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# Identifying and exploiting genes that potentiate the evolution of antibiotic resistance

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**One Sentence Summary:** Here we identify potentiator genes and pathways that make bacteria prone to evolving antibiotic resistance, and we exploit this to design treatment strategies for preventing resistance evolution.

**Introductory paragraph**: There is an urgent need to develop novel approaches for predicting and preventing the evolution of antibiotic resistance. Here we show that the ability to evolve *de novo* resistance to a clinically important β-lactam antibiotic, ceftazidime, varies drastically across the genus *Pseudomonas*. This variation arises because strains possessing the *ampR* global transcriptional regulator evolve resistance at a high rate. This does not arise because of mutations in *ampR*. Instead, this regulator potentiates evolution by allowing mutations in conserved peptidoglycan biosynthesis genes to induce high levels of β-lactamase expression. Crucially, blocking this evolutionary pathway by co-administering ceftazidime with the β-lactamase inhibitor avibactam can be used to eliminate pathogenic *P. aeruginosa* populations before they can evolve resistance. In summary, our study shows that identifying potentiator genes that act as evolutionary catalysts can be used to both predict and prevent the evolution of antibiotic resistance.

44

45 **Main text:** Antibiotic resistance in pathogenic bacteria poses a growing threat to human health,
46 by increasing the mortality rate and economic burden associated with bacterial infections[1]. In
47 light of this threat, there is an urgent need to develop new tools for predicting when resistance is
48 likely to evolve in pathogen populations[2]. Research in this area has largely focused on
49 understanding how differing antibiotic treatment strategies, such as mixtures and cycles,
50 influence the evolutionary dynamics of resistance[3-5]. An alternative approach is to ask if there are
51 specific genes that make bacteria more likely to evolve resistance to antibiotics[6]. Whole genome
52 sequencing has highlighted the incredible genetic diversity of pathogenic bacteria[7], but the
53 impact of this diversity on the evolution of antibiotic resistance remains poorly understood. For
54 example, recent work in *Streptococcus pneumoniae* has shown that genes that are important for
55 resistance in one strain may be completely dispensable in another[8]. Although many genes are
56 associated with clinical resistance, it is unclear to what extent other genes in the genome
57 influence the evolution of resistance. For example, recent work has shown that some genes
58 'potentiate' the evolution of novel bacterial phenotypes by opening otherwise inaccessible routes
59 to adaptation[9,10]. The existence of potentiator genes suggests that genomic background may play
60 a key role in the evolution of antibiotic resistance.

61 *In vitro* selection experiments have emerged as an important tool for studying the
62 evolution of antibiotic resistance[1,3,5]. However, these studies typically use selection lines derived
63 from a single ancestral clone, making it difficult to understand the role that genetic background
64 itself plays in the evolution of resistance. One approach to circumvent this difficulty is to use
65 comparative experimental evolution, where a diverse collection of strains are challenged with
66 adapting to a common selective pressure[6]. Using this approach, we recently demonstrated that
67 genetic background influences the evolution of resistance to rifampicin by altering the spectrum
68 and fitness effects of mutations in a highly conserved domain of RNA polymerase that confer
69 resistance to rifampicin[6,11]. In this paper, we extend this approach to uncover resistance
70 potentiator genes by challenging 8 strains that span the genus *Pseudomonas* with the β-lactam
71 antibiotic ceftazidime.

72 *Pseudomonas* is a diverse genus of bacteria that includes *P. aeruginosa*, an important
73 opportunistic pathogen of humans that is the primary cause of mortality in patients who suffer
74 from cystic fibrosis. Crucially, it is possible to culture a wide range of *Pseudomonas* strains
75 under a common set of lab conditions, making it possible to study evolutionary responses to
76 antibiotics in these bacteria using tightly controlled and replicated experiments. We chose to
77 study the evolution of resistance to ceftazidime for two reasons. First, ceftazidime is a clinically
78 relevant antibiotic that is commonly used to treat *Pseudomonas* infections[12] and ceftazidime
79 resistance is common in clinical isolates of *P. aeruginosa.* Second, the mechanisms of
80 ceftazidime action and resistance are well characterized. Ceftazidime inhibits cell wall
81 biosynthesis by irreversibly binding to periplasmic penicillin-binding proteins, ultimately leading
82 to cell death. In spite of this simple mechanism of action, *Pseudomonas* can use at least 4 routes
83 to evolve resistance to ceftazidime: altering the structure of penicillin-binding proteins,
84 upregulating the expression of efflux pumps, reducing permeability of the outer membrane and
85 upregulating the expression of β-lactamase enzymes that break down the antibiotic[13-15] (Figure
86 S1). Mutations altering the structure of the β-lactamase enzyme itself do occur, but provide much
87 lower increases in resistance[16].

88 Here we use a serial passage experiment to challenge close to 1,000 populations of
89 *Pseudomonas* with doses of ceftazidime that increased from sub-lethal to lethal concentrations
90 over the course of 1 week. We then use extensive whole genome re-sequencing of evolved
91 clones to identify genes and pathways that contribute to the rapid evolution of elevated
92 ceftazidime resistance. Using selection experiments and competition assays with defined
93 mutants, we directly test the evolutionary impact of key pathways to resistance identified from
94 whole genome sequencing. Finally, we demonstrate that understanding the genetic drivers of
95 resistance evolution can be used to design a simple drug mixture, consisting of ceftazidime
96 coupled to a β-lactamase inhibitor, to prevent the evolution of resistance *in vitro*.
97

## 98 Results and discussion

99 ***Strain-specific variation in resistance evolution*** To test the role of genetic background in the
100 evolution of antibiotic resistance, we challenged 120 populations of each of 8 strains that span
101 the diversity of the genus *Pseudomonas* with ceftazidime (Figure 1a). This breadth of
102 phylogenetic coverage allowed us to explore the impact of genome content on resistance
103 evolution, and strains were chosen on the basis of variation in genome size, experimental
104 tractability, and the availability of high-quality published reference genomes. Populations were
105 serially passaged in standard lab culture medium supplemented with ceftazidime, the
106 concentration of which was doubled daily from sub-lethal (1/8 minimal inhibitory concentration,
107 or 'MIC') to super-lethal (8× MIC) levels over a 7 day selection experiment. The MIC of the
108 parental strains varies (0.65-8 mg/L) and we controlled for this variation by standardizing
109 antibiotic doses of selection lines to their appropriate parental strains. In this experimental
110 design, populations can only avoid extinction if they evolve elevated antibiotic resistance, and
111 we measured population survival at each day of the experiment. We define the rate of population
112 extinction within strains as a measure of adaptive potential for resistance evolution, or
113 'evolvability'. The rate of population extinction varied profoundly between strains (Figure 1b;
114 Cox's proportional hazard, likelihood ratio=1930, df=7, $P<10^{-6}$). For example, all of the replicate
115 populations went extinct in some strains, such as *P. mendocina* CCUG1781 and *P. fulva*
116 CCUG12537, while at the other extreme, every population of *P. protegens* Pf-5 survived at up to
117 8× the MIC of the parental strain. Given that resistance evolved by selection on spontaneous
118 mutations, one potential explanation for this result is that the ability to evolve ceftazidime
119 resistance correlates to the mutation rate. However, evolvability does not correlate with mutation
120 rate (r=0.33, $F_{1,6}$=0.74, P=0.42, see supplementary table S1 for calculations) or mutation supply
121 rate, which is the product of initial population size and mutation rate (r=0.22, $F_{1,5}$=0.27, P=0.62).
122 Additionally, there was no correlation between survival and the absolute difference between the
123 temperature of the selection experiment (30° C) and published optimal growth temperatures for
124 each strain (r=0.06, $F_{1,6}$=0.027, P=0.88).

125 ***Genomics of resistance evolution*** To determine the genetic basis of resistance evolution, we
126 sequenced the genomes of 100 independently evolved clones from populations that survived
127 selection for elevated resistance (*n*=14–24 clones/strain). We identified a total of 196 novel
128 mutations in 69 unique genes (i.e. orthologs across strains are each counted once). Mutations
129 included SNPs (*n*=80), short indels (*n*=71), insertion element insertions (*n*=15), larger insertions
130 and deletions (*n*=7), and intergenic mutations (*n*=23). Several lines of evidence indicate that the
131 mutations that we identified were predominantly beneficial. First, parallel evolution occurred
132 both within and across strains. We identified a total of 25 genes mutated in two or more

independent clones, and 76% of mutations occurred in these 25 genes. Second, all 80 SNPs observed in coding regions were non-synonymous, which is a clear hallmark of positive selection. A full list of the mutations we identified is given in supplementary data table S2.We categorized mutations according to known resistance mechanisms: (i) porin genes, (ii) penicillin binding proteins (PBPs), (iii) peptidoglycan biosynthesis genes and (iv) multidrug efflux pumps[13-15]. Almost all of the evolved clones (88/100) carry mutations in previously established ceftazidime resistance pathways. However, the distribution of mutations across these resistance pathways differs profoundly between strains, demonstrating strain-specific mechanisms of resistance evolution (Figure 2; $\chi^2$=139, df=12, P<$10^{-6}$). *P. protegens* Pf-5 and *P. fluorescens* Pf0-1 adapt by mutations in genes involved in peptidoglycan biosynthesis and recycling (*ampD* and *mpl*), knockouts of which are known to increase the expression of the chromosomal *ampC* β-lactamase gene[14,17]. In addition to mutations in *ampD* and *mpl*, 21 of 24 clones of *P. aeruginosa* PAO1 carry mutations in a non-essential PBP (*dacB*/PBP4) that has also been shown to increase *ampC* expression when knocked out[18]. Consistent with this genetic data, clones from these strains have increased resistance to a broad spectrum of β-lactams, but retain sensitivity to imipenem, which is a poor substrate for the AmpC β-lactamase. In contrast, *P. stutzeri* ATCC17588 and *P. putida* KT2440 evolve resistance by mutations in efflux pump genes and, to a lesser extent, porins. Mutations in efflux pumps are associated with small increases in ceftazidime resistance and a multi-drug resistant phenotype, while porin mutations are predominantly associated with elevated β-lactam resistance (Figure 2). A substantial fraction (33.3%) of clones from these strains lack mutations in known resistance genes; however, these clones have resistance profiles that are similar to those of clones carrying mutations in known efflux pumps or porins.

***The AmpR transcription factor increases evolvability*** The key insight from whole genome sequencing, and phenotypic analysis of evolved clones, is that large increases in ceftazidime resistance are associated with mutations in the peptidoglycan biosynthesis pathway associated with increased β-lactamase production[14]. Importantly, the relevant peptidoglycan biosynthesis genes (*ampD, mpl* and *dacB*) are present in all of the strains, and the *ampC* β-lactamase gene is present in all of the strains except *P. stutzeri* ATCC17588 (which possesses another β-lactamase gene, *blaZ)*.

These observations raise an interesting puzzle: if the key genes involved in adaptation are largely maintained, then why does evolvability vary across strains? An alternative approach to understanding why evolvability varies across strains is to take a functional approach to characterizing the effects of beneficial mutations. Inactivation of the peptidoglycan biosynthesis genes involved in adaptation in our experiment has been shown to increase *ampC* expression by causing an intracellular accumulation of peptidoglycan catabolites[14,17]. However, *ampC* induction via this mechanism requires the AmpR transcription factor; inactivation of *ampR* removes the ability to increase *ampC* expression in response to β-lactams[17]. Crucially, among our strains *ampR* is only present in the genomes of *P. aeruginosa* PAO1, *P. protegens* Pf-5, and the two *P. fluorescens* Pf0-1 and SBW25, and not found in the others. This simple association between the presence of the AmpR transcription factor and the probability of survival to the end of the experiment through adaptation suggests that regulation of *ampC* expression is key.

How does *ampR* increase evolvability? One simple possibility is that this regulator potentiates evolution by opening up new genetic paths to evolving elevated ceftazidime resistance[9,10]. Specifically, *ampR* could potentiate the evolution of ceftazidime resistance by allowing mutations in peptidoglycan biosynthesis genes, such as *ampD*, *mpl,* and *dacB* to increase levels

178 of *ampC* expression. Consistent with this hypothesis, mutations in peptidoglycan biosynthesis
179 genes and *dacB* are known to only increase resistance in the presence of *ampR*[17,18]. This
180 hypothesis generates two simple predictions that can be tested using our method. First, if
181 elevated expression of *ampC i*s a key mechanism for evolving ceftazidime resistance, then
182 deleting *ampC s*hould decrease evolvability. Second, if the AmpR regulator is required to drive
183 the evolution of increased *ampC e*xpression, then deleting *ampR s*hould reduce evolvability by
184 the same amount as deleting the *ampC.* To test these predictions, we challenged populations of
185 Δ*ampR* and Δ*ampC* mutants of *P. aeruginosa* PAO1 with increasing doses of ceftazidime, as in
186 our initial experiment (Figure 3a). Both of the mutants have dramatically reduced evolvability
187 compared to their isogenic *P. aeruginosa* PAO1 control (Cox's proportional hazard, likelihood
188 ratio=23.82, df=2, $P=6\times10^{-6}$), providing conclusive evidence that both the β-lactamase (*ampC)*
189 and its regulator (*ampR*) play key roles in driving the evolution of elevated ceftazidime
190 resistance.

191 The low survival probability of *P. aeruginosa* PAO1 in comparison with the other strains that
192 carry both *ampR* and *ampC* is also consistent with this hypothesis. Strains of *P. fluorescens and*
193 *P. prote*gens carry 2 homologs of *ampD*, which represses the expression of *ampC*, whereas *P.*
194 *aerugi*nosa PAO1 carries 3 homologs of this gene. The additional copy of *ampD* found in *P.*
195 *aeruginosa* ensures that *ampD* mutations lead to weaker de-repression of *ampC* expression, and
196 this is likely to translate into reduce evolvability in comparison to strains with onl*y* 2 *ampD*
197 homologs; the *ampD* dosage effect has been demonstrated experimentally[20]. Consequently, most
198 surviving *P. aeruginosa* strains possessed two loss of function mutations in the peptidoglycan
199 biosynthesis pathway, in comparison with one only in the other *ampR/ampC* possessing strains
200 (Figure 2).

201 Additionally, it is possible that adaptive plasticity in *ampC* expression mediated by *ampR* could
202 increase evolvability[22]. Exposure to β-lactam antibiotics interferes with peptidoglycan
203 biosynthesis by inhibiting PBPs, causing an AmpR-mediated increase in *ampC* expression[17,19].
204 This, in turn, may accelerate the genetic evolution of resistance by providing bacterial
205 populations with the time to acquire ceftazidime resistance mutations. According to this
206 explanation, *ampR* increases evolvability through ecological potentiation. The key assumption of
207 this hypothesis is that the plasticity in *ampC* expression mediated by *ampR* must provide a
208 benefit in the presence of ceftazidime. To test this hypothesis, we measured the effect of deleting
209 *ampR* and *ampC* on fitness using short-term competition experiments (Figure 3b). Deleting
210 *ampC* leads to a decrease in fitness the presence of ceftazidime, demonstrating that induced
211 expression of this gene is beneficial. However, deleting *ampR* actually increases fitness in the
212 presence of sub-MIC concentrations of ceftazidime, demonstrating that plasticity in gene
213 expression cannot explain the link between *ampR* and increased evolvability. Indeed, as *ampR*
214 expression is not particularly strongly induced by ceftazidime[23], this suggests that *ampR* does not
215 simply allow populations to 'buy time' to wait for an adaptive mutation. Although this result is
216 counter-intuitive, it is important to emphasize that *ampR* is a global transcriptional regulator that
217 affects the expression of 100s of genes[24,25], including repressing another chromosomal β-
218 lactamase, *poxB*[25]. In particular, *ampR* is involved in regulating quorum sensing factors,
219 including *lasR*, several metabolic pathways, and the *rpoS*-mediated stress response pathway[25].
220 Although it is clear that inducing elevated levels of *ampC* expression in the presence of
221 ceftazidime is beneficial, the fitness cost associated with the *ampR* regulator implies that the net
222 fitness effect of all of the changes in gene expression caused by this regulator in the presence of
223 ceftazidime is deleterious. The importance of *ampR* as a global regulator of expression perhaps

224 explains why increased *ampC* expression did not arise through mutations in *ampR* itself, and
225 why *ampR* mutations are not typically observed in clinical *P. aeruginosa* isolates[26].

226 ***Inhibiting the evolution of ceftazidime resistance*** Given the important role that *ampR* mediated
227 induction of *ampC* expression plays in the evolution of resistance, our results suggest that one
228 possible strategy to prevent the evolution of cephalosporin resistance in *P. aeruginosa* infections
229 would be to co-administer ceftazidime with AmpC β-lactamase inhibitors[27]. The rationale for this
230 strategy is that a combination of a β-lactam and β-lactamase inhibitor will be active against both
231 wild-type bacterial strains and mutants with elevated β-lactamase secretion. In other words, this
232 strategy should effectively block a major evolutionary path to elevated resistance. To test this
233 idea, we challenged *P. aeruginosa* PAO1 with ceftazidime in the presence of avibactam[28], a
234 recently developed AmpC inhibitor (Figure 4a). Unlike most β-lactamase inhibitors, avibactam
235 does not possess any toxic effects on *Pseudomonas*[28] and we did not detect any population
236 extinction in the avibactam treated control populations. In support of our hypothesis, avibactam
237 increased the rate of population extinction in the presence of ceftazidime compared to
238 ceftazidime treated control populations (Cox's proportional hazard, likelihood ratio test=78.968,
239 df=1, $P<10^{-6}$). We failed to detect any viable cells in 59 out of 60 populations of *P. aeruginosa*
240 that were selected in 8× MIC ceftazidime supplemented with avibactam demonstrating that the
241 effect of avibactam suppresses the evolution of elevated β-lactamase secretion just as effectively
242 as knocking out *ampC* or *ampR* (Figure 3a). Importantly, this effect does not arise because
243 avibactam increases the potency of ceftazidime. Surprisingly, we found that avibactam treatment
244 actually increased the MIC of ceftazidime from 0.76 mg/L to 1.14 mg/L (Figure S2).

245 As a final test of the role of *ampR* in evolvability, we challenged populations of Δ*ampR*
246 (Figure 4b) and Δ*ampC* (Figure 4c) mutants of *P. aeruginosa* PAO1 with a combination of
247 ceftazidime and avibactam, as in our experiment with wild-type *P. aeruginosa* PAO1. If our
248 hypothesis is correct, then avibactam should have no effect on evolvability in these mutant
249 strains, because they are effectively unable to increase *ampC* expression under our experimental
250 conditions. Consistent with this idea, we found that avibactam does not have an effect on
251 evolvability in either Δ*ampR* or Δ*ampC* mutants (Cox's proportional hazard, likelihood ratio
252 test=3.25, df=1, P= 0.071 and likelihood ratio test=0.02, df=1, P=0.876, respectively).

253

## 254 Conclusion

255 Whole genome sequencing is revolutionizing our understanding of the evolution and
256 ecology of bacterial pathogens. One of the challenges that has arisen from this revolution is to
257 understand the consequences of genetic diversity in pathogen populations. Here we show that
258 comparative experimental evolution can be used to identify genes and pathways that influence
259 the rate and mechanisms of adaptation to antibiotics. Our experiment addressed this problem at a
260 fairly broad scale, by comparing the evolutionary responses of strains from different species. Our
261 initial reasoning for working at this scale was that comparing divergent strains effectively
262 maximizes the number of genes and SNPs that are included in the experiment, therefore
263 maximizing the likelihood of detecting an impact of genetic background on evolvability.
264 However, the sheer number of genetic differences between even the most closely related strains
265 used in this study may have hindered our ability to detect more subtle genomic effects on
266 evolvability. While it is clear that inducible *ampC* β-lactamase expression is an important driver
267 of evolvability in this genus, it is clear that other genes must influence the ability to evolve

268 ceftazidime resistance. For example, *P. stutzeri* ATCC17588 and *P. putida* KT2440, both of
269 which lack *ampR,* have similar evolvability to *P. aeruginosa* PAO1. We are currently extending
270 this research program by focusing on studying variation in evolvability between clones from the
271 same species, and we hope that this approach will enable us to identify genetic drivers of
272 evolvability in greater depth.

273 The differing modes of *ampC* expression among the pseudomonads affect their ability to evolve
274 resistance to β-lactams by interacting with genes in the peptidoglycan biosynthesis pathway. In
275 strains possessing *ampR*, the intracellular accumulation of peptidoglycan catabolites converts the
276 AmpR transcription factor into an activator of *ampC* expression in response to peptidoglycan
277 damage. Mechanistically, *ampR* increases evolvability by allowing mutations in peptidoglycan
278 biosynthesis genes to induce high levels of β-lactamase expression, which effectively amplifies
279 the *ampC* expression plasticity that occurs when cell wall synthesis is compromised by β-
280 lactams[20]. From a more conceptual perspective, *ampR* can be thought of as a conduit that
281 translates genetic variation in the peptidoglycan biosynthesis gene network into phenotypic
282 variation in *ampC* expression. This suggests that response pathways that are involved in sensing
283 environmental change may have a general role as evolutionary catalysts, linking plastic and
284 mutational responses to environmental change. Intriguingly, these alternative expression modes
285 are disseminated among the enterobacteria; however, insertion of the *ampR* gene into
286 constitutive producers is not sufficient to restore inducible expression, suggesting a distinct
287 regulatory mechanism in constitutive producers[29]. To avoid the evolution of high levels of
288 ceftazidime resistance, and subsequent treatment failure, treatment with ceftazidime should be
289 avoided in infections caused by strains with inducible *ampC* expression.

290 Understanding the evolutionary trajectory to high levels of ceftazidime resistance makes it
291 possible to design a simple two-drug mixture consisting of ceftazidime and avibactam that can be
292 used to effectively eliminate populations of the pathogen *P. aeruginosa*. We argue that this
293 strategy is successful because avibactam effectively prevents mutations in peptidoglycan
294 biosynthesis genes and *dacB* from increasing ceftazidime resistance, eliminating their fitness
295 benefit. One possible solution to this evolutionary challenge would be to first evolve avibactam
296 resistance, and then evolve ceftazidime resistance. However, avibactam does not have any
297 detectable toxic effects on *Pseudomonas* at concentrations where it is able to effectively inhibit
298 AmpC, rendering this evolutionary pathway to combined avibactam/ceftazidime resistance
299 inaccessible. Although these results are encouraging, we emphasize that there are a number of
300 confounding factors that may affect the efficacy of this combination of drugs *in vivo*. For
301 example, the pharmacokinetic properties of the two drugs may make it difficult to effectively
302 maintain the drug mixtures at the site of bacterial infections, and it is also possible that
303 avibactam resistant alleles of *ampC* or other β-lactamases capable of hydrolyzing ceftazidime are
304 already present in pathogen populations.

305 Predicting the evolution of antibiotic resistance is a challenging and important objective. Here
306 we show that comparative experimental evolution can be used to identify genes and pathways
307 that make some bacterial strains prone to evolving resistance, and to exploit this to design
308 treatment strategies for preventing resistance evolution. High throughput sequencing is
309 revolutionizing clinical microbiology[30,31], and it may be possible to identify such potentiator
310 genes in clinical pathogen populations and to use this information to optimize antimicrobial
311 treatment strategies.

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316 **Statement of author contributions:** This study was designed by RCM. Experiments were
317 carried out by VF, AP and TV. Bioinformatics were done by DRG. VF, AP, DRG and RCM
318 analyzed data. AO contributed reagents and expertise. The manuscript was written by RCM,
319 DRG and VF.

320 **Competing interests:** The authors declare the absence of any competing interests.

321

## 322 Materials and Methods

323 *MIC Determination for parental strains*

324 Three independent estimations of the MIC for each parental strain were determined in 96-well
325 plates using the broth microdilution method. Briefly, 5-10 morphologically similar colonies of
326 each strain were resuspended in sterile saline solution (NaCl 0.9%). The solution was adjusted to
327 the adequate optical density so that it would contain approximately $1.5 \times 10^8$ cells/mL. This
328 standardized inoculum and was diluted a further 200-fold in Mueller-Hinton 2 (MH2, Sigma-
329 Aldrich, United Kingdom) broth containing ceftazidime (Sigma-Aldrich) at a concentration
330 between 64 mg/L and 0.0625 mg/L. After 24h of incubation at 30 °C with shaking at 250 rpm,
331 optical density at 595nm was determined for each well with a Synergy 2 plate reader (Biotek,
332 Winooski, USA). We considered that bacterial growth had been inhibited if the optical density
333 was less than 25% of that of antibiotic-free cultures. The lowest antibiotic concentration at which
334 growth had been inhibited was considered the MIC. The measured MIC was used to calculate the
335 ramping ceftazidime concentration regime in the selection experiment (see "Experimental
336 evolution").

337 *Effect of avibactam on MIC*

338 The effect of avibactam on MIC was evaluated by measuring growth inhibition by ceftazidime at
339 the presence/absence of avibactam. The procedure was identical to MIC determination described
340 above, except that one group of replicates was supplemented with 4 mg/L of avibactam
341 (BioVision Inc. USA). No avibactam was added to a control group. The avibactam treatment and
342 control groups were tested at concentrations ranging from 0.1 mg/L to 3.8 mg/L of ceftazidime
343 with 4 replicates each.

344 *Mutation rate estimation*

345 Mutation rates were estimated by fluctuation assays, with the antibiotic rifampicin as the
346 selection agent, using the method of Luria and Delbruck[32]. 480 replicate cultures were inoculated
347 with approximately 50 cells from an overnight culture of each parental strain and incubated for
348 48 hours in 200 µl of KB media at 30 °C with constant shaking at 200 rpm. Approximately $10^7$
349 cells from each culture were then plated onto KB-agar containing rifampicin at the appropriate
350 MIC (minimum inhibitory concentration) for each strain (60 mg/L for *P. aeruginosa* PAO1, 30
351 mg/L for all other strains). For each strain the proportion of cultures yielding no mutants was
352 scored, from which the mutation per culture was calculated using the negative natural logarithm.
353 This value was then divided by the number of cells plated, which provides an estimate of the
354 mutation rate per cell division.

355 *Experimental evolution*

356 To initiate the selection experiment, all parental strains were recovered from −80 °C stocks and
357 cultured overnight in MH2 broth at 30 °C for 24 h with shaking at 250 rpm. Next, the cultures
358 were diluted by $10^{-6}$ in MH2 broth and distributed on 96-well plates (200 µL per well). After 48 h
359 of incubation at 30°C, we initiated the first transfer by diluting these cultures 1:100 in MH2
360 broth containing 1/8 MIC of ceftazidime, relative to the measured MIC of each strain. Bacterial
361 populations were incubated for 24 h at 30°C with shaking at 250 rpm and diluted 1:100 for the
362 next transfer. Every transfer ceftazidime concentration was doubled, reaching 8× MIC in the
363 final transfer. Population survival was monitored during the course of the selection experiment
364 by measuring optical density at 595 nm using a Synergy 2 microtiter plate reader (BioTek,
365 Winooski, VT, USA). We additionally confirmed population survival after the last transfer by
366 plating a 1 uL sample of each population on antibiotic-free MH2 agar plates that were scored for
367 growth after overnight incubation at 30 °C. We performed the evolution experiment in two
368 independent blocks. In each block we propagated 60 replicates populations of each strain that
369 were challenged with increasing doses of ceftazidime and 12 replicate controls populations of
370 each strain that were allowed to evolve in antibiotic-free MH2. At the end of the experiment, a
371 maximum of 20 population per strain were streaked in MH2 agar plates and a clone was picked
372 for each population was picked and amplified for further analyses. To avoid bias by conducting
373 the experiment at different temperatures and incubators, a common growth environment (i.e. 30
374 °C, MH2) and growth medium (Mueller-Hinton 2) that supports the growth of all strains was
375 chosen for all strains. Although the strains have different optimal growth temperatures (28 °C for
376 *P. protegens*, *P. fluorescens*, and *P. fulva*; 30 °C for *P. putida* and *P. mendocina*, 35 °C for *P.*
377 *stutzeri* and 37°C for *P. aeruginosa*), all were capable of vigorous growth in this environment,
378 hence the number of generations per day (6-7) is instead dictated by the dilution factor (1/100).

379 *Experimental evolution with ΔampC and ΔampR mutants*

380 We obtained Δ*ampC* and Δ*ampR* mutants of *P. aeruginosa* PAO1 that were constructed
381 following well-established procedures based on the *cre-lox* system for gene deletion and
382 antibiotic resistance marker recycling[33]. We determined the MIC of these mutants using the
383 microbroth dilution method, as above. To test evolvability of Δ*ampC* and Δ*ampR* mutants, we
384 followed the same protocol as the main selection experiment, as outlined above. We challenged
385 90 replicate populations of each deletion mutant and 30 replicate populations of PAO1 wild-type
386 with increasing doses of ceftazidime. In addition, we evolved 18 control populations per strain in
387 antibiotic-free culture medium. This experiment was carried out in a single block.

388 *Experimental evolution to test the effect of avibactam*

389 The effect of avibactam on evolvability was tested for Δ*ampC* and Δ*ampR* mutants and for wild-
390 type PAO1. 120 replicate populations of each strain were passaged following exactly the same
391 procedure as in the two previous experiments. The ceftazidime concentration was doubled every
392 transfer from 1/8 to 8× MICs. For each strain, half of the populations (60 replicates) were
393 additionally challenged with avibactam (always 4 mg/L, BioVision Inc. USA). Population
394 survival was monitored for 7 serial transfers by measuring optical density. We also included 20
395 control populations evolving at the presence of avibactam but without the antibiotic. There was
396 no extinction observed in the control treatment.

397 *Inhibition zone assays*

Evolved clones were cultured in MH2 broth overnight (30 °C, 250 rpm). A sterile swab was dipped then into a $10^{-3}$ dilution of this overnight culture to and the swab was used to inoculate the surface of three MH2 agar plates. Then we placed four different antibiotic susceptibility testing discs (Oxoid) on each plate, testing a total of 12 antibiotics: ceftazidime, piperacillin, meropenem, imipenem, aztreonam, cloramphenicol, tetracycline, rifampicin, amikacin, tobramycin, ciprofloxacin and levofloxacin. After 24 h of incubation at 30 °C, the diameter of the different inhibition zones was measured with a ruler taking the average of three measurements in different axis. Assays were performed in 4 randomized blocks containing a similar number of evolved clones for each strain, and all ancestral strains were tested in each block as a control. Change in antibiotic sensitivity was estimated as the difference in diameter of the inhibition zone of each clone compared to its ancestor for each antibiotic.

*DNA extraction and sequencing*

DNA from the evolved clones surviving the duration of the experiment was extracted using the Wizard Genomic DNA Purification Kit (Promega, UK) as per the manufacturer's instructions. To maximize phylogenomic coverage and reduce bias toward *P. fluorescens* strains, *P. fluorescens* SBW25 was excluded from sequencing due to being highly similar to *P. fluorescens* Pf0-1. We assessed the purity of DNA extractions by measuring absorbance at 230, 260, and 280 nm and by visualizing migration on a 0.7% agarose gel. The concentration of each genomic DNA in each sample was then accurately determined using QuantiFluordsDNA System (Promega, UK) and samples were diluted to 30 ng/µL in TE Buffer before sequencing.

Resequencing was done using Illumina HiSeq2000 with 100bp paired-end reads (Wellcome Trust Centre for Human Genetics, Oxford, UK). Sequencing analysis was performed using the pipeline first described in San Millan et al.[34]. Read filtering was done using the NIH-QCToolkit[35]. Read ends were trimmed if the Phred quality score was less than 20. We discarded reads <50bp after trimming, with >2% ambiguous bases, or with >20% bases of Phred score <20. BWA was used to map reads to the reference genome of each strain. Mapped reads were processed to increase the quality of the variant calling: 1) reads with multiple best hits were discarded; 2) duplicated reads were discarded using MarkDuplicates from the Picard package (http://picard.sourceforge.net); 3) reads around indels were locally realigned using RealignerTargetCreator and IndelRealigner from the GATK package to correct for misalignment; and 4) mate pairs were sorted using FixMateInformation in the Picard package. Variant calling was performed with GATK UnifiedGenotyper[36]and Samtools mpileup[37]. VCFtools vcf-annotate[38], and GATK toolkit VariantFiltration[39], were used to filter the raw variants for strand bias, end distance bias, base quality bias, SNPs around gaps, low coverage and erroneously high coverage. Variants were combined using GATK's CombineVariants (keeping any unfiltered). High quality variants not filtered were annotated using SnpEff[40]. Three approaches were used to detect structural variants: BreakDancer[41] (indels, inversions and translocations), Pindel[42](indels, inversions, tandem duplications and breakpoints), and ControlFREEC (copy number variants[43]with mappability tracks generated by gem-mappability (GEM library[44]).

*Comparative genomics of resistance pathways*

Using pairwise reciprocal BLAST between the reference sequences of the sequenced strains, we determined their similarity in genome content. This approach was taken because the strains differ in the extent to which their genomes are annotated. Using the KEGG database[45], we compared

442 the genes in the β-lactam resistance and peptidoglycan recycling pathways (irrespective of
443 whether they had mutated during selection).

444 *Competition experiment with ΔampC and ΔampR mutants*

445 To measure relative fitness of the deletion mutants, we performed a competition experiment.
446 Δ*ampC*, Δ*ampR* and their isogenic PAO1 wild-type were competed against a YFP-marked tester
447 strain PAO1 strain that carries a constitutively expressed YFP integrated at the mini-Tn7
448 insertion site[14]. Competition experiments were carried out in MH2 broth containing ceftazidime
449 at a concentration of 0, 0.25 or 0.5 mg/L. All competition experiments were replicated 9 times.
450 First, the strains were recovered from -80 °C stock and cultured overnight in MH2 broth medium
451 at 30 °C with shaking at 250 rpm. The overnight cultures were diluted 1:50 in MH2 broth and
452 used to prepare 1:1 mixtures of PAO1-YFP with each of the 3 strains to be tested. Before starting
453 competition, we first estimated the exact starting proportion of strains using flow cytometry (for
454 details see below). Next, we combined 10 µL of these mixtures and 190 µL of MH2 with a
455 corresponding ceftazidime concentration (0, 1/4 and 1/2 MICs). This resulted in an additional
456 1:20 dilution. The bacterial strains were let to compete in 96-well plates for 24 h at 30 °C. The
457 next day, the cultures were diluted 1:50 in saline solution (0.9% NaCl) and analyzed on a flow
458 cytometer in order to estimate the resulting proportion of the YFP-labeled versus unlabelled cells
459 after competition (see below).

460 Flow cytometry was performed on Accuri C6 (BD Biosciences, UK). The cell densities were
461 adjusted to give around 1000 events per second. During data acquisition, a lower cut off was set
462 at 10,000 for FSC-H and at 8000 for SSC-H. The data were exported as FCS-files and processed
463 in R using a custom pipeline based on flowCore and flowViz packages[46-48]. In the pipeline, the
464 events were automatically gated on size by retaining the cells within 2 standard deviations
465 around the median in the bivariate normal distribution of FSC-A and SSC-A. Then, k-mean
466 clustering algorithm was applied on fluorescence intensity FL1-H to differentiate fluorescent
467 versus non-fluorescent cells. For each antibiotic concentration, we ensured that YFP-expressing
468 strain can be well separated from non-fluorescent strains by overlaying non-mixed controls
469 (overlap is usually less than 2% of the cells). Figure S3 shows a representative plot of the gating
470 strategy.

471 Relative fitness was calculated according to the formula

472 $w = \log_2[\ p_1/(p_0/1000)\ ] / \log_2[\ (1-p_1)/[(1-p_0)/1000]\ ]$,

473 where $p_0$ is an initial proportion of an unlabelled stain, and $p_1$ is a final proportion of an
474 unlabelled stain after competition. 1000 is a dilution factor, which reflects a difference in cell
475 density at the beginning and at the end of the competition.

476 **Data availability**

477 Data generated or analysed during this study are included in this published article (and its
478 supplementary information files), with the exception of sequence data, which are deposited in
479 Eurpoean Nucleotide Archive (PRJEB20060).

480

481 **Figure legends**

482

483 **Figure 1:** Responses of *Pseudomonas* to ceftazidime. **a** Phylogeny of the strains used in this
484 study, all nodes were supported with >99% confidence and the scale bar shows genetic distance
485 (adapted from ref. 11 and 49 with permission under Creative Commons licence CC-BY-4.0). **b**
486 The proportion of populations ($n$ = 120 populations/strain) of each strain that survived exposure
487 to increasing doses of ceftazidime. Doses were standardized relative to the MIC of the ancestral
488 clone of each strain, and doses increased 2 fold daily up to 8× MIC. Evolvability differs between
489 strains that are not connected by red lines (Post-hoc test on Cox's proportional hazard, P<0.05).

490

491 **Figure 2: Resistance in evolved clones**. Each column in this figure represents a single,
492 randomly chosen clone from a population that survived until the end of the selection experiment
493 (8× MIC). **a** Black boxes show the presence of mutations in known ceftazidime resistance genes,
494 as determined by whole genome resequencing. Note that some clones carry mutations in multiple
495 resistance genes, and that some clones lack mutations in known resistance genes (online
496 supplementary data table S2). **b** Coloured boxes show the change in ceftazidime MIC of evolved
497 clones (mean of $n$=3 replicates), and **c** changes in the zone of inhibition for a large panel of
498 antibiotics, as determined by disc diffusion assay (mean of $n$=3 replicates).

499

500 **Figure 3: The AmpR transcription factor potentiates the evolution of ceftazidime resistance**
501 **in *P. aeruginosa* PAO1**. **a** The survival of populations of an *ampR* deletion strain
502 (PAO1::Δ*ampR; n* = 90) relative to an isogenic PAO1 control ($n$ = 30) under increasing doses of
503 ceftazidime. The *ampR* deletion reduces evolvability to levels comparable to those observed in a
504 mutant lacking the *ampC* β-lactamase (PAO1::Δ*ampC*; $n$=90). **b** Relative fitness (mean +/- s.e; $n$
505 = 9) of the PAO1::Δ*ampR* mutant (grey triangles) and the PAO1::Δ*amp*C mutant (blue circles) in
506 direct competition with a PAO1 reference strain carrying a neutral YFP marker. Symbols denote
507 statistical significance, as determined by a Bonferroni-corrected Wilcoxon rank sum test (N.S. =
508 P>0.05,* = P<0.05, *** = P<0.001).

509

510 **Figure 4: Blocking the evolution of ceftazidime resistance. a** Survival of populations of *P.*
511 *aeruginosa* PAO1 that were challenged with increasing doses of ceftazidime in either the
512 presence or absence of the AmpC-inhibitor avibactam ($n$ = 60 populations/treatment). Avibactam
513 was administered at a constant, non-inhibitory dose (4 mg/L). Avibactam increases the rate of
514 population extinction in the presence of increasing doses of ceftazidime. **b** and **c** The survival of
515 *ampR* or *ampC* deletion strains (PAO1::Δ*ampR* and PAO1::Δ*ampC*) under the same
516 experimental conditions as for the isogenic wild-type POA1 ($n$ = 60 populations/treatment for
517 each strain). Avibactam had no effect on the survival of *ampR* or *ampC* deletion mutants.

518

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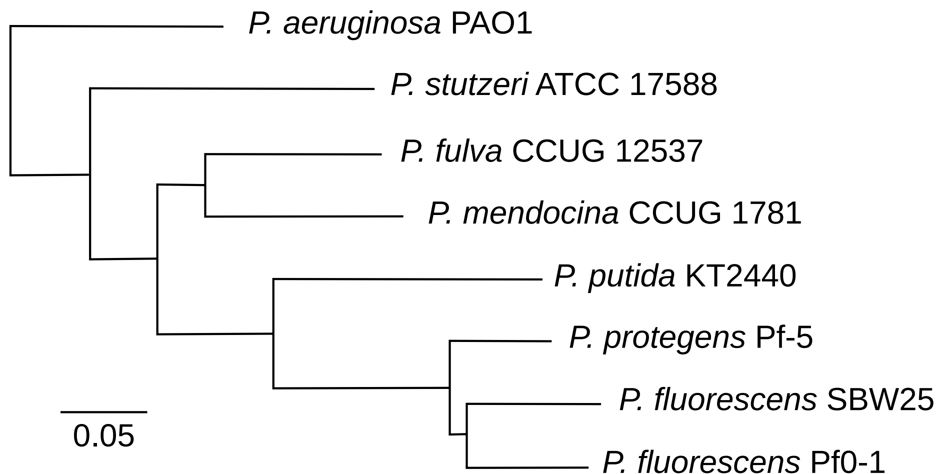
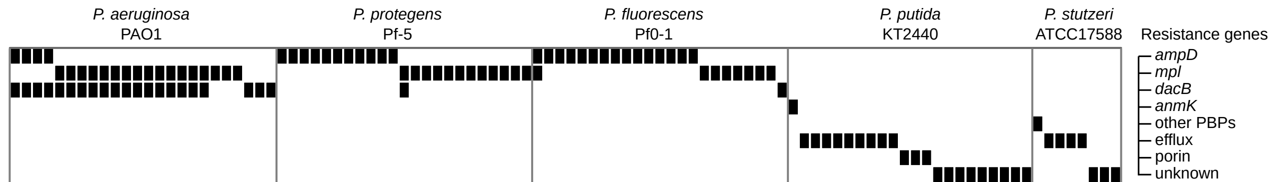
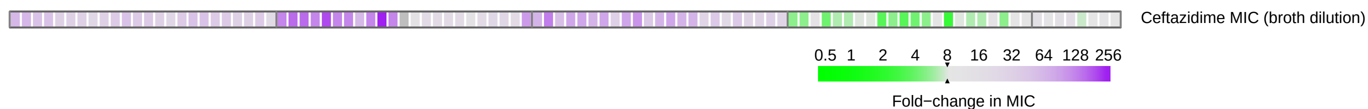
660


a
P. aeruginosa PAO1
P. stutzeri ATCC 17588
P. fulva CCUG 12537
P. mendocina CCUG 1781
P. putida KT2440
P. protegens Pf-5
P. fluorescens SBW25
P. fluorescens Pf0-1
0.05
b
Ceftazidime concentration in MIC
⅛
¼
½
1
2
4
8
Proportion populations suriving
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Day
P. protegens Pf−5
P. fluorescens Pf0−1
P. fluorescens SBW25
P. aeruginosa PAO1
P. putida KT2440
P. stutzeri ATCC 17588
P. mendocina CCUG 1781
P. fulva CCUG 12537

a




b




c

Beta lactams (disc diffusion)

ceftazidime
piperacillin
meropenem
aztreonam
imipenem (AmpC-stable)

Other antibiotics (disc diffusion)

chloramphenicol
tetracycline
levofloxacin
rifampicin
amikacin
ciprofloxacin
tobramycin

15 10 5 0 −5 −10 −15 −20 −25 −30

Change in zone of inhibition (mm)

a

Ceftazidime concentration (factors of MIC)

⅛ ¼ ½ 1 2 4 8

Proportion populations suriving

1.0 0.8 0.6 0.4 0.2 0

PAO1

PAO1::Δ*ampC*

PAO1::Δ*ampR*

1 2 3 4 5 6 7

Day

b

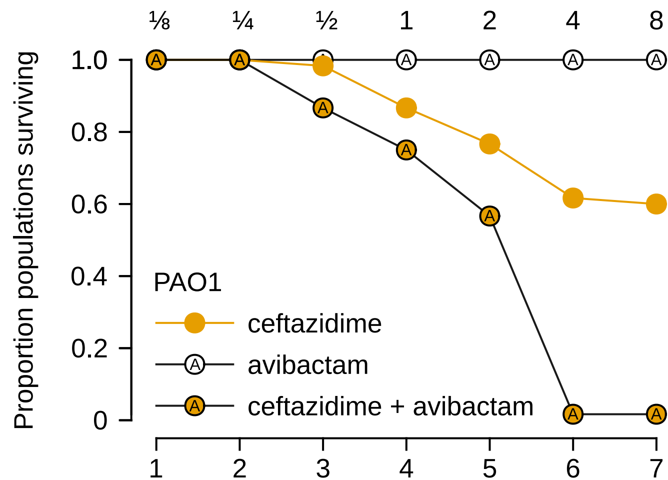
Relative fitness ±SE

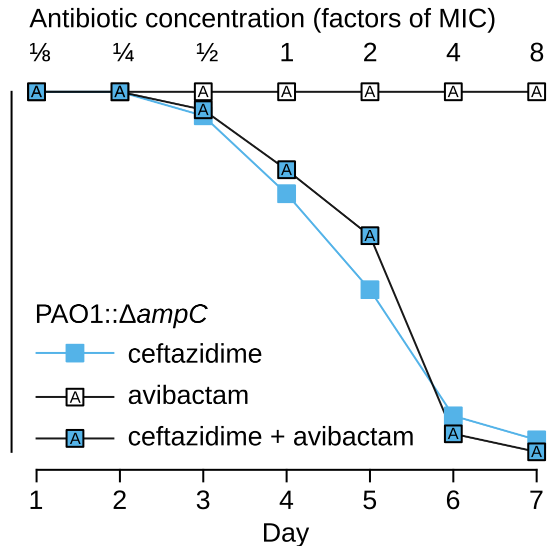
1.15 1.10 1.05 1.00 0.95 0.90

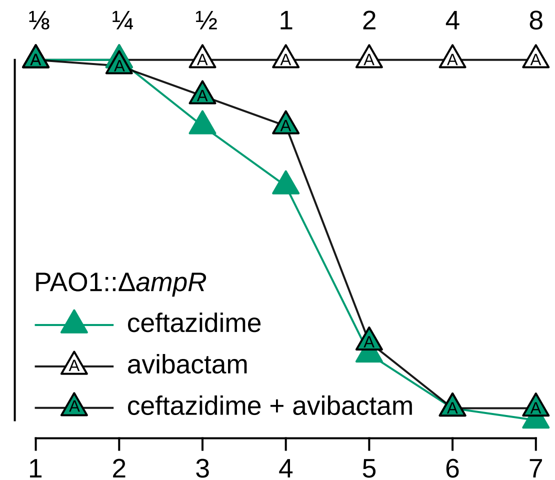
*** * *** N.S. *** ***

0 ¼ ½

Ceftazidime concentration (factors of MIC)


a
Proportion populations surviving
⅛
¼
½
1
2
4
8
1.0
0.8
0.6
0.4
0.2
0
PAO1
ceftazidime
avibactam
ceftazidime + avibactam
1
2
3
4
5
6
7


b
Antibiotic concentration (factors of MIC)
⅛
¼
½
1
2
4
8
PAO1::Δ*ampC*
ceftazidime
avibactam
ceftazidime + avibactam
1
2
3
4
5
6
7
Day


c
⅛
¼
½
1
2
4
8
PAO1::Δ*ampR*
ceftazidime
avibactam
ceftazidime + avibactam
1
2
3
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6
7

**Identifying and exploiting genes that potentiate the evolution of antibiotic resistance**

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**Table S1: Mutation rate estimates for the 8 strain used.** Mutation rates were estimated by Luria-Delbrück fluctuation test using the $p_0$ method.

| | Mean density | Cultures | No. without mutants | Proportion ($p_0$) | $-\ln(p_0)$ | Mutation rate (95% upper bound) |
|---|---|---|---|---|---|---|
| *P. aeruginosa* PAO1 | $3.65\times10^{6}$ | 480 | 367 | 0.765 | 0.268 | $7.36\times10^{-8}$ |
| *P. fluorescens* Pf0-1 | $5.20\times10^{6}$ | 480 | 421 | 0.877 | 0.131 | $2.52\times10^{-8}$ |
| *P. fluorescens* SBW25 | $6.20\times10^{6}$ | 480 | 465 | 0.969 | 0.032 | $5.12\times10^{-9}$ |
| *P. fulva* CCUG 12573 | $4.10\times10^{7}$ | 480 | 168 | 0.350 | 1.050 | $2.56\times10^{-8}$ |
| *P. mendocina* CCUG 1781 | $1.30\times10^{7}$ | 480 | 186 | 0.388 | 0.948 | $7.29\times10^{-8}$ |
| *P. protegens* Pf-5 | $2.80\times10^{6}$ | 480 | 368 | 0.767 | 0.266 | $9.49\times10^{-8}$ |
| *P. putida* KT2440 | $1.70\times10^{6}$ | 480 | 380 | 0.792 | 0.234 | $1.37\times10^{-7}$ |
| *P. stutzeri* ATCC 17588 | $8.50\times10^{6}$ | 480 | 49 | 0.102 | 2.282 | $2.68\times10^{-7}$ |

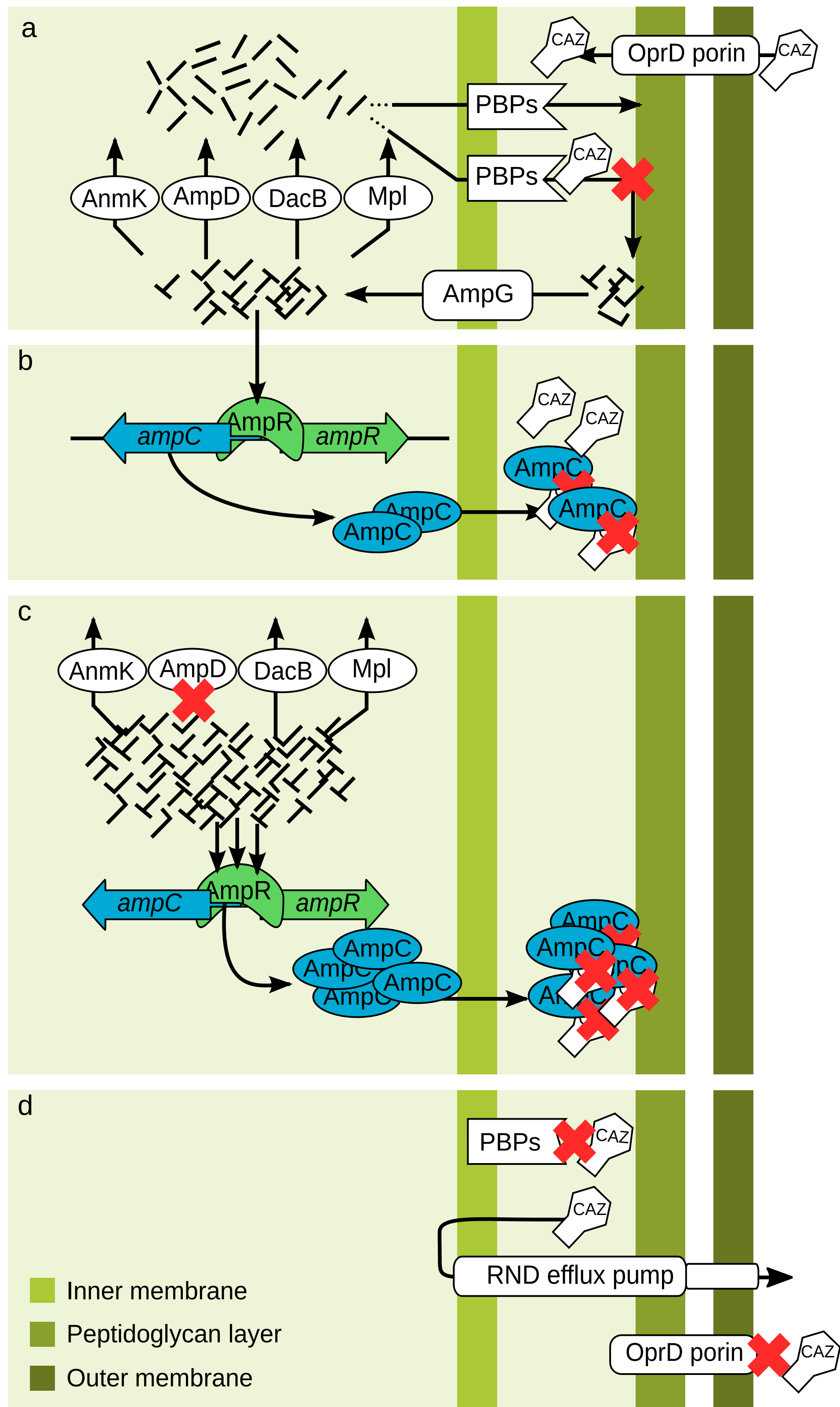


**Figure S1: Mechanisms of ceftazidime action and resistance a** Ceftazidime (CAZ) enters into the periplasm by diffusion through outer membrane porins. CAZ inhibits penicillin binding proteins (PBPs), resulting in an incrased accumulation of peptidoglycan catabolites. **b** The accumulation of peptidoglycan catabolites in the cytoplasm converst the AmpR transcription factor into an activator of *ampC* expression. AmpC is secreted into the periplasm, where it hydrolyses CAZ. **c** Loss of function mutations in peptidoglycan recycling genes lead to increased resistance by increasing *ampC* expression. **d** Resistance can also be caused by structural alterations to PBPs, elevated expression of RND efflux pumps, or mutations in porin genes.

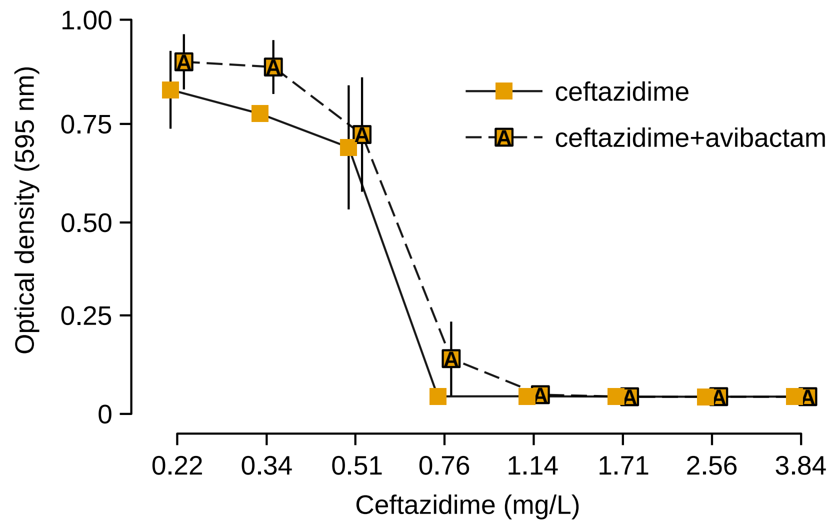


**Figure S2: Ceftazidime and ceftazidime+avibactam MIC in *Pseudomonas aeruginosa* PAO1.** The addition of avibactam increased the MIC of ceftazidime from 0.76 mg/L to 1.14 mg/L.

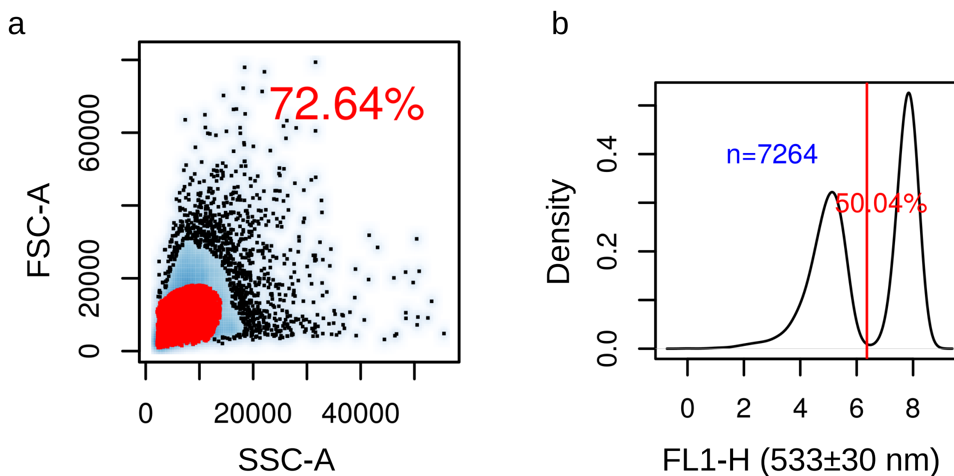


**Figure S3: Flow cytometry gating strategy. a** Around 10000 events were acquired per sample. The gating procedure described below would typically preserve 7000-8000 events for subsequent analysis. During data acquisition, a lower cut off was set at 10,000 for FSC-H and at 8000 for SSC-H. In the gating pipeline, the events were automatically gated on size/shape by retaining the cells within 2 standard deviations around the median in the bivariate normal distribution of FSC-A and SSC-A ("norm2Filter" from flowCore package, red area). **b** A k-means clustering algorithm was applied on fluorescence intensity FL1-H to differentiate YFP-expressing versus non-fluorescent cells ("kmeansFilter" from flowCore package, red horizontal bar). For each antibiotic concentration, we ensured that YFP-expressing strain could be well separated from non-fluorescent strains by overlaying non-mixed controls (overlap is usually less than 2% of the cells).