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 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 is likely to impact on levels of pathogenicity and the efficacy of antibiotic treatment [16, 20], and could potentially impact how other species of microbe 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, whilst 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 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

76 environmental conditions [35]. Our study differs from this previous work, in that our major
77 focus was on community function within populations rather than on specific isolates and
78 defining ecological niches. We grew our bead associated biofilms in a synthetic CF sputum
79 media (SCFM), which recapitulates the chemical environment found in CF sputum [36-38].
80 Our study therefore generated phenotypically and genotypically heterogeneous populations of
81 *P. aeruginosa* in a spatially structured environment chemically relevant to CF sputum. We
82 utilized these heterogenous evolved populations to study the changes in the functional
83 community phenotypes instead of the traditional approach of working with single evolved
84 isolates. We used a metagenomic approach to assess genetic alterations within evolving
85 populations, and we monitored fluctuations in allele frequency during the selection process. To
86 determine the impact of genomic heterogeneity within populations on various phenotypes, we
87 assessed collective phenotypic traits (community function) of the evolved populations.
88
89 One of the most commonly described adaptations of *P. aeruginosa* to the CF lung is the loss
90 of the *las* QS system, predominantly through point mutations, frameshifts and deletions in the
91 *lasR* gene [17, 39-41]. We used our evolved populations to specifically focus on the impact of
92 *lasR* mutation frequency on QS phenotypes. The *lasR* gene encodes the LasR transcriptional
93 regulator, which binds the QS signal *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C12-
94 HSL) [42-45]. LasR-bound 3O-C12-HSL controls the transcription of approximately 10 % of
95 the *P. aeruginosa* genome, including a number of genes involved in social behaviors,
96 pathogenesis, antibiotic resistance and biofilm formation [46-49]. Despite a number of
97 previous studies which describe the changes and adaptation of various lineages of *P.*
98 *aeruginosa* in CF lungs [14, 50-53], it remains unclear how polymorphisms in the *lasR* gene
99 impacts on community function within evolving heterogenous *P. aeruginosa* populations. This
100 is because most previous studies that focused on within host adaptation of *P. aeruginosa* used

101 single colonies isolated from temporal CF sputum samples and not whole populations [12, 14,
102 15, 19, 54].
103
104 Overall we found that (i) evolutionary trajectories were reproducible between independently
105 evolving populations and that over 60% of genomic changes in populations occurred within
106 the first 10 days of selection; (ii) after 30 days of evolution in SCFM, the evolved communities
107 displayed an increase in *lasR* mutant frequency and a decrease in QS-dependent traits; (iii)
108 there was a significant correlation between *lasR* mutant frequency, the loss of social traits and
109 an increase in tolerance to β-lactam antibiotics. Our findings provide insights into how allelic
110 polymorphism and population heterogeneity in general, can impact on phenotypes and
111 community functions within evolving *P. aeruginosa* populations. Further, we demonstrate that
112 changes in *P. aeruginosa* population dynamics can alter factors associated with virulence and
113 provide explanations for increased antibiotic tolerance, even in situations when antibiotics have
114 not been used.
115
116 **Results**
117 **Genomic variation in evolving biofilm populations over 50 days selection in SCFM.** We
118 evolved the *P. aeruginosa* strain PAO1 for 50 days (≈ 800 generations) in biofilms using a
119 previously described biofilm bead method [34], and a growth medium that chemically mimics
120 CF sputum (SCFM), and where the physiology of *P. aeruginosa* is similar to when grown in
121 human sputum [36-38]. Our experimental evolution approach contained four-independent
122 replicate lines (Fig. S1). We collected and stored biofilm evolved populations after 10, 20, 30,
123 40 and 50 days of evolution (Rounds 1 to 5: R1 – R5). We used the Illumina MiSeq platform
124 to deep sequence evolved populations in order to determine genomic changes through time.
125 We also sequenced our laboratory PAO1 ancestral strain, and after *de novo* genome assembly

126 of this strain, we mapped the sequence reads of the evolved populations to the ancestor in order
127 to detect SNPs [55]. Our SNP calling analysis, combined with an analysis of allele frequency,
128 revealed that in all four independent replicate lines, an average of 282 ± 13 SNPs occurred in
129 the populations after 10 days of selection (Fig. 1A). We found that around 60 % of these SNPs,
130 were present through all other rounds of selection (Fig. 1B; Fig. S2). This suggests that the
131 evolutionary trajectories of biofilm growth in SCFM are similar in independently evolving
132 populations, and that the major genetic heterogeneity in evolving populations occurs during
133 the early phases of selection.
134
135 **SNP frequency in genes involved in social traits fluctuates over time.** In our evolution
136 experiment, we found emergence of polymorphisms in 45 genes involved in various
137 physiological functions (Fig. S3; Table S1). We found that between 10-25% of SNPs were
138 fixed (frequency of 1) in the populations over 50 days of selection across all four replicate
139 evolution lines (Fig. S4). When we focused on the allele frequency and not the number of
140 positions altered in each coding region, we found that the frequency of SNPs in genes involved
141 in different traits changed during the course of the experiment (Fig. 2; Fig. S3; Fig. S4 and Fig.
142 S5). The genes highlighted in Fig. 2 are genes that have previously been shown to be
143 commonly mutated in *P. aeruginosa* CF isolates [9, 15, 16, 29, 30, 56].
144 The allele frequencies of nonsynonymous SNPs in *ccoN2* (PA1557), and synonymous SNPs
145 in *pvdJ* (PA2400) and *tufA* (PA4265) became fixed in the population at a frequency of 1, while
146 the frequency of nonsynonymous SNPs in *phzC2* (PA1901) and *pvdD* (PA2399) fluctuated
147 between 0.4-0.5 in different rounds of selection. We detected a number of SNPs occurring in
148 *mutS* (PA3620), *pilQ* (PA5040), *pilN* (PA5043) at 10, 20 and 30 days of selection in all four
149 independent evolved lines. We observed an increase in *lasR* (PA1430) mutant allele frequency
150 between 30-40 days of selection (Fig. 2; Fig. S3 and Fig. S5).

151 **Accumulation of SNPs shapes community functions in evolved *P. aeruginosa* populations.**
152 We next examined the production of phenotypic social traits in evolved populations in order to
153 determine changes in community function of the genetically heterogenous evolving
154 populations. We measured levels of biofilm formation, QS signals, total protease and the
155 siderophores pyoverdine and pyochelin. We observed a small but significant increase in
156 biofilm formation by evolved populations when compared to the PAO1 ancestor (Fig. 3A).
157 After 30 days of selection, production of total protease (Fig. 3B) and the 3O-C12-HSL QS
158 signal (Fig. 3C) decreased in evolved populations, however, the levels of C4-HSL signal (Fig.
159 3C), and pyochelin and pyoverdine (Fig. 3D) did not follow this trend, and any changes were
160 generally not significantly different from the values of the ancestor strain. We also observed
161 an increase in colony morphology types (morphotypes) in the evolved populations starting after
162 20 days of selection (Fig. S6).
163
164 To determine whether the emergence and increase in frequency of SNPs in the populations
165 impact upon community function, we used a linear regression model. We focused on changes
166 in the allele frequency detected in the QS regulator LasR. We chose *lasR* because the genes
167 and phenotypes it regulates in *P. aeruginosa* are well understood [30, 40, 44, 47, 57] and also,
168 *lasR* mutants are regularly isolated from CF sputum [13, 50, 58]. We detected a non-
169 synonymous SNP (V208G) in the DNA binding domain of LasR between 30-40 days of
170 selection (Fig. S5). Interestingly, SNPs in the same position (V208) have been identified in *P.*
171 *aeruginosa* isolates collected from CF sputum samples [40]. V208 is adjacent to the D209
172 residue in the LasR DNA-binding domain [59, 60], suggesting an impact on the structure of
173 LasR and its DNA binding affinity. We assessed the impact of *lasR* V208G SNP frequency on
174 changes on two QS-dependent phenotypic traits; production of 3O-C12-HSL signal and total
175 protease. We found a significant negative correlation between the frequency of the V208G *lasR*

176 SNP in the whole-evolved populations and the total protease activity of evolved populations.
177 We found that 87% (R^2=0.8704, F=20.15; p=0.0206) of the decreased protease activity was
178 correlated with the accumulation of the *lasR* mutation in the populations (Fig. 4A). We used
179 the same analysis to determine whether *lasR* mutant accumulation impacts on 3O-C12-HSL
180 production within populations. We found that only 53% of the decreased 3O-C12-HSL levels
181 (R^2=0.5363, F=3.469; p=0.1594) can be explained by the accumulation of *lasR* mutation in the
182 populations (Fig. 4B). However, when we only included the last 30 days of selection in our
183 analysis, the decrease in 3O-C12-HSL levels could be fully (100%) explained by the
184 accumulation of *lasR* mutants (R^2=1.0, p=0.0034) (Fig. 4B). Our analysis did not show any
185 correlation between the frequency of the *lasR* SNP and changes in the production of C4-HSL
186 (Fig. 4C) or biofilm formation (Fig. 4D).
187
188 **Accumulation of *lasR* mutants in evolved populations leads to increased tolerance to β-**
189 **lactam antibiotics.** Previously it has been shown that *lasR* mutants display increased β-
190 lactamase activity, and therefore increased resistance to β-lactam antibiotics (such as
191 ceftazidime) [61] which are routinely used in CF clinics. To determine possible links between
192 the loss of *lasR* function and changes in tolerance to routinely used antibiotics, we first assessed
193 the antimicrobial susceptibility levels of the evolved populations. When we tested levels of
194 antimicrobial susceptibility to six routinely used antibiotics for chronic CF lung infection, after
195 30 days of selection the evolved populations showed an increased tolerance (indicated by a
196 decrease in the zone of inhibition) to three antibiotics; ceftazidime, piperacillin/tazobactam and
197 meropenem which are all β-lactam class antibiotics (Fig. 5; Fig. S7). We then tested for
198 correlations between frequencies of the *lasR* V208G SNP and resistance to ceftazidime and
199 piperacillin/tazobactam. We observed a positive and significant correlation between
200 accumulation of *lasR* mutants and the increased resistance to both ceftazidime and

201 piperacillin/tazobactam (Fig. 5A and B). We also tested whether the increase in tolerance could
202 be explained by an increase in biofilm formation. We found there was no significant
203 correlation, suggesting that increases in biofilm formation do not necessarily translate to an
204 increase in drug tolerance (Fig. S7C).
205
206 To confirm that our evolved populations contained *lasR* mutants with increased β-lactam
207 tolerance and that *lasR* frequency affects community function, we first selected and sequenced
208 an evolved isolate (BB8) from the final round of selection (50 days) with no protease activity
209 and which demonstrated increased antibiotic tolerance compared to the ancestor PAO1 (Fig.
210 5C). The sequencing of isolate BB8 revealed the same SNP in the *lasR* gene that we detected
211 in the evolved populations (V208G). We complemented BB8 with an intact copy of the PAO1
212 *lasR* gene *in trans* on a plasmid and then assessed β-lactam tolerance levels. We observed a
213 decrease in tolerance to ceftazidime and piperacillin/tazobactam in the *lasR* complemented
214 BB8 isolate (Fig. 5C). Finally we tested whether changes in the starting frequency of *lasR*
215 mutants in mixed populations impacted on community functions. We found that when BB8
216 was mixed with wild type cells, this resulted in an increased tolerance to ceftazidime (Fig. 5C)
217 and a reduction in 3O-C12-HSL but not C4-HSL in mixed populations (Fig. S8).
218
219 **Discussion**
220 Despite a number of recent studies focused on adaptive changes in evolved populations of *P.*
221 *aeruginosa* in environments designed to mimic CF sputum [35, 62-66], there remain significant
222 gaps in knowledge about how genomic and phenotypic diversity impacts upon on interactions
223 within populations and on community-level phenotypes. In our current study, we focused on
224 understanding how the evolution and coexistence of multiple lineages of *P. aeruginosa* shape
225 community functions. To generate diversity, we performed a 50-day selection experiment,

226 evolving PAO1 as biofilms in an artificial sputum medium (SCFM) [36-38]. We observed (i)
227 a rise in genetic diversity after 10 days of selection in biofilms grown in SCFM; (ii) up to 25%
228 of SNPs became fixed in the population and carried in evolved populations through 50 days of
229 selection; (iii) emergence and accumulation of SNPs in genes involved in motility, respiration,
230 DNA mismatch repair, transcription and QS emerged between 10 and 40 days of selection and
231 (iv) accumulation of *lasR* SNPs correlated with decreased protease activity, 3O-C12-HSL
232 production and increased tolerance to β-lactam antibiotics despite no prior treatment with any
233 antibiotic.
234
235 The divergent evolution of *P. aeruginosa* during chronic infection of CF lungs has been the
236 focus of numerous studies on longitudinal collections of sputum samples. Analysis of single *P.*
237 *aeruginosa* isolates from these collections has identified genomic signatures for adaptation to
238 the CF lung environment [9, 14, 29, 50, 53, 67, 68]. Similar studies on collections of *P.*
239 *aeruginosa* isolates sourced from single sputum samples, have shown considerable phenotypic
240 and genetic diversification of *P. aeruginosa* strains which group into clades [16, 17], and which
241 may colonize different ecological niches in CF lungs [28, 69]. All of these studies focused on
242 the diversification, adaptation and heterogeneity of *P. aeruginosa* during chronic CF lung
243 infection. However, the impact that this heterogeneity may have on collective phenotypes has
244 been largely over-looked. We hypothesized that the combination of wild-type and mutated
245 alleles in populations shapes collective phenotypes. We observed that the accumulation and
246 frequency of various SNPs in heterogenous populations of *P. aeruginosa,* significantly impacts
247 the collective phenotypes of evolved populations (Fig. 2; Fig. 3). Although we observed that
248 the majority of genomic changes occurred during the first 10 days of selection, the functional
249 outcome was only impactful after 20-30 days, where we observed an increase in biofilm

250 formation and colony morphotypes and a significant decrease in total protease activity and
251 production of 3O-C12-HSL by evolved populations.

252 The biofilm bead system has previously been used to examine the evolution of *P. aeruginosa*
253 in biofilms [35], although there are significant differences between this and our study. First,
254 the environmental conditions were different, with the previous study growing in a minimal
255 media (M63) whilst we used SCFM. Secondly, we evolved PAO1 whereas the previous study
256 evolved PA14 [35]. Whilst both are popular model strains for *P. aeruginosa* study, they differ
257 in a number of factors including virulence [70]. Third, we focused primarily on the emergent
258 properties of the whole community rather than individual isolates, which we show has
259 important implications for signaling and antimicrobial tolerance. Finally, the previous study
260 studied the emergence of *mutL* and *mutS* genes which may have contributed to the emergence
261 of different individual morphotypes [35]. We observed low frequency mutations in the *mutS*
262 gene after 30 days (Fig. S3). As these were synonymous mutations, they did not appear to
263 significantly increase mutation rate after 30 days of selection.

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

270 Our study identified genomic changes often observed in *P. aeruginosa* isolates taken from CF
271 lungs including changes in secretion genes (*hcpA, hcpB, vgrG2a, vgrG4b*), motility genes (*pilQ*
272 and *pilN*), iron aquisition genes (*pvdD, pvdJ*) and biofilm formation (*cdrA*). We also observed
273 a consistent change in all lines and rounds of selection in anaerobic respiratory pathways (Fig.

274 2; Fig. S3). *ccoN2* (PA1557) encodes the cytochrome C oxidase subunit (ccb3 type). Increased
275 expression of *ccoN2* in small colony variant has been previously noted during microaerophilic
276 conditions [71, 72].

277 One of best-known signatures of *P. aeruginosa* adaptation to the CF lung environment, is
278 mutation in the QS regulator LasR. Several studies on *P. aeruginosa* strains isolated from CF
279 sputum samples, found significant genomic changes in *lasR,* including truncation, deletion,
280 frame shifts and SNPs, with many resulting in a loss of function [30, 40, 41, 50, 73]. Although
281 there can be a high percentage of *lasR*-deficient isolates collected from patients, studies have
282 also shown differential frequencies of functional and intact *lasR* alleles within patients [13,
283 74]. Considering the importance of *lasR*-dependent social interactions for the fitness of *P.*
284 *aeruginosa,* mutation-frequency of *lasR* within populations of *P. aeruginosa* could
285 significantly impact on QS-dependent phenotypes and fitness at the population level [75-77].
286 Previously we have demonstrated that an increase in the frequency of QS social cheats (*lasR*
287 mutants) in defined populations of wild-type and *lasR* mutants, leads to a reduction in
288 cooperation and virulence in mouse models of infection [75]. These studies showed that even
289 simple mixed genotype populations of *P. aeruginosa* can have a significant impact on
290 community function, virulence and the outcome of infection.

291 In our current study, we observed a negative correlation between an increase in the frequency
292 of the *lasR* V208G SNP in evolved populations and levels of protease activity and 3O-C12-
293 HSL production (Fig. 4). Mutation of *lasR* in *P. aeruginosa* isolates from CF lungs has also
294 previously been shown to be important for increased tolerance to β-lactam antibiotics such as
295 ceftazidime [19, 56, 61]. Here we examined whether the increase of *lasR* mutants in our
296 evolved populations had an impact on levels of antimicrobial tolerance. Despite no prior
297 treatments with antibiotics, we observed an increased tolerance to three antibiotics from the β-
298 lactam family (Fig. 5). In contrast, we found no correlation between increased biofilm

299 production by evolved populations and antibiotic resistance (Fig. S7). Resistance of *P.*
300 *aeruginosa* to antibiotics in chronic infections such as CF or wounds, is often thought to be
301 due to specific mechanisms such as efflux pumps or via production of excess polysaccharides
302 such as alginate. Our findings suggest that the accumulation and frequency of genetic variants
303 that might not traditionally be associated with resistance to drugs (e.g. QS mutants) within a
304 heterogenous population, can alter phenotypes within populations that can result in important
305 clinical repercussions.

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

318 **Materials and methods**

319 **Bacterial strains and growth conditions.** For our experimental evolution, we used the PAO1
320 (University of Nottingham) strain of *P. aeruginosa.* For SCFM we followed the protocol
321 provided in [36, 38]. Briefly for the buffer base we prepared NaH_2PO_4 (1.3 mM), Na_2HPO4
322 (1.2 mM), KNO_3 (0.348 mM), K_2SO_4 (0.271 mM), NH_4Cl (2.28 mM), KCl (14.9 mM), NaCl

323 (51.8 mM) was prepared in 10mM of MOPS at pH=6.8, then the amino acids were added[36,
324 38] . The Dextrose (3mM), L-lactic acid (9.3 mM), $CaCl_2$*2 H_2O (1.75 mM), $MgCl_2$* 6H_2O
325 (0.606 mM) and Fe.SO_4*7H_2O (0.0036 mM) was added fresh every time the media was
326 prepared.
327
328 **Long term experimental evolution.** To assess how genomic diversity impacts on *P.*
329 *aeruginosa* populations, we generated a diverse population using a long-term evolution
330 experimental approach. We evolved the *P. aeruginosa* strain PAO1 in biofilms using plastic
331 beads [34, 35, 78] (9x6 mm width) suspended in SCFM, in order to mimic a biofilm life cycle
332 and a chemical environment similar to that found in CF lung sputum. To start the experimental
333 evolution process, we first grew PAO1 on an LB agar plate. Then we inoculated a single colony
334 of PAO1 into 3 ml of fresh SCFM [38], and incubated for up to 6 h to grow up to mid-log
335 phase. We stored this mid-log phase cells as the ancestral PAO1 and compared all further
336 phenotypic and genomic properties of the evolved populations to this. We inoculated mid-log
337 phase cells to $OD_{600} \approx 0.05$ into 4 tubes (in order to evolve separate independent lines named
338 A-D) containing 3 ml of SCFM and a plastic bead. We incubated cultures for 24 h at 37 °C/200
339 rpm. After 24 h of incubation, we transferred bacterial covered beads into fresh tubes
340 containing 3 ml of SCFM and a new bead. After each round the biofilms that formed on beads
341 were composed of approximately 10^8 cells. We then incubated again for 24 h at 37 °C/200 rpm.
342 We continued the bead transfers for 50 days and stored biofilm portion of populations (attached
343 to plastic beads) every 10 days (Rounds 1 to 5: R1-R5) (Fig. S1). We did not transfer or store
344 the planktonic fractions at any point during the selection experiment.

345 **Deep sequencing of evolved populations.** We extracted Genomic DNA from evolved
346 populations after 18 h growth in SCFM; using DNeasy® Blood & Tissue Kit (QIAGEN) by
347 following the manufacturer's instructions. We prepared sequencing libraries using the

348 NexteraXT protocol (Illumina), and sequenced in 24-plex on the Illumina MiSeq platform to
349 obtain an approximate calculated level of coverage of 220-600× for each evolved population.
350 A *de novo* assembly of the ancestral strain genome (*P. aeruginosa* PAO1 ancestor) was
351 obtained using Spades with the –careful flag, and annotated using Prokka. We mapped reads
352 of the evolved populations against the ancestral PAO1 genome using BWA[79]. For SNP
353 calling, the sequences were summarized using a MATLAB script for base quality, genomic
354 position and mapping quality [55] script using bowtie2 [80], SAMtools [81] and BAMtools.
355 To determine the allele frequency, we applied the breseq consensus model [82] to each of the
356 samples collected. Later, for the variant sites, we used the composition in each nucleotide as
357 the surrogacy for the allele frequency, and we excluded any allele frequency below 10% in
358 each evolved population. Our deep sequence analysis was not designed to detect any insertions
359 or deletions (INDELS), only SNPs. It is likely that INDELs were present in genes in our
360 evolved populations.

361 **Measurement of biofilms formed on beads.** To determine the levels of biofilm formation by
362 each evolved population, we grew biofilms on plastic beads as previously described [34, 78].
363 For each set of biofilm assays, we directly inoculated a 10 μl loop of frozen evolved
364 populations (approximately 10^6 cells) into 3 ml of SCFM and incubated at 37°C/200 rpm for
365 16 h. Then we measured the OD_{600}, and diluted in 3ml SCFM to $OD_{600} \approx 0.05$. Then 3 plastic
366 beads were added to each tube. After 24 h growth at 37°C/200 rpm and biofilm formation. The
367 beads were then washed 3× with 10 ml of PBS to remove any residual planktonic cells not
368 bound to the plastic beads. Then we transferred each plastic bead into 1ml of PBS and sonicated
369 the beads for 10 mins, using a bath sonicator to detach biofilm forming cells from the beads.
370 We then serially diluted the cells and plated out onto LB agar plates for colony forming unit
371 (CFU) calculations. To assess other phenotypic traits, a cell free supernatant of biofilm forming
372 cells was prepared from liquid part of the cultures and corrected values for $OD_{600} \approx 1$.

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

378 **Total protease activity**. To assess the total protease activity of evolved populations, we used
379 skimmed milk agar plates. We inoculated 10 µl of cell free supernatant from each evolved
380 population onto skimmed milk agar plates (1.2% Bacto Agar, 0.015% of skimmed milk)
381 alongside 10µl of 10µg/ml of proteinase K and supernatant of PAO1 as controls. The zone of
382 clearance was scored based on appearance and measured with a ruler (in mm). We then imaged
383 each plate using an Epson scanner at 800dpi. We then compared it to the zone of clearance
384 produced by the PAO1 ancestor [83].

385 **Siderophore production**. To measure the levels of the two main siderophores produced by *P.*
386 *aeruginosa*, we used the cell free supernatants. 100 µl aliquots of cell free supernatant from
387 evolved populations and the PAO1 ancestor was transferred into a black clear bottom 96 well
388 plate (Corning). We measured the emission as Relative Fluorescent Units (RFU) using a Multi
389 microplate reader (Tecan Infinite® M200 Pro). We measured the wavelengths at excitation of
390 400 nm/emission460 nm for Pyoverdine and 350/430 nm for Pyochelin [30, 84, 85]. We
391 corrected the values for pyoverdine and pyochelin to the absorption at (OD_{600}) of the original
392 cultures.

393 **Measurement of C4-HSL and 3O-C12-HSL produced by evolved populations**. The cell
394 free supernatants were used to determine the concentration of QS signals. We used two *E. coli*
395 bioreporter strains to measure production of the two main signal molecules by the evolved
396 population. The *E. coli* reporters pSB536 and pSB1142 were used to detect C4-HSL and 3O-

397 C12-HSL respectively [86]. We calculated signal levels based on standard curves fitted to the
398 concentrations of synthetic 3O-C12-HSL and C4-HSL standards (Sigma) [87, 88].

399 **Mixed isolate experiments.** For mixed constructed populations, we grew PAO1, the BB8
400 evolved isolate and BB8 complemented with *lasR* (BB8:*lasR*) and empty vector (BB8:vector)
401 in SCFM to $OD_{600} \approx 0.5$. We then mixed PAO1 and BB8 at two different starting frequencies
402 (50:50 and 30:70) in 3 ml of SCFM. The cultures were then incubated for 16 h at 37°C/200
403 rpm. We measured and adjusted the OD_{600} of each culture to $OD_{600} \approx 1$ and prepared a cell
404 free supernatant for QS signal activity measurements.

405 **Antibiotic susceptibility assay**. To determine the antibiotic susceptibility in evolved
406 populations, we followed the British Society for Antimicrobial Chemotherapy (BSAC)
407 guidelines (Version.14, 05-01-2015) using Isosensitest agar plates (Oxoid). We tested the
408 susceptibility of evolved populations, PAO1 ancestral strain and the NCTC (10662) PAO1
409 strain to Gentamicin (10 µg), Meropenem (10 µg), Ciprofloxacin (1 µg), Ceftazidime (30 µg),
410 Piperacillin/Tazobactam (85 µg) and Amikacin (30 µg) (Oxoid). The zone of inhibition and
411 clearance in this method was compared to the available zone of inhibition breakpoints for
412 susceptibility (mm) for each tested antibiotic based on BSAC guidelines.

413

414 **Determining colony morphology diversity in evolved populations.** To determine the
415 diversity in colony morphology in the biofilm evolved population, we used a Congo Red based
416 agar media (1% agar, 1xM63 salts (3g monobasic KHPO4, 7g K2PO4, 2g NH4.2SO4, pH
417 adjusted to 7.4), 2.5mM magnesium chloride, 0.4 mM calcium chloride, 0.1% casamino acids,
418 0.1% yeast extracts, 40 mg/L Congo red solution, 100 µM ferrous ammonium sulphate and
419 0.4% glycerol) [83]. We recovered the evolved populations from beads and serially diluted the
420 populations and then inoculated onto CRA plates alongside the PAO1 ancestor. We incubated

421 the plates overnight at 37°C, and for a further 4 days at 22°C. The colonies were imaged using
422 an Epson scanner at 800dpi.
423
424 **Complementation of *lasR*.** To complement an evolved isolate (BB8) containing a *lasR*
425 mutation with a functional *lasR* allele, we amplified a 920 bp product comprising *lasR* and 200
426 bp upstream of the *lasR* start codon that includes its native promoter, from genomic DNA
427 isolated from wildtype ancestral PAO1. We cloned this PCR product into the shuttle vector
428 pME6032, which replicates in both *E. coli* and *P.aeruginosa*, [89] by Gibson assembly [90]
429 using the commercially available NEBuilder HiFi DNA Assembly Cloning Kit (New England
430 Biolabs, Ipswich, MA). We introduced the *lasR* complementation construct into BB8 by
431 electroporation [91] and plating on selective media containing 300 ug/mL tetracycline. We
432 then tested susceptibility of the *lasR* complemented strains to ceftazidime and
433 piperacillin/tazobactam using the BSAC method. For mixed constructed populations, we grew
434 PAO1, the BB8 evolved isolate and BB8 complemented with *lasR* (BB8:*lasR*) and empty
435 vector (BB8:vector) in SCFM to $OD_{600} \approx 0.5$. We then mixed PAO1 and BB8 at two different
436 starting frequencies (50:50 and 30:70) in 1 ml of PBS and tested for antibiotic tolerance by
437 following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Version.14,
438 05-01-2015) using Isosensitest agar plates (Oxoid).
439
440 **Statistical analysis.** For statistical analysis of the phenotypic assays, we used GraphPad Prism
441 8.0. For analysis of SNP frequency, we used R package 3.6. We used the Interactive Venn [92]
442 to analyze shared SNPs within and between evolved populations.
443
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450 comments on the work.
451

452 **Figure legends**

453 **Figure 1. The evolutionary trajectory of *P. aeruginosa* during biofilm growth in SCFM.**
454 The evolution trajectories were similar in four independent replicate lines, and the major
455 genetic heterogeneity in the evolved populations occurred during the first 10 days of selection.
456 (A) SNP calling analysis showed that the four independently evolved populations had more
457 than 80% similarity (267 shared SNPs) in genomic changes in the populations over the 50 days
458 of selection. (B) The Venn diagram shows the shared SNPs between all 4 replicate lines, over
459 50 days of selection. Numbers in brackets, (i.e 1354 for 10 days), represent the combined
460 number of SNPs in each of the four evolved lines. The majority of genetic variation within the
461 populations occurred within the first 10 days of selection. Each circle represents the total
462 number SNPs for each independently evolved line.

463 **Figure 2. Allele frequency of SNPs changes over the course of selection.** Emergence of
464 nonsynonymous SNPs in genes involved in social traits (QS: *lasR*), oxidative respiration and
465 DNA mismatch repair, motility and iron chelation occurred after 20 and 30 days of selection;
466 and fixed SNPs in genes such as elongation factor *tufA,* Cytochrome c oxidase subunit (*ccoN2*)
467 and pyoverdine biosynthesis protein *pvdJ* emerged after 10 days of selection and were fixed in
468 the populations over 50 days (A-D represents replicate evolving lines).
469

470 **Figure 3. Phenotypes of evolved populations of PAO1.** (A) Increase in biofilm formation by
471 evolved populations of PAO1 (Kurskal-Wallis, Dunn's multiple comparison to PAO1, $p < 0.05$,
472 n=5. Errors bars indicate the standard error of the mean, SEM); (B) The total protease activity
473 was reduced at 30 days of selection; (C) Production of 3O-C12-HSL significantly decreased at
474 30 days of selection (Kruskal-Wallis, uncorrected Dunn's test multiple comparison to PAO1,
475 $p < 0.05$, n=5. Errors bars indicate the SEM) and there were no significant changes in the levels
476 of C4-HSL; (D) There were no significant changes in siderophore production in evolved
477 populations compared to the PAO1 ancestor (Kruskal-Wallis, uncorrected Dunn's test multiple
478 comparison to PAO1, $p > 0.05$, n=5). Each dot represents the mean value of one independent
479 experiment for each evolved population at each round of selection. All experiments were
480 performed in 3 technical replicates.

481 **Figure 4. Loss of protease activity and a decrease in QS signal production in evolved**
482 **populations can be explained by an accumulation of *lasR* SNPs.** A linear regression model
483 showed that the loss of total protease activity and the decrease in 3O-C12-HSL levels can be
484 explained by the accumulation of *lasR* SNPs. (A) There is a significant negative correlation
485 between *lasR* SNP frequency and total protease activity of the evolved populations (R^2 =
486 0.7298, p = 0.04; n=8); (B) There is no significant correlation between 3O-C12-HSL levels
487 and accumulation of the *lasR* SNP in the evolved populations (R^2 = 0.5363, p = 0.1594; n=8).
488 However, if only changes in 3O-C12-HSL levels after 30 days of selection is considered, there
489 is a strong correlation between emergence and accumulation of *lasR* SNP in evolved
490 populations (small inset: R^2=1.0; p = 0.0034; n=8); (C) There are no significant correlations
491 between accumulation of *lasR* SNPs in the evolved populations and levels of C4-HSL (R^2 =
492 0.1187, p = 0.5702; n=8); (D) There is no significant correlation between *lasR* frequency and
493 biofilm formation (R^2 = 0.0004, p = 0.9062; n=8).

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

507 **Supplementary figure legends**

508 **Figure S1.** Long term evolution of PAO1 in biofilms and SCFM. PAO1 was grown in SCFM
509 and inoculated into four independent replicate lines in the presence of one plastic bead (6×7
510 mm) to enable the bacteria to form biofilms on the surface. Every 24 hours the beads were
511 transferred to a new tube with fresh SCFM media and a new bead. After 10 days, the bacterial
512 populations were sonicated off the beads and stored as round 1 (R1). To start the next 10 days
513 of selection, a 10 µl loop of bacteria (approximately 10^6 cells) was used. This whole process
514 was repeated 5 times to give 50 days (rounds 1 to 5) of selection.

515

516 **Figure S2. Number of SNPs shared in all evolved lines during 50 days of evolution in**
517 **SCFM.** We tested the number and percentages of the SNPS that occurred and are shared
518 between various evolution lines at different rounds of selections. There are 207 SNPs that are

519 shared between all 4 lines of selection (A-D) at day 10, 205 on day 20, 206 on day 30, 172 on
520 day 40 and 169 on day 50 (upper panels). We observed that the majority of SNPs occurred
521 during the first 10 days of selection and are shared and carried over to subsequent rounds of
522 selection in all evolution lines.
523
524 **Figure S3. Number of SNPs in genes over 50 days of selection in SCFM.** The emergence
525 of polymorphisms in 45 genes involved in various physiological functions (Evolved line A
526 shown as a representative for all evolved lines). The shaded boxes represent the abundance of
527 SNPs in each gene.
528
529 **Figure S4. Abundance of differential allele frequencies in each evolved population over**
530 **five rounds of selection.** Different SNPS emerged after 10 days of selection at varying
531 frequencies within the populations. Each round of selection contained varying frequencies of
532 SNPs. Over 10-25% of SNPs were fixed in populations after 10 days of selection, across all
533 four independently evolving populations (A-D).
534
535 **Figure S5. Number of SNPs in various genes after 50 days of selection.** We found high
536 levels of similarity in number and frequency of SNPs in all independent evolved lines after 50
537 days of selection. Each data point is representative of SNPs in each gene, at various frequencies
538 in all four evolved lines.
539
540 **Figure S6. Increase in diversity of colony morphologies in evolving populations over time.**
541 Diversity of colony morphology increased over time in evolutionary lines. We used Congo Red
542 Agar plates to highlight differences in morphologies between haplotypes.
543

544 **Figure S7. Increased resistance to β-lactam antibiotics in evolved populations.** (A) There
545 is a decrease in the ZOI to all β-lactam antibiotics after 30 days of selection in evolved
546 populations. (B) There is an increase in resistance to meropenem after 30 days of selection in
547 the evolved population, which significantly correlates to accumulation of the *lasR* SNP in the
548 populations. However, the increased resistance does not pass the threshold to be considered
549 clinically resistant to meropenem. (C) There is no correlation between the levels of biofilm
550 formation or antibiotic resistance at the population level.
551
552 **Figure S8. Increase in *lasR* mutant frequency changes the functional phenotype of**
553 **synthetic mixed populations.** Varying frequencies of BB8 isolate (V208G *lasR* allele) with
554 the PAO1 ancestor, significantly alters the levels of 3O-C12-HSL and has no impact C4-HSL
555 levels (Multiple comparison, One Way ANOVA, n=5).
556
557 **Table S1.** SNP positions and altered allele frequencies in four evolved lines over 5 rounds of
558 selection.
559 **Table S2.** SNPs in the 50 day evolved isolate BB8.
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796 *kilobases.* Nature Methods, 2009. **6**(5): p. 343-U41.
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798 *highly electrocompetent Pseudomonas aeruginosa cells: application for DNA*
799 *fragment transfer between chromosomes and plasmid transformation.* J Microbiol
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801 92. Heberle, H., et al., *InteractiVenn: a web-based tool for the analysis of sets through*
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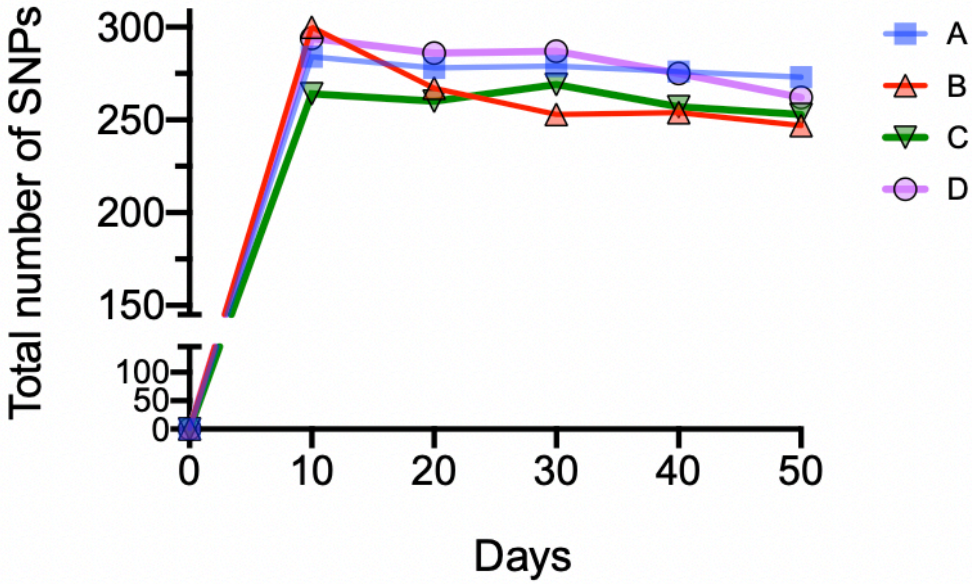
803

A


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

B

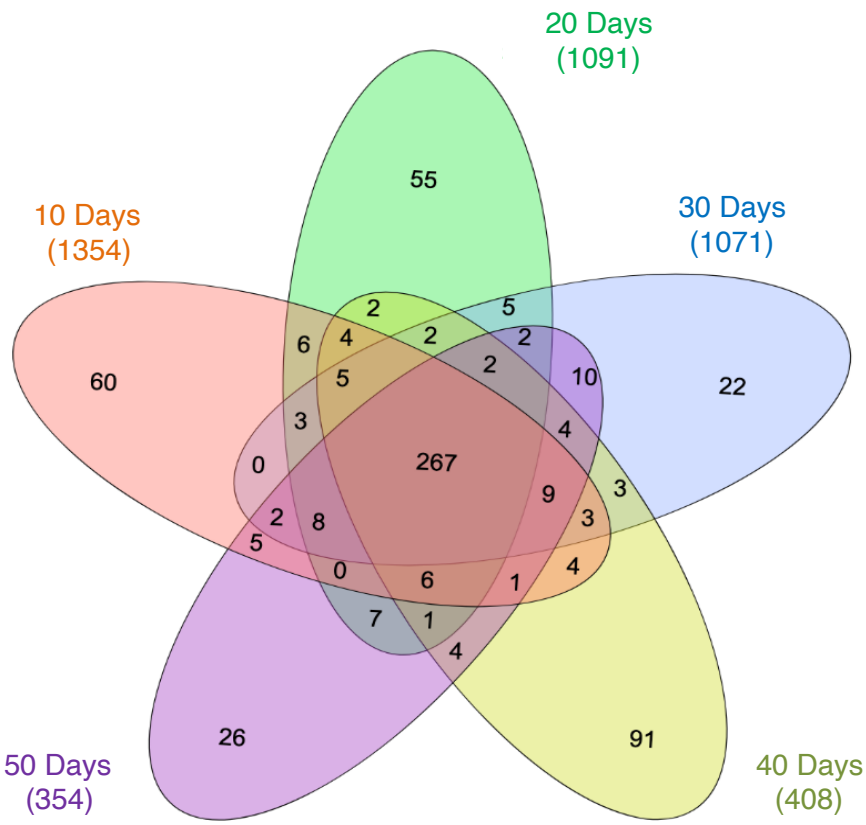

20 Days
(1091)
10 Days
(1354)
30 Days
(1071)
50 Days
(354)
40 Days
(408)
55
60
22
26
91
267

Figure 1

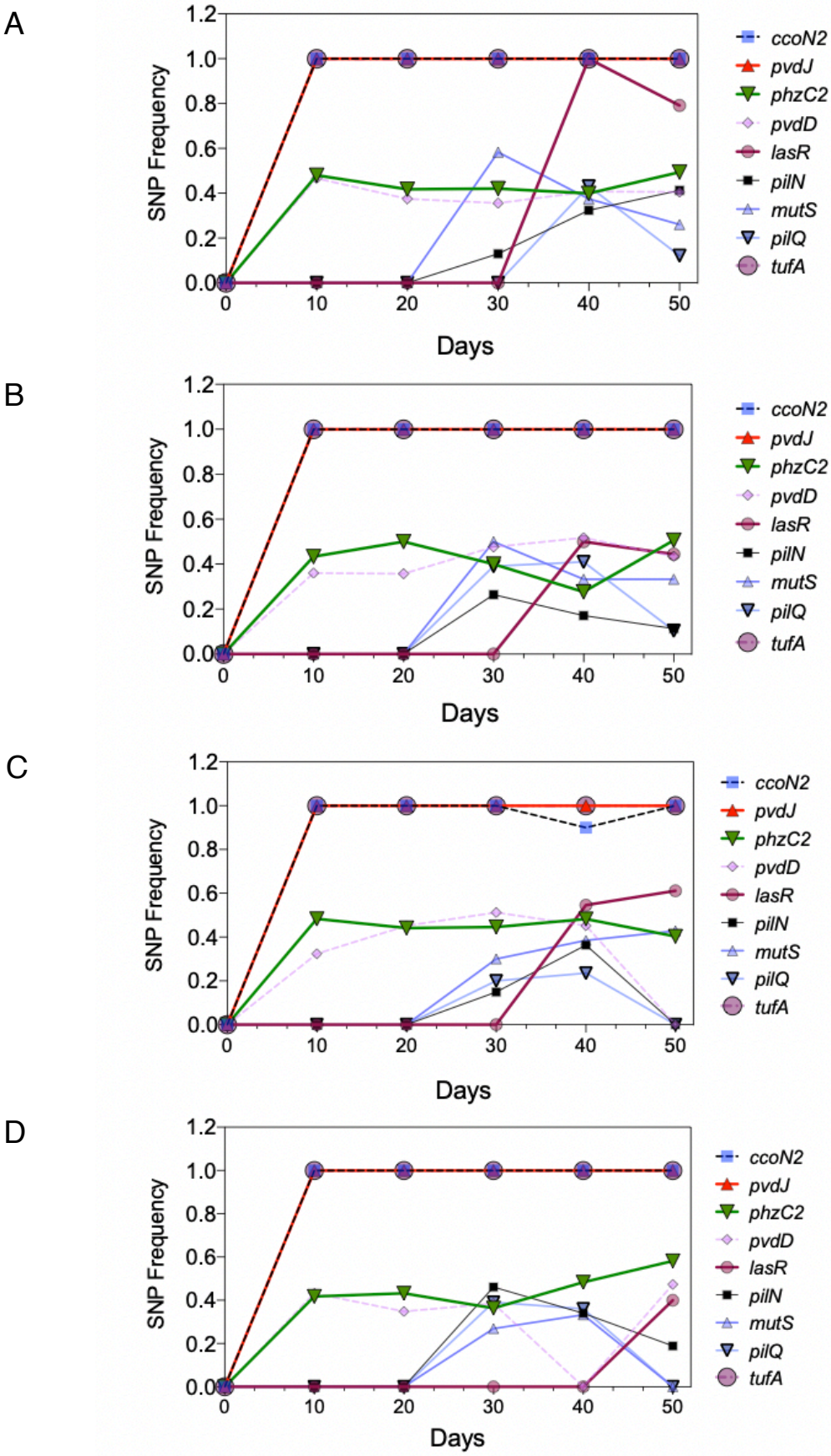

A
B
C
D
SNP Frequency
Days
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
10
20
30
40
50
ccoN2
pvdJ
phzC2
pvdD
lasR
pilN
mutS
pilQ
tufA

Figure 2

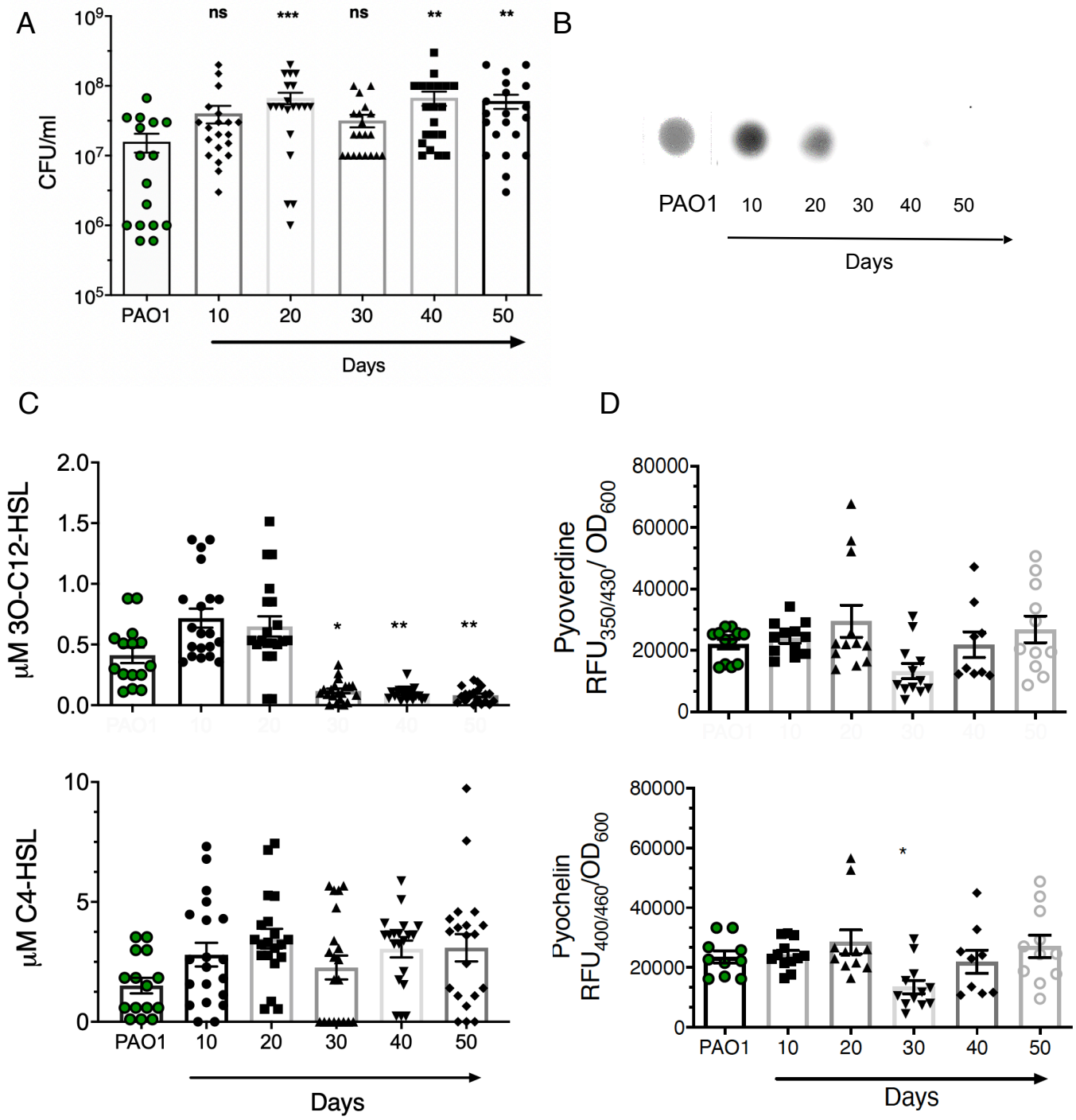

A
CFU/ml
10^9
10^8
10^7
10^6
10^5
ns
***
ns
**
**
PAO1
10
20
30
40
50
Days
B
PAO1
10
20
30
40
50
Days
C
µM 3O-C12-HSL
2.0
1.5
1.0
0.5
0.0
*
**
**
µM C4-HSL
10
5
0
PAO1
10
20
30
40
50
Days
D
Pyoverdine
$RFU_{350/430}$ / OD_{600}
80000
60000
40000
20000
0
Pyochelin
$RFU_{400/460}$/OD_{600}
80000
60000
40000
20000
0
*
PAO1
10
20
30
40
50
Days

Figure 3

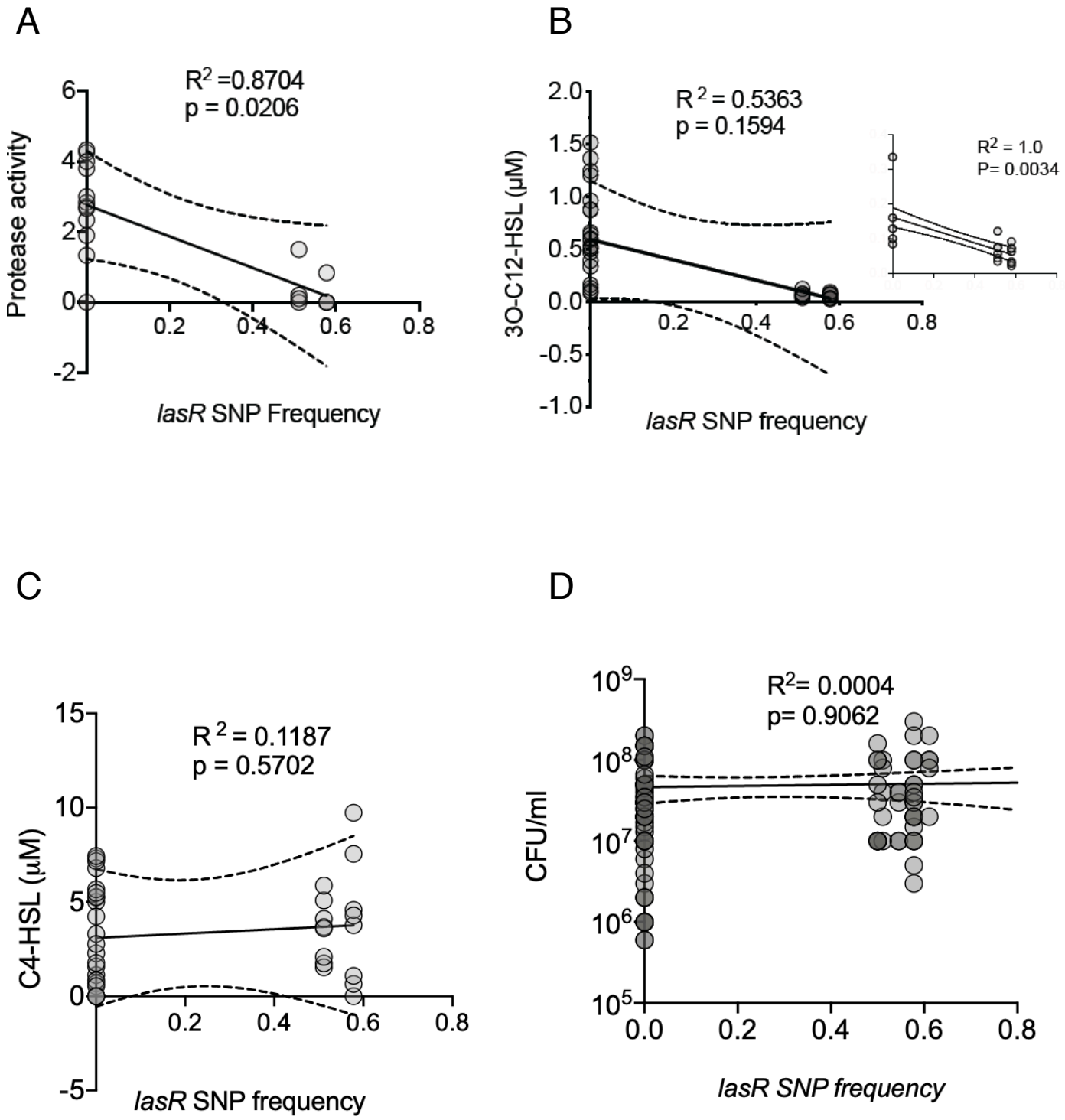
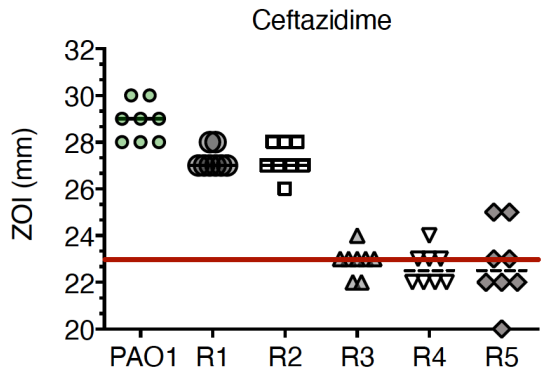
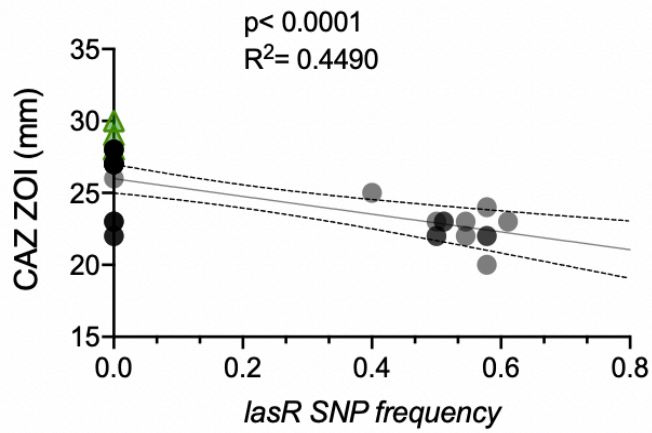

A
R2 =0.8704
p = 0.0206
Protease activity
lasR SNP Frequency
B
R 2 = 0.5363
p = 0.1594
3O-C12-HSL (μM)
lasR SNP frequency
R2 = 1.0
P= 0.0034
C
R 2 = 0.1187
p = 0.5702
C4-HSL (μM)
lasR SNP frequency
D
R2= 0.0004
p= 0.9062
CFU/ml
lasR SNP frequency

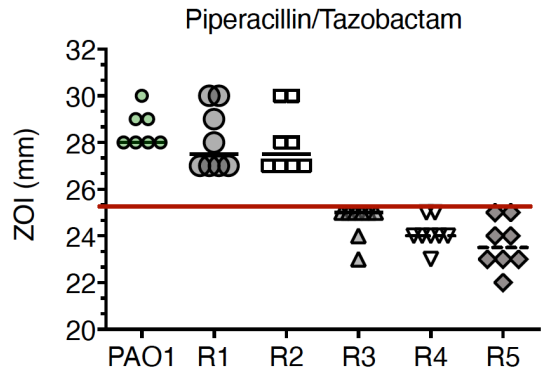
Figure 4

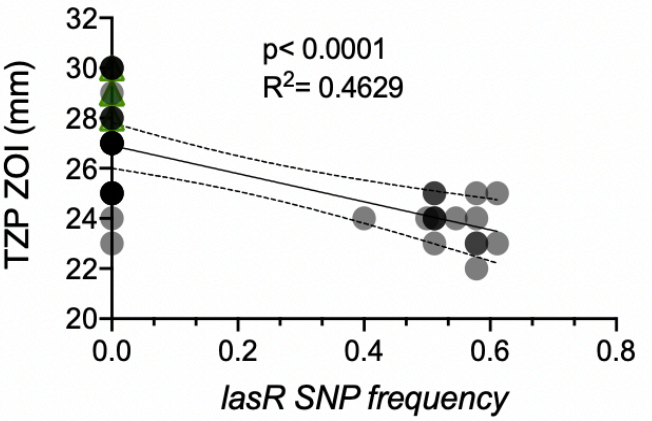
A


Ceftazidime
ZOI (mm)
32
30
28
26
24
22
20
PAO1
R1
R2
R3
R4
R5


p< 0.0001
R2= 0.4490
CAZ ZOI (mm)
35
30
25
20
15
0.0
0.2
0.4
0.6
0.8
lasR SNP frequency

B


Piperacillin/Tazobactam
ZOI (mm)
32
30
28
26
24
22
20
PAO1
R1
R2
R3
R4
R5


p< 0.0001
R2= 0.4629
TZP ZOI (mm)
32
30
28
26
24
22
20
0.0
0.2
0.4
0.6
0.8
lasR SNP frequency

C

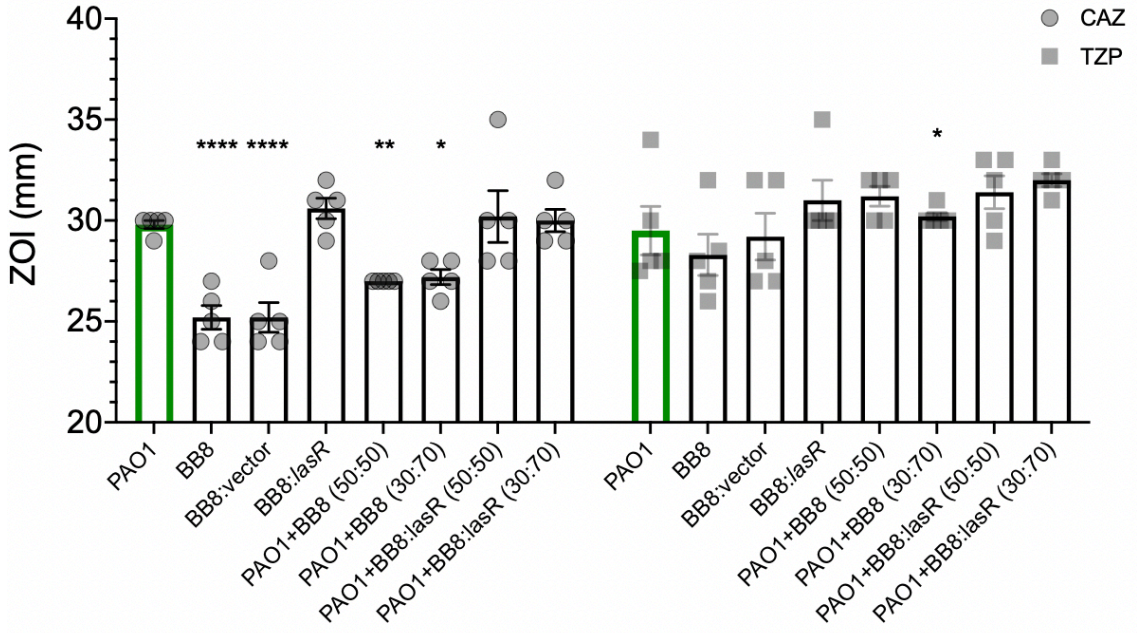

CAZ
TZP
ZOI (mm)
40
35
30
25
20
**** ****
**
*
*
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+BB8 (30:70)
PAO1+BB8:lasR (50:50)
PAO1+BB8:lasR (30:70)
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+BB8 (30:70)
PAO1+BB8:lasR (50:50)
PAO1+BB8:lasR (30:70)

Figure 5

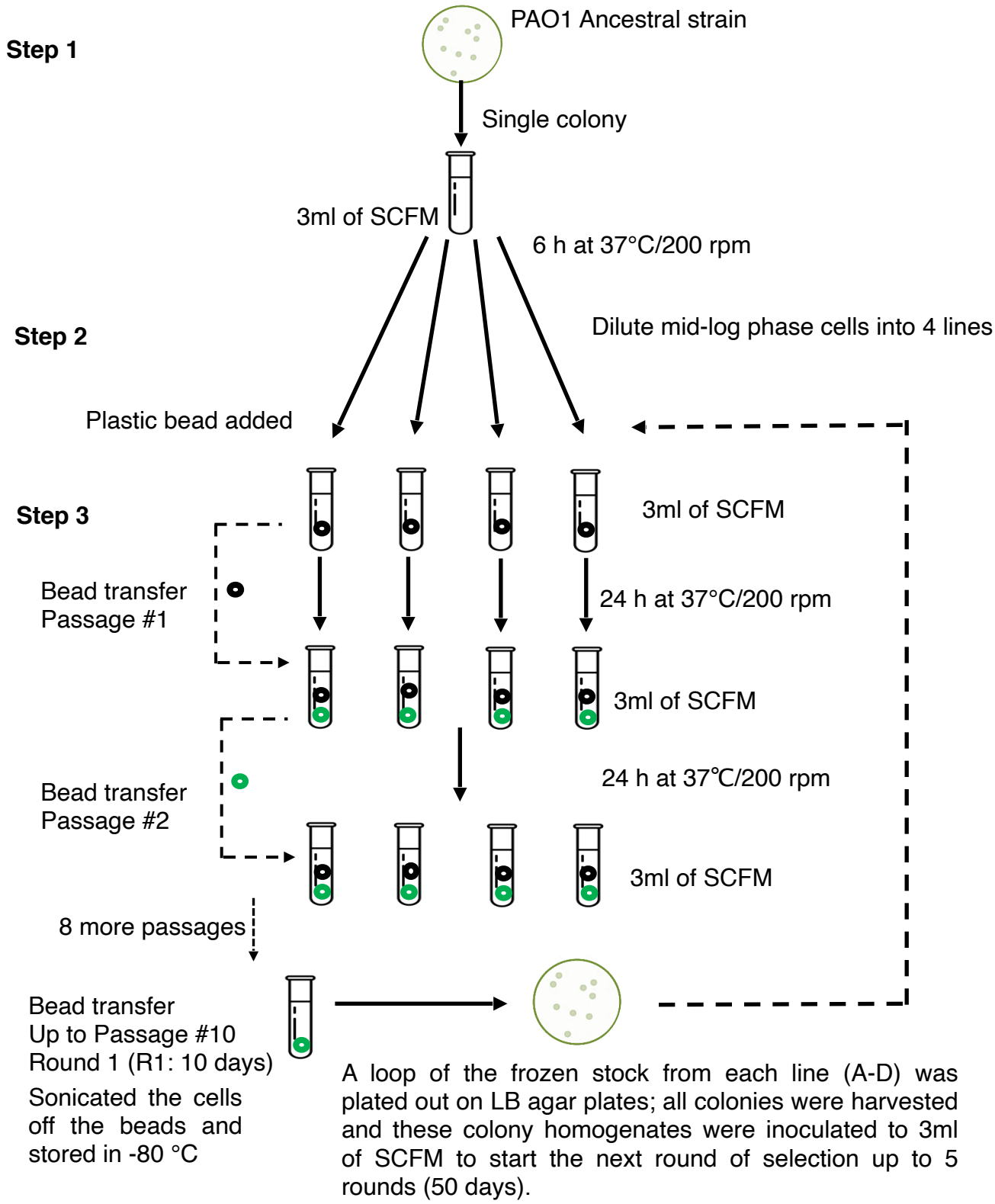

Step 1
PAO1 Ancestral strain
Single colony
3ml of SCFM
6 h at 37°C/200 rpm
Step 2
Dilute mid-log phase cells into 4 lines
Plastic bead added
Step 3
3ml of SCFM
Bead transfer
Passage #1
24 h at 37°C/200 rpm
3ml of SCFM
Bead transfer
Passage #2
24 h at 37°C/200 rpm
3ml of SCFM
8 more passages
Bead transfer
Up to Passage #10
Round 1 (R1: 10 days)
Sonicated the cells off the beads and stored in -80 °C
A loop of the frozen stock from each line (A-D) was plated out on LB agar plates; all colonies were harvested and these colony homogenates were inoculated to 3ml of SCFM to start the next round of selection up to 5 rounds (50 days).

Figure S1

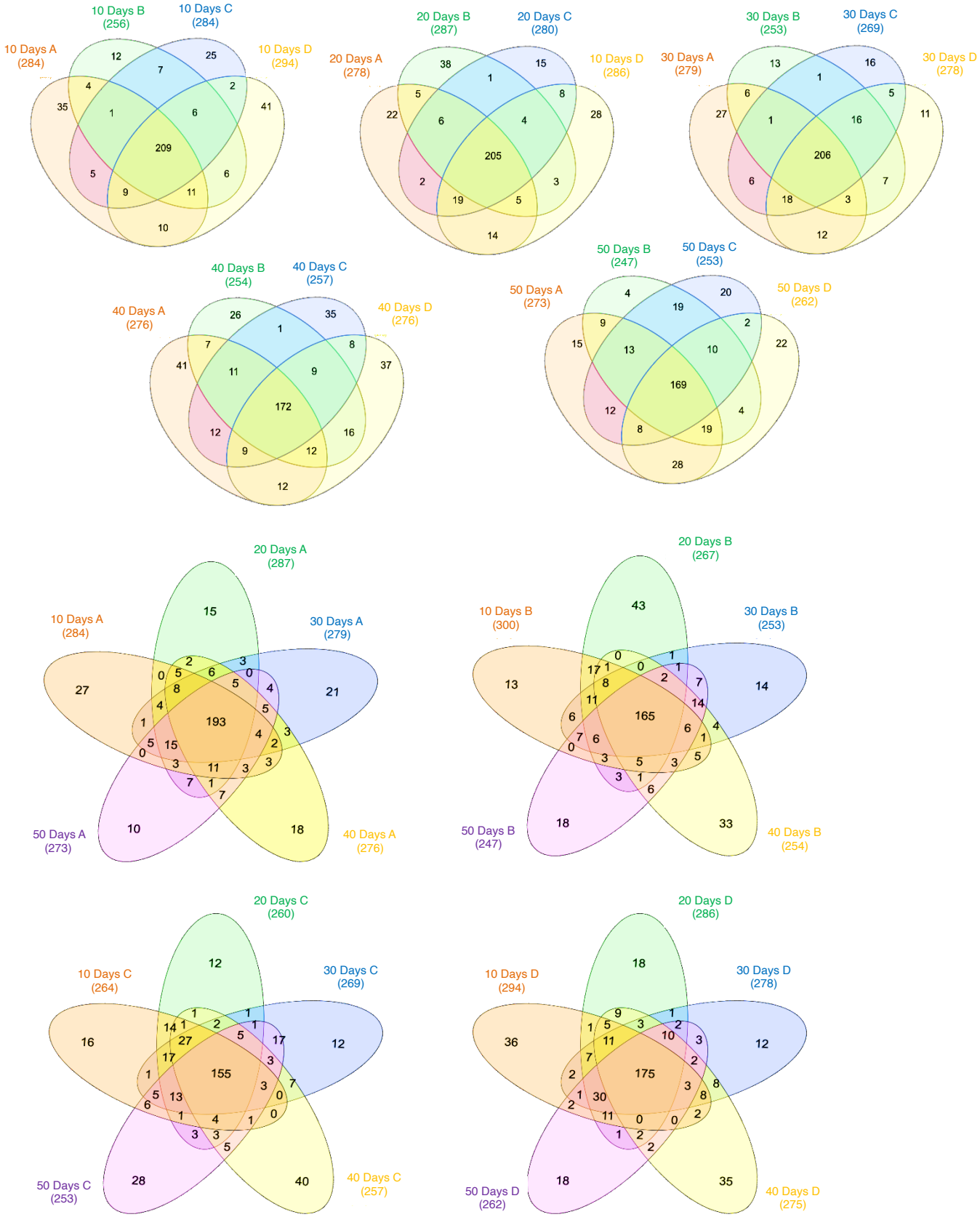

10 Days A (284)
10 Days B (256)
10 Days C (284)
10 Days D (294)
20 Days A (278)
20 Days B (287)
20 Days C (280)
10 Days D (286)
30 Days A (279)
30 Days B (253)
30 Days C (269)
30 Days D (278)
40 Days A (276)
40 Days B (254)
40 Days C (257)
40 Days D (276)
50 Days A (273)
50 Days B (247)
50 Days C (253)
50 Days D (262)
20 Days A (287)
10 Days A (284)
30 Days A (279)
50 Days A (273)
40 Days A (276)
20 Days B (267)
10 Days B (300)
30 Days B (253)
50 Days B (247)
40 Days B (254)
20 Days C (260)
10 Days C (264)
30 Days C (269)
50 Days C (253)
40 Days C (257)
20 Days D (286)
10 Days D (294)
30 Days D (278)
50 Days D (262)
40 Days D (275)

Figure S2

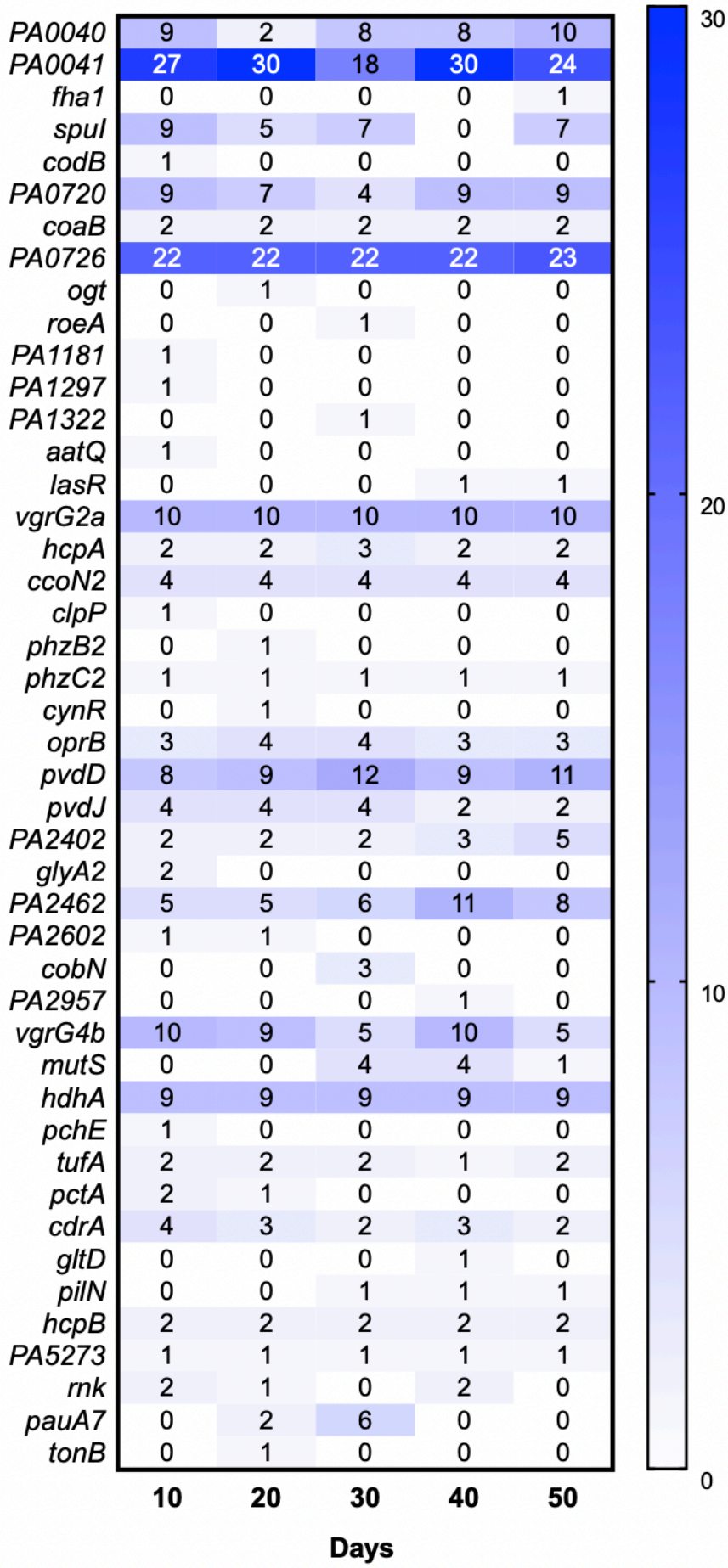

PA0040 9 2 8 8 10
PA0041 27 30 18 30 24
fha1 0 0 0 0 1
spuI 9 5 7 0 7
codB 1 0 0 0 0
PA0720 9 7 4 9 9
coaB 2 2 2 2 2
PA0726 22 22 22 22 23
ogt 0 1 0 0 0
roeA 0 0 1 0 0
PA1181 1 0 0 0 0
PA1297 1 0 0 0 0
PA1322 0 0 1 0 0
aatQ 1 0 0 0 0
lasR 0 0 0 1 1
vgrG2a 10 10 10 10 10
hcpA 2 2 3 2 2
ccoN2 4 4 4 4 4
clpP 1 0 0 0 0
phzB2 0 1 0 0 0
phzC2 1 1 1 1 1
cynR 0 1 0 0 0
oprB 3 4 4 3 3
pvdD 8 9 12 9 11
pvdJ 4 4 4 2 2
PA2402 2 2 2 3 5
glyA2 2 0 0 0 0
PA2462 5 5 6 11 8
PA2602 1 1 0 0 0
cobN 0 0 3 0 0
PA2957 0 0 0 1 0
vgrG4b 10 9 5 10 5
mutS 0 0 4 4 1
hdhA 9 9 9 9 9
pchE 1 0 0 0 0
tufA 2 2 2 1 2
pctA 2 1 0 0 0
cdrA 4 3 2 3 2
gltD 0 0 0 1 0
pilN 0 0 1 1 1
hcpB 2 2 2 2 2
PA5273 1 1 1 1 1
rnk 2 1 0 2 0
pauA7 0 2 6 0 0
tonB 0 1 0 0 0
10 20 30 40 50
Days
30
20
10
0

Figure S3

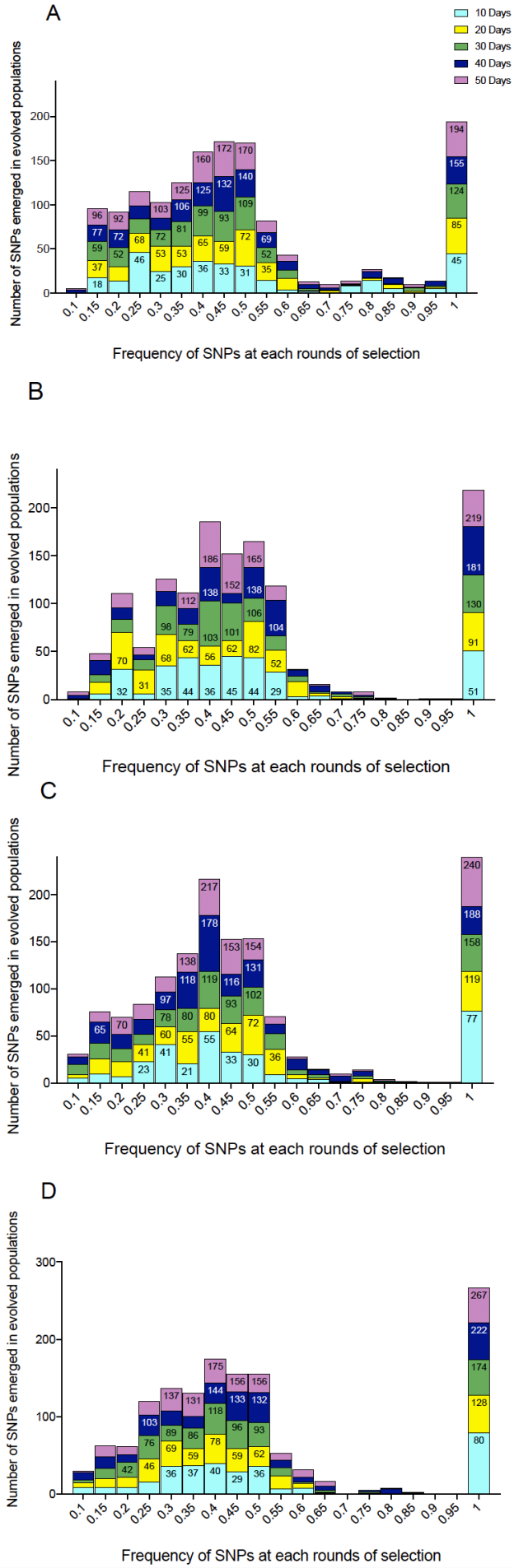

A
10 Days
20 Days
30 Days
40 Days
50 Days
Number of SNPs emerged in evolved populations
Frequency of SNPs at each rounds of selection
B
Number of SNPs emerged in evolved populations
Frequency of SNPs at each rounds of selection
C
Number of SNPs emerged in evolved populations
Frequency of SNPs at each rounds of selection
D
Number of SNPs emerged in evolved populations
Frequency of SNPs at each rounds of selection

Figure S4

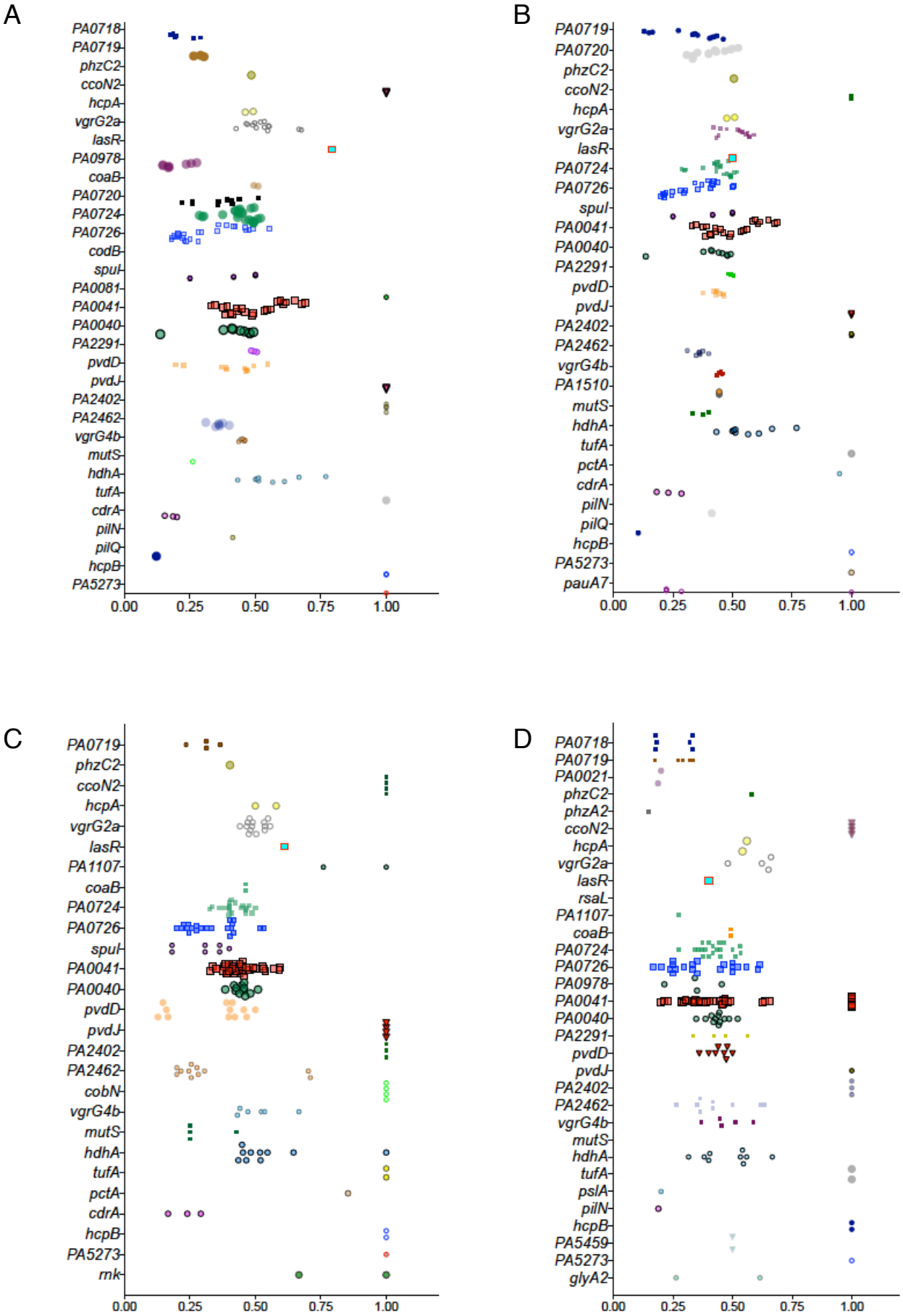

A
PA0718
PA0719
phzC2
ccoN2
hcpA
vgrG2a
lasR
PA0978
coaB
PA0720
PA0724
PA0726
codB
spuI
PA0081
PA0041
PA0040
PA2291
pvdD
pvdJ
PA2402
PA2462
vgrG4b
mutS
hdhA
tufA
cdrA
pilN
pilQ
hcpB
PA5273
0.00
0.25
0.50
0.75
1.00
B
PA0719
PA0720
phzC2
ccoN2
hcpA
vgrG2a
lasR
PA0724
PA0726
spuI
PA0041
PA0040
PA2291
pvdD
pvdJ
PA2402
PA2462
vgrG4b
PA1510
mutS
hdhA
tufA
pctA
cdrA
pilN
pilQ
hcpB
PA5273
pauA7
0.00
0.25
0.50
0.75
1.00
C
PA0719
phzC2
ccoN2
hcpA
vgrG2a
lasR
PA1107
coaB
PA0724
PA0726
spuI
PA0041
PA0040
pvdD
pvdJ
PA2402
PA2462
cobN
vgrG4b
mutS
hdhA
tufA
pctA
cdrA
hcpB
PA5273
rnk
0.00
0.25
0.50
0.75
1.00
D
PA0718
PA0719
PA0021
phzC2
phzA2
ccoN2
hcpA
vgrG2a
lasR
rsaL
PA1107
coaB
PA0724
PA0726
PA0978
PA0041
PA0040
PA2291
pvdD
pvdJ
PA2402
PA2462
vgrG4b
mutS
hdhA
tufA
pslA
pilN
hcpB
PA5459
PA5273
glyA2
0.00
0.25
0.50
0.75
1.00

Figure S5

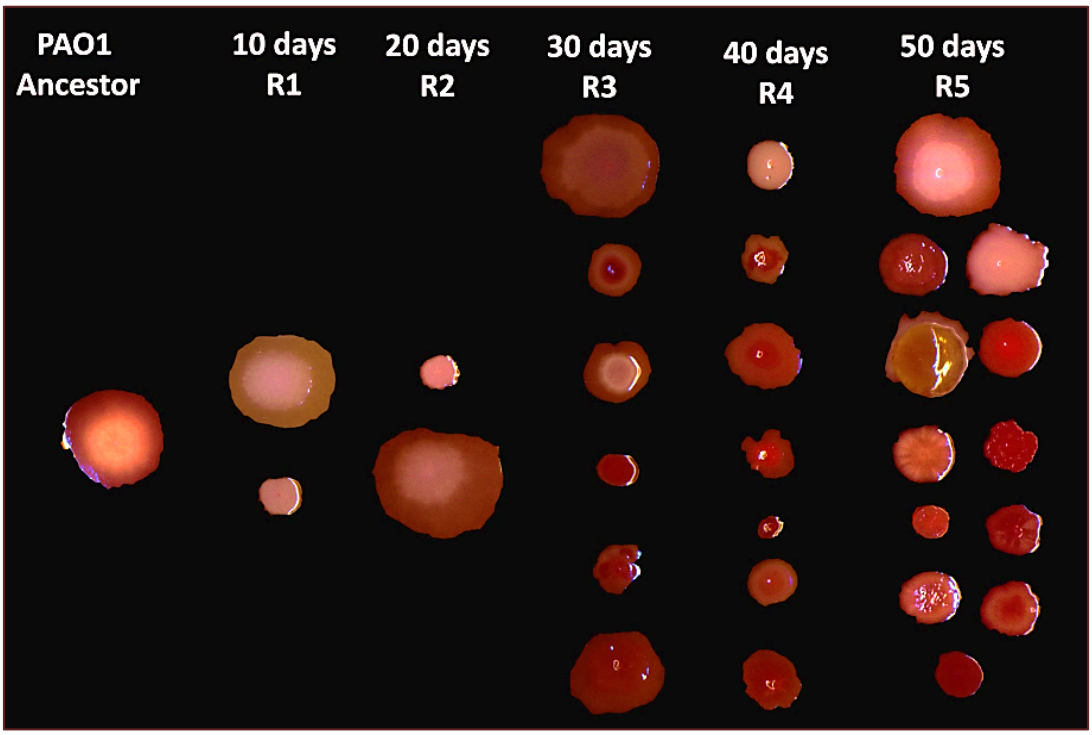

PAO1
Ancestor
10 days
R1
20 days
R2
30 days
R3
40 days
R4
50 days
R5

Figure S6

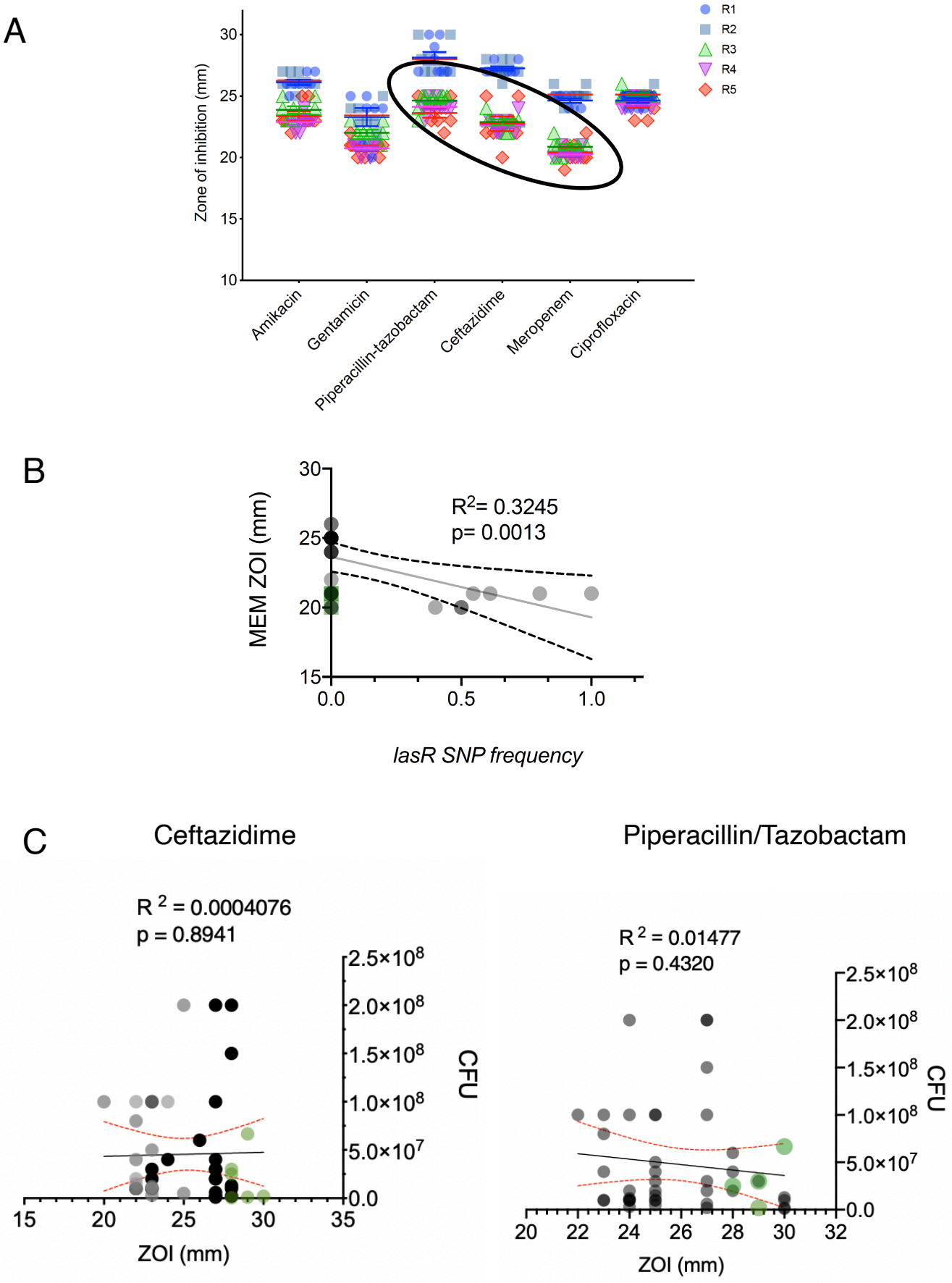
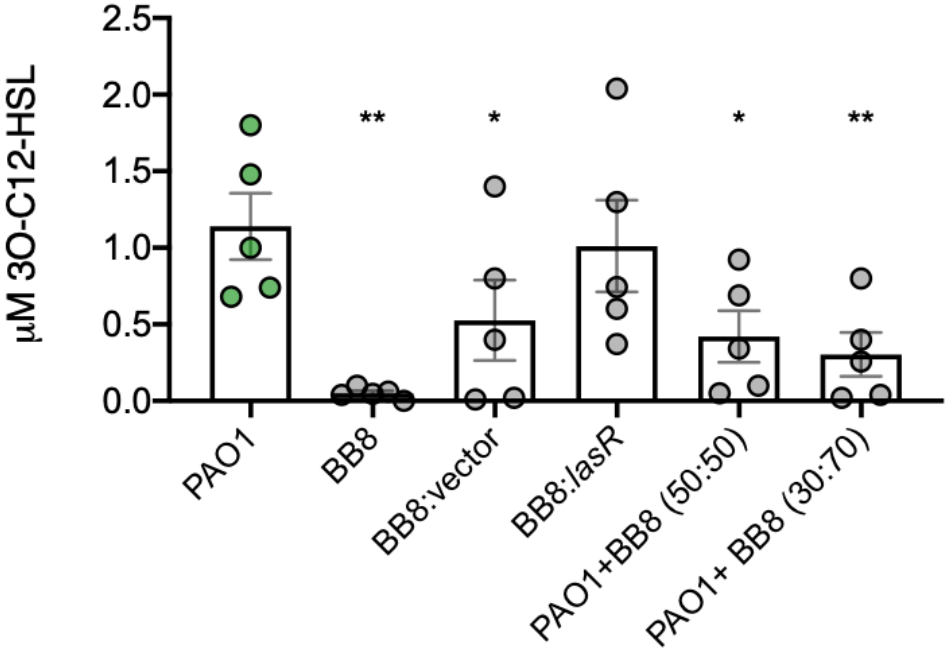

A
Zone of inhibition (mm)
30
25
20
15
10
R1
R2
R3
R4
R5
Amikacin
Gentamicin
Piperacillin-tazobactam
Ceftazidime
Meropenem
Ciprofloxacin
B
MEM ZOI (mm)
30
25
20
15
R2= 0.3245
p= 0.0013
0.0
0.5
1.0
lasR SNP frequency
C
Ceftazidime
R 2 = 0.0004076
p = 0.8941
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
CFU
15
20
25
30
35
ZOI (mm)
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 S7


μM 3O-C12-HSL
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
**
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+ BB8 (30:70)

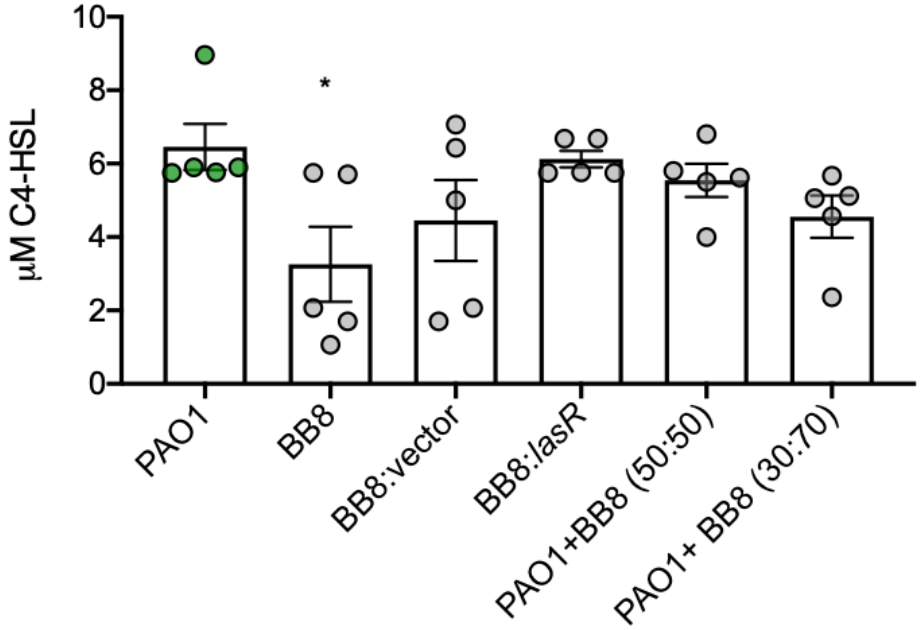
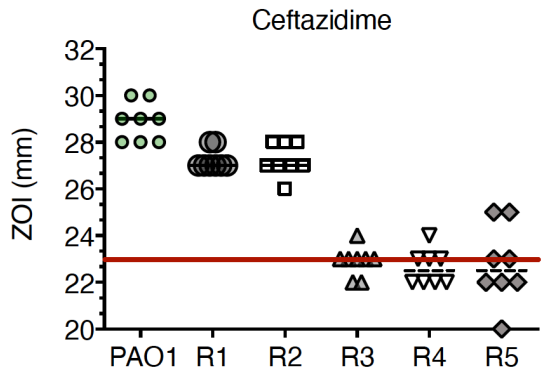
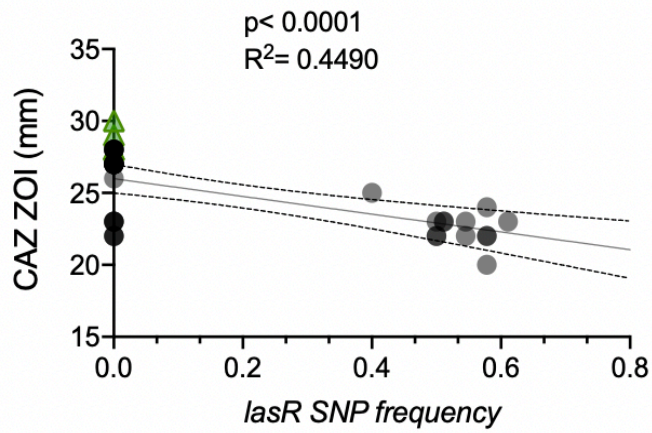

μM C4-HSL
10
8
6
4
2
0
*
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+ BB8 (30:70)

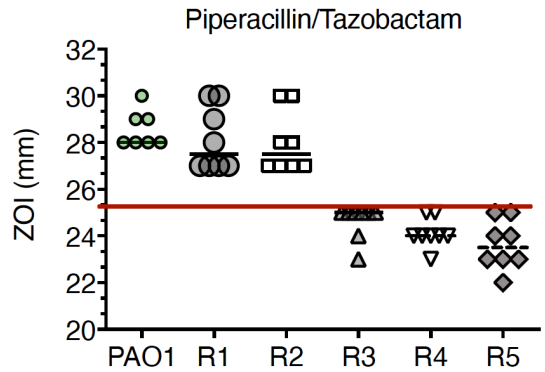
Figure S8

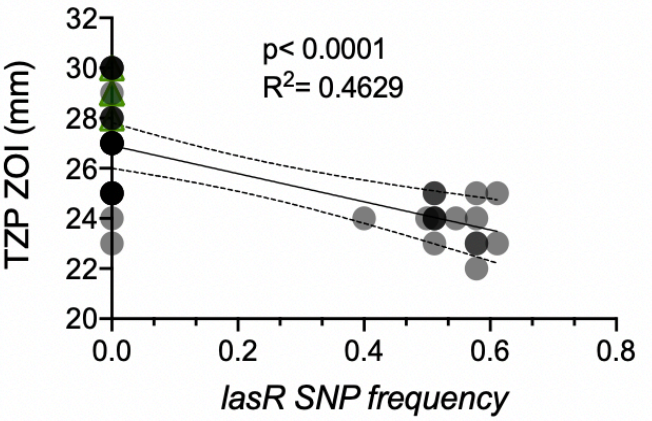
A


Ceftazidime
ZOI (mm)
32
30
28
26
24
22
20
PAO1
R1
R2
R3
R4
R5


p< 0.0001
R2= 0.4490
CAZ ZOI (mm)
35
30
25
20
15
0.0
0.2
0.4
0.6
0.8
lasR SNP frequency

B


Piperacillin/Tazobactam
ZOI (mm)
32
30
28
26
24
22
20
PAO1
R1
R2
R3
R4
R5


p< 0.0001
R2= 0.4629
TZP ZOI (mm)
32
30
28
26
24
22
20
0.0
0.2
0.4
0.6
0.8
lasR SNP frequency

C

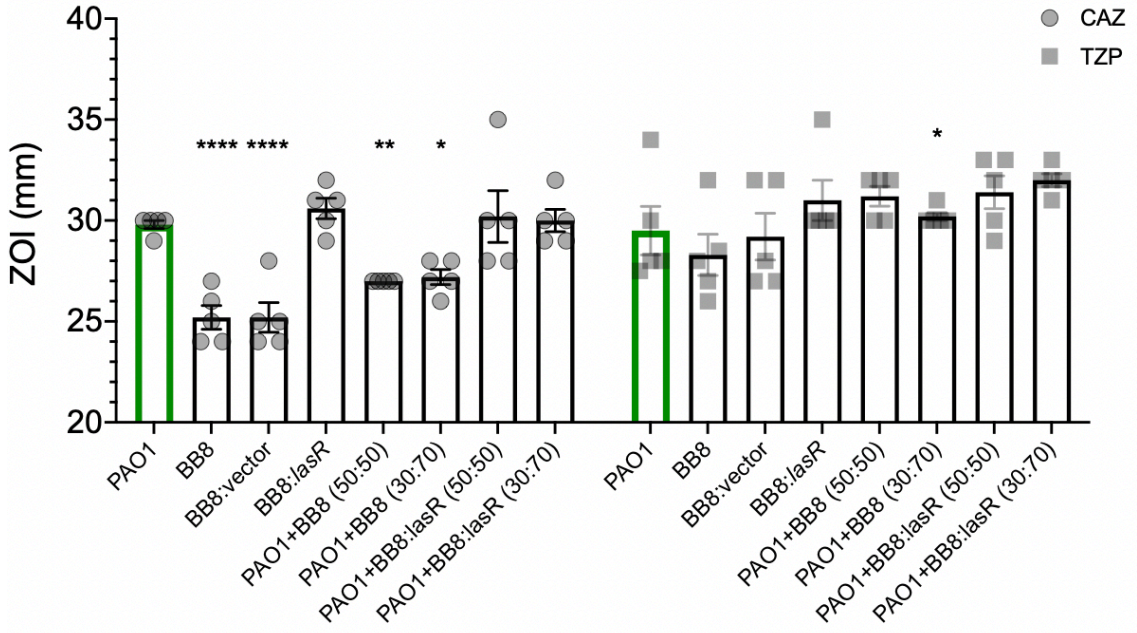

CAZ
TZP
ZOI (mm)
40
35
30
25
20
**** ****
**
*
*
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+BB8 (30:70)
PAO1+BB8:lasR (50:50)
PAO1+BB8:lasR (30:70)
PAO1
BB8
BB8:vector
BB8:lasR
PAO1+BB8 (50:50)
PAO1+BB8 (30:70)
PAO1+BB8:lasR (50:50)
PAO1+BB8:lasR (30:70)

Figure 5