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Identifying and exploiting genes that potentiate the evolution of antibiotic resistance

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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 infections[1]. In light of this threat, there is an urgent need to develop new tools for predicting when resistance is likely to evolve in pathogen populations[2]. Research in this area has largely focused on understanding how differing antibiotic treatment strategies, such as mixtures and cycles, influence the evolutionary dynamics of resistance[3–5]. An alternative approach is to ask whether there are specific genes that make bacteria more likely to evolve resistance to antibiotics[6]. Whole-genome sequencing has highlighted the incredible genetic diversity of pathogenic bacteria[7], 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 another[8]. 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 adaptation[9,10]. 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 resistance[1,3,5]. 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 pressure[6,11]. 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 rifampicin[6,12]. In this paper, we extend this approach to uncover resistance potentiator genes by challenging eight 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 laboratory conditions, allowing us 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* infections[13] 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 (PBPs), ultimately leading to cell death. In spite of this simple mechanism of action, *Pseudomonas* can use at least four routes to evolve resistance to ceftazidime: altering the structure of PBPs, upregulating the expression of efflux pumps, reducing the permeability of the outer membrane and upregulating the expression of β-lactamase enzymes that break down the antibiotic[14–16] (Supplementary Fig. 1). Mutations altering the structure of the β-lactamase enzyme itself do occur, but provide much lower increases in resistance[17].

We used 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 used extensive whole-genome resequencing 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 tested 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

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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 (Fig. 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 laboratory culture medium supplemented with ceftazidime, the concentration of which was doubled daily from sub-lethal (1/8 minimum inhibitory concentration (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^{-1}) 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 on 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 (Fig. 1b; Cox's proportional hazard, likelihood ratio = 1,930, d.f. = 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 with the mutation rate. However, evolvability does not correlate with mutation rate (Pearson correlation coefficient $r = 0.33$, $F_{1,6} = 0.74$, $P = 0.42$; see Supplementary Table 1 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 per strain). We identified a total of 196 novel mutations in 69 unique genes (that is, orthologues across strains were each counted once). Mutations included single nucleotide polymorphisms (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 Table 2. We categorized mutations according to known resistance mechanisms: (1) porin genes, (2) PBPs, (3) peptidoglycan biosynthesis genes and (4) multidrug efflux pumps[14–16]. Almost all of the evolved clones (88 out of 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

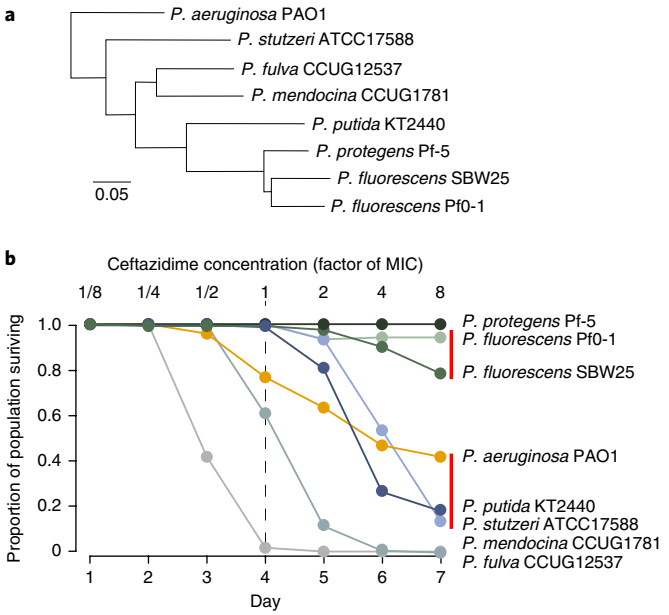

Fig. 1 | Responses of *Pseudomonas* to ceftazidime. a, Phylogeny of the strains used in this study. All nodes were supported with >99% confidence and the scale bar shows genetic distance. **b**, Proportion of populations ($n = 120$ populations per strain) of each strain that survived exposure to increasing doses of ceftazidime. Doses were standardized relative to the MIC of the ancestral clone of each strain, and doses increased twofold daily up to 8× MIC. Evolvability differs between strains that are not connected by red lines (post-hoc test on Cox's proportional hazard, $P < 0.05$). Panel **a** adapted from ref. [12] and Dryad data from ref. [50] under a Creative Commons licence CC BY 4.0.

(Fig. 2; $\chi^2 = 139$, d.f. = 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[15,18]. 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 these 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 multidrug-resistant phenotype, while porin mutations are predominantly associated with elevated β-lactam resistance (Fig. 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[15]. 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, why does evolvability

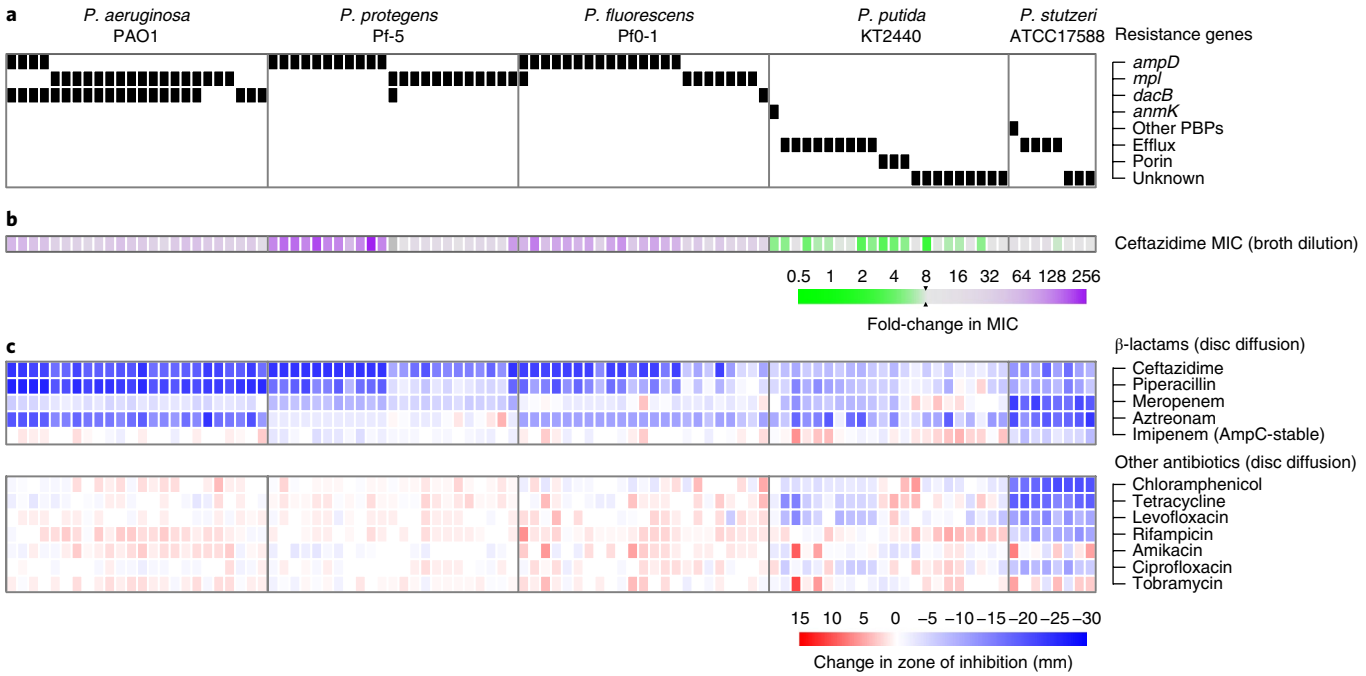

Fig. 2 | Resistance in evolved clones. Each column represents a single, randomly chosen clone from a population that survived until the end of the selection experiment (8× MIC). **a**, Black boxes show the presence of mutations in known ceftazidime resistance genes, as determined by whole-genome resequencing. Note that some clones carry mutations in multiple resistance genes and some clones lack mutations in known resistance genes (Supplementary Table 2). **b**, Coloured boxes show the change in ceftazidime MIC of evolved clones (mean of $n=3$ replicates). **c**, Coloured boxes show changes in the zone of inhibition for a large panel of antibiotics, as determined by disc diffusion assay (mean of $n=3$ replicates).

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 intracellular accumulation of peptidoglycan catabolites[15,18]. 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[18]. Crucially, among our strains, *ampR* is only present in the genomes of *P. aeruginosa* PAO1, *P. protegens* Pf-5, *P. fluorescens* Pf0-1 and *P. fluorescens* SBW25. 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 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*[18,19]. This hypothesis generates two simple predictions that can be tested using our method. First, if elevated expression of *ampC* is a key mechanism for evolving ceftazidime resistance, deleting *ampC* should decrease evolvability. Second, if the AmpR regulator is required to drive the evolution of increased *ampC* expression, deleting *ampR* should reduce evolvability by the same amount as deleting *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 (Fig. 3a). Both mutants have dramatically reduced evolvability compared with their isogenic *P. aeruginosa* PAO1 control

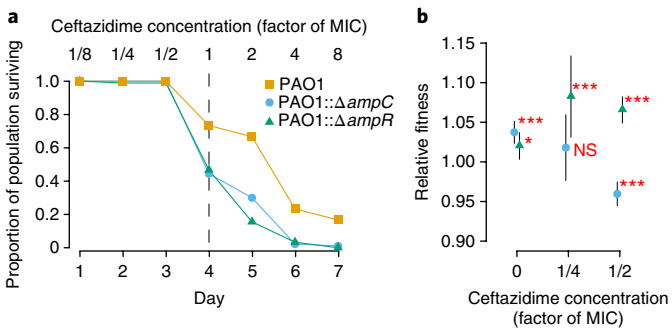

Fig. 3 | The AmpR transcription factor potentiates the evolution of ceftazidime resistance in *P. aeruginosa* PAO1. a, Survival of populations of an *ampR* deletion strain (PAO1::Δ*ampR*; $n=90$) relative to an isogenic PAO1 control ($n=30$) under increasing doses of ceftazidime. The *ampR* deletion 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.m.; $n=9$) of the PAO1::Δ*ampR* mutant (green triangles) and the PAO1::Δ*ampC* 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 (NS, not significant ($P>0.05$); $^{*}P<0.05$; $^{***}P<0.001$).

(Cox's proportional hazard, likelihood ratio = 23.82, d.f. = 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. protegens* carry two homologues of *ampD*, which represses the expression of *ampC*, whereas *P. aeruginosa* PAO1 carries three

homologues of this gene. The additional copy of *ampD* found in *P. aeruginosa* ensures that *ampD* mutations lead to weaker derepression of *ampC* expression, and this is likely to translate into reduced evolvability compared with strains with only two *ampD* homologues; the *ampD* dosage effect has been demonstrated experimentally[19]. Consequently, most surviving *P. aeruginosa* strains possessed two loss of function mutations in the peptidoglycan biosynthesis pathway, compared with one only in the other *ampR*/*ampC*-possessing strains (Fig. 2).

Additionally, it is possible that adaptive plasticity in *ampC* expression mediated by *ampR* could increase evolvability[21]. Exposure to β-lactam antibiotics interferes with peptidoglycan biosynthesis by inhibiting PBPs, causing an AmpR-mediated increase in *ampC* expression[18,22]. 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 (Fig. 3b). Deleting *ampC* leads to a decrease in fitness in 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 ceftazidime[23], 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 hundreds of genes[24,25], 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 pathway[25]. 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 gene 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* isolates[26].

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 inhibitors[27]. 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 avibactam[28], a recently developed AmpC inhibitor (Fig. 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 with ceftazidime-treated control populations (Cox's proportional hazard, likelihood ratio test = 78.968, d.f. = 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* (Fig. 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 to 1.14 mg l^{-1} (Supplementary Fig. 2).

As a final test of the role of *ampR* in evolvability, we challenged populations of Δ*ampR* (Fig. 4b) and Δ*ampC* (Fig. 4c) mutants of *P. aeruginosa* PAO1 with a combination of ceftazidime and avibactam, as in our experiment with wild-type *P. aeruginosa* PAO1. Avibactam had no effect on evolvability in either Δ*ampR* or Δ*ampC* mutants (Cox's proportional hazard, likelihood ratio test = 3.25, d.f. = 1, $P = 0.071$; likelihood ratio test = 0.02, d.f. = 1, $P = 0.876$, respectively), showing that these strains were effectively unable to increase *ampC* expression under our experimental conditions.

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 the ability 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 also 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 programme 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-lactams[20]. 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 producers[29]. To avoid the evolution of high

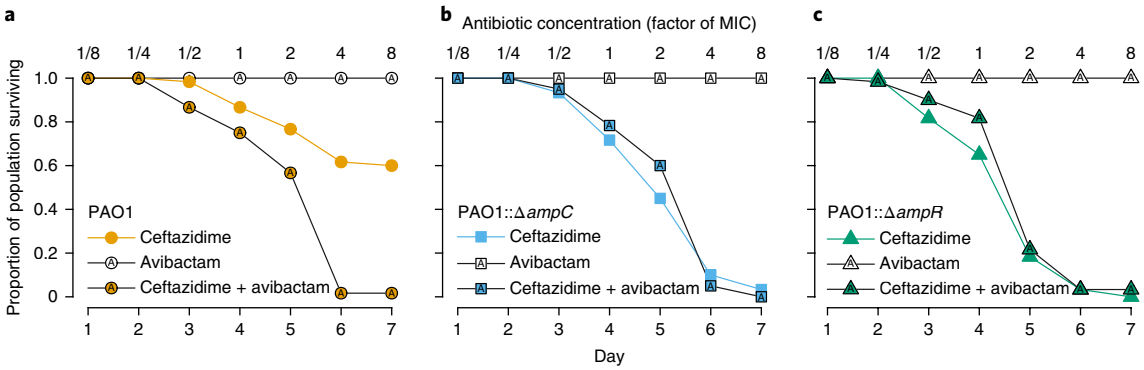

Fig. 4 | Blocking the evolution of ceftazidime resistance. a, Survival of populations of *P. aeruginosa* PAO1 that were challenged with increasing doses of ceftazidime in either the presence or absence of the AmpC inhibitor avibactam ($n = 60$ populations per treatment). Avibactam was administered at a constant, non-inhibitory dose (4 mg l^{-1}). **b**,**c**, Survival of *ampR* or *ampC* deletion strains (PAO1::Δ*ampR* and PAO1::Δ*ampC*, respectively) under the same experimental conditions as for the isogenic wild-type POA1 in **a** ($n = 60$ populations per treatment for each strain).

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 hydrolysing 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 microbiology[30,31], and it may be possible to identify such potentiator genes in clinical pathogen populations and to use this information to optimize antimicrobial treatment strategies.

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^{-1}. This standardized inoculum was diluted a further 200-fold in Mueller–Hinton 2 (MH2; Sigma–Aldrich) broth containing ceftazidime (Sigma–Aldrich) at a concentration between 64 mg and 0.0625 mg l^{-1}. After 24 h of incubation at 30 °C with shaking at 250 r.p.m., optical density at 595 nm was determined for each well with a Synergy 2 plate reader (BioTek). 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 in the presence/absence of avibactam. The procedure was identical to MIC determination, as described above, except that one group of replicates was supplemented with 4 mg l^{-1} of avibactam (BioVision). No avibactam was added to the control group. The avibactam treatment and control groups were tested at concentrations ranging from 0.1 to 3.8 mg l^{-1} 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 described in ref. [32]. Replicate cultures ($n = 480$) were inoculated with approximately 50 cells from an overnight culture of each parental strain and incubated for 48 h in 200 µl of King's B media at 30 °C with constant shaking at 200 r.p.m. Approximately 10^7 cells from each culture were then plated onto King's B agar containing rifampicin at the appropriate MIC for each strain (60 mg l^{-1} for *P. aeruginosa* PAO1; 30 mg l^{-1} 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 provided 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 r.p.m. 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 r.p.m. 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 Microplate Reader (BioTek). We additionally confirmed population survival after the last transfer by plating a 1 µl 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 replicate populations of each strain that were challenged with increasing doses of ceftazidime, as well as 12 replicate control populations of each strain that were allowed to evolve in antibiotic-free MH2. At the end of the experiment, a maximum of 20 populations per strain were streaked in MH2 agar plates and a clone was picked for each population and amplified for further analyses. To avoid bias by conducting the experiment at different temperatures and incubators, a common growth environment (that is, 30 °C, MH2) and growth medium (MH2) 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) was 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 recycling[33]. We determined the MIC of these mutants using the microbroth dilution method, as above. To test evolvability of Δ*ampC* and Δ*ampR* mutants, 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. Replicate populations ($n=120$) 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^{-1}; BioVision). Population survival was monitored for seven serial transfers by measuring optical density. We also included 20 control populations evolving in the presence of avibactam but without the antibiotic. No extinction was observed in the control treatment.

Inhibition zone assays. Evolved clones were cultured in MH2 broth overnight (30 °C, 250 r.p.m.). A sterile swab was then dipped into a 10^{-3} dilution of this overnight culture and used to inoculate the surface of three MH2 agar plates. Then, we placed 4 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 along different axes. Assays were performed in four 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 with 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) as per the manufacturer's instructions. To maximize phylogenomic coverage and reduce bias towards *P. fluorescens* strains, *P. fluorescens* SBW25 was excluded from sequencing due to it 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 visualizing migration on a 0.7% agarose gel. The concentration of each genomic DNA in each sample was then accurately determined using a QuantiFluor dsDNA System (Promega), and samples were diluted to 30 ng μl^{-1} in Tris-EDTA buffer before sequencing.

Resequencing was performed using an Illumina HiSeq 2000 system with 100-base pair (bp) paired-end reads (Wellcome Trust Centre for Human Genetics). Sequencing analysis was performed using the pipeline first described in ref. [34]. Read filtering was done using the NGS QC Toolkit[35]. Read ends were trimmed if the Phred quality score was less than 20. We discarded reads <50 bp after trimming, with >2% ambiguous bases or with >20% bases of Phred score <20. The Burrows–Wheeler Aligner (http://bio-bwa.sourceforge.net/)[36] 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 (https://broadinstitute.github.io/picard/); (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[37] and SAMtools mpileup[38]. VCFtools vcf-annotate[39] and GATK toolkit VariantFiltration[40] 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[41]. Three approaches were used to detect structural variants: BreakDancer[42] (indels, inversions and translocations), Pindel[43] (indels, inversions, tandem duplications and breakpoints) and Control-FREEC (copy number variants[44] with mappability tracks generated by gem-mappability (GEM library[45]).

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 differed in the extent to which their genomes were annotated. Using the KEGG database[46], we compared the genes in the β-lactam resistance and peptidoglycan recycling pathways (irrespective of whether they had mutated during selection).

Competition experiment with Δ*ampC* and Δ*ampR* mutants. To measure the 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 yellow fluorescent protein (YFP)-marked tester PAO1 strain that carries a constitutively expressed YFP integrated at the mini-Tn7 insertion site[15].

Competition experiments were carried out in MH2 broth containing ceftazidime at a concentration of 0, 0.25 or 0.5 mg l^{-1}. All competition experiments were replicated nine times. First, the strains were recovered from −80 °C stock and cultured overnight in MH2 broth medium at 30 °C with shaking at 250 r.p.m. The overnight cultures were diluted 1:50 in MH2 broth and used to prepare 1:1 mixtures of PAO1-YFP with each of the three 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 left 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 analysed on a flow cytometer to estimate the resulting proportion of the YFP-labelled versus unlabelled cells after competition (see below).

Flow cytometry was performed on an Accuri C6 flow cytometer (BD Biosciences). The cell densities were adjusted to give around 1,000 events per second. During data acquisition, a lower cut-off was set at 10,000 for forward scatter height and at 8,000 for side scatted height. The data were exported as flow cytometry standard files (FCS version 3.0) and processed in R using a custom pipeline based on flowCore and flowViz packages[47–49]. In the pipeline, the events were automatically gated on size by retaining the cells within two s.d. around the median in the bivariate normal distribution of forward scatter area and side scatter area. Then, the k-mean clustering algorithm was applied on fluorescence intensity height, excited by a 488 nm laser and measured through a 533 ± 30 nm bandpass filter, to differentiate fluorescent versus non-fluorescent cells. 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). Supplementary Fig. 3 shows a representative plot of the gating strategy.

Relative fitness was calculated according to the formula

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

where p_0 is an initial proportion of an unlabelled stain and p_1 is a final proportion of an unlabelled stain after competition. The number $D=1{,}000$ is a dilution factor, which reflects a difference in cell density at the beginning and at the end of the competition.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are included within the paper and its Supplementary Information, with the exception of sequence data, which are deposited in the Eurpoean Nucleotide Archive (PRJEB20060).

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Author contributions

R.C.M. designed the study. V.F., A.P. and T.V. conducted the experiments. D.R.G. performed the bioinformatics analyses. V.F., A.P., D.R.G. and R.C.M. analysed the data. A.O. contributed reagents and expertise. R.C.M., D.R.G. and V.F. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Experimental design

1. Sample size

Describe how sample size was determined.

Sample size was chosen to optimize detection of differences in proportion, such that e.g. the minimum difference that could be detected with power 0.8 was 0.07 for proportions at the extremes where the variance is lowest (e.g. or p=1 vs p=0.93), and 0.18 for proportions around p=0.5 where variance is highest (e.g. p=0.41, p=0.59)

2. Data exclusions

Describe any data exclusions.

To increase the breadth of phylogenetic coverage, genomes from the strain SBW25 were excluded due to its similarity with strain Pf0-1

3. Replication

Describe whether the experimental findings were reliably reproduced.

All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Bacterial cells were distributed randomly from a larger population of cells to initiate selection lines.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Control (i.e. no antibiotic) and treatment groups within each strain were transferred blinded.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
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See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Statistics were performed in R with published open-source packages. Bioinformatics analysis was performed using a published pipeline incorporating published open-source tools. All details are described in the methods.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	No restrictions

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	N/A
b. Describe the method of cell line authentication used.	N/A
c. Report whether the cell lines were tested for mycoplasma contamination.	N/A
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	N/A

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Describe the covariate-relevant population characteristics of the human research participants.	N/A

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Flow Cytometry Reporting Summary

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Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	Flow cytometry was performed on Accuri C6 (BD Biosciences, UK). The cell densities were adjusted to give around 1000 events per second.
6. Identify the instrument used for data collection.	BD Accuri C6 with CSampler attachment for 96 well plates
7. Describe the software used to collect and analyze the flow cytometry data.	BD Accuri C6 Software was used to collect the data. R flowCore and flowViz from Bioconductor were used to import, visualize and gate raw cytometry data.
8. Describe the abundance of the relevant cell populations within post-sort fractions.	Around 10000 events were acquired per sample. The gating procedure described below would typically preserve 7000-8000 events for subsequent analysis
9. Describe the gating strategy used.	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). Then, k-mean clustering algorithm was applied on fluorescence intensity FL1-H to differentiate fluorescent versus non-fluorescent cells ("kmeansFilter" from flowCore package). 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).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒