Allelic polymorphism shapes collective phenotypes in evolving *Pseudomonas aeruginosa* populations

Sheyda Azimi[a], Aled E. L. Roberts[b], Shengyun Peng[a], Joshua S. Weitz[a,c], Alan McNally[d], Samuel P. Brown[a] and Stephen P. Diggle[a*]

[a] *Center for Microbial Dynamics & Infection, School of Biological Sciences, Georgia Institute of Technology, Atlanta, U.S.A.;* [b]*Microbiology & Infectious Diseases Group, Institute of Life Science, Swansea University Medical School, Swansea, U.K.;* [c] *School of Physics, Georgia Institute of Technology, Atlanta, U.S.A;* [d] *Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, U.K.*

*Correspondence: stephen.diggle@biosci.gatech.edu

Keywords: diversity, quorum sensing, *Pseudomonas aeruginosa*, population phenotype

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that chronically infects cystic fibrosis (CF) lungs and chronic wounds by forming antibiotic resistant multi-cellular biofilms. Emergence of phenotypically diverse isolates within *P. aeruginosa* infections has previously been reported, however, the impact of this heterogeneity on social behaviors is poorly understood. Here we test how this phenotypic and genomic diversity specifically impacts on traits by evolving the strain PAO1 in biofilms grown in a synthetic sputum medium for 50 days. We measured social trait production and used a metagenomic approach to analyze and assess phenotypic and genomic changes over the duration of the evolution experiment. We hypothesized that we would observe parallel phenotypic and genomic diversification in independently evolving lines, and that diversity would have an impact on collective phenotypes in evolved populations. 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 more specifically on quorum sensing (QS), a well-studied *P. aeruginosa* social 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 production of QS and QS-dependent traits due to accumulation of a SNP in the *lasR* gene; (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 the clinically relevant β-lactam antibiotics ceftazidime and piperacillin/tazobactam, despite no previous antibiotic exposure. Overall, our findings provide insights into the impact of allele polymorphism on the collective phenotypes of diverse *P. aeruginosa* populations. Further, we demonstrate that *P. aeruginosa* population and evolutionary dynamics can impact on traits important for virulence, and could also provide reasons for antibiotic resistance found in CF infections.

Introduction

The cystic fibrosis (CF) lung is a spatially complex and inflamed environment that also provides beneficial growth conditions for a number of bacterial species, including the opportunistic pathogen *Pseudomonas aeruginosa* (1-5). Long-term and chronic infection of CF lungs with highly adapted and antibiotic resistant biofilms of *P. aeruginosa* is a major cause of decline in lung function, which results in a concomitant increase in morbidity and mortality in individuals with CF (5-15). 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 (16-20). Studies on *P. aeruginosa* populations isolated from individual lungs, have demonstrated divergent evolution, resulting in heterogeneous populations of *P. aeruginosa* within individual patients (19, 21, 22). This genetic adaptation and diversification is likely to impact on levels of pathogenicity and the efficacy of antibiotic treatment (19, 23, 24), and could potentially impact how other species of microbe colonize the CF lung (25-33).

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 (34, 35). Major adaptations of *P. aeruginosa* to the CF lung include alginate production, loss of quorum sensing (QS), hypermutability and increased resistance to antimicrobials (7, 9, 17, 36-39). Heterogeneity in *P. aeruginosa* populations has also been explained by early divergent evolution and adaptation to differential ecological niches (40, 41) and recombination between isolates residing in the airways (19, 42), but the exact mechanisms leading to heterogeneity have yet to be fully elucidated. Further, whilst it is accepted that genomic heterogeneity arises, it remains unknown how genotypic changes shape collective phenotypes of whole *P. aeruginosa* populations.

We hypothesized that the combination of wild-type and mutated alleles in populations shapes collective phenotypes, which could result in clinically-relevant outcomes such as antibiotic resistance. To test this, we first evolved *P. aeruginosa* PAO1 in biofilms on plastic beads (43) for up to 50 days, in a synthetic CF sputum media (SCFM), which recapitulates the chemical environment found in CF sputum (44-46). Our experimental plan allowed us to generate phenotypically and genotypically diverse populations of *P. aeruginosa* in a spatially structured environment chemically relevant to CF sputum. We then 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 measured collective phenotypic traits of the evolved populations.

One of the most common known 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 (22, 47-49). 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) (50-53). LasR-bound 3O-C12-HSL controls the transcription of 5-10 % of the *P. aeruginosa* genome, including a number of genes involved in social behaviours, pathogenesis, antibiotic resistance and biofilm formation (10, 54-59). Despite our knowledge of the changes and adaptation of various lineages of *P. aeruginosa* in CF lungs, it remains unclear how polymorphisms in the *lasR* gene impacts on the overall phenotypes of evolving heterogenous

P. aeruginosa populations. This is generally 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 (16-18, 60).

Overall we found that (i) evolutionary trajectories were reproducible between independently evolving populations and that over 60% of genomic diversity 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 the increase in tolerance to β-lactam antibiotics. Our findings provide insights into how allelic polymorphism and diversity in general, can impact on phenotypes within evolving *P. aeruginosa* populations. Further, we demonstrate that changes in *P. aeruginosa* population dynamics can potentially impact on virulence, and provide explanations for antibiotic resistance even in situations when antibiotics have not been used.

Results

Genomic variation in evolving biofilm populations over 50 days selection in SCFM. We evolved the *P. aeruginosa* strain PAO1 for 50 days (≈ 800 generations) in biofilms using a previously described bead method (43), 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 (44-46). Our experimental evolution approach contained four-independent replicate lines (Fig. S1). We collected and stored evolved biofilm populations after 10, 20, 30, 40 and 50 days of evolution (Rounds 1 to 5: R1 – R5). We used the Illumina MiSeq platform to deep sequence evolving populations in order to determine genomic changes through time. We also sequenced the 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 (61). 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; Fig. S2). We found that around 60 % of these SNPs, were present through all other rounds of selection (Fig. 1B). 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). We found that between 25-40% of all SNPs were fixed (frequency of 1) in the populations over 50 days of selection across all four replicate evolution lines (Fig. 2A-D). 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 (Fig. 2A-D; Fig. S3). 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 0.4-0.5 in different rounds of selection. We detected a number of SNPs occurring in *mutS* (PA3620), *pilQ* (PA5040), *pilN* (PA5043) between 20 and 30 days of selection in all four independent evolved lines. We observed an increase in *lasR* (PA1430) mutant allele frequency after 30 days (Fig. 2A-D; Fig. S3).

Accumulation of SNPs shapes the collective phenotype of evolved *P. aeruginosa* populations. We next examined the production of phenotypic social traits in evolved populations in order to determine changes in collective phenotypes of the genetically heterogenous evolving populations. We measured 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 to the PAO1 ancestor (Fig. 3A). After 30 days of selection, 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. S4).

To determine whether the emergence and increase in frequency of SNPs in the populations impact upon collective phenotypes, we utilized 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 (36, 48, 52, 58, 62) and also, *lasR* mutants are regularly isolated from CF sputum (63-65). We detected a nonsynonymous SNP (V208G) in the DNA binding domain of LasR after 30 days of selection. Interestingly, SNPs in the same position (V208) have been identified in *P. aeruginosa* isolates collected from CF sputum samples (48). V208 is adjacent to the D209 residue in the LasR DNA-binding domain (66, 67), 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).

Increased tolerance to β-lactams is significantly correlated with increased frequency of *lasR* mutants in evolved populations. Previously it has been shown that *lasR* mutants display an increased β-lactamase activity and therefore increased resistance to β-lactam antibiotics (such as ceftazidime) (68) which 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 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 resistance (indicated by a decrease in the zone of inhibition) to three antibiotics; ceftazidime, piperacillin/tazobactam and meropenem which are all β-lactam class antibiotics (Fig. 5; Fig. S5). We then tested for correlations between 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 and B). We also tested whether the increased resistance could

be due to an increase in biofilm production (Fig. S6). We found there was no significant correlation, suggesting that increases in biofilm formation do not necessarily translate to an increase in drug resistance.

Discussion

Despite a number of recent studies focused on adaptive changes in evolved populations of *P. aeruginosa* in environments designed to mimic CF sputum (69-73), there remain significant gaps in knowledge about how genomic and phenotypic diversity impacts upon on interactions within populations and on population-level phenotypes. In our current study, we focused on understanding how the evolution and coexistence of multiple lineages of *P. aeruginosa* shape collective phenotypes. To generate diversity, we performed a 50-day selection experiment, evolving PAO1 as biofilms in an artificial sputum medium (SCFM) (44-46). We observed (i) a rise in genetic diversity after 10 days of selection in biofilms grown in SCFM; (ii) up to 40 % 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 20 and 30 days of selection and (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. Analysis of single *P. aeruginosa* isolates from these collections has identified genomic signatures for adaptation to the CF lung environment (10, 17, 35, 37, 63, 74-76). Similar studies on collections of *P. aeruginosa* isolates sourced from single sputum samples, have shown considerable phenotypic and genetic diversification of *P. aeruginosa* strains which group into clades (19, 22) and which may colonize different ecological niches in CF lungs (34, 77). 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 over-looked. 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 heterogenous populations of *P. aeruginosa,* significantly impacts the collective phenotypes of evolved populations (Fig. 2; Fig. 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 a significant increase in biofilm formation and colony morphotypes and a significant decrease in total protease activity and production of 3O-C12-HSL by evolved populations.

One of 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, frame shifts and SNPs, with many resulting in a loss of function (36, 48, 49, 63, 78, 79). 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 (53, 65, 80). 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 (81-83). 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 (81, 84). These studies showed that even simple mixed genotype populations of *P. aeruginosa* can have a significant impact on population phenotypes, 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 (21, 68, 85). Here we examined whether the increase of *lasR* mutants in our evolved populations had an impact on 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; Fig. S5). In contrast, we found no correlation between increased biofilm production by evolved populations and antibiotic resistance (Fig. S6). 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 heterogenous population, can alter phenotypes within populations that can result in important clinical repercussions.

The 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 mispresents 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 non-healing chronic wounds and they may also extend 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 (44, 46). Briefly for the buffer base we prepared NaH_2PO_4 (1.3 mM), Na_2HPO4 (1.2 mM), KNO_3 (0.348 mM), K_2SO_4 (0.271 mM), NH_4Cl (2.28 mM), KCl (14.9 mM), NaCl (51.8 mM) was prepared in 10mM of MOPS at pH=6.8, then the amino acids were added(44, 46) . The Dextrose (3mM), 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) was 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 (43, 86) 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 (46), and incubated for up to 6 h to grow up to mid-log phase. We diluted mid-log phase cells to $OD_{600} \approx 0.05$ into 4 tubes (in order to evolve separate independent lines: A-D) containing 3 ml of SCFM and a plastic bead (9x6 mm width); and incubated for 24 h at 37 °C/200 rpm. We stored the rest of the mid-log phase cells as the ancestor culture and used it to measure ancestor phenotypes. After 24 h of incubation, we transferred beads into fresh tubes containing 3 ml of SCFM and a new bead. We then incubated again for 24 h at 37 °C/200 rpm. We continued the bead transfers for 50 days and stored samples from populations every 10 days (Rounds 1 to 5: R1-R5) (Fig. S1).

Deep sequencing of evolved populations. We extracted Genomic DNA from evolved populations after 18 h 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 250× for each evolved population. A de novo assembly of the ancestral strain genome 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 (87), and the sequences were summarized using a MATLAB for base quality, genomic position and mapping quality (61) script. To determine the allele frequency, we applied the breseq consensus model (88) to each of the samples collected. Specifically, we used *Pseudomonas aeruginosa* PAO1 (accession: NC_002516.2) as the reference genome and we mapped sequences from both ends to the reference genome. Later, for the variant sites, we used the composition in each nucleotide as the surrogacy for the allele frequency.

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 (43, 86). For each set of biofilm assays, we directly inoculated a 10 µl loop of frozen evolved populations into 3 ml of SCFM and incubated at 37°C/200 rpm for 16 h. Then we measured the OD_{600}, and diluted in 3ml SCFM to $OD_{600} \approx 0.05$. Then 3 plastic beads were added to each tube. After 24 h growth at 37°C/200 rpm and biofilm formation; we removed the planktonic liquid part of the cultures to prepare the cell free supernatants that we used to measure other phenotypic traits. The beads were then washed 3× with 10 ml of PBS to remove the cells that were not bound to the plastic beads. Then we transferred each plastic bead into 1ml of PBS and sonicated the

beads for 10 mins, 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.

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 for the formation of biofilms on beads. We 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 10µl of 10µg/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 800dpi. We then compared it to the zone of clearance produced by the PAO1 ancestor (89).

Siderophore production. To measure the levels of the two main siderophores produced by *P. aeruginosa*, we used the cell free supernatants. 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/emission460 nm for Pyoverdine and 350/430 nm for Pyochelin (36, 90, 91). 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 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 (92). We calculated signal levels based on standard curves fitted to the concentrations of synthetic 3O-C12-HSL and C4-HSL standards (Sigma) (93, 94).

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 to 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, 1xM63 salts (3g monobasic KHPO4, 7g K2PO4, 2g NH4.2SO4, pH adjusted to 7.4), 2.5mM magnesium chloride, 0.1% casamino acids, 0.1% yeast extracts, 40 mg/L Congo red solution, 100 µM ferrous ammonium sulphate and 0.4% glycerol) (89). 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 800dpi.

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 (95) to analyze shared SNPs within and between evolved populations.

Acknowledgements. For funding we thank the Human Frontier Science Program (RGY0081/2012) and Georgia Institute of Technology; The Cystic Fibrosis Foundation (DIGGLE18IO) to SPD; Cystic Fibrosis Foundation for a Fellowship to SA; CF@latna for a Fellowship to SA. The National Heart Lung Blood Institute (R56HL142857) and The Simons Foundation (396001) to SPB. We acknowledge Freya Harrison and James Gurney for helpful comments on the work.

Figure legends

Figure 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. The Venn diagram shows the shared SNPs between all 4 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 independently evolved lines; (B) The majority of genetic variation within the populations occurred within the first 10 days of selection. Each circle represents the total number SNPs for each independently evolved line.

Figure 2. Allele frequency of SNPs changes over the course of selection. Allele frequency analysis of all evolved populations showed that 45 genes were affected in the selection experiments (Fig. S3). Different haplotypes emerged after 10 days of selection at varying frequencies within the populations. Each round of selection contained varying frequencies of SNPs. Over 25-40% of SNPs emerged at 10 days of selection were fixed in populations, across all four independently evolving populations (A-D). 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 represents replicate evolving lines).

Figure 3. Phenotypes of evolved populations of PAO1. (A) Increase in biofilm formation by evolved populations of PAO1 (Kurskal-Wallis, Dunn's multiple comparison to PAO1, $p < 0.05$, n=5); (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) 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 to the PAO1 ancestor (Kruskal- Wallis, uncorrected Dunn's test multiple comparison to PAO1, $p > 0.05$, n=5).

Figure 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 ($R^2 = 0.8704$, $p = 0.0206$; n=5); (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 is 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).

Figure 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 indicates resistance. There is a correlation between increased resistance and the frequency of the *lasR* SNP in the evolved populations.

Supplementary figure legends

Figure S1. Long term evolution of PAO1 in biofilms and SCFM. PAO1 was grown in SCFM and inoculated into four independent replicate lines in the presence of one plastic bead (6×7 mm) to enable the bacteria to form biofilms on the surface. Every 24 hours the beads were transferred to a new tube with fresh SCFM media and a new bead. After 10 days, the bacterial populations were sonicated off the beads and stored as round 1 (R1) and used as the ancestor population for the next round (10 days) of selection. This was repeated for 50 days (5 rounds of selection).

Figure S2. Number of SNPs shared in all evolved lines during 50 days of evolution in SCFM. We tested the number and percentages of the SNPS that occurred and are shared between various evolution lines at different rounds of selections. There are 207 SNPs that are shared between all 4 lines of selection (A-D) at day 10, 205 on day 20, 206 on day 30, 172 on day 40 and 169 on day 50 (upper panels). We observed that the majority of SNPs occurred during the first 10 days of selection and are shared and carried over to subsequent rounds of selection in all evolution lines.

Figure S3. Number of SNPs in genes over 50 days of selection in SCFM. The emergence of polymorphisms in 45 genes involved in various physiological functions. The shaded boxes represent the abundance of SNPs in each gene.

Figure S4. Increase in diversity of colony morphologies in evolving populations over time. Diversity of colony morphology increased over time in evolutionary lines. We used Congo Red Agar plates to highlight differences in morphologies between haplotypes.

Figure S5. Increased resistance to β-lactam antibiotics in evolved populations after 30 days of selection. (A) There was a decrease in the ZOI to all β-lactam antibiotics after 30 days of selection in evolved populations; (B) There is an increase in resistance to meropenem after 30 days of selection in the evolved population, which significantly correlates to accumulation of the *lasR* SNP in the populations. However, the increased resistance does not pass the threshold to be considered clinically resistant to meropenem.

Figure S6. Biofilm tolerance and antibiotic resistance are not linked. There was no correlation with the levels of biofilm formation or antibiotic resistance at the population level.

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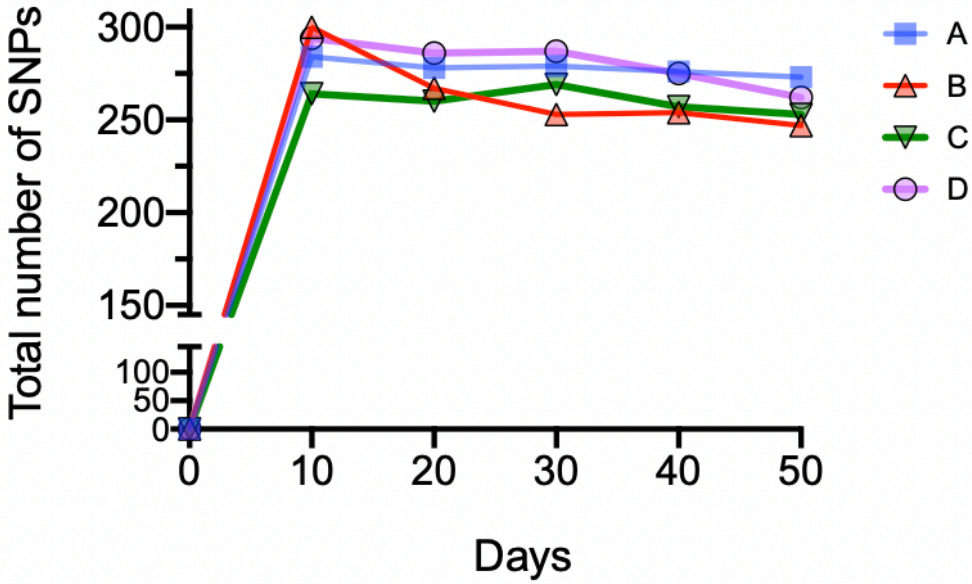
A


Total number of SNPs
300
250
200
150
100
50
0
0
10
20
30
40
50
Days
A
B
C
D

B

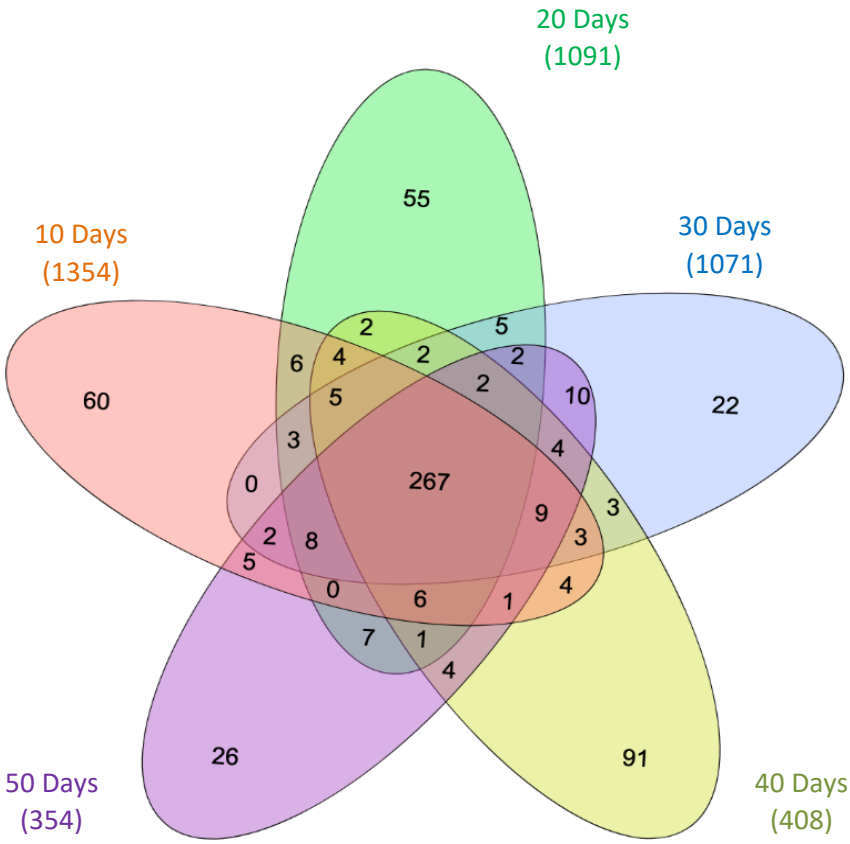

20 Days
(1091)
10 Days
(1354)
30 Days
(1071)
50 Days
(354)
40 Days
(408)
55
60
22
26
91
267

Figure 1

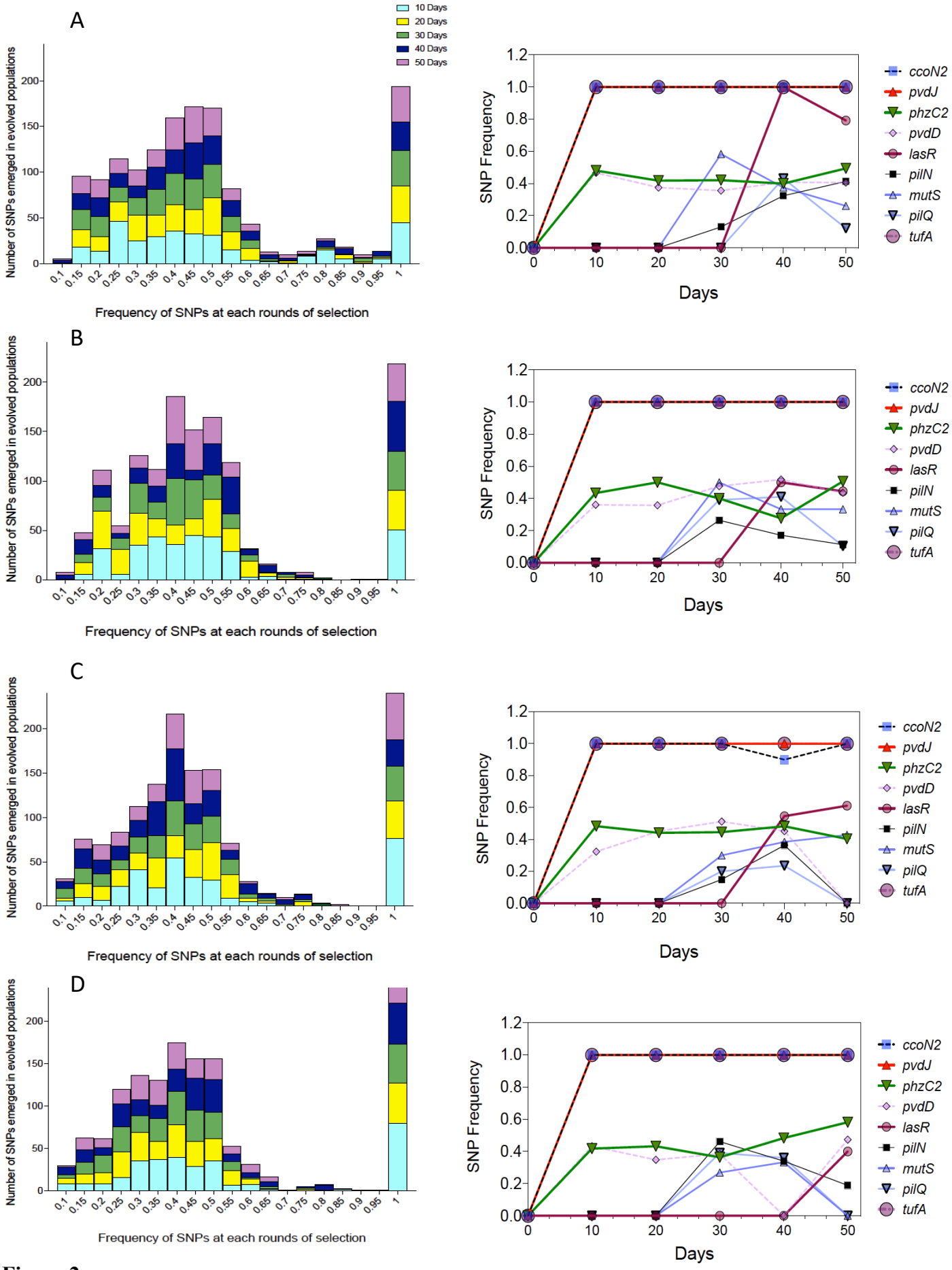

A
B
C
D
10 Days
20 Days
30 Days
40 Days
50 Days
Number of SNPs emerged in evolved populations
Frequency of SNPs at each rounds of selection
SNP Frequency
Days
ccoN2
pvdJ
phzC2
pvdD
lasR
pilN
mutS
pilQ
tufA

Figure 2

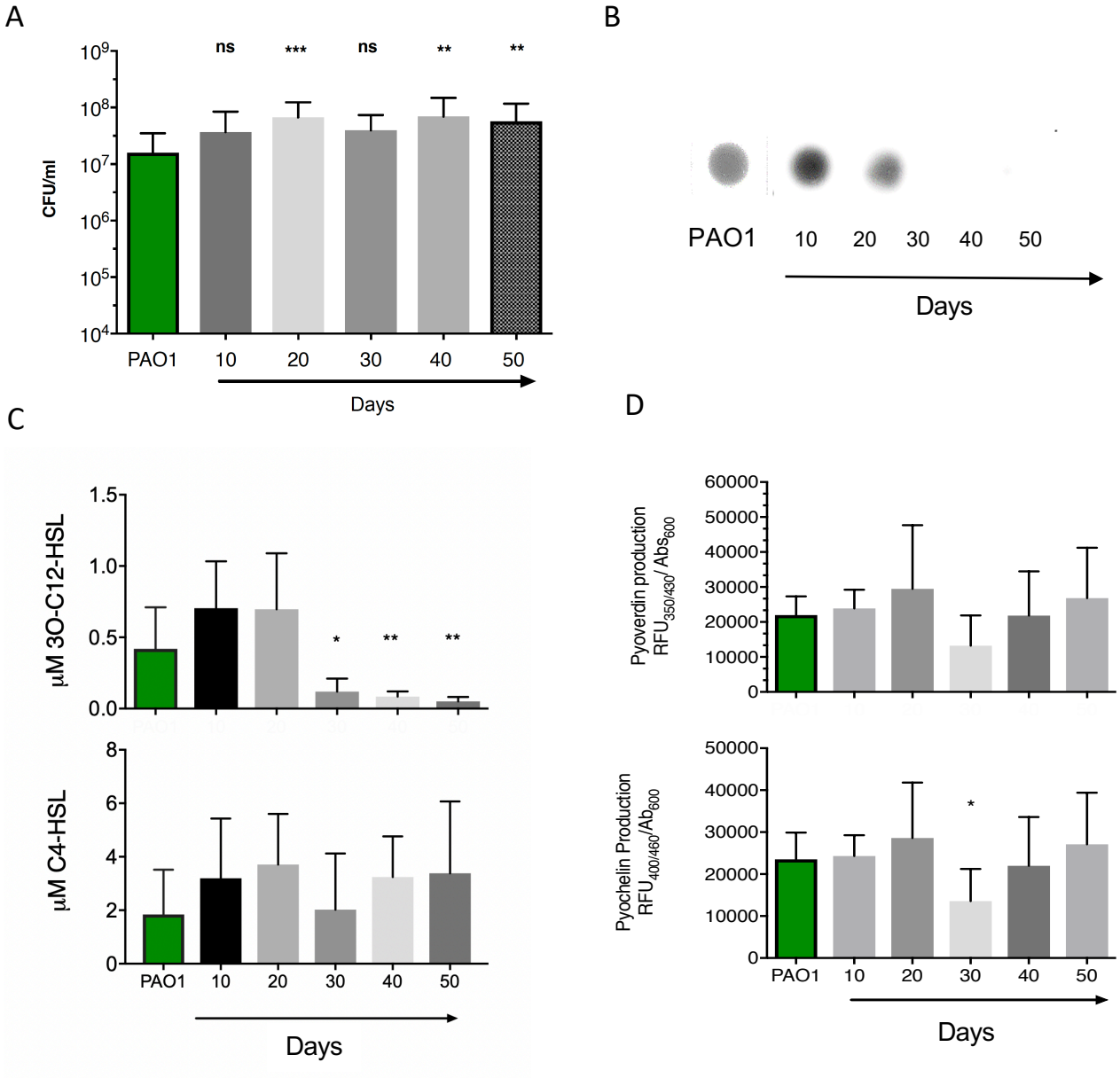
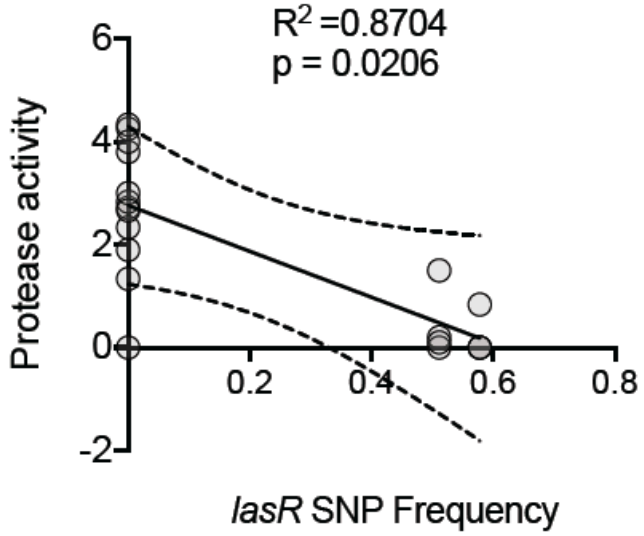
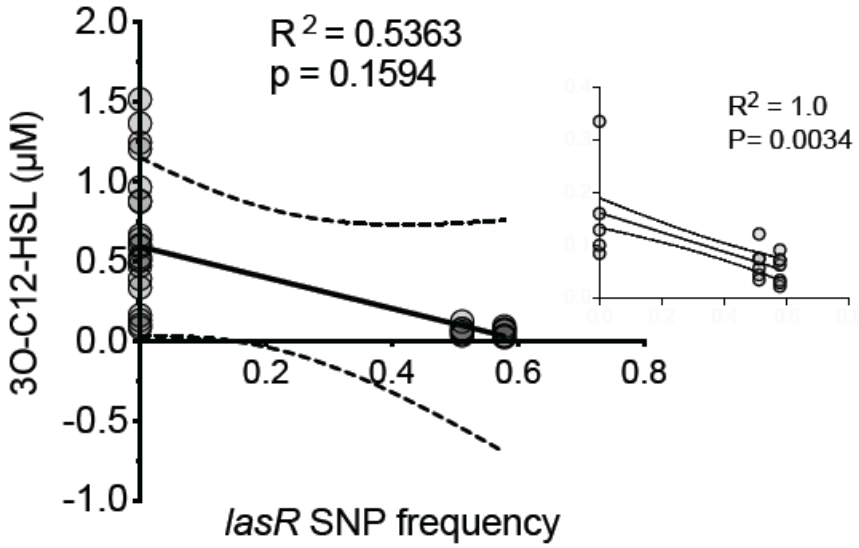

A
ns
***
ns
**
**
CFU/ml
PAO1
10
20
30
40
50
Days
B
PAO1
10
20
30
40
50
Days
C
µM 3O-C12-HSL
*
**
**
µM C4-HSL
PAO1
10
20
30
40
50
Days
D
Pyoverdin production
RFU350/430/ Abs600
Pyochelin Production
RFU400/460/Ab600
*
PAO1
10
20
30
40
50
Days

Figure 3

A


R2 =0.8704
p = 0.0206
Protease activity
6
4
2
0
-2
0.2
0.4
0.6
0.8
lasR SNP Frequency

B


R 2 = 0.5363
p = 0.1594
3O-C12-HSL (μM)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0.2
0.4
0.6
0.8
lasR SNP frequency
R2 = 1.0
P= 0.0034

C

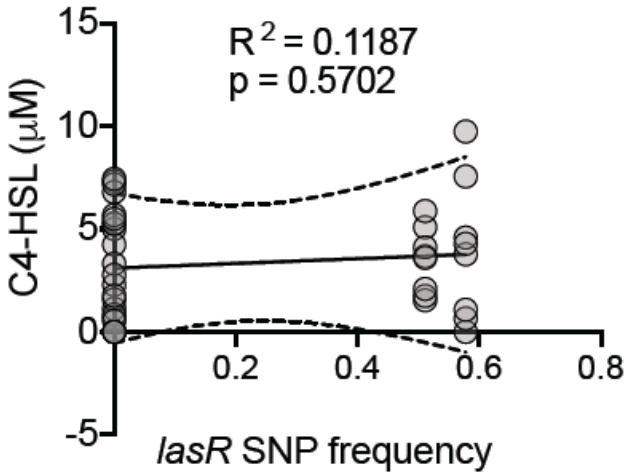

R 2 = 0.1187
p = 0.5702
C4-HSL (μM)
15
10
5
0
-5
0.2
0.4
0.6
0.8
lasR SNP frequency

Figure 4

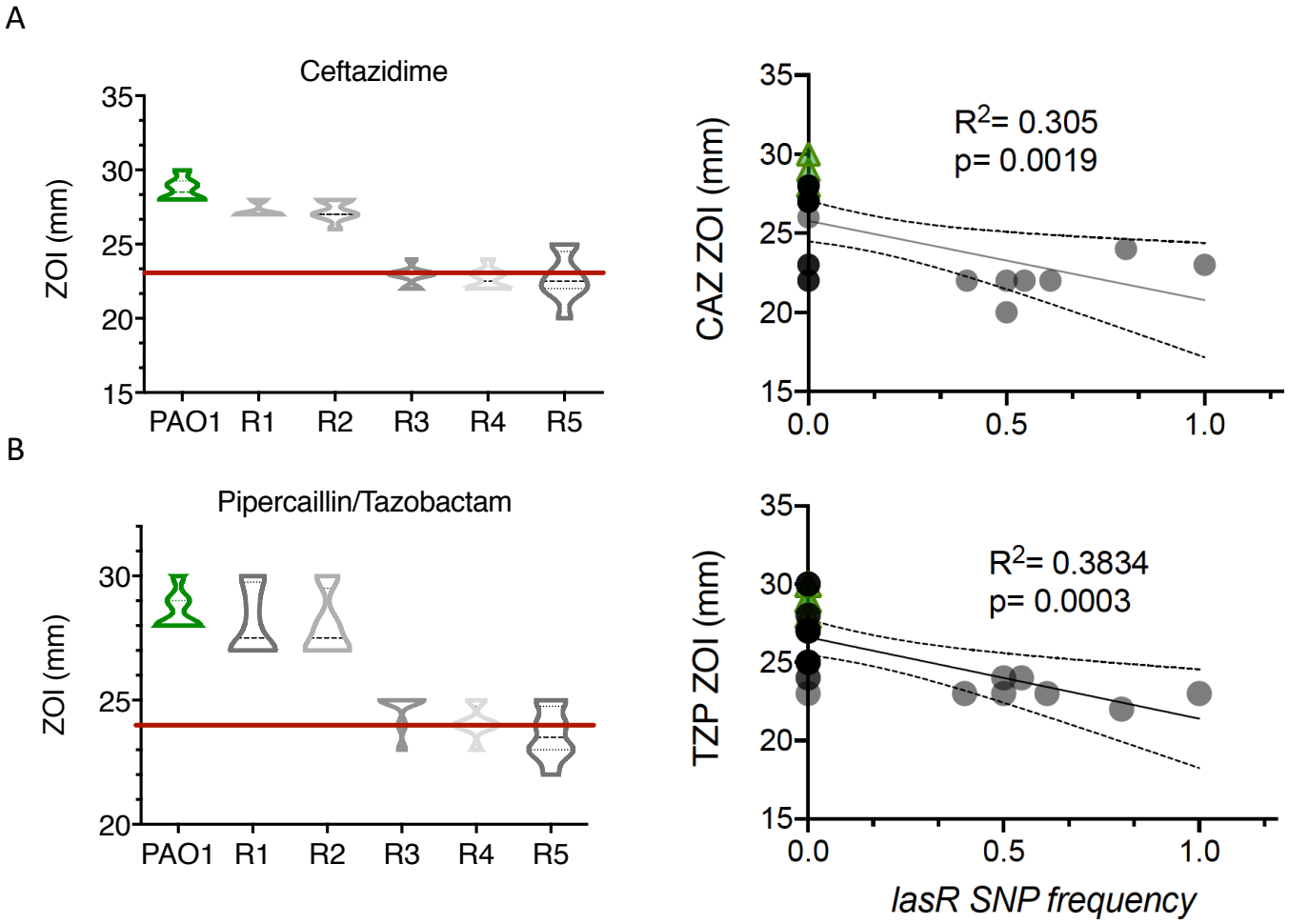

A
Ceftazidime
ZOI (mm)
35
30
25
20
15
PAO1
R1
R2
R3
R4
R5
CAZ ZOI (mm)
R2= 0.305
p= 0.0019
0.0
0.5
1.0
B
Pipercaillin/Tazobactam
ZOI (mm)
30
25
20
PAO1
R1
R2
R3
R4
R5
TZP ZOI (mm)
R2= 0.3834
p= 0.0003
lasR SNP frequency

Figure 5

Supplementary figures

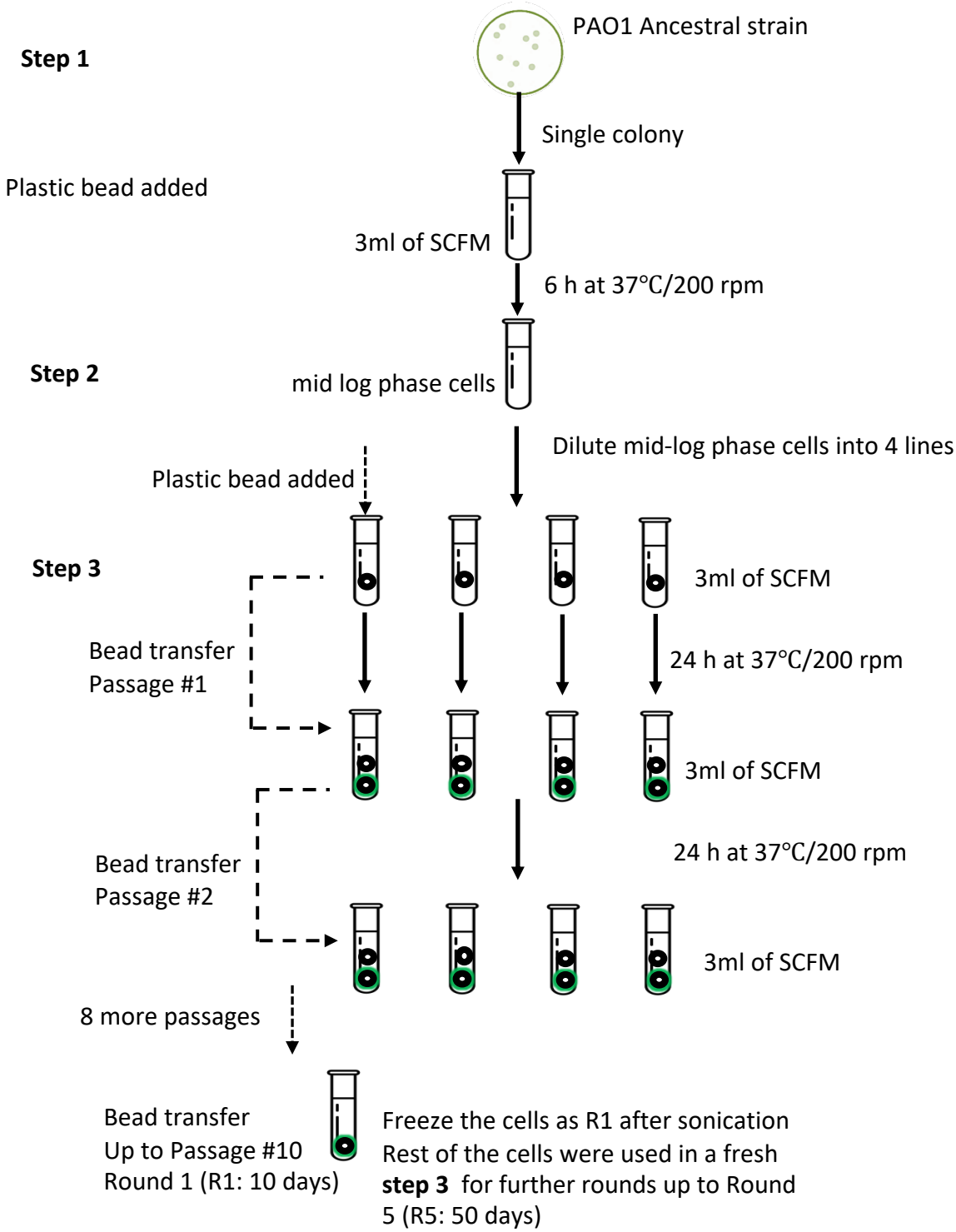

Step 1
PAO1 Ancestral strain
Single colony
Plastic bead added
3ml of SCFM
6 h at 37°C/200 rpm
Step 2
mid log phase cells
Dilute mid-log phase cells into 4 lines
Plastic bead added
Step 3
3ml of SCFM
Bead transfer
Passage #1
24 h at 37°C/200 rpm
3ml of SCFM
Bead transfer
Passage #2
24 h at 37°C/200 rpm
3ml of SCFM
8 more passages
Bead transfer
Up to Passage #10
Round 1 (R1: 10 days)
Freeze the cells as R1 after sonication
Rest of the cells were used in a fresh
step 3 for further rounds up to Round
5 (R5: 50 days)

Figure S1

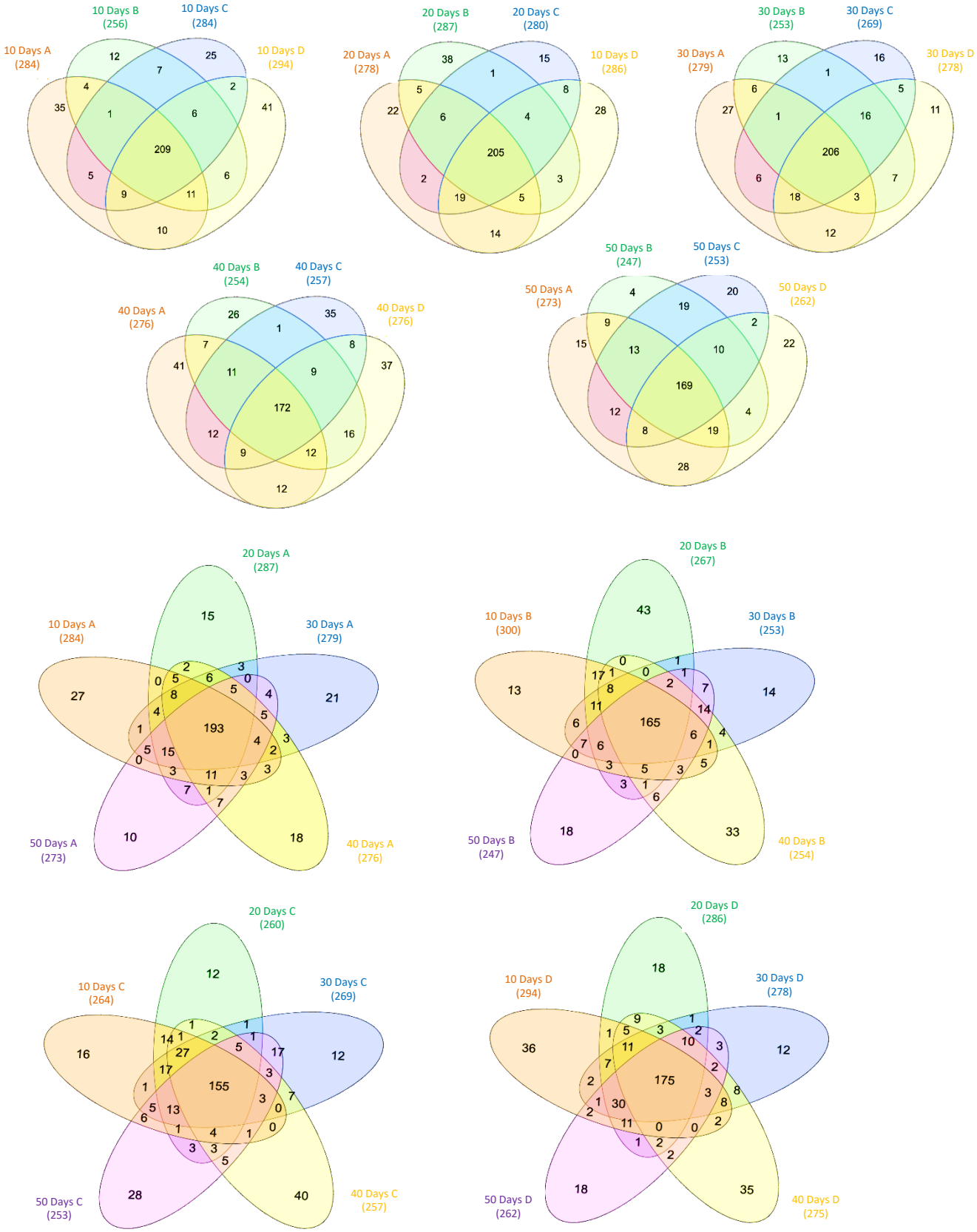

10 Days A (284)
10 Days B (256)
10 Days C (284)
10 Days D (294)
12 25 7 4 2 35 1 6 41 209 5 6 9 11 10
20 Days A (278)
20 Days B (287)
20 Days C (280)
10 Days D (286)
38 15 1 5 8 22 6 4 28 205 2 3 19 5 14
30 Days A (279)
30 Days B (253)
30 Days C (269)
30 Days D (278)
13 16 1 6 5 27 1 16 11 206 6 7 18 3 12
40 Days A (276)
40 Days B (254)
40 Days C (257)
40 Days D (276)
26 35 1 7 8 41 11 9 37 172 12 16 9 12 12
50 Days A (273)
50 Days B (247)
50 Days C (253)
50 Days D (262)
4 20 19 9 2 15 13 10 22 169 12 4 8 19 28
10 Days A (284)
20 Days A (287)
30 Days A (279)
50 Days A (273)
40 Days A (276)
15 27 21 193 10 18
10 Days B (300)
20 Days B (267)
30 Days B (253)
50 Days B (247)
40 Days B (254)
43 13 14 165 18 33
10 Days C (264)
20 Days C (260)
30 Days C (269)
50 Days C (253)
40 Days C (257)
12 16 12 155 28 40
10 Days D (294)
20 Days D (286)
30 Days D (278)
50 Days D (262)
40 Days D (275)
18 36 12 175 18 35

Figure S2

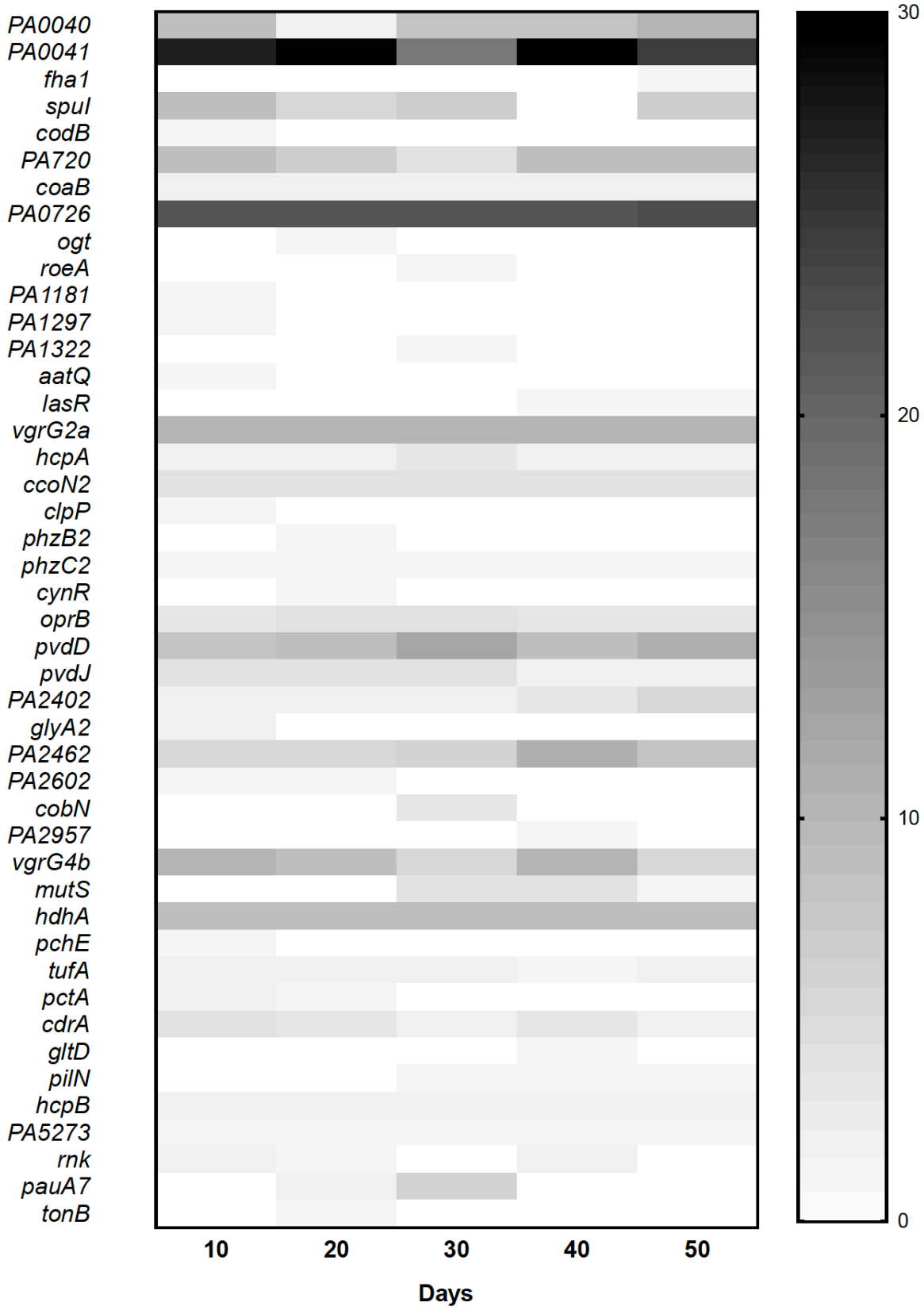

PA0040
PA0041
fha1
spuI
codB
PA720
coaB
PA0726
ogt
roeA
PA1181
PA1297
PA1322
aatQ
lasR
vgrG2a
hcpA
ccoN2
clpP
phzB2
phzC2
cynR
oprB
pvdD
pvdJ
PA2402
glyA2
PA2462
PA2602
cobN
PA2957
vgrG4b
mutS
hdhA
pchE
tufA
pctA
cdrA
gltD
pilN
hcpB
PA5273
rnk
pauA7
tonB
10
20
30
40
50
Days
30
20
10
0

Figure S3

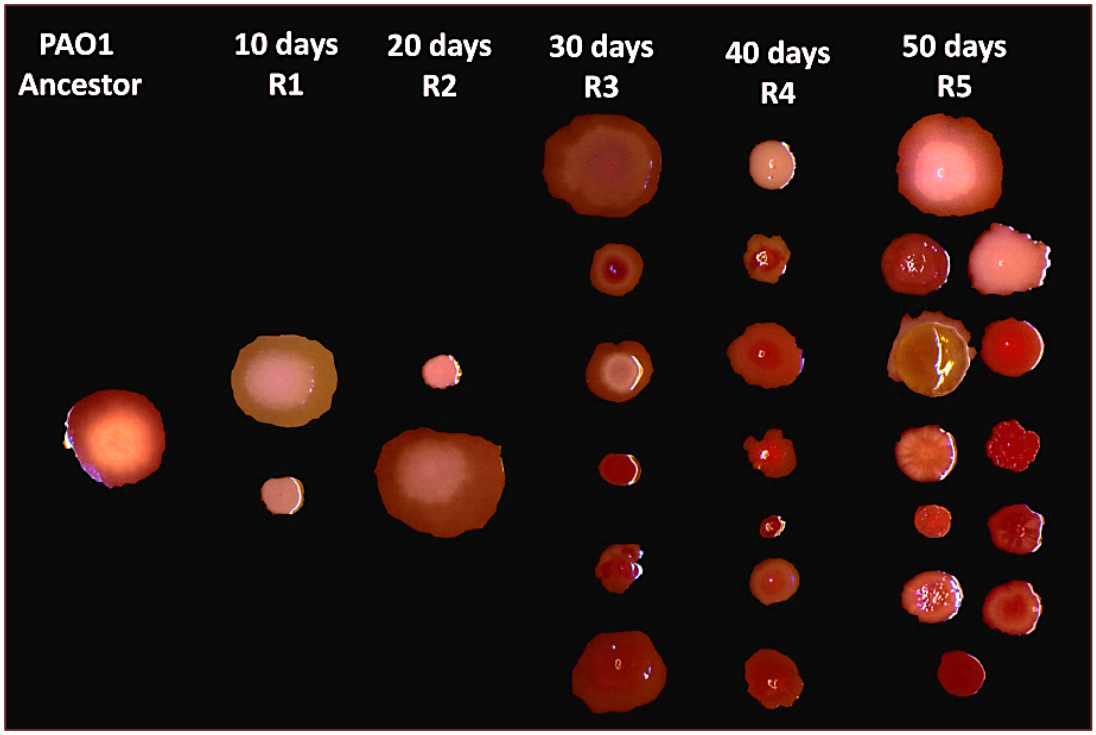
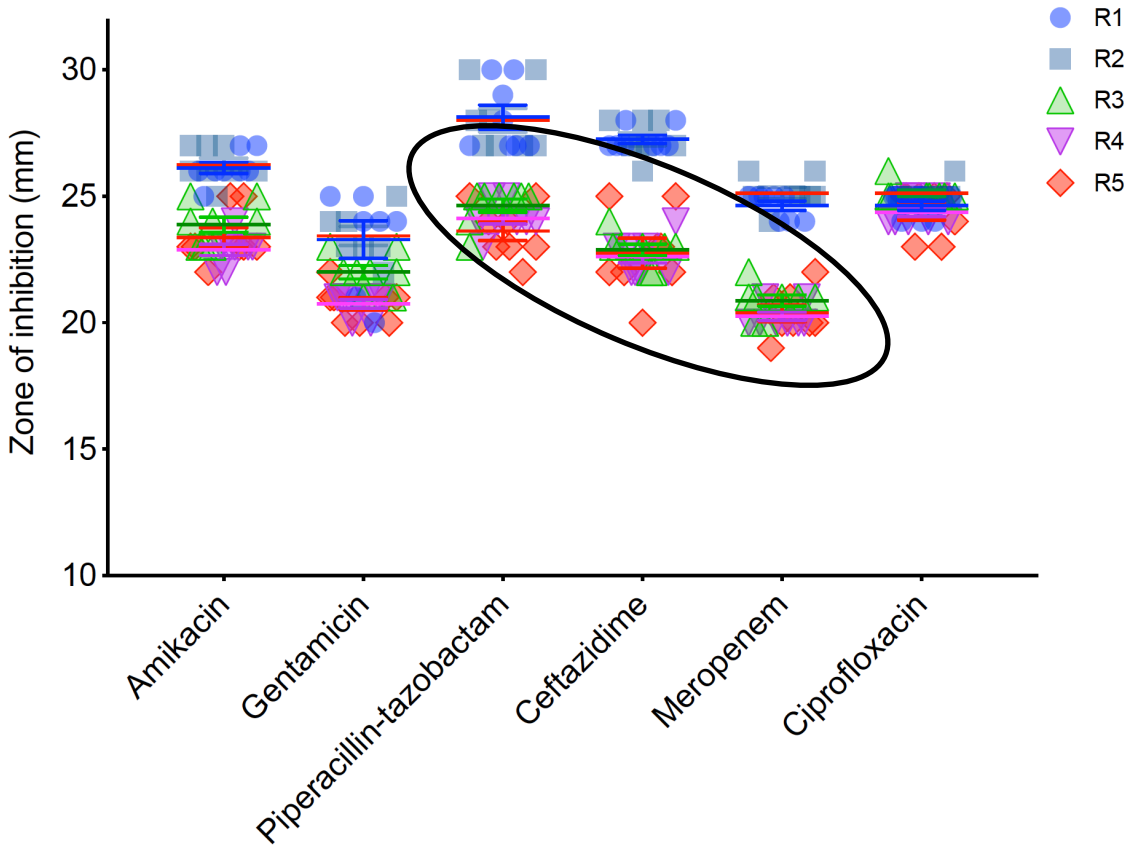

PAO1
Ancestor
10 days
R1
20 days
R2
30 days
R3
40 days
R4
50 days
R5

Figure S4

A


R1
R2
R3
R4
R5
Zone of inhibition (mm)
30
25
20
15
10
Amikacin
Gentamicin
Piperacillin-tazobactam
Ceftazidime
Meropenem
Ciprofloxacin

B

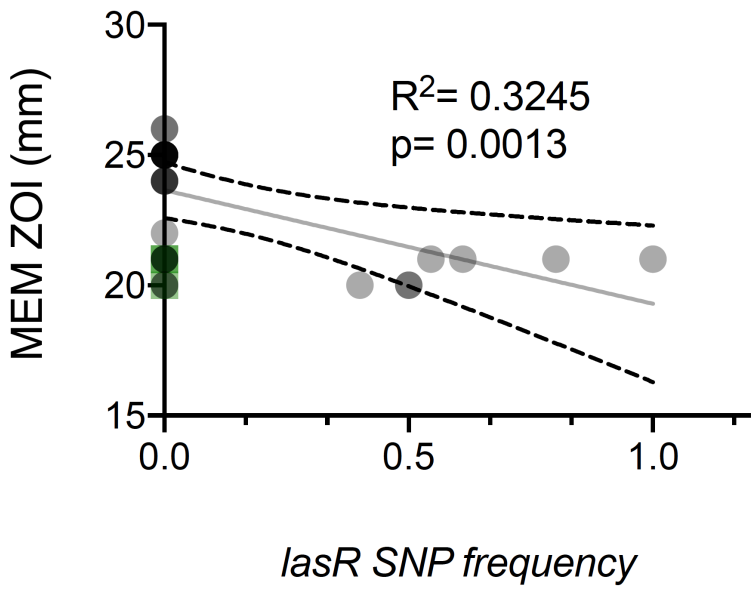

MEM ZOI (mm)
30
25
20
15
R2= 0.3245
p= 0.0013
0.0
0.5
1.0
lasR SNP frequency

Figure S5

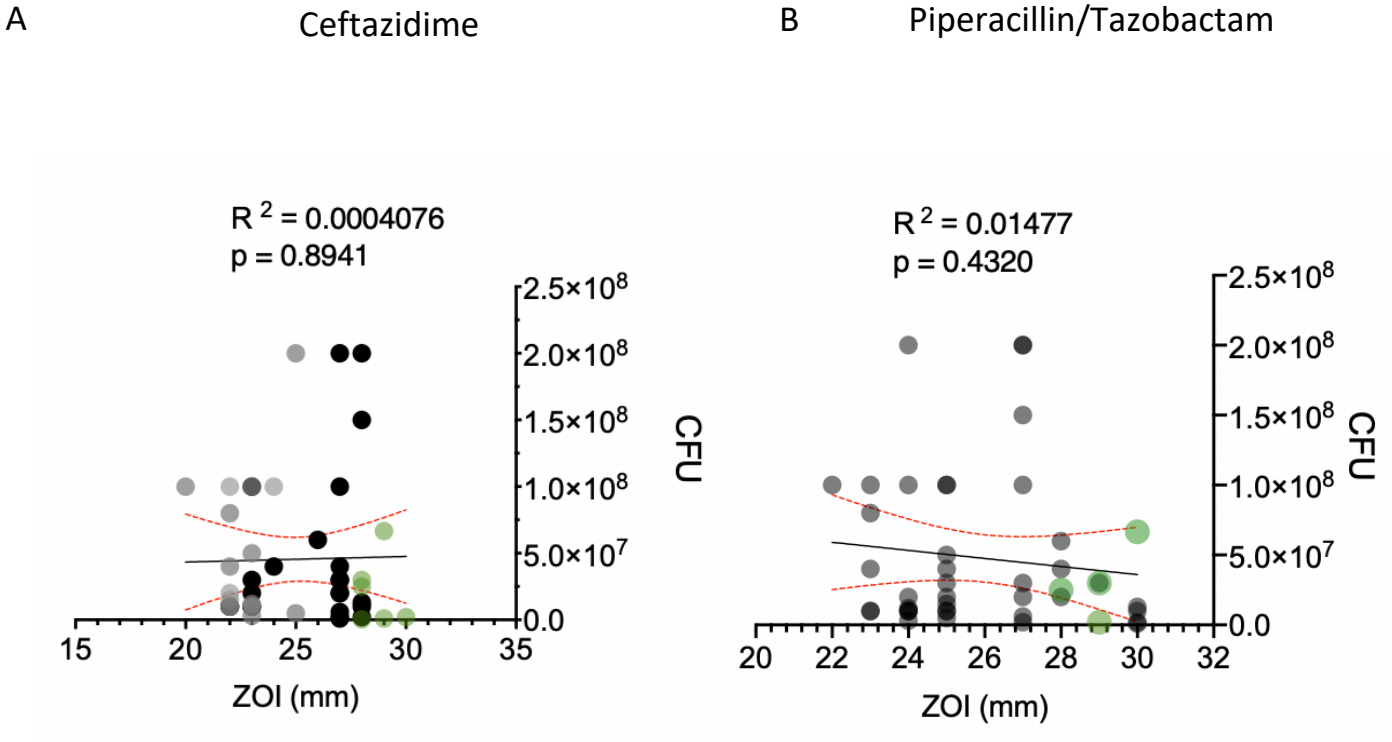

A
Ceftazidime
R 2 = 0.0004076
p = 0.8941
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
CFU
15
20
25
30
35
ZOI (mm)
B
Piperacillin/Tazobactam
R 2 = 0.01477
p = 0.4320
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
CFU
20
22
24
26
28
30
32
ZOI (mm)

Figure S6