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ARTICLE

Allelic polymorphism shapes community function in evolving *Pseudomonas aeruginosa* populations

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that chronically infects the lungs of individuals with cystic fibrosis (CF) by forming antibiotic-resistant biofilms. Emergence of phenotypically diverse isolates within CF *P. aeruginosa* populations has previously been reported; however, the impact of heterogeneity on social behaviors and community function is poorly understood. Here we describe how this heterogeneity impacts on behavioral traits by evolving the strain PAO1 in biofilms grown in a synthetic sputum medium for 50 days. We measured social trait production and antibiotic tolerance, and used a metagenomic approach to analyze and assess genomic changes over the duration of the evolution experiment. We found that (i) evolutionary trajectories were reproducible in independently evolving populations; (ii) over 60% of genomic diversity occurred within the first 10 days of selection. We then focused on quorum sensing (QS), a well-studied *P. aeruginosa* trait that is commonly mutated in strains isolated from CF lungs. We found that at the population level, (i) evolution in sputum medium selected for decreased the production of QS and QS-dependent traits; (ii) there was a significant correlation between *lasR* mutant frequency, the loss of protease, and the 3O-C12-HSL signal, and an increase in resistance to clinically relevant β-lactam antibiotics, despite no previous antibiotic exposure. Overall, our findings provide insights into the effect of allelic polymorphism on community functions in diverse *P. aeruginosa* populations. Further, we demonstrate that *P. aeruginosa* population and evolutionary dynamics can impact on traits important for virulence and can lead to increased tolerance to β-lactam antibiotics.

Introduction

The cystic fibrosis (CF) lung is a spatially complex and inflamed environment that provides beneficial growth conditions for a number of bacterial species, including the opportunistic pathogen *Pseudomonas aeruginosa* [1–5]. Chronic infection of CF lungs with highly adapted and antibiotic-resistant biofilms of *P. aeruginosa* is a major cause of lung function decline, which results in a concomitant increase in morbidity and mortality in individuals with CF [3, 4, 6–11]. Longitudinal studies of chronic CF infections with *P. aeruginosa* have revealed that patients become infected at a young age with an environmental or transmissible isolate that evolves and adapts over time to the lung environment [12–18]. Studies on *P. aeruginosa* populations isolated from individual lungs, have demonstrated divergent evolution, resulting in heterogeneous populations of *P. aeruginosa* within patients [16, 17, 19]. This genetic adaptation and diversification are likely to impact on levels of pathogenicity and the efficacy of antibiotic treatment

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[16, 20], and could potentially impact how other species of microbes colonize the CF lung [4, 21–27].

Studies on explanted CF lungs have shown that the spatial structure found within lungs, and physical separation of infecting isolates, plays a role in generating the vast phenotypic and genotypic heterogeneity seen within *P. aeruginosa* populations in individual patients [16, 17, 28, 29]. Major adaptations of *P. aeruginosa* to the CF lung include alginate production, loss of quorum sensing (QS), hypermutability, and increased resistance to antimicrobials [7, 8, 14, 30, 31]. Heterogeneity in *P. aeruginosa* populations has also been explained by early divergent evolution and adaptation to differential ecological niches [32] and recombination between isolates residing in the airways [16, 33]; however, the exact mechanisms leading to heterogeneity have yet to be fully elucidated. Further, while it is accepted that genomic heterogeneity arises in CF chronic lung infections, it remains unknown how genotypic changes shape community functions within whole *P. aeruginosa* populations. Understanding what drives community structure and function remains a key goal in microbial ecology, because the overall community function is determined by all the individuals in the population.

In this study, we hypothesized that the combination of wild-type and mutated alleles in populations shapes community functions, which could result in clinically relevant outcomes such as increased or decreased antibiotic tolerance and traits important for virulence. To test this, we first evolved *P. aeruginosa* PAO1 in biofilms on plastic beads [34] for up to 50 days. This bead biofilm system has previously been successfully used to study genetic adaptation and phenotypic diversity of *Burkholderia cenocepacia* and *P. aeruginosa* to different environmental conditions [35]. Our study differs from this previous work, in that our major focus was on community function within populations rather than on specific isolates and defining ecological niches. We grew our bead-associated biofilms in a synthetic CF sputum media (SCFM), which recapitulates the chemical environment found in CF sputum [36–38]. Our study therefore generated phenotypically and genotypically heterogeneous populations of *P. aeruginosa* in a spatially structured environment chemically relevant to CF sputum. We utilized these heterogeneous evolved populations to study the changes in the functional community phenotypes instead of the traditional approach of working with single-evolved isolates. We used a metagenomic approach to assess genetic alterations within evolving populations, and we monitored fluctuations in allele frequency during the selection process. To determine the impact of genomic heterogeneity within populations on various phenotypes, we assessed collective phenotypic traits (community function) of the evolved populations.

One of the most commonly described adaptations of *P. aeruginosa* to the CF lung is the loss of the *las* QS system, predominantly through point mutations, frameshifts, and deletions in the *lasR* gene [17, 39–41]. We used our evolved populations to specifically focus on the impact of *lasR* mutation frequency on QS phenotypes. The *lasR* gene encodes the LasR transcriptional regulator, which binds the QS signal *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C12-HSL) [42–45]. LasR-bound 3O-C12-HSL controls the transcription of ~10% of the *P. aeruginosa* genome, including a number of genes involved in social behaviors, pathogenesis, antibiotic resistance, and biofilm formation [46–49]. Despite a number of previous studies that describe the changes and adaptation of various lineages of *P. aeruginosa* in CF lungs [14, 50–53], it remains unclear how polymorphisms in the *lasR* gene impact on community function within evolving heterogeneous *P. aeruginosa* populations. This is because most previous studies that focused on within-host adaptation of *P. aeruginosa* used single colonies isolated from temporal CF sputum samples and not whole populations [12, 14, 15, 19, 54].

Overall, we found that (i) evolutionary trajectories were reproducible between independently evolving populations, and that over 60% of genomic changes in populations occurred within the first 10 days of selection; (ii) after 30 days of evolution in SCFM, the evolved communities displayed an increase in *lasR* mutant frequency and a decrease in QS-dependent traits; (iii) there was a significant correlation between *lasR* mutant frequency, the loss of social traits, and an increase in tolerance to β-lactam antibiotics. Our findings provide insights into how allelic polymorphism and population heterogeneity, in general, can impact on phenotypes and community functions within evolving *P. aeruginosa* populations. Further, we demonstrate that changes in *P. aeruginosa* population dynamics can alter factors associated with virulence and provide explanations for increased antibiotic tolerance, even in situations when antibiotics have not been used.

Results

Genomic variation in evolving biofilm populations over 50 days of selection in SCFM

We evolved the *P. aeruginosa* strain PAO1 for 50 days (≈800 generations) in biofilms using a previously described biofilm bead method [34], and a growth medium that chemically mimics CF sputum (SCFM), and where the physiology of *P. aeruginosa* is similar to when grown in human sputum [36–38]. Our experimental evolution approach contained four independent replicate lines (Fig. S1). We collected and stored biofilm-evolved populations after 10,

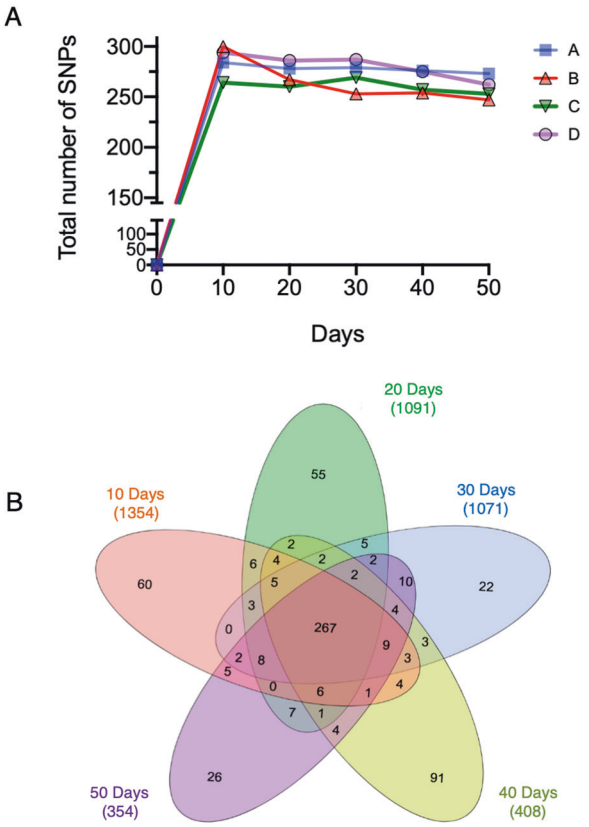


Fig. 1 The evolutionary trajectory of *P. aeruginosa* during biofilm growth in SCFM. The evolution trajectories were similar in four independent replicate lines, and the major genetic heterogeneity in the evolved populations occurred during the first 10 days of selection. **a** SNP calling analysis showed that the four independently evolved populations had more than 80% similarity (267 shared SNPs) in genomic changes in the populations over the 50 days of selection. **b** The Venn diagram shows the shared SNPs between all four replicate lines, over 50 days of selection. Numbers in brackets (i.e., 1354 for 10 days) represent the combined number of SNPs in each of the four evolved lines. The majority of genetic variation within the populations occurred within the first 10 days of selection. Each circle represents the total number of SNPs for each independently evolved line.

20, 30, 40, and 50 days of evolution (Rounds 1–5: R1–R5). We used the Illumina MiSeq platform to deep-sequence evolved populations in order to determine genomic changes through time. We also sequenced our laboratory PAO1 ancestral strain, and after de novo genome assembly of this strain, we mapped the sequence reads of the evolved populations to the ancestor in order to detect SNPs [55]. Our SNP calling analysis, combined with an analysis of allele frequency, revealed that in all four independent replicate lines, an average of 282 ± 13 SNPs occurred in the populations after 10 days of selection (Fig. 1a). We found that around 60% of these SNPs were present through all other rounds of selection (Figs. 1b and S2). This suggests that the evolutionary trajectories of biofilm growth in SCFM are similar in independently evolving populations, and that the major genetic heterogeneity in evolving populations occurs during the early phases of selection.

SNP frequency in genes involved in social traits fluctuates over time

In our evolution experiment, we found emergence of polymorphisms in 45 genes involved in various physiological functions (Fig. S3 and Table S1). We found that between 10 and 25% of SNPs were fixed (frequency of 1) in the populations over 50 days of selection across all four replicate evolution lines (Fig. S4). When we focused on the allele frequency and not the number of positions altered in each coding region, we found that the frequency of SNPs in genes involved in different traits changed during the course of the experiment (Figs. 2, S3, S4, and S5). The genes highlighted in Fig. 2 are genes that have previously been shown to be commonly mutated in *P. aeruginosa* CF isolates [9, 15, 16, 29, 30, 56].

The allele frequencies of nonsynonymous SNPs in *ccoN2* (PA1557), and synonymous SNPs in *pvdJ* (PA2400) and *tufA* (PA4265) became fixed in the population at a frequency of 1, while the frequency of nonsynonymous SNPs in *phzC2* (PA1901) and *pvdD* (PA2399) fluctuated between 0.4 and 0.5 in different rounds of selection. We detected a number of SNPs occurring in *mutS* (PA3620), *pilQ* (PA5040), and *pilN* (PA5043) at 10, 20, and 30 days of selection in all four independent evolved lines. We observed an increase in *lasR* (PA1430) mutant allele frequency between 30 and 40 days of selection (Figs. 2, S3, and S5).

Accumulation of SNPs shapes community functions in evolved *P. aeruginosa* populations

We next examined the production of phenotypic social traits in evolved populations in order to determine changes in community function of the genetically heterogeneous evolving populations. We measured the levels of biofilm formation, QS signals, total protease, and the siderophores pyoverdine and pyochelin. We observed a small but significant increase in biofilm formation by evolved populations when compared with the PAO1 ancestor (Fig. 3a). After 30 days of selection, the production of total protease (Fig. 3b) and the 3O-C12-HSL QS signal (Fig. 3c) decreased in evolved populations; however, the levels of C4-HSL signal (Fig. 3c), and pyochelin and pyoverdine (Fig. 3d), did not follow this trend, and any changes were generally not significantly different from the values of the ancestor strain. We also observed an increase in colony morphology types (morphotypes) in the evolved populations starting after 20 days of selection (Fig. S6).

To determine whether the emergence and increase in the frequency of SNPs in the populations impact upon

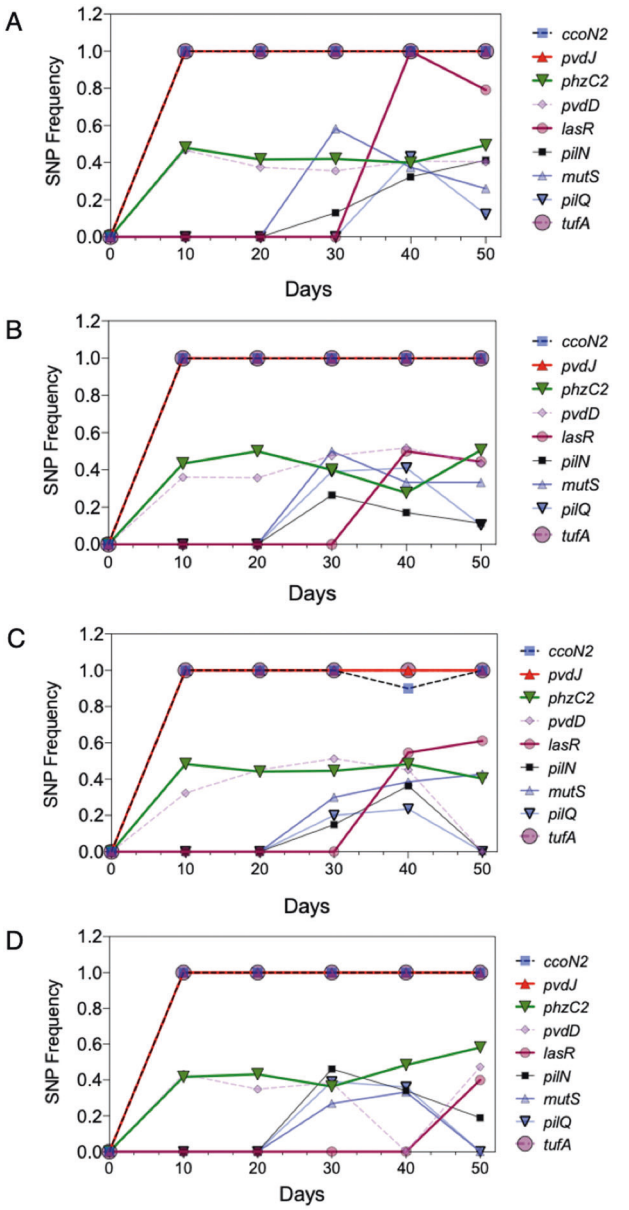


Fig. 2 Allele frequency of SNPs changes over the course of selection. Emergence of nonsynonymous SNPs in genes involved in social traits (QS: *lasR*), oxidative respiration and DNA mismatch repair, motility, and iron chelation occurred after 20 and 30 days of selection, and fixed SNPs in genes such as elongation factor *tufA*, Cytochrome c oxidase subunit (*ccoN2*), and pyoverdine biosynthesis protein *pvdJ* emerged after 10 days of selection, and were fixed in the populations over 50 days (**a**–**d** represent replicate evolving lines).

community function, we used a linear regression model. We focused on changes in the allele frequency detected in the QS regulator LasR. We chose *lasR* because the genes and phenotypes it regulates in *P. aeruginosa* are well understood [30, 40, 44, 47, 57] and also, *lasR* mutants are regularly isolated from CF sputum [13, 50, 58]. We detected a nonsynonymous SNP (V208G) in the DNA-binding domain of LasR between 30 and 40 days of selection (Fig. S5). Interestingly, SNPs in the same position (V208) have been identified in *P. aeruginosa* isolates collected from CF sputum samples [40]. V208 is adjacent to the D209 residue in the LasR DNA-binding domain [59, 60], suggesting an impact on the structure of LasR and its DNA-binding affinity. We assessed the impact of *lasR* V208G SNP frequency on changes on two QS-dependent phenotypic traits: production of 3O-C12-HSL signal and total protease. We found a significant negative correlation between the frequency of the V208G *lasR* SNP in the whole-evolved populations and the total protease activity of evolved populations. We found that 87% ($R^2 = 0.8704$, $F = 20.15$; $p = 0.0206$) of the decreased protease activity was correlated with the accumulation of the *lasR* mutation in the populations (Fig. 4a). We used the same analysis to determine whether *lasR* mutant accumulation impacts on 3O-C12-HSL production within populations. We found that only 53% of the decreased 3O-C12-HSL levels ($R^2 = 0.5363$, $F = 3.469$; $p = 0.1594$) can be explained by the accumulation of *lasR* mutation in the populations (Fig. 4b). However, when we only included the last 30 days of selection in our analysis, the decrease in 3O-C12-HSL levels could be fully (100%) explained by the accumulation of *lasR* mutants ($R^2 = 1.0$, $p = 0.0034$) (Fig. 4b). Our analysis did not show any correlation between the frequency of the *lasR* SNP and changes in the production of C4-HSL (Fig. 4c) or biofilm formation (Fig. 4d).

Accumulation of *lasR* mutants in evolved populations leads to increased tolerance to β-lactam antibiotics

Previously, it has been shown that *lasR* mutants display increased β-lactamase activity, and therefore increased resistance to β-lactam antibiotics (such as ceftazidime) [61] that are routinely used in CF clinics. To determine possible links between the loss of *lasR* function and changes in tolerance to routinely used antibiotics, we first assessed the antimicrobial susceptibility levels of the evolved populations. When we tested the levels of antimicrobial susceptibility to six routinely used antibiotics for chronic CF lung infection, after 30 days of selection, the evolved populations showed an increased tolerance (indicated by a decrease in the zone of inhibition) to three antibiotics: ceftazidime, piperacillin/tazobactam, and meropenem that are all β-lactam-class antibiotics (Figs. 5 and S7). We then tested for correlations between the frequencies of the *lasR* V208G SNP and resistance to ceftazidime and piperacillin/tazobactam. We observed a positive and significant correlation between accumulation of *lasR* mutants and the increased resistance to both ceftazidime and piperacillin/tazobactam (Fig. 5a, b). We also tested whether the increase in tolerance could be explained by an increase in biofilm formation. We

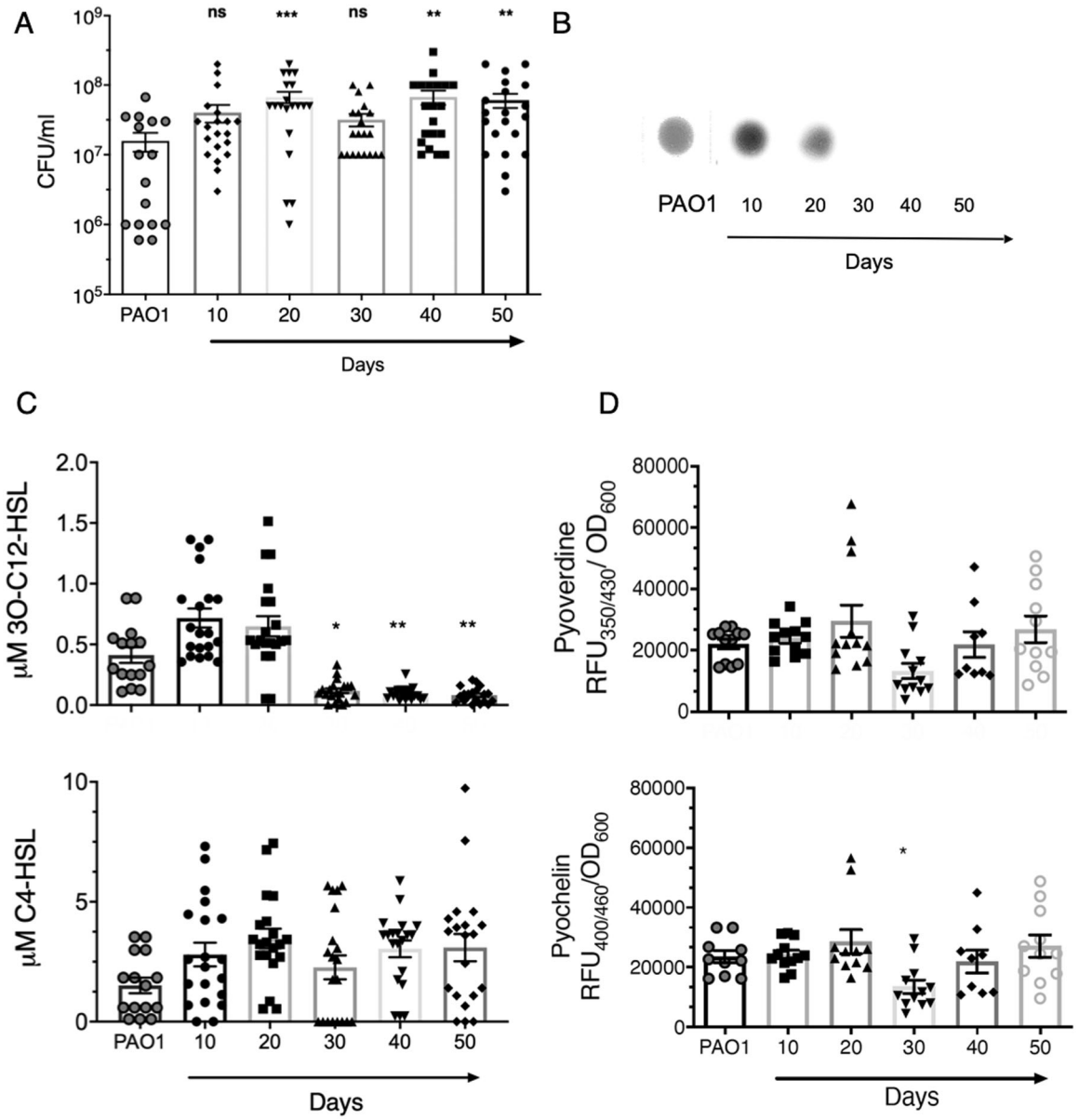


Fig. 3 Phenotypes of evolved populations of PAO1. a Increase in biofilm formation by evolved populations of PAO1 (Kruskal–Wallis, Dunn's multiple comparison with PAO1, $p < 0.05$, $n = 5$. Errors bars indicate the standard error of the mean, SEM). **b** The total protease activity was reduced at 30 days of selection. **c** Production of 3O-C12-HSL significantly decreased at 30 days of selection (Kruskal–Wallis, uncorrected Dunn's test multiple comparison to PAO1, $p < 0.05$, $n = 5$. Errors bars indicate the SEM), and there were no significant changes in the levels of C4-HSL. **d** There were no significant changes in siderophore production in evolved populations compared with the PAO1 ancestor (Kruskal–Wallis, uncorrected Dunn's test multiple comparison to PAO1, $p > 0.05$, $n = 5$). Each dot represents the mean value of one independent experiment for each evolved population at each round of selection. All experiments were performed in three technical replicates.

found that there was no significant correlation, suggesting that increases in biofilm formation do not necessarily translate to an increase in drug tolerance (Fig. S7C).

To confirm that our evolved populations contained *lasR* mutants with increased β-lactam tolerance, and that *lasR* frequency affects community function, we first selected and sequenced an evolved isolate (BB8) from the final round of selection (50 days) with no protease activity, and which demonstrated increased antibiotic tolerance compared with the ancestor PAO1 (Fig. 5c). The sequencing of isolate BB8 revealed the same SNP in the *lasR* gene that we detected in the evolved populations (V208G) (Table S2). We complemented BB8 with an intact copy of the PAO1 *lasR* gene in *trans* on a plasmid and then assessed β-lactam tolerance levels. We observed a decrease in tolerance to ceftazidime and piperacillin/tazobactam in the *lasR*-complemented BB8

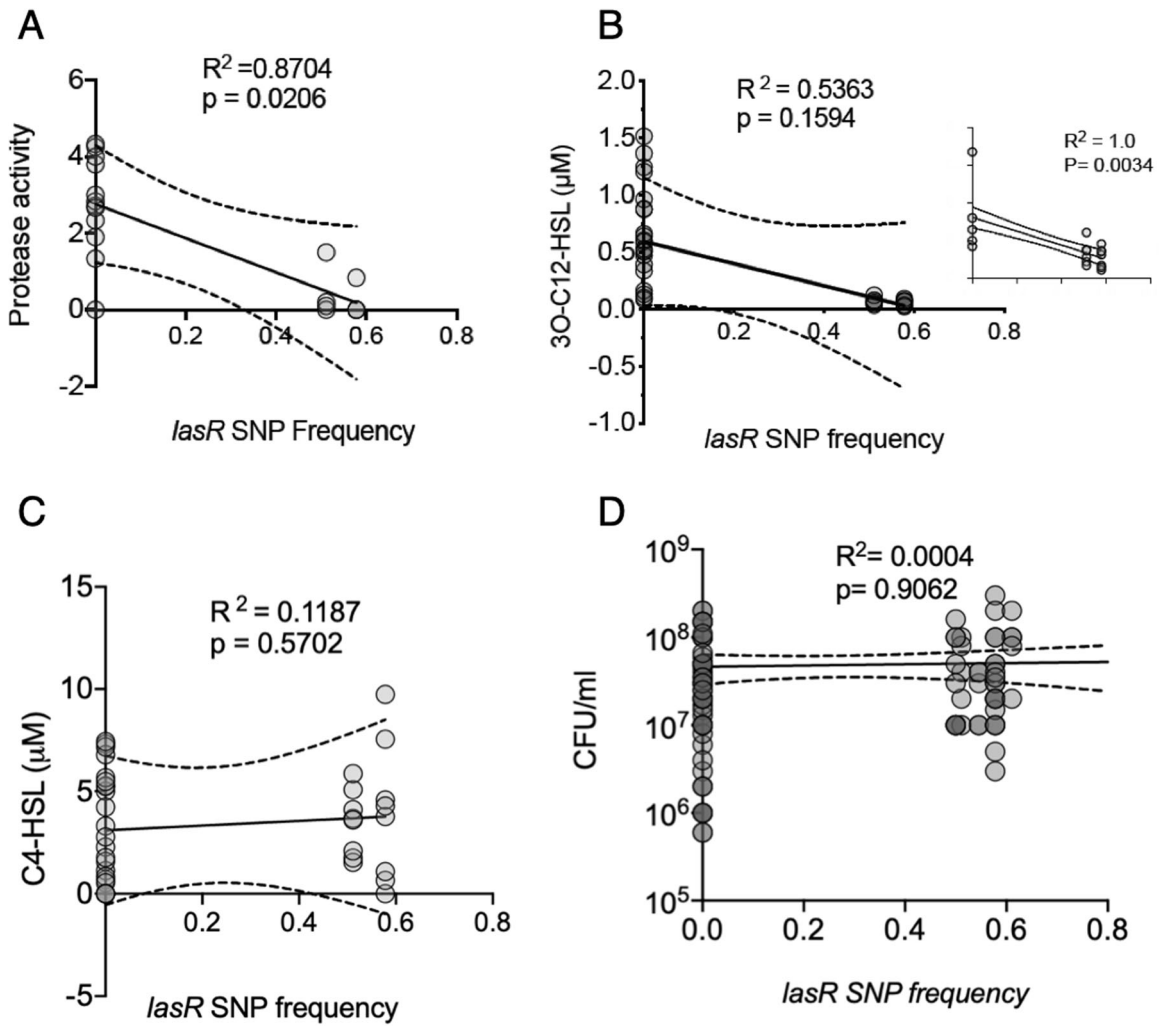
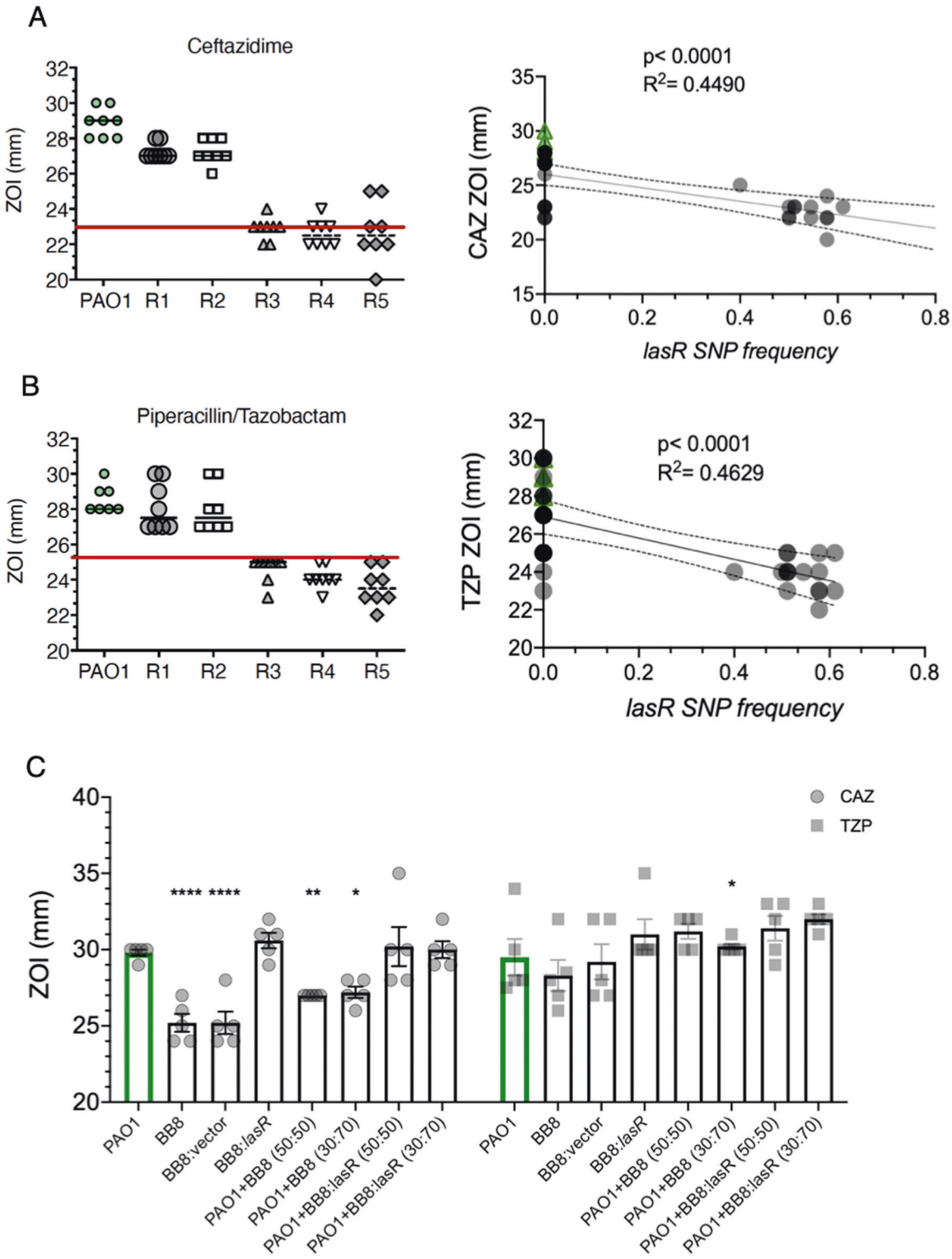


Fig. 4 Loss of protease activity and a decrease in QS signal production in evolved populations can be explained by an accumulation of *lasR* SNPs. A linear regression model showed that the loss of total protease activity and the decrease in 3O-C12-HSL levels can be explained by the accumulation of *lasR* SNPs. **a** There is a significant negative correlation between *lasR* SNP frequency and total protease activity of the evolved populations ($R^2 = 0.7298$, $p = 0.04$; $n = 8$). **b** There is no significant correlation between 3O-C12-HSL levels and accumulation of the *lasR* SNP in the evolved populations ($R^2 = 0.5363$, $p = 0.1594$; $n = 8$). However, if only changes in 3O-C12-HSL levels after 30 days of selection are considered, there is a strong correlation between emergence and accumulation of *lasR* SNP in evolved populations (small inset: $R^2 = 1.0$; $p = 0.0034$; $n = 8$). **c** There are no significant correlations between accumulation of *lasR* SNPs in the evolved populations and levels of C4-HSL ($R^2 = 0.1187$, $p = 0.5702$; $n = 8$). **d** There is no significant correlation between *lasR* frequency and biofilm formation ($R^2 = 0.0004$, $p = 0.9062$; $n = 8$).

isolate (Fig. 5c). Finally, we tested whether changes in the starting frequency of *lasR* mutants in mixed populations impacted on community functions. We found that when BB8 was mixed with wild-type cells, this resulted in an increased tolerance to ceftazidime (Fig. 5c) and a reduction in 3O-C12-HSL but not C4-HSL in mixed populations (Fig. S8).

Discussion

Despite a number of recent studies focused on adaptive changes in evolved populations of *P. aeruginosa* in environments designed to mimic CF sputum [35, 62–66], there remain significant gaps in knowledge about how genomic and phenotypic diversity impacts upon interactions within populations and on community-level phenotypes. In our current study, we focused on understanding how the evolution and coexistence of multiple lineages of *P. aeruginosa* shape community functions. To generate diversity, we performed a 50-day selection experiment, evolving PAO1 as biofilms in an artificial sputum medium (SCFM) [36–38]. We observed (i) a rise in genetic diversity after 10 days of selection in biofilms grown in SCFM; (ii) up to 25% of SNPs became fixed in the population and carried in evolved populations through 50 days of selection; (iii)



emergence and accumulation of SNPs in genes involved in motility, respiration, DNA mismatch repair, transcription, and QS emerged between 10 and 40 days of selection; (iv) accumulation of *lasR* SNPs correlated with decreased protease activity, 3O-C12-HSL production, and increased tolerance to β-lactam antibiotics, despite no prior treatment with any antibiotic.

The divergent evolution of *P. aeruginosa* during chronic infection of CF lungs has been the focus of numerous studies on longitudinal collections of sputum samples.

◀ **Fig. 5 Increased resistance to ceftazidime and piperacillin/tazobactam in evolved populations is correlated with *lasR* SNP frequency.** There is an increased resistance, indicated by a decrease in the zone of inhibition (ZOI) to ceftazidime (**a**) and piperacillin/tazobactam (**b**) in evolved populations after 30 days of selection (R3). The red lines represent the ZOI breakpoint (mm) for each antibiotic (BSAC guidelines). Below, the lines indicate resistance. There is a positive correlation between increased resistance and the frequency of the *lasR* SNP in the evolved populations ($n = 8$, Pearson correlation. Each dot represents the mean value for the zone of inhibition at each round of selection in all eight independent experiments). **c** Complementation of a 50-day evolved isolate (BB8) containing a SNP in *lasR* with an intact *lasR* in *trans*, restores sensitivity to ceftazidime and piperacillin/tazobactam. Differential frequencies of B8 mixed with ancestral PAO1 significantly increased tolerance to ceftazidime ($n = 5$, two-way ANOVA, multiple comparison to PAO1, Fisher's LSD test, $p < 0.05$. Errors bars indicate SEM).

Analysis of single *P. aeruginosa* isolates from these collections has identified genomic signatures for adaptation to the CF lung environment [9, 14, 29, 50, 53, 67, 68]. Similar studies on collections of *P. aeruginosa* isolates sourced from single-sputum samples, have shown considerable phenotypic and genetic diversification of *P. aeruginosa* strains that group into clades [16, 17], and which may colonize different ecological niches in CF lungs [28, 69]. All of these studies focused on the diversification, adaptation, and heterogeneity of *P. aeruginosa* during chronic CF lung infection. However, the impact that this heterogeneity may have on collective phenotypes has been largely overlooked. We hypothesized that the combination of wild-type and mutated alleles in populations shapes collective phenotypes. We observed that the accumulation and frequency of various SNPs in heterogeneous populations of *P. aeruginosa*, significantly impact the collective phenotypes of evolved populations (Figs. 2, 3). Although we observed that the majority of genomic changes occurred during the first 10 days of selection, the functional outcome was only impactful after 20–30 days, where we observed an increase in biofilm formation and colony morphotypes, and a significant decrease in total protease activity and production of 3O-C12-HSL by evolved populations.

The biofilm bead system has previously been used to examine the evolution of *P. aeruginosa* in biofilms [35], although there are significant differences between this and our study. First, the environmental conditions were different, with the previous study growing in a minimal media (M63) while we used SCFM. Second, we evolved PAO1, whereas the previous study evolved PA14 [35]. While both are popular model strains for *P. aeruginosa* study, they differ in a number of factors including virulence [5]. Third, we focused primarily on the emergent properties of the whole community rather than individual isolates, which we show have important implications for signaling and antimicrobial tolerance. Finally, the previous study studied the emergence of *mutL* and *mutS* genes that may have contributed to the emergence of different individual morphotypes [35]. We observed low-frequency mutations in the *mutS* gene after 30 days (Fig. S3). As these were synonymous mutations, they did not appear to significantly increase the mutation rate after 30 days of selection.

Our study also shows similarities with another recent study. Here the authors evolved PA14 in SCFM supplemented with mucin to create a structured environment [62]. The main findings of this study were that mucin promoted diversification of *P. aeruginosa* between and not within populations. The authors found increases in tolerance to antibiotics in evolved populations, and changes in growth, motility, pyocyanin production, and biofilm formation. They did not present genomic evidence that underpinned these changes [62].

Our study identified genomic changes often observed in *P. aeruginosa* isolates taken from CF lungs, including changes in secretion genes (*hcpA, hcpB, vgrG2a,* and *vgrG4b*), motility genes (*pilQ* and *pilN*), iron aquisition genes (*pvdD* and *pvdJ*), and biofilm formation (*cdrA*). We also observed a consistent change in all lines and rounds of selection in anaerobic respiratory pathways (Figs. 2 and S3). *ccoN2* (PA1557) encodes the cytochrome C oxidase subunit (ccb3 type). Increased expression of *ccoN2* in a small colony variant has been previously noted during microaerophilic conditions [70, 71].

One of the best-known signatures of *P. aeruginosa* adaptation to the CF lung environment is mutation in the QS regulator LasR. Several studies on *P. aeruginosa* strains isolated from CF sputum samples found significant genomic changes in *lasR*, including truncation, deletion, frameshifts, and SNPs, with many resulting in a loss of function [30, 40, 41, 50, 72]. Although there can be a high percentage of *lasR*-deficient isolates collected from patients, studies have also shown differential frequencies of functional and intact *lasR* alleles within patients [13, 73]. Considering the importance of *lasR*-dependent social interactions for the fitness of *P. aeruginosa*, mutation frequency of *lasR* within populations of *P. aeruginosa* could significantly impact on QS-dependent phenotypes and fitness at the population level [74–76]. Previously, we have demonstrated that an increase in the frequency of QS social cheats (*lasR* mutants) in defined populations of wild-type and *lasR* mutants, leads to a reduction in cooperation and virulence in mouse models of infection [74]. These studies showed that even simple mixed genotype populations of *P. aeruginosa* can have a significant impact on community function, virulence, and the outcome of infection.

In our current study, we observed a negative correlation between an increase in the frequency of the *lasR* V208G SNP in evolved populations and levels of protease activity and 3O-C12-HSL production (Fig. 4). Mutation of *lasR* in

P. aeruginosa isolates from CF lungs has also previously been shown to be important for increased tolerance to β-lactam antibiotics such as ceftazidime [19, 56, 61]. Here we examined whether the increase of *lasR* mutants in our evolved populations had an impact on the levels of antimicrobial tolerance. Despite no prior treatments with antibiotics, we observed an increased tolerance to three antibiotics from the β-lactam family (Fig. 5). In contrast, we found no correlation between increased biofilm production by evolved populations and antibiotic resistance (Fig. S7). Resistance of *P. aeruginosa* to antibiotics in chronic infections such as CF or wounds, is often thought to be due to specific mechanisms such as efflux pumps, or via production of excess polysaccharides such as alginate. Our findings suggest that the accumulation and frequency of genetic variants that might not traditionally be associated with resistance to drugs (e.g., QS mutants) within a heterogeneous population, can alter phenotypes within populations that can result in important clinical repercussions.

Our work suggests that in the future, we should consider metagenomic and metaphenotypic assessments of *P. aeruginosa* populations collected from CF patients, rather than focusing on single colonies. This is because the phenotype of populations is dictated by the frequencies of various alleles in the populations. Focusing on just single isolates sourced from infections or long-term evolution experiments, results in particular strains being characterized with certain phenotypes, which misrepresents what is found in the population as a whole. It becomes particularly problematic in studies focusing on single colonies from longitudinal samples, and when genomic sequencing predicts how a strain genetically evolves over time during an infection. Our findings may also be particularly relevant when considering whether a *P. aeruginosa* infection is resistant or sensitive to antibiotic treatments. Our findings may extend to other infections caused by *P. aeruginosa*, such as nonhealing chronic wounds, and they may also be relevant to other species of bacteria.

Materials and methods

Bacterial strains and growth conditions

For our experimental evolution, we used the PAO1 (University of Nottingham) strain of *P. aeruginosa*. For SCFM, we followed the protocol provided in refs. [36, 38]. Briefly for the buffer base, we prepared NaH_2PO_4 (1.3 mM), Na_2HPO_4 (1.2 mM), KNO_3 (0.348 mM), K_2SO_4 (0.271 mM), NH_4Cl (2.28 mM), and KCl (14.9 mM); NaCl (51.8 mM) was prepared in 10 mM of MOPS at pH = 6.8; then the amino acids were added [36, 38]. Dextrose (3 mM), L-lactic acid (9.3 mM), $CaCl_2$*2 H_2O (1.75 mM), $MgCl_2$*$6H_2O$ (0.606 mM), and $Fe.SO_4$*$7H_2O$ (0.0036 mM) were added fresh every time the media was prepared.

Long-term experimental evolution

To assess how genomic diversity impacts on *P. aeruginosa* populations, we generated a diverse population using a long-term evolution experimental approach. We evolved the *P. aeruginosa* strain PAO1 in biofilms using plastic beads [34, 35, 77] (9 × 6-mm width) suspended in SCFM, in order to mimic a biofilm life cycle and a chemical environment similar to that found in CF lung sputum. To start the experimental evolution process, we first grew PAO1 on an LB agar plate. Then we inoculated a single colony of PAO1 into 3 ml of fresh SCFM [38], and incubated for up to 6 h to grow up to the mid-log phase. We stored this mid-log-phase cells as the ancestral PAO1 and compared all further phenotypic and genomic properties of the evolved populations with this. We inoculated the mid-log-phase cells to $OD_{600} \approx 0.05$ into four tubes (in order to evolve separate independent lines named A–D) containing 3 ml of SCFM and a plastic bead. We incubated the cultures for 24 h at 37 °C/200 rpm. After 24 h of incubation, we transferred bacterial covered beads into fresh tubes containing 3 ml of SCFM and a new bead. After each round, the biofilms that formed on beads were composed of ~10^8 cells. We then incubated again for 24 h at 37 °C/200 rpm. We continued the bead transfers for 50 days and stored the biofilm portion of populations (attached to plastic beads) every 10 days (Rounds 1–5: R1–R5) (Fig. S1). We did not transfer or store the planktonic fractions at any point during the selection experiment.

Deep sequencing of evolved populations

We extracted genomic DNA from evolved populations after 18 h of growth in SCFM, using DNeasy® Blood & Tissue Kit (QIAGEN) by following the manufacturer's instructions. We prepared sequencing libraries using the NexteraXT protocol (Illumina), and sequenced in 24-plex on the Illumina MiSeq platform to obtain an approximate calculated level of coverage of 220–600× for each evolved population. A de novo assembly of the ancestral strain genome (*P. aeruginosa* PAO1 ancestor) was obtained using Spades with the –careful flag, and annotated using Prokka. We mapped reads of the evolved populations against the ancestral PAO1 genome using BWA [78]. For SNP calling, the sequences were summarized using a MATLAB script for base quality, genomic position, and mapping-quality [55] script using bowtie2 [79], SAMtools [80], and BAMtools. To determine the allele frequency, we applied the breseq polymorphism mode [81] to each of the samples collected. Later, for the variant sites, we used the composition in each nucleotide as the surrogacy for the allele

frequency, and we excluded any allele frequency below 10% in each evolved population. Our deep-sequence analysis was not designed to detect any insertions or deletions (INDELS), only SNPs. It is likely that INDELs were present in genes in our evolved populations.

Measurement of biofilms formed on beads

To determine the levels of biofilm formation by each evolved population, we grew biofilms on plastic beads as previously described [34, 77]. For each set of biofilm assays, we directly inoculated a 10-µl loop of frozen evolved populations (~10^6 cells) into 3 ml of SCFM and incubated at 37 °C/200 rpm for 16 h. Then we measured the OD_{600}, and diluted in 3 ml of SCFM to $OD_{600} \approx 0.05$. Then three plastic beads were added to each tube. Growth was monitored at 24 h at 37 °C/200 rpm and biofilm formation. The beads were then washed 3× with 10 ml of PBS to remove any residual planktonic cells not bound to the plastic beads. Then we transferred each plastic bead into 1 ml of PBS and sonicated the beads for 10 min, using a bath sonicator to detach biofilm-forming cells from the beads. We then serially diluted the cells and plated out onto LB agar plates for colony-forming unit (CFU) calculations. To assess other phenotypic traits, a cell-free supernatant of biofilm-forming cells was prepared from the liquid part of the cultures and corrected values for $OD_{600} \approx 1$.

Preparation of cell-free supernatants

To assess the levels of protease, QS signals, and siderophore production during biofilm formation, we collected 3 ml of the SCFM used in the biofilm assays. We used the media surrounding the bead biofilms, and measured the OD_{600} and adjusted it to 1 for all the cultures with SCFM. We then filtered the supernatants using 0.22-µm filters and used these cell-free supernatants to measure phenotypic traits.

Total protease activity

To assess the total protease activity of evolved populations, we used skimmed milk agar plates. We inoculated 10 µl of cell-free supernatant from each evolved population onto skimmed milk agar plates (1.2% Bacto Agar, 0.015% of skimmed milk) alongside 10 µl of 10 µg/ml of proteinase K and supernatant of PAO1 as controls. The zone of clearance was scored based on appearance and measured with a ruler (in mm). We then imaged each plate using an Epson scanner at 800 dpi. We then compared it with the zone of clearance produced by the PAO1 ancestor [82].

Siderophore production

To measure the levels of the two main siderophores produced by *P. aeruginosa*, we used the cell-free supernatants. In total, 100-µl aliquots of cell-free supernatant from evolved populations and the PAO1 ancestor was transferred into a black clear-bottom 96-well plate (Corning). We measured the emission as relative fluorescent units (RFU) using a multi microplate reader (Tecan Infinite® M200 Pro). We measured the wavelengths at excitation of 400 nm/emission 460 nm for pyoverdine and 350/430 nm for pyochelin [30, 83, 84]. We corrected the values for pyoverdine and pyochelin to the absorption at (OD_{600}) of the original cultures.

Measurement of C4-HSL and 3O-C12-HSL produced by evolved populations

The cell-free supernatants were used to determine the concentration of QS signals. We used two *E. coli* bioreporter strains to measure the production of the two main signal molecules by the evolved population. The *E. coli* reporters pSB536 and pSB1142 were used to detect C4-HSL and 3O-C12-HSL, respectively [85]. We calculated signal levels based on standard curves fitted to the concentrations of synthetic 3O-C12-HSL and C4-HSL standards (Sigma) [86, 87].

Mixed isolate experiments

For mixed constructed populations, we grew PAO1, the BB8-evolved isolate, and BB8 complemented with *lasR* (BB8:*lasR*) and empty vector (BB8:vector) in SCFM to $OD_{600} \approx 0.5$. We then mixed PAO1 and BB8 at two different starting frequencies (50:50 and 30:70) in 3 ml of SCFM. The cultures were then incubated for 16 h at 37 °C/200 rpm. We measured and adjusted the OD_{600} of each culture to $OD_{600} \approx 1$, and prepared a cell-free supernatant for QS signal activity measurements.

Antibiotic susceptibility assay

To determine the antibiotic susceptibility in evolved populations, we followed the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Version 14, 05-01-2015) using Isosensitest agar plates (Oxoid). We tested the susceptibility of evolved populations, PAO1 ancestral strain and the NCTC (10662) PAO1 strain to Gentamicin (10 µg), Meropenem (10 µg), Ciprofloxacin (1 µg), Ceftazidime (30 µg), Piperacillin/Tazobactam (85 µg), and Amikacin (30 µg) (Oxoid). The zone of inhibition and clearance in this method was compared with the available zone of inhibition

breakpoints for susceptibility (mm) for each tested antibiotic based on BSAC guidelines.

Determining colony morphology diversity in evolved populations

To determine the diversity in colony morphology in the biofilm-evolved population, we used a Congo Red-based agar media (1% agar, 1×M63 salts (3 g of monobasic $KHPO_4$, 7 g of K_2PO_4, and 2 g of $NH4.2SO_4$, pH adjusted to 7.4), 2.5 mM magnesium chloride, 0.4 mM calcium chloride, 0.1% casamino acids, 0.1% yeast extracts, 40 mg/l Congo red solution, 100 µM ferrous ammonium sulfate, and 0.4% glycerol) [82]. We recovered the evolved populations from beads and serially diluted the populations and then inoculated onto CRA plates alongside the PAO1 ancestor. We incubated the plates overnight at 37 °C, and for a further 4 days at 22 °C. The colonies were imaged using an Epson scanner at 800 dpi.

Complementation of *lasR*

To complement an evolved isolate (BB8) containing a *lasR* mutation with a functional *lasR* allele, we amplified a 920-bp product comprising *lasR* and 200 bp upstream of the *lasR* start codon that includes its native promoter, from genomic DNA isolated from wild-type ancestral PAO1. We cloned this PCR product into the shuttle vector pME6032, which replicates in both *E. coli* and *P. aeruginosa* [88] by Gibson assembly [89] using the commercially available NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, MA). We introduced the *lasR* complementation construct into BB8 by electroporation [90] and plating on selective media containing 300 µg/ml tetracycline. We then tested the susceptibility of the *lasR*-complemented strains to ceftazidime and piperacillin/tazobactam using the BSAC method. For mixed constructed populations, we grew PAO1, the BB8-evolved isolate, and BB8 complemented with *lasR* (BB8:*lasR*) and empty vector (BB8:vector) in SCFM to $OD_{600} \approx 0.5$. We then mixed PAO1 and BB8 at two different starting frequencies (50:50 and 30:70) in 1 ml of PBS, and tested for antibiotic tolerance by following the BSAC guidelines (Version 14, 05-01-2015) using Isosensitest agar plates (Oxoid).

Statistical analysis

For statistical analysis of the phenotypic assays, we used GraphPad Prism 8.0. For analysis of SNP frequency, we used R package 3.6. We used the Interactive Venn [91] to analyze shared SNPs within and between evolved populations.

Publication of genome sequencing

All sequences described in this paper have been uploaded to the NCBI SRA database (accession number PRJNA613708).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Adler FR, Liou TG. The dynamics of disease progression in cystic fibrosis. PLoS ONE. 2016;11:e0156752.
2. Cantin AM, Hartl D, Konstan MW, Chmiel JF. Inflammation in cystic fibrosis lung disease: pathogenesis and therapy. J Cyst Fibros. 2015;14:419–30.
3. Elborn JS. Cystic fibrosis. Lancet 2016;388:2519–31.
4. Jorth P, Ehsan Z, Rezayat A, Caldwell E, Pope C, Brewington JJ, et al. Direct lung sampling indicates that established pathogens dominate early infections in children with cystic fibrosis. Cell Rep. 2019;27:1190–204 e3.
5. Diggle SP, Whiteley M. Microbe profile: *Pseudomonas aeruginosa:* opportunistic pathogen and lab rat. Microbiology. 2020;166:30–3.
6. Frederiksen B, Koch C, Hoiby N. Antibiotic treatment of initial colonization with *Pseudomonas aeruginosa* postpones chronic infection and prevents deterioration of pulmonary function in cystic fibrosis. Pediatr Pulmonol. 1997;23:330–5.
7. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, Rosenfeld M, et al. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis. 2001;183:444–52.
8. Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol. 2009;44:547–58.
9. Hogardt M, Heesemann J. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. Int J Med Microbiol. 2010;300:557–62.
10. Doring G, Parameswaran IG, Murphy TF. Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. FEMS Microbiol Rev. 2011;35:124–46.
11. Filkins LM, O'Toole GA. Cystic fibrosis lung infections: polymicrobial, complex, and hard to treat. PLoS Pathog. 2015;11:e1005258.
12. Jelsbak L, Johansen HK, Frost AL, Thogersen R, Thomsen LE, Ciofu O, et al. Molecular epidemiology and dynamics of

Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients. Infect Immun. 2007;75:2214–24.
13. Wilder CN, Allada G, Schuster M. Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. Infect Immun. 2009;77:5631–9.
14. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, et al. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol. 2012;10:841–51.
15. Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. Nat Genet. 2015;47:57–64.
16. Darch SE, McNally A, Harrison F, Corander J, Barr HL, Paszkiewicz K, et al. Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. Sci Rep. 2015;5:7649.
17. Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, et al. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. Am J Respir Crit Care Med. 2015;191:775–85.
18. Wee BA, Tai AS, Sherrard LJ, Ben Zakour NL, Hanks KR, Kidd TJ, et al. Whole genome sequencing reveals the emergence of a *Pseudomonas aeruginosa* shared strain sub-lineage among patients treated within a single cystic fibrosis centre. BMC Genomics. 2018;19:644.
19. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, et al. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci USA. 2006;103:8487–92.
20. Khaledi A, Schniederjans M, Pohl S, Rainer R, Bodenhofer U, Xia B, et al. Transcriptome profiling of antimicrobial resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2016;60:4722–33.
21. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, et al. Adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-infection. PLoS ONE. 2014;9:e89614.
22. O'Toole GA. Cystic fibrosis airway microbiome: overturning the old, opening the way for the new. J Bacteriol. 2018;200:e00561–17.
23. Ahlgren HG, Benedetti A, Landry JS, Bernier J, Matouk E, Radzioch D, et al. Clinical outcomes associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* airway infections in adult cystic fibrosis patients. BMC Pulm Med. 2015;15:67.
24. Limoli DH, Whitfield GB, Kitao T, Ivey ML, Davis MR Jr., Grahl N, et al. *Pseudomonas aeruginosa* Alginate overproduction promotes coexistence with *Staphylococcus aureus* in a model of cystic fibrosis respiratory infection. MBio. 2017;8:e00186–17.
25. Radlinski L, Rowe SE, Kartchner LB, Maile R, Cairns BA, Vitko NP, et al. *Pseudomonas aeruginosa* exoproducts determine antibiotic efficacy against *Staphylococcus aureus*. PLoS Biol. 2017;15:e2003981.
26. O'Brien S, Fothergill JL. The role of multispecies social interactions in shaping *Pseudomonas aeruginosa* pathogenicity in the cystic fibrosis lung. FEMS Microbiol Lett. 2017;364:fnx128. https://doi.org/10.1093/femsle/fnx128.
27. Limoli DH, Hoffman LR. Help, hinder, hide and harm: what can we learn from the interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during respiratory infections? Thorax. 2019;74:684–92.
28. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, et al. Regional isolation drives bacterial diversification within cystic fibrosis lungs. Cell Host Microbe. 2015;18:307–19.
29. Marvig RL, Dolce D, Sommer LM, Petersen B, Ciofu O, Campana S, et al. Within-host microevolution of *Pseudomonas aeruginosa* in Italian cystic fibrosis patients. BMC Microbiol. 2015;15:218.
30. Jiricny N, Molin S, Foster K, Diggle SP, Scanlan PD, Ghoul M, et al. Loss of social behaviours in populations of *Pseudomonas aeruginosa* infecting lungs of patients with cystic fibrosis. PLoS ONE. 2014;9:e83124.
31. Bartell JA, Sommer LM, Haagensen JAJ, Loch A, Espinosa R, Molin S, et al. Evolutionary highways to persistent bacterial infection. Nat Commun. 2019;10:629.
32. Markussen T, Marvig RL, Gomez-Lozano M, Aanaes K, Burleigh AE, Hoiby N, et al. Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. MBio. 2014;5:e01592–14.
33. Didelot X, Walker AS, Peto TE, Crook DW, Wilson DJ. Within-host evolution of bacterial pathogens. Nat Rev Microbiol. 2016;14:150–62.
34. Poltak SR, Cooper VS. Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. ISME J. 2011;5:369–78.
35. Flynn KM, Dowell G, Johnson TM, Koestler BJ, Waters CM, Cooper VS. Evolution of ecological diversity in biofilms of *Pseudomonas aeruginosa* by altered cyclic diguanylate signaling. J Bacteriol. 2016;198:2608–18.
36. Palmer KL, Mashburn LM, Singh PK, Whiteley M. Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. J Bacteriol. 2005;187:5267–77.
37. Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. Proc Natl Acad Sci USA. 2015;112:4110–5.
38. Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol. 2007;189:8079–87.
39. Varga JJ, Barbier M, Mulet X, Bielecki P, Bartell JA, Owings JP, et al. Genotypic and phenotypic analyses of a *Pseudomonas aeruginosa* chronic bronchiectasis isolate reveal differences from cystic fibrosis and laboratory strains. BMC Genomics. 2015;16:883.
40. Feltner JB, Wolter DJ, Pope CE, Groleau MC, Smalley NE, Greenberg EP, et al. LasR variant cystic fibrosis isolates reveal an adaptable quorum-sensing hierarchy in *Pseudomonas aeruginosa*. MBio. 2016;7:e01513–16.
41. Kostylev M, Kim DY, Smalley NE, Salukhe I, Greenberg EP, Dandekar AA. Evolution of the *Pseudomonas aeruginosa* quorum-sensing hierarchy. Proc Natl Acad Sci USA. 2019;116:7027–32.
42. Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol. 1996;21:1137–46.
43. Gambello MJ, Iglewski BH. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J Bacteriol. 1991;173:3000–9.
44. Pearson JP, Pesci EC, Iglewski BH. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol. 1997;179:5756–67.
45. Gilbert KB, Kim TH, Gupta R, Greenberg EP, Schuster M. Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. Mol Microbiol. 2009;73:1072–85.
46. Diggle SP, Gardner A, West SA, Griffin AS. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos Trans R Soc Lond B Biol Sci. 2007;362:1241–9.
47. Bjarnsholt T, Jensen PO, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, et al. Quorum sensing and virulence of *Pseudomonas*

aeruginosa during lung infection of cystic fibrosis patients. PLoS ONE. 2010;5:e10115.
48. Bartell JA, Blazier AS, Yen P, Thogersen JC, Jelsbak L, Goldberg JB, et al. Reconstruction of the metabolic network of *Pseudomonas aeruginosa* to interrogate virulence factor synthesis. Nat Commun. 2017;8:14631.
49. Whiteley M, Diggle SP, Greenberg EP. Progress in and promise of bacterial quorum sensing research. Nature. 2017;551:313–20.
50. Ciofu O, Mandsberg LF, Bjarnsholt T, Wassermann T, Hoiby N. Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in mucA and/or lasR mutants. Microbiology. 2010;156:1108–19.
51. Diaz Caballero J, Clark ST, Coburn B, Zhang Y, Wang PW, Donaldson SL, et al. Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. MBio. 2015;6:e00981–15.
52. Feliziani S, Lujan AM, Moyano AJ, Sola C, Bocco JL, Montanaro P, et al. Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. PLoS ONE. 2010;5:e12669.
53. Nguyen D, Singh PK. Evolving stealth: genetic adaptation of *Pseudomonas aeruginosa* during cystic fibrosis infections. Proc Natl Acad Sci USA. 2006;103:8305–6.
54. Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. PLoS Genet. 2013;9:e1003741.
55. Lieberman TD, Flett KB, Yelin I, Martin TR, McAdam AJ, Priebe GP, et al. Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. Nat Genet. 2014;46:82–7.
56. LaFayette SL, Houle D, Beaudoin T, Wojewodka G, Radzioch D, Hoffman LR, et al. Cystic fibrosis-adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyperinflammatory responses. Sci Adv. 2015;1:e1500199.
57. Rumbaugh KP, Griswold JA, Hamood AN. Contribution of the regulatory gene lasR to the pathogenesis of *Pseudomonas aeruginosa* infection of burned mice. J Burn Care Rehabil. 1999;20:42–9.
58. Storey DG, Ujack EE, Rabin HR, Mitchell I. *Pseudomonas aeruginosa lasR* transcription correlates with the transcription of *lasA, lasB*, and *toxA* in chronic lung infections associated with cystic fibrosis. Infect Immun. 1998;66:2521–8.
59. Chowdhury N, Bagchi A. Molecular insight into the activity of LasR protein from *Pseudomonas aeruginosa* in the regulation of virulence gene expression by this organism. Gene 2016;580:80–7.
60. Kafle P, Amoh AN, Reaves JM, Suneby EG, Tutunjian KA, Tyson RL, et al. Molecular insights into the impact of oxidative stress on the quorum-sensing regulator protein LasR. J Biol Chem. 2016;291:11776–86.
61. D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Deziel E, Smith EE, et al. Growth phenotypes of *Pseudomonas aeruginosa lasR* mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol. 2007;64:512–33.
62. Schick A, Kassen R. Rapid diversification of *Pseudomonas aeruginosa* in cystic fibrosis lung-like conditions. Proc Natl Acad Sci USA. 2018;115:10714–9.
63. Fung C, Naughton S, Turnbull L, Tingpej P, Rose B, Arthur J, et al. Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum. J Med Microbiol. 2010;59:1089–100.
64. Behrends V, Geier B, Williams HD, Bundy JG. Direct assessment of metabolite utilization by *Pseudomonas aeruginosa* during growth on artificial sputum medium. Appl Environ Microbiol. 2013;79:2467–70.
65. Melnyk AH, McCloskey N, Hinz AJ, Dettman J, Kassen R. Evolution of cost-free resistance under fluctuating drug selection in *Pseudomonas aeruginosa*. mSphere. 2017;2:e00158–17.
66. Wong A, Rodrigue N, Kassen R. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Genet. 2012;8:e1002928.
67. Bielecki P, Komor U, Bielecka A, Musken M, Puchalka J, Pletz MW, et al. Ex vivo transcriptional profiling reveals a common set of genes important for the adaptation of *Pseudomonas aeruginosa* to chronically infected host sites. Environ Microbiol. 2013;15:570–87.
68. Ciofu O, Riis B, Pressler T, Poulsen HE, Hoiby N. Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrob Agents Chemother. 2005;49:2276–82.
69. Manfredi P, Jenal U. Bacteria in the CF lung: isolation drives diversity. Cell Host Microbe. 2015;18:268–9.
70. Kirisits MJ, Prost L, Starkey M, Parsek MR. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol. 2005;71:4809–21.
71. Arai H. Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. Front Microbiol. 2011;2:103.
72. Wang C, McPherson JR, Zhang LH, Rozen S, Sabapathy K. Transcription-associated mutation of lasR in *Pseudomonas aeruginosa*. DNA Repair. 2016;46:9–19.
73. Tai AS, Bell SC, Kidd TJ, Trembizki E, Buckley C, Ramsay KA, et al. Genotypic diversity within a single *Pseudomonas aeruginosa* strain commonly shared by Australian patients with cystic fibrosis. PLoS ONE. 2015;10:e0144022.
74. Rumbaugh KP, Trivedi U, Watters C, Burton-Chellew MN, Diggle SP, West SA. Kin selection, quorum sensing and virulence in pathogenic bacteria. Proc Biol Sci. 2012;279:3584–8.
75. Kohler T, Buckling A, van Delden C. Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations. Proc Natl Acad Sci USA. 2009;106:6339–44.
76. Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, West SA. Quorum sensing and the social evolution of bacterial virulence. Curr Biol. 2009;19:341–5.
77. Traverse CC, Mayo-Smith LM, Poltak SR, Cooper VS. Tangled bank of experimentally evolved Burkholderia biofilms reflects selection during chronic infections. Proc Natl Acad Sci USA. 2013;110:E250–9.
78. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754–60.
79. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9.
80. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25:2078–9.
81. Barrick JE, Colburn G, Deatherage DE, Traverse CC, Strand MD, Borges JJ, et al. Identifying structural variation in haploid microbial genomes from short-read resequencing data using breseq. BMC Genomics. 2014;15:1039.
82. Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-Bogart GZ, et al. *Pseudomonas aeruginosa* in vitro phenotypes distinguish cystic fibrosis infection stages and outcomes. Am J Respir Crit Care Med. 2014;190:289–97.
83. Kummerli R, Jiricny N, Clarke LS, West SA, Griffin AS. Phenotypic plasticity of a cooperative behaviour in bacteria. J Evol Biol. 2009;22:589–98.
84. Ankenbauer R, Sriyosachati S, Cox CD. Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect Immun. 1985;49:132–40.
85. Winson MK, Camara M, Latifi A, Foglino M, Chhabra SR, Daykin M, et al. Multiple N-acyl-L-homoserine lactone signal

molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA. 1995;92:9427–31.
86. Cornforth DM, Popat R, McNally L, Gurney J, Scott-Phillips TC, Ivens A, et al. Combinatorial quorum sensing allows bacteria to resolve their social and physical environment. Proc Natl Acad Sci USA. 2014;111:4280–4.
87. Popat R, Pollitt EJ, Harrison F, Naghra H, Hong KW, Chan KG, et al. Conflict of interest and signal interference lead to the breakdown of honest signaling. Evolution. 2015;69:2371–83.
88. Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, et al. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. Mol Plant Microbe. 2000;13:232–7.
89. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6:343–U41.
90. Choi KH, Kumar A, Schweizer HP. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods. 2006;64:391–7.
91. Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinforma. 2015;16:169.