When cheating becomes too much of a burden, a population may undergo collapse. Here, we show that population collapse could be triggered by adaptive mutations acquired as a result of tobramycin selection. We identified three genes which cause population collapse when inactivated **(Fig. 1, 2)**. The genes *ptsP*, *ygdP*, and *mdpA* are tobramycin-adapted mutations identified from a laboratory evolution experiment (16). PtsP inactivation is quite interesting because this genetic change also has a role in suppressing *lasR* mutant cheaters (16). Thus, population collapse is triggered even in populations where *lasR* cheaters are suppressed.

The mechanistic pathway by which *ptsP*-, *ygdP*-, and *mdpA*-null mutations triggers a population collapse remains unknown. It is possible that PtsP inactivation leads to dysregulation of toxin production that results in killing of both cooperators and cheaters. Since it is playing a role in the PTS^{Ntr} system, inactivation of *ptsP* may also result in enhanced competition between the cooperators and cheaters, leading to population collapse. The gene *ygdP*, also known as *rppH*, is playing a role in RNA decay (45). It is predicted to form an operon with *ptsP*, but the connection between these two genes is unknown. YgdP could also be regulated by quorum sensing because it is downregulated in a PqsR mutant in a transcriptomics study (42). How a *ygdP* inactivation could trigger is unclear. Inactivation of *mdpA* may be causing starvation of both cheaters and cooperators as *mdpA* is playing a role in catabolism of dipeptides.

Social traits, which are highly influenced by evolutionary forces, include virulence factors and exoproducts in *P. aeruginosa* (10, 46-48). Thus, social dynamics of this pathogen

may also impact pathogenesis. The cheater's ability to invade *P. aeruginosa* populations could be exploited in finding new strategies to treat *P. aeruginosa* infections. Evolution favors genes for cheating, which may lead to a decline in population fitness, and in turn may result in a higher susceptibility to the host immune response or antibiotic therapy (15, 49-53). For example, mutations that cause cheaters to accelerate population collapse could be used as a Trojan horse to destabilize fitness of the bacterial population. In the Trojan horse concept, the cheaters could also be used to introduce other gene products that are detrimental to the target bacterial population (49).

Previously, we showed how pleiotropic effects of *ptsP* inactivation caused suppression of cheaters by policing. The *ptsP* mutations were initially selected by tobramycin due to their role in increasing tobramycin resistance. Here, we show that inactivation of *ptsP,* and also mutations in *ygdP* and *mdpA,* can trigger population collapse. Thus, the results of this study illustrate another example of pleiotropic effects of antibiotic selection on bacterial sociality. In particular, these results show how antibiotic selection can lead to heritable changes in cooperator-cheater dynamics. These studies may give insight in addressing concerns related to medical, agriculture, and industry-related challenges.

Materials and Methods

Culture conditions and reagents

Bacteria were routinely grown in Luria-Bertani broth (LB), 50 mM 3-(morpholino)-propanesulfonic acid (MOPS)-buffered LB (pH 7), or on LB agar (LBA) with 1.5% weight per volume Bacto-Agar. For specific experiments, M9-caseinate (16), MOPS minimal medium (16, 54), Pyocyanin Producing Media (PPM) (55), and 4% skim milk agar (SMA) were used (11). All *P. aeruginosa* broth cultures were grown at 37°C with shaking at 250 rpm using 18 mm test tubes (for 2 ml cultures) or 125 ml baffled flasks (10 ml cultures). For *E. coli*, 100 μg ml^{-1} carbenicillin and 15 μg ml^{-1} gentamicin were used while 300 μg ml^{-1} carbenicillin and 200 μg ml^{-1} gentamicin were used for *P. aeruginosa*. Genomic or plasmid DNA was extracted using Qiagen Puregene Core A kit (Hilden, Germany) or IBI Scientific plasmid purification mini-prep kit (IA, USA) while PCR products were purified using IBI Scientific PCR clean-up/gel extraction kits, according to the manufacturer's protocol. All antibiotics were purchased from GoldBio (MO, USA).

Bacterial strains and strain construction

All bacterial strains and plasmids used in this study are listed in **Tables 1–2**. *P. aeruginosa* strain UCBPP-PA14 ('PA14') and PA14 derivatives were used for these studies. Markerless deletions in specific loci of *P. aeruginosa* PA14 were generated using allelic exchange as described previously (56). To generate plasmids for allelic exchange, DNA fragments with the mutated or deleted gene plus 500 bp flanking DNA (Δ*mdpA*, *mdpA* A646T, Δ*ygdP,* Δ*pqsA*, Δ*pqsH*, Δ*pqsR*) were generated by DNA synthesis (Genscript, NJ, USA), creating an unmarked, non-polar deletion of the gene with incorporated restriction sites. The

DNA fragment was digested and ligated to pEXG2 and transformed into the appropriate *P. aeruginosa* strain. The plasmids for Δ*lasR* (16), Δ*hcnC* (22), and Δ*ptsP* (34) were described elsewhere. Merodiploids were selected on Pseudomonas Isolation Agar (PIA)-carbenicillin (300 μg ml^{-1}) for Δ*lasR* or PIA-gentamicin (200 μg ml^{-1}) for Δ*mdpA*, *mdpA* A646T, Δ*ygdP*, Δ*pqsA*, Δ*pqsH*, Δ*pqsR*, Δ*hcnC*, and Δ*ptsP*. Deletion mutants were counterselected using NaCl-free 15% sucrose. Putative mutants were verified through antibiotic sensitivity tests and gene-targeted Sanger sequencing.

Table 1. Bacterial strains used in this study.

Strain	Relevant properties	Reference or source
P. aeruginosa		
UCBPP-PA14	Ancestral wild type	(57)
PA14 Δ*lasR*	PA14 with a deletion of *lasR*	(16)
PA14 Δ*ptsP*	PA14 with a deletion of *ptsP*	(16)
PA14 Δ*ptsP* Δ*lasR*	PA14 Δ*ptsP* with a deletion of *lasR*	(16)
PA14 Δ*ygdP*	PA14 with a deletion of *ygdP*	This study
PA14 Δ*ygdP* Δ*lasR*	PA14 Δ*ygdP* with a deletion of *lasR*	This study
PA14 Δ*mdpA*	PA14 with a deletion of *mdpA*	This study
PA14 Δ*mdpA* Δ*lasR*	PA14 Δ*mdpA* with a deletion of *lasR*	This study
PA14 *mdpA* A646T	PA14 with *mdpA* A646T mutation	This study
PA14 *mdpA* A646T Δ*lasR*	PA14 *mdpA* A646T with a deletion of *lasR*	This study
PA14 Δ*ptsP* Δ*phz1* Δ*phz2*	PA14 Δ*ptsP* with a deletion of Δ*phz1* Δ*phz2*	(16)
PA14 Δ*ptsP* Δ*phzM*	PA14 Δ*ptsP* with a deletion of *phzM*	(16)

PA14 Δ*ptsP* Δ*phzS*	PA14 Δ*ptsP* with a deletion of *phzS*	(16)
PA14 Δ*ptsP* Δ*phzH*	PA14 Δ*ptsP* with a deletion of *phzH*	(16)
PA14 Δ*ptsP* Δ*phz1* Δ*phz2* Δ*lasR*	PA14 Δ*ptsP* Δ*phz1* Δ*phz2* with a deletion of *lasR*	This study
PA14 Δ*ptsP* Δ*phzM* Δ*lasR*	PA14 Δ*ptsP* Δ*phzM* with a deletion of *lasR*	This study
PA14 Δ*ptsP* Δ*phzS* Δ*lasR*	PA14 Δ*ptsP* Δ*phzS* with a deletion of *lasR*	This study
PA14 Δ*ptsP* Δ*phzH* Δ*lasR*	PA14 Δ*ptsP* Δ*phzH* with a deletion of *lasR*	This study
PA14 Δ*lasR* Δ*hcnC*	PA14 Δ*lasR* with a deletion of *hcnC*	This study
PA14 Δ*ptsP* Δ*lasR* Δ*hcnC*	PA14 Δ*ptsP* Δ*lasR* with a deletion of *hcnC*	This study
PA14 Δ*ptsP* Δ*lasR* Δ*pqsA*	PA14 Δ*ptsP* Δ*lasR* with a deletion of *pqsA*	This study
PA14 Δ*ptsP* Δ*lasR* Δ*pqsH*	PA14 Δ*ptsP* Δ*lasR* with a deletion of *pqsH*	This study
PA14 Δ*ptsP* Δ*lasR* Δ*pqsR*	PA14 Δ*ptsP* Δ*lasR* with a deletion of *pqsR*	This study
Escherichia coli		
DH5α	F^- Φ80*lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17*(r_K^- m_K^+) *recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
S17-1	*recA pro hsdR RP4-2-Tc::Mu-km::Tn7*	(58)

Table 2. Plasmids used in this study.

Plasmid	Relevant properties	Reference or source
pEX18Ap	Suicide vector, Ap^r	(59)
pDA8	pEX18Ap containing flanking sequences of *lasR,* Ap^r	(16)
pEXG2	Suicide vector, Gm^r	(60)
pEXG2-Δ*ptsP*	pEXG2 containing Δ*ptsP* with flanking sequences, Gm^r	(34)
pEXG2-Δ*ygdP*	pEXG2 containing Δ*ygdP* with flanking sequences, Gm^r	This study
pEXG2-Δ*mdpA*	pEXG2 containing Δ*mdpA* with flanking sequences, Gm^r	This study
pEXG2-*mdpA* A646T	pEXG2 containing *mdpA* A646T with flanking sequences, Gm^r	This study
pEXG2-Δ*pqsA*	pEXG2 containing Δ*pqsA* with flanking sequences, Gm^r	This study
pEXG2-Δ*pqsH*	pEXG2 containing Δ*pqsH* with flanking sequences, Gm^r	This study
pEXG2-Δ*pqsR*	pEXG2 containing Δ*pqsR* with flanking sequences, Gm^r	This study
pEXG2-Δ*hcnC*	pEXG2 containing Δ*hcnC* with flanking sequences, Gm^r	(22)
pPROBE-GT	Broad-host-range pVS1/p15a GFP reporter, Gm^r	(61)
pBS351	pPROBE-GT with -1 through-501 5' region of *lasI*, Gm^r	(43)

Evolution experiments in casein

Evolution experiments were carried out as described previously (16). Overnight cultures were diluted 1:40 into 2 ml LB-MOPS, grown to OD_{600} ~3.5, and inoculated 1:50 into 2 ml casein broth. All cultures were passaged in casein broth at 24 h intervals for 32 days. CFU ml^{-1} for each lineage was determined by viable plate counts while the % *lasR* cheater was determined

by patching 50 colonies onto SMA where *lasR* cheaters form distinct colony phenotypes (11, 16, 17, 62).

Coculture assays

Overnight (18 h) pure cultures were grown in LB-MOPS, diluted to OD_{600} of 0.025 for cheaters or 0.1–0.15 for cooperators into LB-MOPS, and grown to an OD_{600} of ~5, unless otherwise specified, before combining at different ratios (9:1, 1:1, or 99:1 cooperator:cheater). This mixture was then diluted 1:40 into casein broth to start the coculture in 18 mm test tubes. Cocultures were diluted 1:40 into fresh casein broth in a new tube every 24 h for 3–7 days. The initial and final total population counts (CFU ml^{-1}) were determined by viable plate counts. The % *lasR* cheater was determined by patching 100 colonies on SMA where *lasR* cheaters form distinct colony phenotypes (11, 16, 17, 62). As controls, all strains were also grown as a monoculture.

Protease assays

Overnight (18 h) pure cultures were grown in LB-MOPS, diluted to OD_{600} of 0.025 for cheaters or 0.1–0.15 for cooperators into LB-MOPS, grown to OD_{600} 5, and diluted 1:40 into casein broth to start the coculture in 18 mm test tubes. Cultures were passaged in casein broth at 24 h intervals for 7 days. Protease was quantified using the Pierce Fluorescent Protease assay kit (Thermo scientific).

Measurements of pyocyanin production

For pyocyanin measurements, we extracted pyocyanin as described previously (63-65). Briefly, cells were grown for 18 h in Pyocyanin-producing media (55), and 5 ml whole culture was extracted with 2 ml chloroform. The organic layer was separated and extracted a second time with 0.2 N HCl. The absorbance of the aqueous layer was measured at 520 nm and multiplied by 17.072 to calculate the pyocyanin concentration (μg ml^{-1}) (64). Pyocyanin measurements were normalized to culture density (optical density at 600 nm) for reporting data.

Antimicrobial susceptibility assay

Tobramycin susceptibility was determined by minimum inhibitory concentration (MIC) according to the 2020 guidelines of the Clinical and Laboratory Standards Institute (CSLI), using a modified dilution method described elsewhere (16). Briefly, two dilution series were made from staggered starting tobramycin concentrations to cover a broader range of concentrations in MOPS minimal medium, and successively diluted 2-fold in a 200 μl volume in 2 ml tubes. The starter cultures were prepared by growing *P. aeruginosa* in LB-MOPS to OD_{600} of 4. Then, the starter cultures were diluted 1:40 into each tube containing tobramycin to start the MIC experiment. After 20 h of incubation with shaking, turbidity was measured using a Biotek Synergy 2 plate reader. The MIC was defined as the lowest concentration of tobramycin (μg ml^{-1}) in which bacterial growth was not measurable.

Transcription reporter assay

To measure LasR activity, we introduced the LasR-responsive plasmid pBS351 to *P. aeruginosa* strains by electroporation (43). Electrocompetent cells were prepared from overnight

cultures using 300 mM sucrose (66). Transformants were selected on LB agar using gentamicin at 50 μg ml^{-1} and routinely grown with gentamicin (50 μg ml^{-1} for agar and 15 μg ml^{-1} for broth) for plasmid maintenance. Overnight cultures of strains with pBS351::P*lasI-gfp* were diluted 1:40 into 2 ml LB-MOPS, grown to OD_{600} ~3.5, and inoculated 1:50 into 2 ml casein broth-gentamicin (15 μg ml^{-1}). Cultures were centrifuged, cell pellets were resuspended in PBS, and diluted 1:10. 200 μl of the diluted sample was aliquoted into clear-bottomed, black-walled, 96-well plates, where fluorescence and OD_{600} were measured using a BioTek Synergy 2 plate reader. Fluorescence was normalized to OD_{600}.

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Supplementary Information

Table S1. Population collapse in Δ*ptsP* evolution experiments.

Lineage	WT		Δ*ptsP*	
	Collapse	% cheater*	Collapse	% cheater*
1	No	>98	No	80
2	No	>98	No	4
3	No	>98	No	0
4	No	>98	No	78
5	No	>98	No	74
6	No	>98	No	86
7	No	>98	No	70
8	No	>98	Yes	92 (24)
9	No	>98	No	84
10	No	>98	Yes	82 (28)
11	No	>98	Yes	>98 (24)
12	No	>98	Yes	88 (20)
13	No	>98	No	86
14	No	>98	No	90

*% *lasR* cheater at the end of the experiment (day 32) or at the number of days of passage (in parenthesis) before collapse has been observed. %*lasR* cheater was determined daily by patching 50 colonies onto 4% skim milk agar until 8% *lasR* cheaters were observed and at 96 h intervals after observing 8% *lasR* cheaters. Each lineage represents an independent experiment.

Table S2. Total population counts in Δ*ptsP* evolution experiments.

Lineage	WT		Δ*ptsP*	
	Collapse	CFU ml^{-1}*	Collapse	CFU ml^{-1}*
1	No	1 X 10^9	No	4 X 10^9
2	No	6 X 10^9	No	4 X 10^9
3	No	2 X 10^9	No	7 X 10^9
4	No	3 X 10^9	No	4 X 10^9
5	No	4 X 10^9	No	4 X 10^9
6	No	2 X 10^9	No	3 X 10^9
7	No	3 X 10^9	No	7 X 10^9
8	No	6 X 10^9	Yes	2 X 10^9 (24)
9	No	3 X 10^9	No	5 X 10^9
10	No	3 X 10^9	Yes	1.1 X 10^9 (28)
11	No	6 X 10^9	Yes	8 X 10^9 (24)
12	No	4 X 10^9	Yes	2 X 10^9 (20)
13	No	1 X 10^9	No	4 X 10^9
14	No	4 X 10^9	No	4 X 10^9

*CFU ml^{-1} at the end of the experiment (day 32) or at the number of days of passage (in parenthesis) before collapse has been observed. Total population was determined daily by viable plate counts until 8% *lasR* cheaters were observed and at 96 h intervals after observing 8% *lasR* cheaters. Each lineage represents an independent experiment.

Table S3. Cocultures involving mutants defective in the production of phenazines, hydrogen cyanide, and PQS.

Cooperator	Cheater	Population collapse
WT	Δ*lasR*	No
WT	Δ*ptsP* Δ*lasR*	Yes
Δ*ptsP*	Δ*lasR*	No
Δ*ptsP*	Δ*ptsP* Δ*lasR*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*phz1* Δ*phz2*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*phzM*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*phzS*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*phzH*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*phz1* Δ*phz2*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*phzM*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*phzS*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*phzH*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*hcnC*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*hcnC*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*pqsA*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*pqsH*	Yes
WT	Δ*ptsP* Δ*lasR* Δ*pqsR*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*pqsA*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*pqsH*	Yes
Δ*ptsP*	Δ*ptsP* Δ*lasR* Δ*pqsR*	Yes

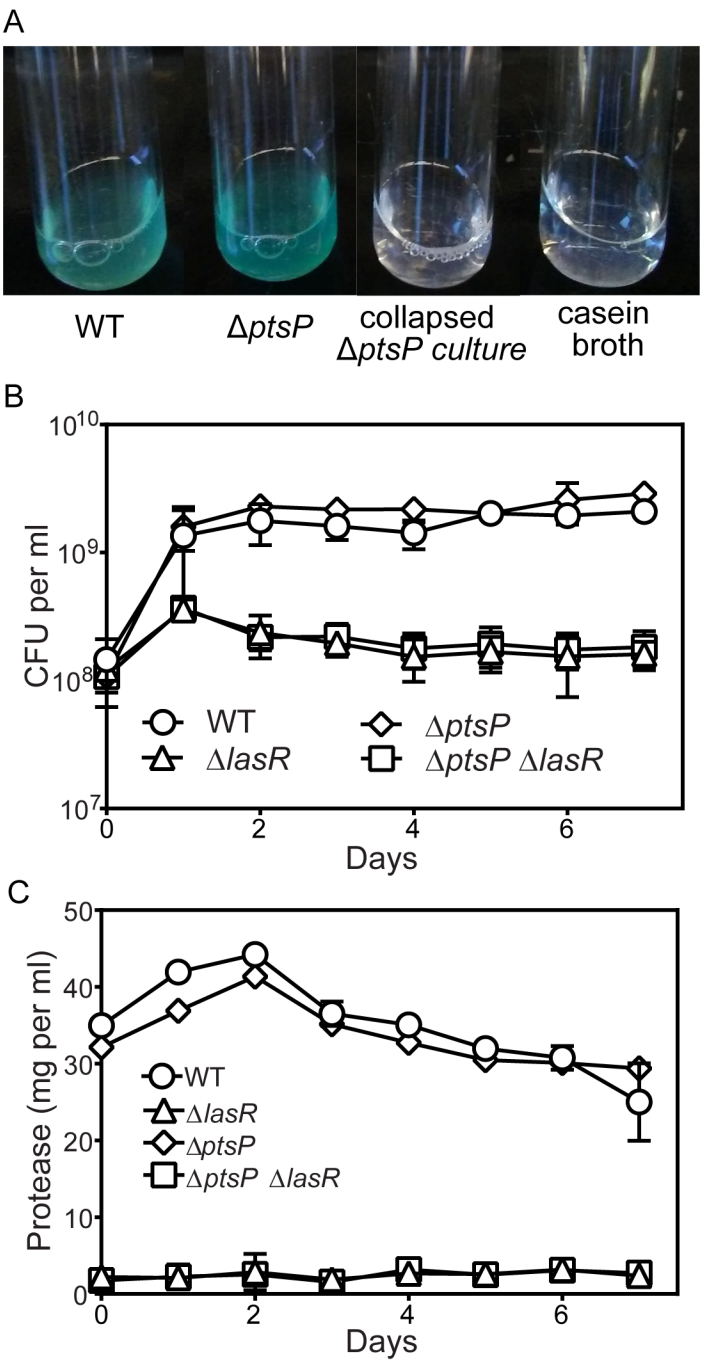

Fig. S1. Pyocyanin production, growth, and protease production of *P. aeruginosa* in casein broth. **A.** Pyocyanin production. Cultures are representative experiment from evolution experiments in **Table S1**, showing differences in pigment formation of growing and collapsed cultures. The uninoculated casein broth is shown as negative control. **B.** Growth of *P. aeruginosa* strains in casein broth. Strains were passaged in 1% casein broth daily for 7 days. Population count was determined daily by viable plate counts. The small increase in growth of Δ*lasR* and Δ*ptsP* Δ*lasR* is probably due to nutrient carryover from the culture used to inoculate the experiment. **C.** Protease production. Cultures were passaged in 1% casein broth daily for 7 days. Protease was quantified as described in the Materials and Methods. The values represent the average of three (B) or two (C) independent experiments. The error bars represent the standard deviation.

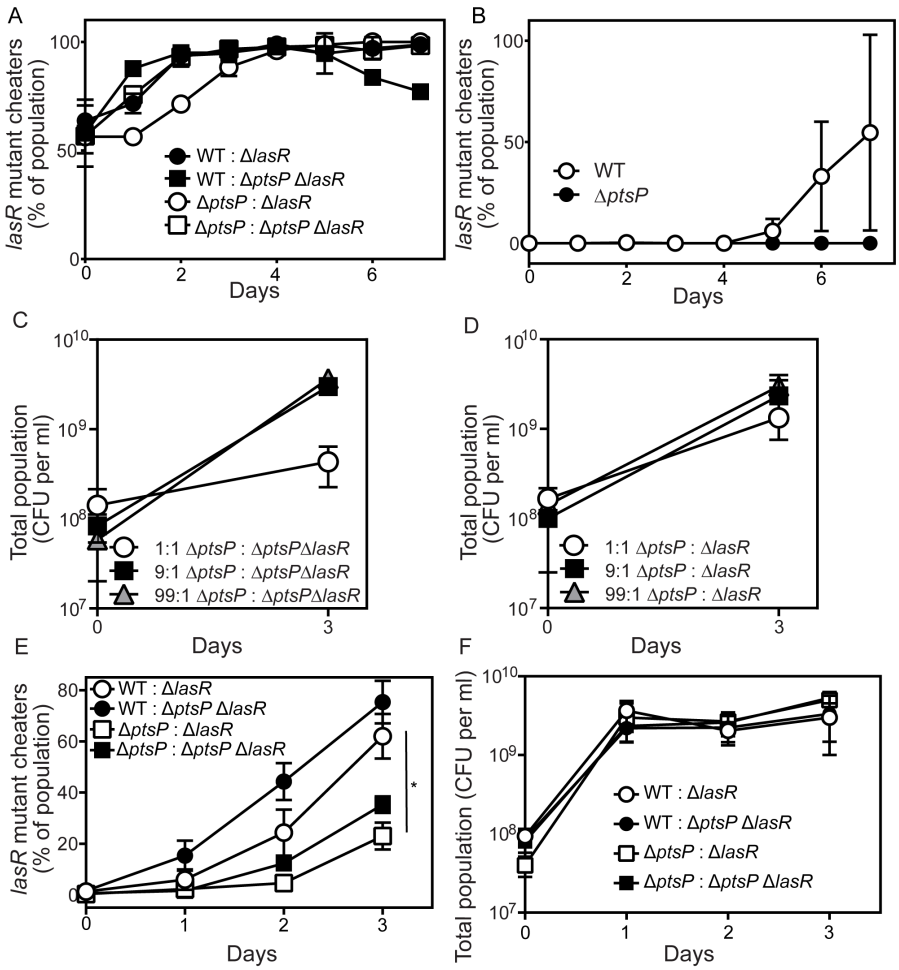

Fig. S2. Frequency of *lasR* cheaters and cell density in monoculture and coculture experiments. **A**. Frequency of *lasR* cheaters in cocultures in **Fig. 2**. Cultures were grown to OD 5 and mixed together at 1:1 (cooperator:cheater) ratio. The cocultures were passaged in 1% casein broth daily for 7 days and frequency of *lasR* cheaters was determined as described in the Materials and Methods. **B.** Frequency of spontaneous *lasR* cheaters that emerged in monocultures. The cultures were passaged in 1% casein broth daily for 7 days and frequency of *lasR* cheaters was determined as described in the Materials and Methods. **C.** Total population counts in Δ*ptsP* and Δ*ptsP* Δ*lasR* cocultures with different starting ratios (1:1, 9:1, or 99:1 cooperator:cheater). **D.** Total population counts in Δ*ptsP* and Δ*lasR* cocultures with different starting ratios (1:1, 9:1, or 99:1 cooperator:cheater). Cocultures were grown to OD 3.5 and mixed together at the specified ratios. The cocultures were passaged in 1% casein broth daily for 3 days. Total population was determined before and after 3 days of incubation by viable plate count. **E.** Frequency of *lasR* cheaters in cocultures started at 9:1 (cooperator:cheater) ratio. Cultures were grown to OD 3.5, mixed together at 9:1 (cooperator:cheater) ratio, and cocultures passaged in 1% casein broth daily for 3 days and frequency of *lasR* cheaters was determined as described in the Materials and Methods. **F.** Total population counts for cocultures in E. Statistical analysis by two-way ANOVA and Dunnett's multiple comparisons test: * $p<0.05$. The values represent the average of three independent experiments. The error bars represent the standard deviation.

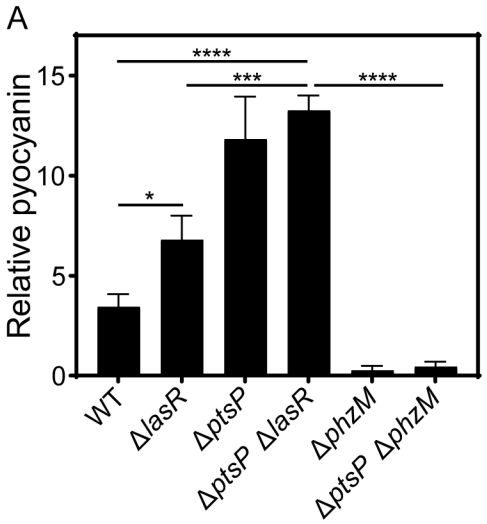

Fig. S3. Effect of *ptsP* and *lasR* inactivation on pyocyanin production. Cultures were inoculated into pyocyanin-producing media, grown for 18 h, and extracted before quantifying pyocyanin as described in Materials and Methods. In all cases, reported values are μg/ml pyocyanin normalized to culture density at the time of measurement. The values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by one-way ANOVA and Tukey's multiple comparisons test: * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

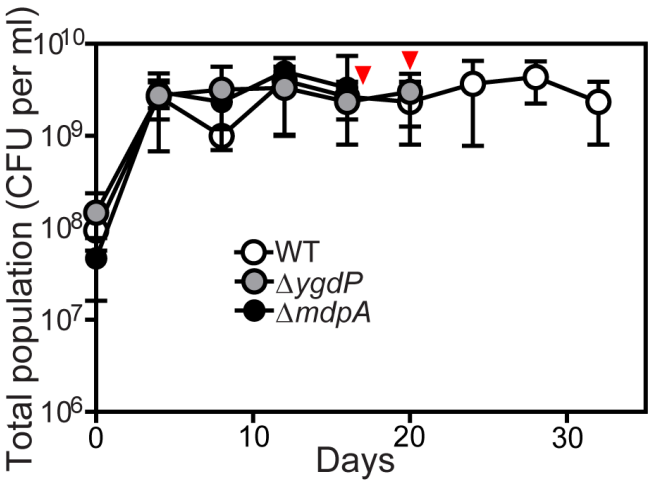

Fig. S4. Cell density of Δ*ygdP* and Δ*mdpA* populations passaged in casein. Collapsed cultures are marked by red inverted triangles. *P. aeruginosa* populations were transferred daily in 1% casein broth for 32 days, and total population count was determined by viable plate count. Values represent the average of three independent experiments and the error bars represent the standard deviation.

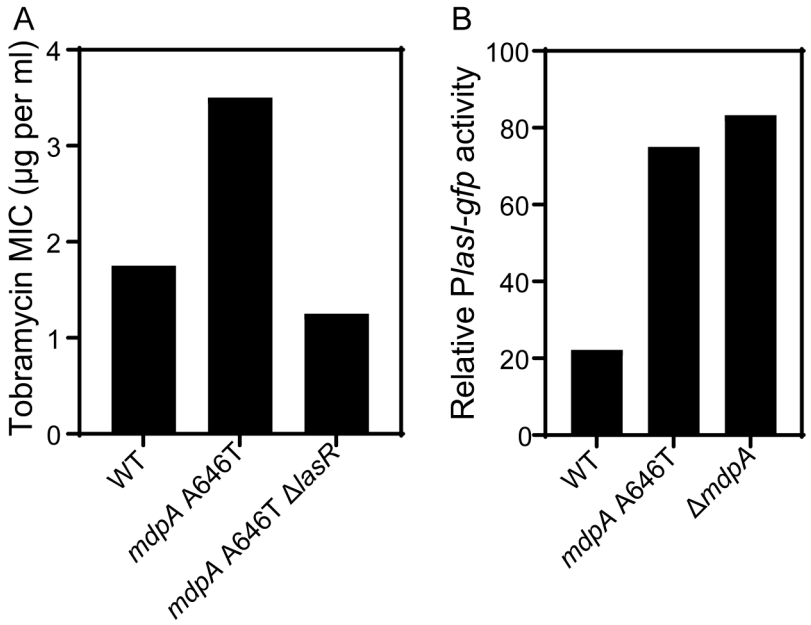

Fig. S5. Tobramycin resistance and LasR activity of *mdpA* mutants. **A**. Tobramycin MIC. The tobramycin MIC was determined as described in Materials and Methods. **B.** LasR activity. *P. aeruginosa* strains were electroporated with a plasmid containing a LasR-responsive GFP reporter (pBS351). Reported values are fluorescence normalized to culture density at 18 h of growth. The values represent one independent experiment.

CHAPTER VI: Discussion

Summary of Findings

This work presents experimental evidence supporting the interplay between quorum sensing and antibiotic resistance in the opportunistic pathogen *Pseudomonas aeruginosa*. In particular, the results illustrate how adaptation to the clinically relevant aminoglycosides tobramycin and gentamicin can have pleiotropic effects on the evolutionary trajectory of quorum sensing. The results of this study show that the LasR-I system plays a small but appreciable role in aminoglycoside resistance. In addition, both tobramycin and gentamicin (1) promote quorum-sensing stability by directly suppressing *lasR* mutant cheaters and enhancing cheater policing through pleiotropic effects of mutations in PtsP and through PtsP-independent mechanisms; (2) select for mutations that alter the role of quorum sensing in antibiotic resistance through epistatic interactions; and (3) induce dynamic changes on social cheating in *P. aeruginosa* including mutations leading to potential escape from antibiotic suppression or acceleration of population collapse. This study also demonstrates how two antibiotics with very similar mode of action select for different sets of genetic mutations in *P. aeruginosa*. Overall, results of this study demonstrate how antibiotics and antibiotic-selected mutations influence the evolution of quorum-sensing cooperation in *P. aeruginosa* have broader implications for our understanding of how communities of pathogens adapt to environmental change.

Significance, Implications, and Limitations of the Study

Quorum sensing and antibiotic resistance

The interplay between antibiotic resistance and quorum sensing in *P. aeruginosa* is not very well understood yet (1-5). Several studies supported quorum sensing-dependent low-level

antibiotic resistance in biofilm conditions (3, 4, 6, 7). The work presented here provided new evidence that quorum sensing-dependent antibiotic resistance is also exhibited by *P. aeruginosa* in planktonic conditions (Chapters II and IV). The role of quorum sensing in antibiotic resistance is subtle, however, low-level antibiotic resistance can cause significant improvement in bacterial fitness as well as equipped a fraction of the population to become tolerant or persister cells (8, 9). As bacteria adapt to their environment, low-level antibiotic resistance may also lead to high-level resistance through accumulation of additional genetic mutations (10). Both of these have been demonstrated in the laboratory evolution experiments conducted in this study (Chapters II, III, and IV).

In addition, quorum sensing increases antibiotic resistance in another Proteobacterium, *Chromobacterium subtsugae* (formerly *C. violaceum*) (11), suggesting this type of regulation might be broadly beneficial in Proteobacteria. There are several potential advantages of placing antibiotic resistance factors under quorum sensing control. For example, by regulating antibiotic resistance, quorum sensing may protect the population against self-produced toxins that are also controlled by quorum sensing (12). Another potential advantage of placing antibiotic resistance under quorum-sensing control is that it could synchronize resistance factor expression across members of the population to protect neighboring cells from exported antibiotics (13-15).

Antibiotic adaptation causes dynamic changes on cooperation and cheating

Antibiotics are well known for their pleiotropic effects, including selection of adaptive mutations that have pleiotropic effects on other phenotypes (16-21). Results of these studies provide examples of how cheater control mechanisms can change under antibiotic selection. Interestingly, aminoglycoside selection does not always lead to stability of cooperation. In some

instances, the adaptation leads the bacterial population to the other end of the spectrum. Chapters IV and V illustrate an example of how gentamicin and tobramycin adaptation could lead to a faster population collapse as well as how cheaters seem to escape suppression imposed directly by antibiotics. The genetic basis for both observations still needs to be explored, but all these results point to the idea that antibiotic selection creates dynamic changes on social cheating in *P. aeruginosa*. By studying the pleiotropic effects of antibiotic-selected mutations on the evolutionary trajectory of quorum sensing in a laboratory setting, we gain a better understanding of how quorum sensing might evolve in natural settings such as multispecies communities or antibiotic-treated infections.

Tight control of gene regulation in P. aeruginosa

Quorum sensing activity is upregulated by *ptsP* inactivation, suggesting that *ptsP* negatively regulates quorum sensing in *P. aeruginosa* (Chapters II and IV) (22, 23). The mechanistic pathway by which *ptsP* regulates the AHL-quorum sensing in *P. aeruginosa* is not yet well understood. We also found other genes that may be involved in quorum sensing regulation (Chapter IV). These results illustrate the intricate gene regulation and regulatory effects on quorum sensing in *P. aeruginosa* (24, 25). Another example of a known quorum sensing regulator is the quorum-sensing-control repressor (QscR), which oversees the timing of quorum-sensing-controlled gene expression (26, 27). This complex gene regulation could help ensure that quorum-sensing-controlled genes are only activated when they are needed, when they offer some selective advantages, or to prevent self-toxicity (25, 26, 28). This may also explain the versatility of *P. aeruginosa*, allowing it to survive various habitats and colonize hosts. The interconnection with global regulators also suggest that quorum sensing is highly influenced by

environmental conditions, metabolism, and other signals produced or encountered by the bacterial cells (25).

Emergence of lasR mutants during infections

The results of this work might have important implications for understanding how *lasR* mutants emerge during chronic *P. aeruginosa* infections. In patients treated with antibiotics, more sensitive *lasR* mutants should be suppressed. Yet, *lasR*-null mutants frequently emerge in these chronic infections (29-33). This study illustrates several ways by which antibiotic adaptation alters the antibiotic susceptibility of *lasR* mutants. Results from these studies show that *lasR* mutants can become more antibiotic resistant through sign epistasis caused by *fusA1* G61A mutations and also through direct acquisition of antibiotic resistance mutations. Both impact the fitness of *lasR* mutants in the presence of antibiotics. A correlation between increased antibiotic resistance and loss of quorum sensing functions has been reported before, however, the underlying mechanism or genetic basis of increased antibiotic resistance in *lasR* mutants is not known (30, 31, 34). Results from this and previous work suggest that *lasR* mutants can develop antibiotic resistance, potentially explaining their emergence during chronic infections of antibiotic-treated patients. It is important to note that the genetic pathways leading to resistance of *lasR* mutants are highly influenced by elements that constrain evolution such as epistasis. For example, sign epistasis constrains the combination of mutations leading to high level antibiotic resistance, which means that antibiotic resistance development could be predictable (35). Negative epistasis has also been observed between *lasR* mutation and antibiotic resistance mutations, suggesting that evolution of antibiotic resistance in *P. aeruginosa* could be influenced

by quorum sensing (36). Epistasis may have implications on how *P. aeruginosa* and other pathogens evolved and how they affect their hosts especially during chronic infections.

Implications for understanding population dynamics in natural environments

This study describes antibiotic adaptations in a well-controlled environment using highly defined growth conditions and a pure laboratory strain. Adaptation could be much more complex in natural environments such as in infections where there are other stressors or in natural habitats where interspecies competitions might exist. The growth condition in the host system may be entirely different that could influence adaptation trajectory. In addition, this study focused on single-clone variants isolated from tobramycin-exposed populations. Adaptive mutations might not be homogeneous throughout the population, and a different behavior may be observed with other clones or with mixed clones at the population level. The antibiotic concentration used in this study is also much lower than what is usually used in a clinical setting, and adaptations at high antibiotic concentrations might be different. Nevertheless, this study identified adaptive events including those detected in clinical settings that aid in discovering important aspects of quorum-sensing biology.

Future Directions

This work provided new insights on the biology of quorum sensing and its interplay with antibiotic resistance. However, there are still queries that remained unanswered. One of the pressing questions that remained unanswered is what is the mechanism of quorum sensing-dependent antibiotic resistance? In *C. subtsugae,* quorum sensing-dependent antibiotic resistance is attributed to an efflux pump (37). In *P. aeruginosa,* there may be multiple factors

contributing to quorum sensing-dependent antibiotic resistance. There are at least three efflux pumps known to have overlapping specificity for aminoglycosides (MexAB (38, 39), MexXY (40, 41), and PA1874-1877 (42)). There are also aminoglycoside-inactivating enzymes (38). Some reports suggest reactive oxygen species (ROS) detoxification systems or regulatory circuits that activate these systems, such as the stringent response alarmone ppGpp, are also important for aminoglycoside resistance (43, 44). Understanding how and why quorum sensing contributes to antibiotic resistance will provide important new information about the biology of quorum sensing and will be relevant to designing new therapies to treat antibiotic-resistant *P. aeruginosa* infections.

One of the genes of interest we found in this study is *ptsP*. The mechanistic pathway by which PtsP (and possibly the PTS^{Ntr} system) contributes to the regulation of quorum sensing and quorum sensing-regulated genes such as pyocyanin, tobramycin resistance, pathogenesis, and biofilm formation are as-yet unknown. In the case of quorum sensing, it has been proposed that PtsP represses LasR activity by somehow increasing production of the antiactivator QscR (22), although it is not clear if PtsP acts through this pathway. It will also be interesting to determine whether *ptsP* mutations are found in clinical strains and what role these mutations play in a clinical setting.

fusA1, another gene of interest in this study, is a mutation hotspot for *P. aeruginosa* clinical isolates (45-49). In this light, sign epistasis between *fusA1* and *lasR* mutations could have important clinical implications. The mechanism behind the increased ribosome stalling in *lasR*-null mutants with *fusA1* G61A mutation that may have been responsible for elevated MexXY expression needs further investigation. Quorum sensing is known to activate ROS detoxification systems such as the enzyme superoxide dismutase (50). We hypothesize *fusA1* G61A mutations

increase production of ROS that could contribute to ribosome stalling. The *fusA1* G61A Δ*lasR* mutant, which does not activate ROS detoxifiers, may have higher levels of ROS and more stalling, which in turn upregulates MexXY. A recent study also showed that a *fusA1* mutation can alter expression of virulence genes in *P. aeruginosa* (19). Whether *fusA1* G61A mutation has the same effect on the virulence of *lasR*-null mutants is yet to be explored.

We observed enhanced quorum sensing activity and delayed cheating phenotypes that occur in a *ptsP*-independent manner. The genetic basis and the mechanistic pathway of these phenotypes still need to be identified. Our genetic screens suggested potential involvement of *lptA* and *phoQ*. *lptA*-null mutations may be involved in enhanced quorum sensing activity and/or delayed cheating because *lptA* inactivation increases transcription of *lasI* in PAO1 (51). Null mutations in *phoQ* can upregulate biosynthesis of pyocyanin (52), which can in turn suppress cheating by policing (23, 53).

We also found several adaptive mutations leading to population collapse. Inactivation of *mdpA* may impair ability to acquire nutrients and could be triggering a population collapse through starvation of both cheaters and cooperators. PtsP inactivation, which upregulates the policing toxin pyocyanin, could be causing dysregulation of toxin production that could lead to collapse. With its role in the PTS^{Ntr} system, *ptsP* inactivation may also be causing enhanced competition for nutrients between the cooperator and cheater, leading to population collapse. Understanding the dynamics and factors contributing to collapse of cooperating populations may provide new insights on developing alternative strategies in treating bacterial diseases.

Mutations in *ptsP*, *fusA1*, *mdpA*, and *ygdP* may be just the tip of the iceberg. We have characterized other variants that appear to have unique adaptive mutations that might also have pleiotropic effects on cheater suppression, sign epistasis, and accelerated collapse. Altogether,

this body of work suggests that there are numerous ways by which adaptive mutations can indirectly alter the evolutionary trajectory of quorum sensing and cooperative behaviors. There are still other mechanisms of quorum sensing evolution to study and interesting quorum sensing biology to discover.

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