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Identifying and exploiting genes that potentiate the evolution of antibiotic resistance

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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.

Antibiotic resistance in pathogenic bacteria poses a growing threat to human health, by increasing the mortality rate and economic burden associated with bacterial infections1. In light of this threat, there is an urgent need to develop new tools for predicting when resistance is likely to evolve in pathogen populations2. Research in this area has largely focused on understanding how differing antibiotic treatment strategies, such as mixtures and

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Data availability

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

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cycles, influence the evolutionary dynamics of resistance3–5. An alternative approach is to ask if there are specific genes that make bacteria more likely to evolve resistance to antibiotics6. Whole genome sequencing has highlighted the incredible genetic diversity of pathogenic bacteria7, but the impact of this diversity on the evolution of antibiotic resistance remains poorly understood. For example, recent work in *Streptococcus pneumoniae* has shown that genes that are important for resistance in one strain may be completely dispensable in another8. Although many genes are associated with clinical resistance, it is unclear to what extent other genes in the genome influence the evolution of resistance. For example, recent work has shown that some genes 'potentiate' the evolution of novel bacterial phenotypes by opening otherwise inaccessible routes to adaptation9,10. The existence of potentiator genes suggests that genomic background may play a key role in the evolution of antibiotic resistance.

In vitro selection experiments have emerged as an important tool for studying the evolution of antibiotic resistance1,3,5. However, these studies typically use selection lines derived from a single ancestral clone, making it difficult to understand the role that genetic background itself plays in the evolution of resistance. One approach to circumvent this difficulty is to use comparative experimental evolution, where a diverse collection of strains are challenged with adapting to a common selective pressure6. Using this approach, we recently demonstrated that genetic background influences the evolution of resistance to rifampicin by altering the spectrum and fitness effects of mutations in a highly conserved domain of RNA polymerase that confer resistance to rifampicin6,11. In this paper, we extend this approach to uncover resistance potentiator genes by challenging 8 strains that span the genus *Pseudomonas* with the β-lactam antibiotic ceftazidime.

Pseudomonas is a diverse genus of bacteria that includes *P. aeruginosa*, an important opportunistic pathogen of humans that is the primary cause of mortality in patients who suffer from cystic fibrosis. Crucially, it is possible to culture a wide range of *Pseudomonas* strains under a common set of lab conditions, making it possible to study evolutionary responses to antibiotics in these bacteria using tightly controlled and replicated experiments. We chose to study the evolution of resistance to ceftazidime for two reasons. First, ceftazidime is a clinically relevant antibiotic that is commonly used to treat *Pseudomonas* infections12 and ceftazidime resistance is common in clinical isolates of *P. aeruginosa.* Second, the mechanisms of ceftazidime action and resistance are well characterized. Ceftazidime inhibits cell wall biosynthesis by irreversibly binding to periplasmic penicillin-binding proteins, ultimately leading to cell death. In spite of this simple mechanism of action, *Pseudomonas* can use at least 4 routes to evolve resistance to ceftazidime: altering the structure of penicillin-binding proteins, upregulating the expression of efflux pumps, reducing permeability of the outer membrane and upregulating the expression of β-lactamase enzymes that break down the antibiotic13–15 (Figure S1). Mutations altering the structure of the β-lactamase enzyme itself do occur, but provide much lower increases in resistance16.

Here we use a serial passage experiment to challenge close to 1,000 populations of *Pseudomonas* with doses of ceftazidime that increased from sub-lethal to lethal concentrations over the course of 1 week. We then use extensive whole genome re-

sequencing of evolved clones to identify genes and pathways that contribute to the rapid evolution of elevated ceftazidime resistance. Using selection experiments and competition assays with defined mutants, we directly test the evolutionary impact of key pathways to resistance identified from whole genome sequencing. Finally, we demonstrate that understanding the genetic drivers of resistance evolution can be used to design a simple drug mixture, consisting of ceftazidime coupled to a β-lactamase inhibitor, to prevent the evolution of resistance *in vitro*.

Results and discussion

Strain-specific variation in resistance evolution

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

Genomics of resistance evolution

To determine the genetic basis of resistance evolution, we sequenced the genomes of 100 independently evolved clones from populations that survived selection for elevated resistance (n=14–24 clones/strain). We identified a total of 196 novel mutations in 69 unique genes (i.e. orthologs across strains are each counted once). Mutations included SNPs (n=80), short indels (n=71), insertion element insertions (n=15), larger insertions and deletions (n=7), and intergenic mutations (n=23). Several lines of evidence indicate that the mutations

that we identified were predominantly beneficial. First, parallel evolution occurred 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 pumps13–15. 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; χ^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 gene14,17. 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 out18. 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 production14. 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 catabolites14,17. 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-lactams17. 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 resistance9,10. 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 of *ampC* expression. Consistent with this hypothesis, mutations in peptidoglycan biosynthesis genes and *dacB* are known to only increase resistance in the presence of *ampR*17,18. This hypothesis generates two simple predictions that can be tested using our method. First, if elevated expression of *ampC i*s a key mechanism for evolving ceftazidime resistance, then deleting *ampC s*hould decrease evolvability. Second, if the AmpR regulator is required to drive the evolution of increased *ampC e*xpression, then deleting *ampR s*hould reduce evolvability by the same amount as deleting the *ampC.* To test these predictions, we challenged populations of Δ*ampR* and Δ*ampC* mutants of *P. aeruginosa* PAO1 with increasing doses of ceftazidime, as in our initial experiment (Figure 3a). Both of the mutants have dramatically reduced evolvability compared to their isogenic *P. aeruginosa* PAO1 control (Cox's proportional hazard, likelihood ratio=23.82, df=2, $P=6\times10^{-6}$), providing conclusive evidence that both the β-lactamase (*ampC)* and its regulator (*ampR*) play key roles in driving the evolution of elevated ceftazidime resistance.

The low survival probability of *P. aeruginosa* PAO1 in comparison with the other strains that carry both *ampR* and *ampC* is also consistent with this hypothesis. Strains of *P. fluorescens and P. prote*gens carry 2 homologs of *ampD*, which represses the expression of *ampC*, whereas *P. aerugi*nosa PAO1 carries 3 homologs of this gene. The additional copy of *ampD* found in *P. aeruginosa* ensures that *ampD* mutations lead to weaker de-repression of *ampC* expression, and this is likely to translate into reduce evolvability in comparison to strains with only 2 *ampD* homologs; the *ampD* dosage effect has been demonstrated experimentally20. Consequently, most surviving *P. aeruginosa* strains possessed two loss of function mutations in the peptidoglycan biosynthesis pathway, in comparison with one only in the other *ampR/ampC* possessing strains (Figure 2).

Additionally, it is possible that adaptive plasticity in *ampC* expression mediated by *ampR* could increase evolvability22. Exposure to β-lactam antibiotics interferes with peptidoglycan biosynthesis by inhibiting PBPs, causing an AmpR-mediated increase in *ampC* expression17,19. This, in turn, may accelerate the genetic evolution of resistance by providing bacterial populations with the time to acquire ceftazidime resistance mutations. According to this explanation, *ampR* increases evolvability through ecological potentiation. The key assumption of this hypothesis is that the plasticity in *ampC* expression mediated by *ampR* must provide a benefit in the presence of ceftazidime. To test this hypothesis, we measured the effect of deleting *ampR* and *ampC* on fitness using short-term competition experiments (Figure 3b). Deleting *ampC* leads to a decrease in fitness the presence of ceftazidime, demonstrating that induced expression of this gene is beneficial. However, deleting *ampR* actually increases fitness in the presence of sub-MIC concentrations of ceftazidime, demonstrating that plasticity in gene expression cannot explain the link between *ampR* and increased evolvability. Indeed, as *ampR* expression is not particularly strongly

induced by ceftazidime23, this suggests that *ampR* does not simply allow populations to 'buy time' to wait for an adaptive mutation. Although this result is counter-intuitive, it is important to emphasize that *ampR* is a global transcriptional regulator that affects the expression of 100s of genes24,25, including repressing another chromosomal β-lactamase, *poxB*25. In particular, *ampR* is involved in regulating quorum sensing factors, including *lasR*, several metabolic pathways, and the *rpoS*-mediated stress response pathway25. Although it is clear that inducing elevated levels of *ampC* expression in the presence of ceftazidime is beneficial, the fitness cost associated with the *ampR* regulator implies that the net fitness effect of all of the changes in gene expression caused by this regulator in the presence of ceftazidime is deleterious. The importance of *ampR* as a global regulator of expression perhaps explains why increased *ampC* expression did not arise through mutations in *ampR* itself, and why *ampR* mutations are not typically observed in clinical *P. aeruginosa* isolates26.

Inhibiting the evolution of ceftazidime resistance

Given the important role that *ampR* mediated induction of *ampC* expression plays in the evolution of resistance, our results suggest that one possible strategy to prevent the evolution of cephalosporin resistance in *P. aeruginosa* infections would be to co-administer ceftazidime with AmpC β-lactamase inhibitors27. The rationale for this strategy is that a combination of a β-lactam and β-lactamase inhibitor will be active against both wild-type bacterial strains and mutants with elevated β-lactamase secretion. In other words, this strategy should effectively block a major evolutionary path to elevated resistance. To test this idea, we challenged *P. aeruginosa* PAO1 with ceftazidime in the presence of avibactam28, a recently developed AmpC inhibitor (Figure 4a). Unlike most β-lactamase inhibitors, avibactam does not possess any toxic effects on *Pseudomonas*28 and we did not detect any population extinction in the avibactam treated control populations. In support of our hypothesis, avibactam increased the rate of population extinction in the presence of ceftazidime compared to ceftazidime treated control populations (Cox's proportional hazard, likelihood ratio test=78.968, df=1, $P<10^{-6}$). We failed to detect any viable cells in 59 out of 60 populations of *P. aeruginosa* that were selected in 8× MIC ceftazidime supplemented with avibactam demonstrating that the effect of avibactam suppresses the evolution of elevated β-lactamase secretion just as effectively as knocking out *ampC* or *ampR* (Figure 3a). Importantly, this effect does not arise because avibactam increases the potency of ceftazidime. Surprisingly, we found that avibactam treatment actually increased the MIC of ceftazidime from 0.76 mg/L to 1.14 mg/L (Figure S2).

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

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Conclusion

Whole genome sequencing is revolutionizing our understanding of the evolution and ecology of bacterial pathogens. One of the challenges that has arisen from this revolution is to understand the consequences of genetic diversity in pathogen populations. Here we show that comparative experimental evolution can be used to identify genes and pathways that influence the rate and mechanisms of adaptation to antibiotics. Our experiment addressed this problem at a fairly broad scale, by comparing the evolutionary responses of strains from different species. Our initial reasoning for working at this scale was that comparing divergent strains effectively maximizes the number of genes and SNPs that are included in the experiment, therefore maximizing the likelihood of detecting an impact of genetic background on evolvability. However, the sheer number of genetic differences between even the most closely related strains used in this study may have hindered our ability to detect more subtle genomic effects on evolvability. While it is clear that inducible *ampC* β-lactamase expression is an important driver of evolvability in this genus, it is clear that other genes must influence the ability to evolve ceftazidime resistance. For example, *P. stutzeri* ATCC17588 and *P. putida* KT2440, both of which lack *ampR,* have similar evolvability to *P. aeruginosa* PAO1. We are currently extending this research program by focusing on studying variation in evolvability between clones from the same species, and we hope that this approach will enable us to identify genetic drivers of evolvability in greater depth.

The differing modes of *ampC* expression among the pseudomonads affect their ability to evolve resistance to β-lactams by interacting with genes in the peptidoglycan biosynthesis pathway. In strains possessing *ampR*, the intracellular accumulation of peptidoglycan catabolites converts the AmpR transcription factor into an activator of *ampC* expression in response to peptidoglycan damage. Mechanistically, *ampR* increases evolvability by allowing mutations in peptidoglycan biosynthesis genes to induce high levels of β-lactamase expression, which effectively amplifies the *ampC* expression plasticity that occurs when cell wall synthesis is compromised by β-lactams20. From a more conceptual perspective, *ampR* can be thought of as a conduit that translates genetic variation in the peptidoglycan biosynthesis gene network into phenotypic variation in *ampC* expression. This suggests that response pathways that are involved in sensing environmental change may have a general role as evolutionary catalysts, linking plastic and mutational responses to environmental change. Intriguingly, these alternative expression modes are disseminated among the enterobacteria; however, insertion of the *ampR* gene into constitutive producers is not sufficient to restore inducible expression, suggesting a distinct regulatory mechanism in constitutive producers29. To avoid the evolution of high levels of ceftazidime resistance, and subsequent treatment failure, treatment with ceftazidime should be avoided in infections caused by strains with inducible *ampC* expression.

Understanding the evolutionary trajectory to high levels of ceftazidime resistance makes it possible to design a simple two-drug mixture consisting of ceftazidime and avibactam that can be used to effectively eliminate populations of the pathogen *P. aeruginosa*. We argue that this strategy is successful because avibactam effectively prevents mutations in peptidoglycan biosynthesis genes and *dacB* from increasing ceftazidime resistance, eliminating their fitness benefit. One possible solution to this evolutionary challenge would be to first evolve

avibactam resistance, and then evolve ceftazidime resistance. However, avibactam does not have any detectable toxic effects on *Pseudomonas* at concentrations where it is able to effectively inhibit AmpC, rendering this evolutionary pathway to combined avibactam/ceftazidime resistance inaccessible. Although these results are encouraging, we emphasize that there are a number of confounding factors that may affect the efficacy of this combination of drugs *in vivo*. For example, the pharmacokinetic properties of the two drugs may make it difficult to effectively maintain the drug mixtures at the site of bacterial infections, and it is also possible that avibactam resistant alleles of *ampC* or other β-lactamases capable of hydrolyzing ceftazidime are already present in pathogen populations.

Predicting the evolution of antibiotic resistance is a challenging and important objective. Here we show that comparative experimental evolution can be used to identify genes and pathways that make some bacterial strains prone to evolving resistance, and to exploit this to design treatment strategies for preventing resistance evolution. High throughput sequencing is revolutionizing clinical microbiology30,31, and it may be possible to identify such potentiator genes in clinical pathogen populations and to use this information to optimize antimicrobial treatment strategies.

Materials and Methods

MIC Determination for parental strains

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

Effect of avibactam on MIC

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

Mutation rate estimation

Mutation rates were estimated by fluctuation assays, with the antibiotic rifampicin as the selection agent, using the method of Luria and Delbruck32. 480 replicate cultures were inoculated with approximately 50 cells from an overnight culture of each parental strain and incubated for 48 hours in 200 μl of KB media at 30 °C with constant shaking at 200 rpm. Approximately 10^7 cells from each culture were then plated onto KB-agar containing rifampicin at the appropriate MIC (minimum inhibitory concentration) for each strain (60 mg/L for *P. aeruginosa* PAO1, 30 mg/L for all other strains). For each strain the proportion of cultures yielding no mutants was scored, from which the mutation per culture was calculated using the negative natural logarithm. This value was then divided by the number of cells plated, which provides an estimate of the mutation rate per cell division.

Experimental evolution

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

Experimental evolution with Δ*ampC* and Δ*ampR* mutants

We obtained Δ*ampC* and Δ*ampR* mutants of *P. aeruginosa* PAO1 that were constructed following well-established procedures based on the *cre-lox* system for gene deletion and antibiotic resistance marker recycling33. We determined the MIC of these mutants using the microbroth dilution method, as above. To test evolvability of Δ*ampC* and Δ*ampR* mutants,

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we followed the same protocol as the main selection experiment, as outlined above. We challenged 90 replicate populations of each deletion mutant and 30 replicate populations of PAO1 wild-type with increasing doses of ceftazidime. In addition, we evolved 18 control populations per strain in antibiotic-free culture medium. This experiment was carried out in a single block.

Experimental evolution to test the effect of avibactam

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

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-QCToolkit35. 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 UnifiedGenotyper36 and Samtools mpileup37. VCFtools vcf-annotate38, and GATK toolkit VariantFiltration39, 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 SnpEff40. Three approaches were used to detect structural variants: BreakDancer41 (indels, inversions and translocations), Pindel42(indels, inversions, tandem duplications and breakpoints), and ControlFREEC (copy number variants43 with mappability tracks generated by gem-mappability (GEM library44).

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 database45, we compared the genes in the β-lactam resistance and peptidoglycan recycling pathways (irrespective of whether they had mutated during selection).

Competition experiment with ΔampC and ΔampR mutants

To measure relative fitness of the deletion mutants, we performed a competition experiment. Δ*ampC*, Δ*ampR* and their isogenic PAO1 wild-type were competed against a YFP-marked tester strain PAO1 strain that carries a constitutively expressed YFP integrated at the mini-Tn7 insertion site14. Competition experiments were carried out in MH2 broth containing ceftazidime at a concentration of 0, 0.25 or 0.5 mg/L. All competition experiments were replicated 9 times. First, the strains were recovered from -80 °C stock and cultured overnight in MH2 broth medium at 30 °C with shaking at 250 rpm. The overnight cultures were diluted 1:50 in MH2 broth and used to prepare 1:1 mixtures of PAO1-YFP with each of the 3 strains to be tested. Before starting competition, we first estimated the exact starting proportion of strains using flow cytometry (for details see below). Next, we combined 10 μL of these mixtures and 190 μL of MH2 with a corresponding ceftazidime concentration (0, 1/4 and 1/2 MICs). This resulted in an additional 1:20 dilution. The bacterial strains were let to compete in 96-well plates for 24 h at 30 °C. The next day, the cultures were diluted 1:50 in saline solution (0.9% NaCl) and analyzed on a flow cytometer in order to estimate the resulting proportion of the YFP-labeled versus unlabelled cells after competition (see below).

Flow cytometry was performed on Accuri C6 (BD Biosciences, UK). The cell densities were adjusted to give around 1000 events per second. During data acquisition, a lower cut off was set at 10,000 for FSC-H and at 8000 for SSC-H. The data were exported as FCS-files and

processed in R using a custom pipeline based on flowCore and flowViz packages46–48. In the pipeline, the events were automatically gated on size by retaining the cells within 2 standard deviations around the median in the bivariate normal distribution of FSC-A and SSC-A. Then, k-mean clustering algorithm was applied on fluorescence intensity FL1-H to differentiate fluorescent versus non-fluorescent cells. For each antibiotic concentration, we ensured that YFP-expressing strain can be well separated from non-fluorescent strains by overlaying non-mixed controls (overlap is usually less than 2% of the cells). Figure S3 shows a representative plot of the gating strategy.

Relative fitness was calculated according to the formula

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

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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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.

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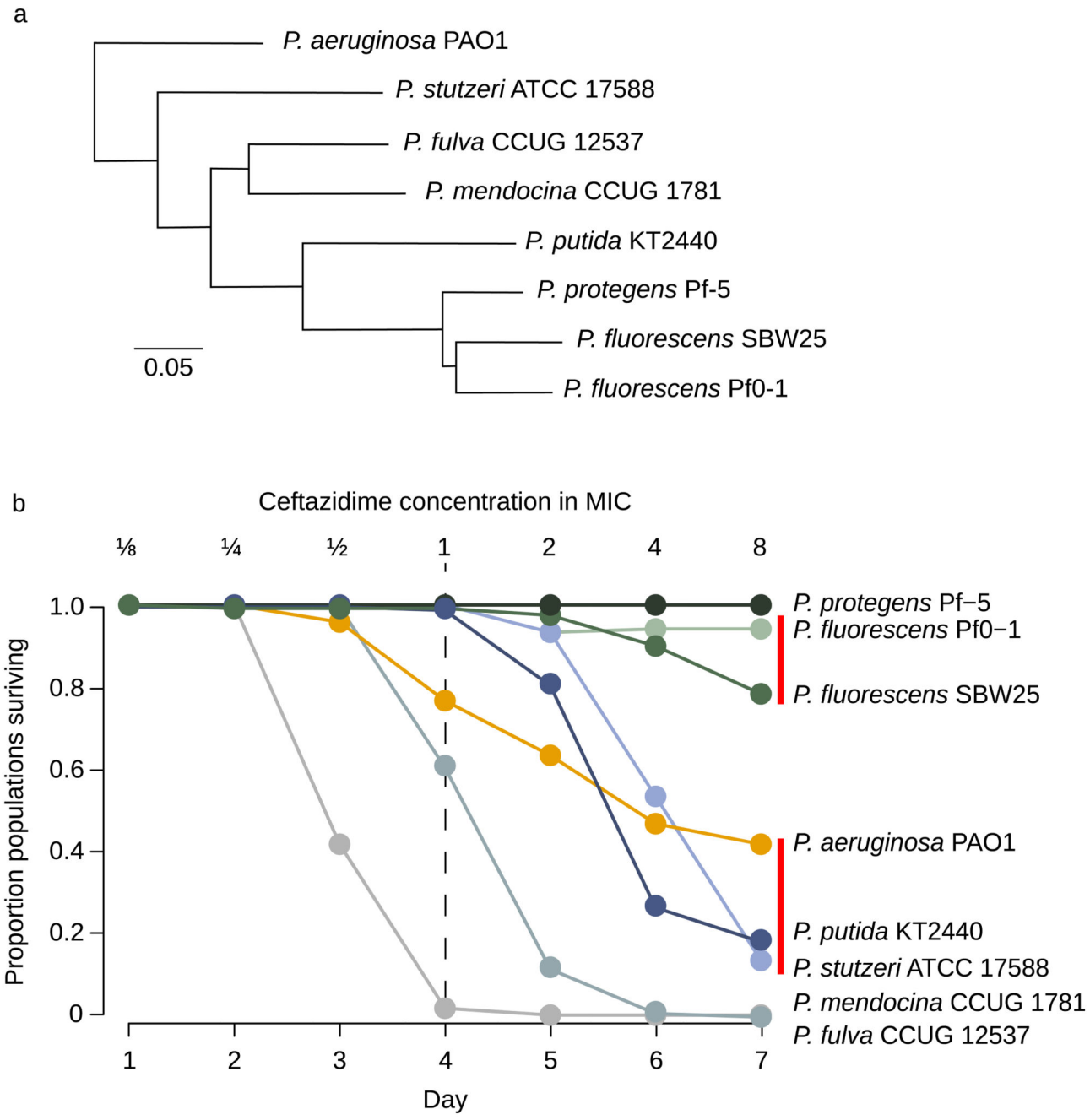

Figure 1. Responses of *Pseudomonas* to ceftazidime.

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

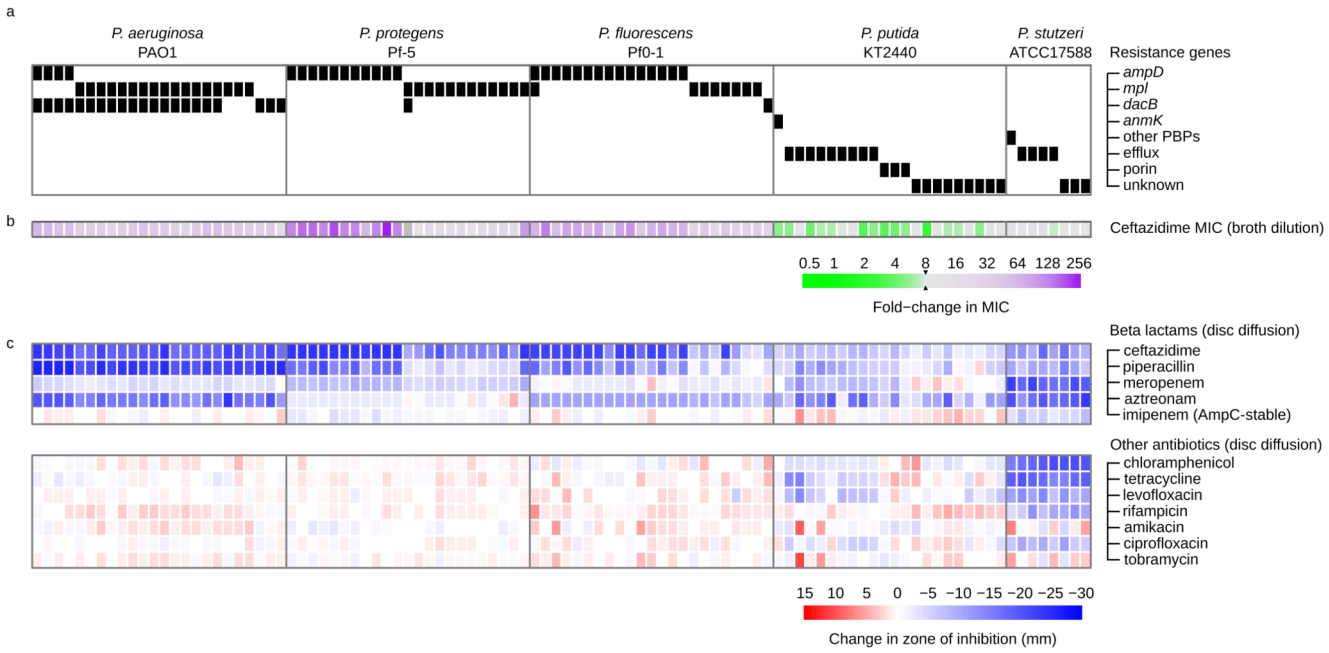

Figure 2. Resistance in evolved clones.

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

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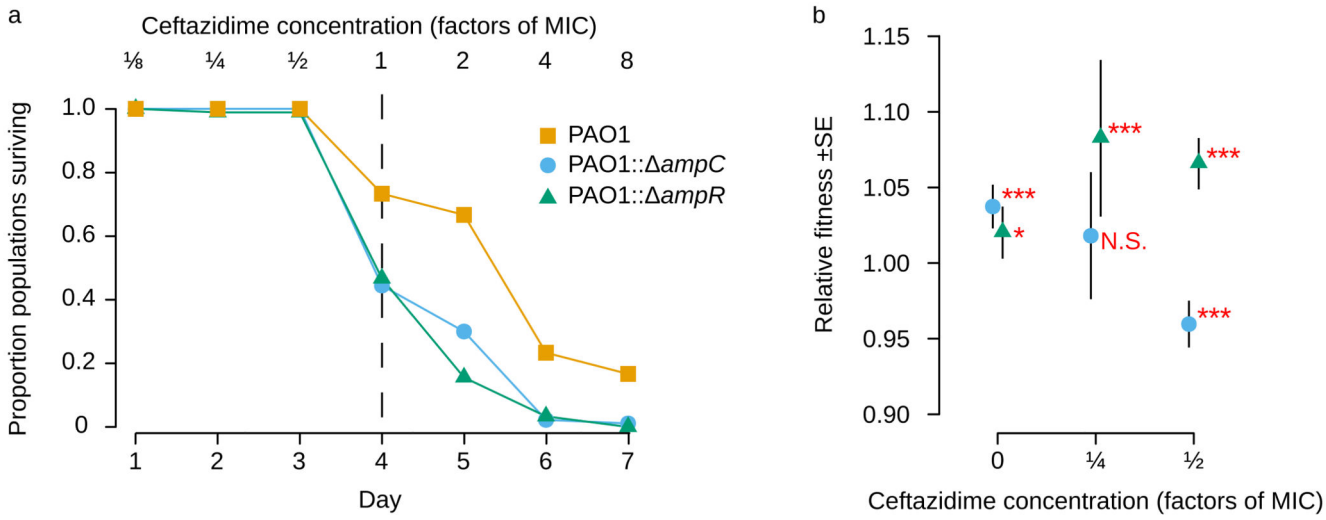

Figure 3. The AmpR transcription factor potentiates the evolution of ceftazidime resistance in *P. aeruginosa* PAO1.

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

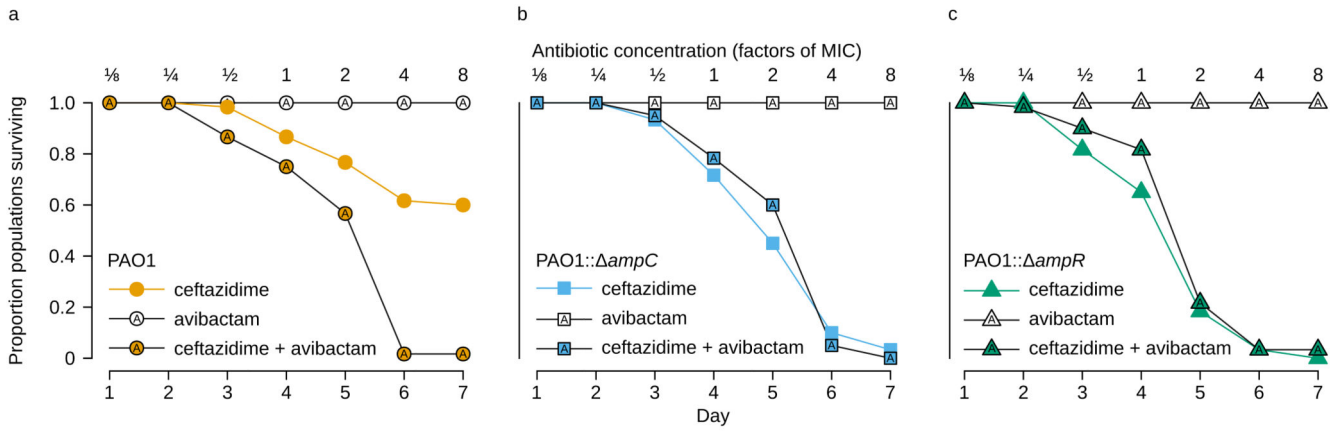

Figure 4. Blocking the evolution of ceftazidime resistance.

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

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