Alternative Evolutionary Paths to Bacterial Antibiotic Resistance Cause Distinct Collateral Effects

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Abstract

When bacteria evolve resistance against a particular antibiotic, they may simultaneously gain increased sensitivity against a second one. Such collateral sensitivity may be exploited to develop novel, sustainable antibiotic treatment strategies aimed at containing the current, dramatic spread of drug resistance. To date, the presence and molecular basis of collateral sensitivity has only been studied in few bacterial species and is unknown for opportunistic human pathogens such as *Pseudomonas aeruginosa*. In the present study, we assessed patterns of collateral effects by experimentally evolving 160 independent populations of *P. aeruginosa* to high levels of resistance against eight commonly used antibiotics. The bacteria evolved resistance rapidly and expressed both collateral sensitivity and cross-resistance. The pattern of such collateral effects differed to those previously reported for other bacterial species, suggesting interspecific differences in the underlying evolutionary trade-offs. Intriguingly, we also identified contrasting patterns of collateral sensitivity and cross-resistance among the replicate populations adapted to the same drug. Whole-genome sequencing of 81 independently evolved populations revealed distinct evolutionary paths of resistance to the selective drug, which determined whether bacteria became cross-resistant or collaterally sensitive towards others. Based on genomic and functional genetic analysis, we demonstrate that collateral sensitivity can result from resistance mutations in regulatory genes such as *nalC* or *mexZ*, which mediate aminoglycoside sensitivity in β-lactam-adapted populations, or the two-component regulatory system gene *pmrB*, which enhances penicillin sensitivity in gentamicin-resistant populations. Our findings highlight substantial variation in the evolved collateral effects among replicates, which in turn determine their potential in antibiotic therapy.

Key words: **antibiotic resistance, *Pseudomonas aeruginosa*, experimental evolution, trade-offs, collateral sensitivity.**

Introduction

Bacteria have the potential to rapidly adapt to virtually any natural or laboratory environment (Kussell 2013). The long-term evolution experiment with *Escherichia coli* (LTEE) has shown that even in simple constant environments, bacteria can achieve comprehensive fitness increases of about 25% within the first 2,000 generations (Lenski et al. 1991). Although the LTEE populations show reduced adaptation rates at later time points (e.g., after 50,000 generations), they still continue to accumulate an almost constant number of new beneficial mutations (Barrick et al. 2009; Tenaillon et al. 2016). Thus, bacteria can adapt rapidly to new challenges and subsequently continue to optimize their fitness. Such remarkable adaptive potential was also observed under more challenging conditions: Using evolution experiments with antibiotics, *E. coli* evolved high levels of drug resistance through the step-wise accumulation of multiple mutations when drug concentrations increased over time (Toprak et al. 2012) or across space (Baym et al. 2016a). Bacteria also readily adapted when they were challenged with two antibiotics simultaneously (Chait et al. 2007; Hegreness et al. 2008; Michel et al. 2008; Pena-Miller et al. 2013), or sequentially (Kim et al. 2014; Fuentes-Hernandez et al. 2015; Roemhild et al. 2015).

Rapid bacterial adaptation to new environments often involves evolutionary trade-offs in the form of reduced fitness under alternative growth conditions (Kussell 2013). In the case of antibiotic resistance evolution, two types of trade-offs (or costs) are commonly observed: i) evolved resistance is costly in the absence of the drugs, thus generating growth deficiencies relative to the susceptible ancestor (Andersson and Hughes 2010; Melnyk et al. 2015), and ii) resistance mutations may exacerbate susceptibility against others (i.e. collateral sensitivity (Szybalski and Bryson 1952; Pál et al. 2015); also referred to as hypersensitivity, or negative cross-resistance in previous publications). However, adaptive mutations do not always entail a cost but instead may increase resistance against other antibiotics (i.e., collateral resistance or cross-resistance); thus favoring multidrug resistance.

The phenomenon of collateral sensitivity was first described in the 1950s in a study by Szybalski and Bryson, in which the authors tested if experimentally evolved resistant *E. coli* was less, equally or more sensitive to previously unmet

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drugs (Szybalski and Bryson 1952). Despite finding that cross-resistance was much more prevalent than collateral sensitivity, the authors hypothesized that these rare cases could then be exploited by rationally using more than one drug during treatment of resistant clinical strains. The employment of drug pairs that produce reciprocal collateral sensitivity might trap bacteria in an evolutionary "double-bind," thus improving treatment efficacy and decreasing the evolution of resistance. This idea was more recently tested by exposing bacteria to such drug pairs being deployed sequentially (Imamovic and Sommer 2013; Kim et al. 2014; Fuentes-Hernandez et al. 2015; Roemhild et al. 2015) or simultaneously (Munck et al. 2014; Evgrafov et al. 2015). Additionally, several other studies have further evaluated what factors could help to predict the changes in drug sensitivity in experimentally evolved resistant *E. coli*. These showed that the strength of selection and the chemogenomic profile similarity between antibiotics play significant roles in the evolution of resistance and hence influence the patterns of cross-resistance and hypersensitivity (Lázár et al. 2013, 2014; Oz et al. 2014).

To fully determine the importance of such trade-offs during bacterial adaptation and also their therapeutic potential, the patterns of collateral resistance/sensitivity observed in *E. coli* need to be assessed in other, clinically relevant bacterial taxa, including those known to possess high adaptive capacity such as members of the genus *Pseudomonas*. This group of bacteria are able to colonize and thrive in a plethora of niches (Nikel et al. 2014), and act as prominent plant and human pathogens (Loper et al. 2012; Rodríguez-Rojas et al. 2012; Balasubramanian et al. 2013). For instance, the opportunistic human pathogen *P. aeruginosa* is commonly associated with hospital-acquired infections, and it is a major cause of chronic lung disease, including the ultimately fatal infections in cystic fibrosis patients (Govan and Deretic 1996; Arruda et al. 1999; Kang et al. 2003; Folkesson et al. 2012). Its success as an opportunistic pathogen can be largely attributed to its vast array of virulence factors, including the production of alginate to form biofilms, its ability to survive oxidative stress, and the availability of various secretion systems (Hauser 2009; Ma et al. 2009; Burrows 2012; Jimenez et al. 2012; Korotkov et al. 2012). Moreover, it carries a large array of intrinsic antibiotic resistance mechanisms and an exceptional potential to acquire resistance, both *de novo* or horizontally transferred (Arruda et al. 1999; Carmeli et al. 1999; Hancock and Speert 2000; Poole 2001; Drenkard and Ausubel 2002; Livermore 2002; Overhage et al. 2008; Breidenstein et al. 2011). To date, there is neither information on the evolution of drug sensitivity trade-offs in *P. aeruginosa*, nor on its genomic underpinnings. To rectify this knowledge gap, we experimentally selected 160 highly resistant populations of *P. aeruginosa* PA14 (plus 20 control populations) and evaluated the possibility of evolutionary trade-offs in the form of fitness deficiencies in the absence of antibiotics and hypersensitivity to other drugs. We assessed the underlying molecular mechanisms of such trade-offs through whole-genome sequencing of 81 evolved resistant populations.

Table 1. List of Antibiotics Used During Selection Experiments.

Functional Target	Class	Drug	Abbreviation
DNA repair	Quinolones	Ciprofloxacin	CIP
Protein synthesis	Aminoglycosides	Gentamicin	GEN
		Streptomycin	STR
Cell wall synthesis	Penicillins	Piperacillin + Tazobactam	PIT
		Carbenicillin	CAR
	Carbapenems	Doripenem	DOR
		Imipenem	IMI
	Cephalosporins	Cefsulodin	CEF

Results and Discussion

Rapid Evolution of Antibiotic Resistance

We used experimental evolution to generate independent populations of *P. aeruginosa* PA14 with significantly increased resistance against eight different antibiotics. We challenged 20 isogenic populations against increasing concentrations of one out of eight different drugs (for abbreviations of all antibiotics see table 1), and included an evolutionary control grown in media only, resulting in a total of 180 independent populations. Experiments were initiated at half the concentration inhibiting >90% of growth (abbreviated IC90), as determined for the ancestral PA14. Populations were then serially transferred every 12 h for 24 days (48 transfers; approximately 58 generations) until reaching around 40 times the IC90, or until less than half of the starting populations from a given antibiotic were still growing (fig. 1A). In the cases of DOR, IMI, and CAR, more than half of the populations went extinct at transfers 20, 28, and 40, respectively; we accordingly isolated ten populations adapted to the 2×, 5× and 17× environments for further analysis. In all other cases, we randomly chose ten surviving populations per antibiotic for subsequent characterization. Frozen populations adapted to GEN could not be recovered after thawing; we could only revive ten of those adapted to the 5× environment. Altogether, we obtained 80 populations adapted to eight different antibiotics and ten additional ones adapted to media only.

For the selected populations, we quantified the changes in resistance to the respective drug used during the evolution experiment (see Materials and Methods). *P. aeruginosa* could rapidly (within 58 generations or less) reach resistance levels of at least 32 times the IC90 of the ancestral PA14 for most antibiotics (fig. 1*B–I*). Interestingly, we observed substantial variation within particular drug treatments, suggesting different routes of adaptation to the antibiotic. Similar increases in drug resistance were found in a recent study with the related *P. aeruginosa* strain PAO1 (Cabot et al. 2016), highlighting the adaptive potential of this species. The same study also revealed in most cases an association of increased resistance with genomic changes in well-known resistance pathways. *P. aeruginosa*'s resistance thus appears to be achieved fast through diverse mechanisms. Several comparable *E. coli* evolution experiments resulted in similar levels of resistance, although variation between populations adapted to the same drug was less pronounced than observed here for *P.*

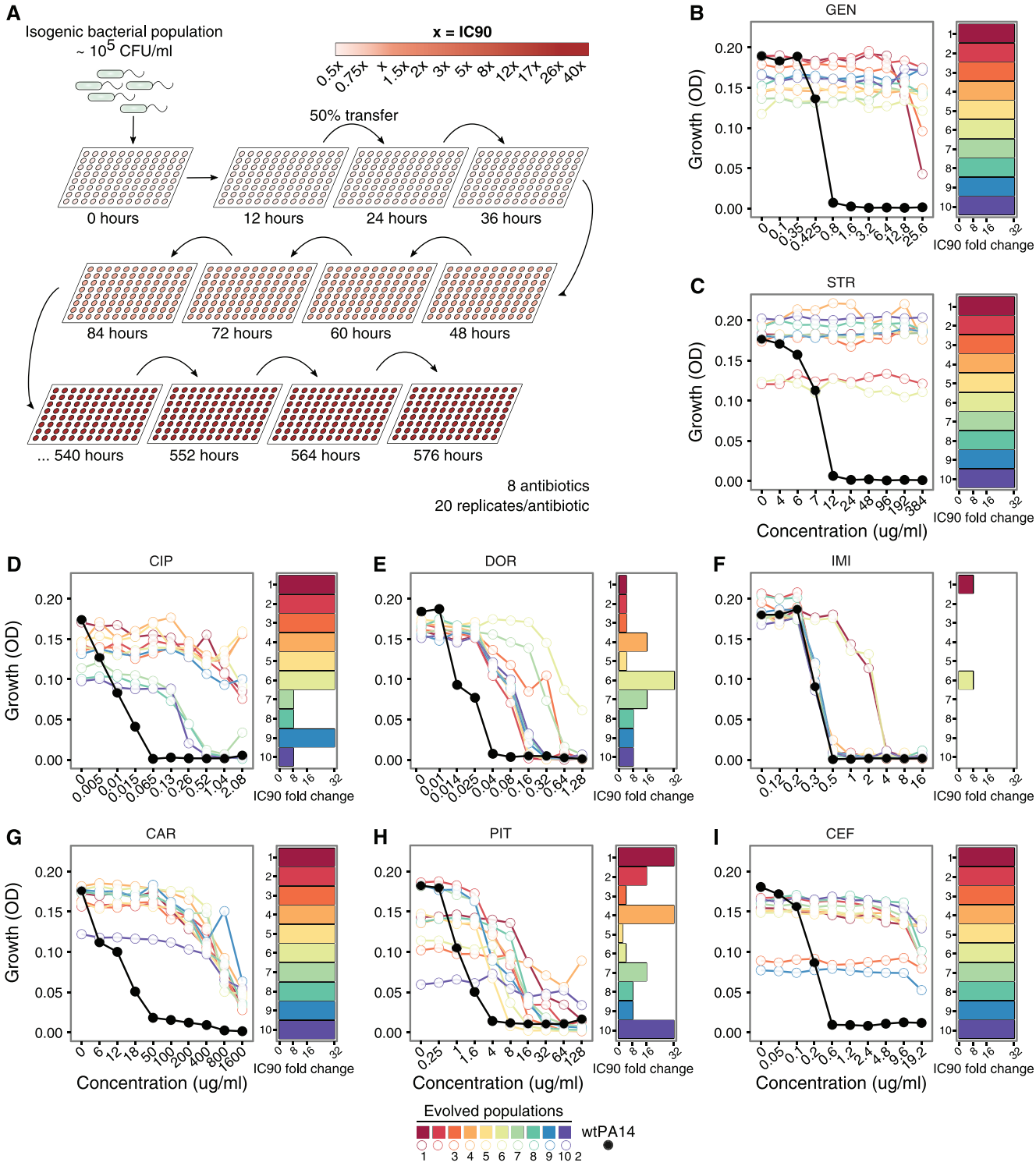

Fig. 1. Directional selection of highly resistant *P. aeruginosa*. (*A*) Illustration of the experimental design used for the selection of resistant populations. Twenty replicate populations for each of the eight included antibiotics (table 1) and a control without antibiotic (a total of 180 populations) were serially transferred every 12 h into fresh medium and, for the drug treatments, increasing concentrations of each drug. Selection was initiated at 0.5 times the concentration inhibiting >90% of growth (IC90) and concluded at ~40 times of the IC90. (*B–I*) Ten populations for each antibiotic were subsequently evaluated for their growth on different concentrations of the drug experienced during the experiment. Dose-response curves are shown in the left panels and IC90 fold changes in the right panels. The ten replicate populations are shown in different colors, whereas the black line represents the ancestral *P. aeruginosa* PA14.

aeruginosa (Imamovic and Sommer 2013; Lázár et al. 2013; Oz et al. 2014).

Rapid Resistance Evolution Entails a Growth Cost

To evaluate whether the evolution of high drug resistance levels was generally associated with a fitness deficiency, we measured different growth parameters of all of the evolved populations, and the ancestral PA14 in a drug-free environment. We found that the control populations, which evolved without antibiotics, had significantly shorter lag phases and lower growth rates relative to the ancestor (supplementary fig. S1, Supplementary Material online). These results suggest that adaptation to the medium alone can have strong effects on fitness. These effects could in turn influence antibiotic resistance. To evaluate this, we compared the sensitivity of the evolved control populations and the ancestor against each of the eight antibiotics. In general, we found no significant differences between the dose response curves of the controls and the ancestor (supplementary fig. S2, Supplementary Material online). The only exceptions refer to cases at subinhibitory drug concentrations where the evolved populations showed higher growth than the ancestral PA14. More importantly, with only a single exception, none of the replicate populations showed a change in the IC90, thus indicating that the fitness alterations of the evolved controls do not translate into changes in drug sensitivity (supplementary fig. S2, Supplementary Material online).

As evolution in the absence of drugs affected growth characteristics, we specifically assessed the costs associated with the adaptation to antibiotics by relating our growth measures of the 80 resistant populations to those of the evolved controls. Resistant populations showed changes in at least one of the measured growth characteristics: lag time, growth rate, and maximum yield (fig. 2). In all cases, we observed a significantly longer time spent in lag phase, and in most cases (five out of eight drugs), a significant reduction of about 20–50% in maximum yield. Only populations adapted to PIT and GEN had significantly reduced growth rates. Overall, 90% of the resistant populations spent longer times in lag phase, 49% produced lower maximum yields, and 36% had lower growth rates (supplementary table S1, Supplementary Material online). Interestingly, 60% of the resistant populations had a significant cost in at least two of the parameters, whereas only five of the populations showed no fitness costs at all.

In most cases, there was substantial variation among populations adapted to the same drug (fig. 2). This might have been the result of at least two paths: a direct outcome of costly adaptation resulting in varying lengths of lag phases depending on the favored resistance mechanism, or, alternatively, an indirect consequence of our experimental design in which selection independently acted in favor of tolerance. In the latter case, an extended lag phase could have allowed *P. aeruginosa* to remain in the environment without immediately changing its initial resistance levels but instead increasing antibiotic tolerance. This could have ultimately resulted in varying levels of measured resistance among the various populations (Levin-Reisman et al. 2017). However, we found no significant correlation between the IC90 fold change and the lag time (or any of the growth parameters) for any of the antibiotics used (supplementary table S2 and fig. S3, Supplementary Material online). This strongly suggests that the differences in resistance are not a result of selection for tolerance, but rather due to underlying differences in the resistance mechanisms that in turn have distinct effects on growth. This finding also means that the extent of the resistance increase does not linearly translate into a fitness cost, but most likely depends on the specific underlying mechanism.

The extent to which growth costs are associated with antibiotic resistance evolution has been evaluated for a variety of combinations of bacteria and drugs, including *P. aeruginosa* (Kugelberg et al. 2005; Andersson and Hughes 2010; Melnyk

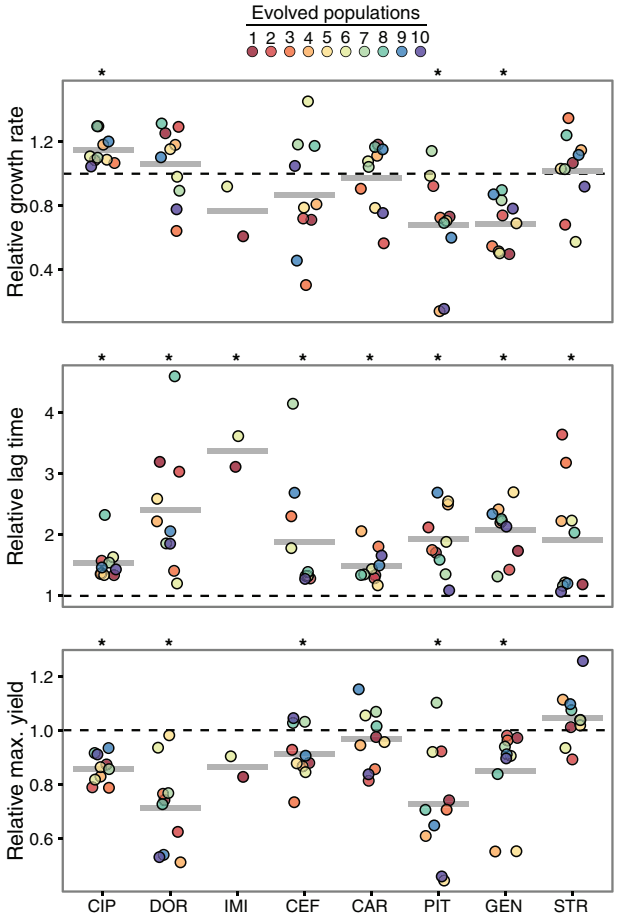

Fig. 2. Relative fitness in the absence of antibiotics. Shown, from top to bottom, is fitness relative to the average of the evolved control populations, calculated for growth rate, lag time, and maximum yield for all populations adapted to one of the eight antibiotics (*X*-axis) after 24 h of growth in antibiotic-free media. Colored points represent the replicate populations and the horizontal grey crossbars indicate the mean for each antibiotic. Black dashed lines highlight equality to the controls; values above indicate a fitness advantage whereas values below denote a cost. Plase note that for the lag phase, this is inverted: values larger than 1 indicate a longer time spent in lag phase and thus a fitness cost. Asterisks on top of each panel indicate significant difference from 1 (i.e., a significant change in fitness) using a Wilcoxon Rank test with probability adjustment based on the false discovery rate (FDR) to account for multiple testing.

et al. 2015). These studies demonstrated that mutations conferring high levels of resistance usually lead to larger fitness costs. Moreover, after adaptation to increasing drug concentrations, a higher number and more complex types of mutations (i.e., large deletions) coincide with larger defects in growth rate (Lázár et al. 2014). Considering such substantial resistance costs, it is surprising that resistant mutants persist both in clinical and experimental environments. Several factors were previously found to ameliorate or completely alleviate the effects of resistance mutations on growth. For instance, quinolone resistant *P. aeruginosa* showed a high prevalence of cost-free mutations as well as the emergence of compensatory mutations in genes that, unexpectedly, are not directly associated to the fitness defect (Kugelberg et al. 2005). In fact, some of our populations showed an increase in fitness in some or all of the growth parameters measured, emphasizing that the costs associated with some resistant mechanisms can be ameliorated rapidly (within 58 generations) or incur no costs at all. Furthermore, the role of epistatic effects, the genetic background in which mutations occur, and the environment also seem to play an important role in the magnitude of the cost (Melnyk et al. 2015). Therefore, there is a lack of predictability, constraining the potential clinical use of such information for a rational design of treatment strategies.

Comprehensive Collateral Effects upon Antibiotic Resistance Evolution

We then tested the evolved populations for the presence of collateral sensitivity or resistance against all other drugs. For this, we challenged each population against various concentrations (in triplicate) of all the antibiotics and compared their growth to the ancestral PA14. To quantify the change in resistance or sensitivity, we considered growth relative to the no-drug environment, which accounts for general fitness changes resulting from adaptation to the experimental environment. We then calculated the area under the curve (AUC) of the ancestral PA14 and subtracted it from that of each population (fig. 3*A* and *B*, for the IC90 fold changes see supplementary fig. S4, Supplementary Material online). Finally, we counted how often adaptation to one antibiotic led to resistance against any other, hereby defined as direct adaptation, and also; how many times resistance to a given drug evolved after adaptation to another one, hereby defined as indirect adaptation (fig. 3*C*). The same process was repeated to calculate the number of cases of sensitivity by direct or indirect adaptation.

Overall, we identified collateral effects in almost all populations with evolved resistances, whereby cross-resistance is generally more common than collateral sensitivity (supplementary fig. S5, Supplementary Material online and fig. 3*B* and *C*; direct adaptation). Essentially identical results are obtained if IC90 fold change is used for the analysis rather than AUC differences (supplementary fig. S4, Supplementary Material online). Five main conclusions can be drawn from our analysis. i) Adaptation to a given antibiotic leads to collateral resistance to drugs of similar classes (see the aminoglycosides STR, and GEN, or the β-lactams CEF, PIT, and CAR). ii) Resistance to most drugs leads to collateral sensitivity against aminoglycosides, whereas resistance to aminoglycosides leads

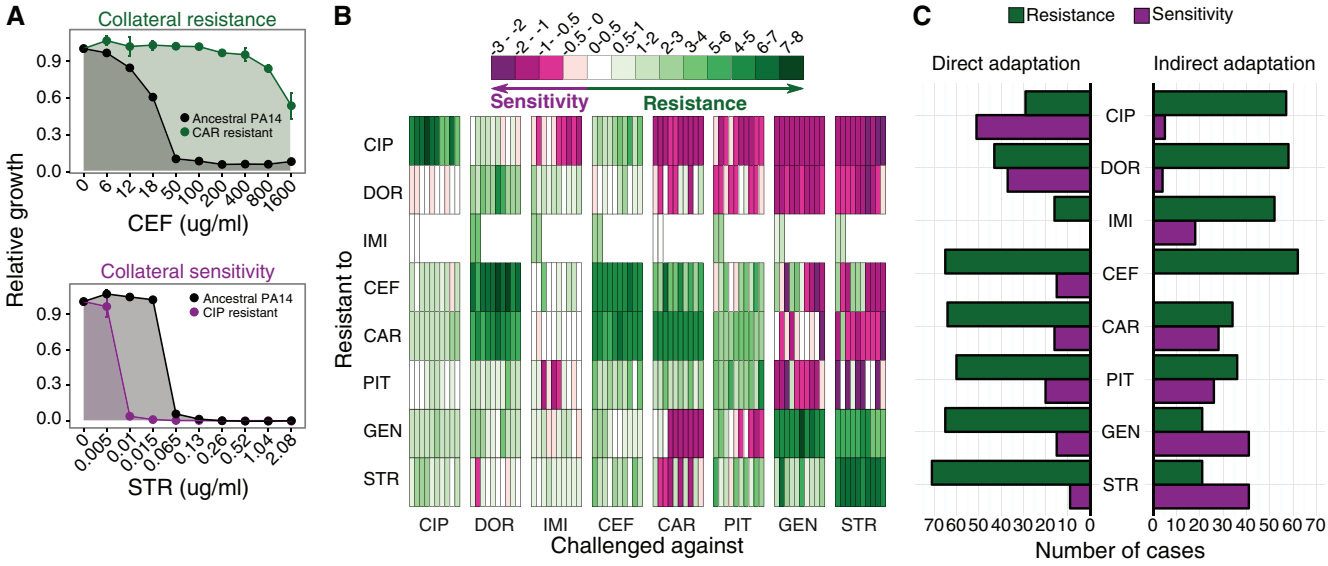

Fig. 3. Evolution of collateral sensitivity and resistance. (*A*) Illustration of the quantification of evolved collateral resistance or sensitivity. Bacterial growth (relative to a no-drug environment) of all evolved populations and the ancestral *P. aeruginosa* PA14 is first measured across concentrations of the various antibiotics. The area under the curve of the ancestor is subsequently subtracted from that of each population; resulting negative values indicate sensitivity (bottom panel), whereas positive values denote resistance (top panel). (*B*) The collateral profiles of all experimentally evolved populations (rows refer to the drugs used during experimental evolution), challenged against all other antibiotics (as indicated by columns). The vertical bars within each block represent the replicate populations. The different shades of purple or green highlight the extent of sensitivity or resistance, respectively. (*C*) We counted the total number of cases, for which adaptation to a particular antibiotic (listed in the middle) led to collateral sensitivity or resistance (direct adaptation; left panel), and also the total number of cases, for which sensitivity or resistance towards the focal antibiotic was observed upon adaptation to any of the other drugs (indirect adaptation; right panel).

to collateral sensitivity almost exclusively to the penicillin-type β-lactams (PIT and CAR; fig. 3*B* and *C*; indirect adaptation). iii) Adaptation to CIP or DOR leads to enhanced sensitivity against most other drugs, but interestingly this effect appears to be unidirectional: adaptation to other antibiotics rarely leads to sensitivity against these two drugs (fig. 3*C*; indirect resistance). iv) Collateral resistance against CEF, a 3rd generation cephalosporin with specific activity against *P. aeruginosa*, is found upon resistance evolution towards all other drugs, suggesting that resistance to some cephalosporins may be readily achieved indirectly, thus compromising its use as a second-line drug. v) In several cases, substantial variation was observed among populations adapted to the same drug, suggesting that different resistance mechanisms may lead to contrasting patterns of collateral effects.

The evolution of collateral resistance and sensitivity seems to be a widespread trade-off in bacteria following drug resistance evolution. For example, in *E. coli*, collateral resistance towards drugs of the same class was repeatedly observed (Imamovic and Sommer 2013; Lázár et al. 2013, 2014; Oz et al. 2014). These previous studies also revealed interesting exceptions. Some drugs, such as CIP, were often targets of indirect multi-drug resistance evolution, regardless of the similarity of the drug used during experimental evolution. Also, the strength of cross-resistance was variable even among drugs with the same cellular targets. For instance, within the cell-wall inhibitors, adaptation to penicillins seems to lead to cross-resistance more often than adaptation to carbapenems.

It is noteworthy that the direction of collateral sensitivity differs in specific cases between *P. aeruginosa* and *E. coli*. In particular, resistance to the aminoglycosides often preceded a 2-fold reduction in MIC against most other drugs in *E. coli* (Imamovic and Sommer 2013; Lázár et al. 2013). In contrast, adaptation to the same drugs in PA14 more often led to cross-resistance, whereas in some individual populations collateral sensitivity was observed almost exclusively towards the penicillins. Moreover, CIP-adapted *E. coli* strains usually exhibited cross-resistance to most other drugs (Imamovic and Sommer 2013; Lázár et al. 2014), whereas our experiments with *P. aeruginosa* produced hypersensitivity in such combinations. These findings highlight the presence of key differences between species in the evolved collateral effects following drug adaptation. Systematic evaluation of different pathogen-drug combinations is thus essential for a full appreciation of incidences and diversity of evolved collateral sensitivity (Imamovic and Sommer 2013; Lázár et al. 2014).

Unexpectedly, our results further demonstrate the presence of substantial variation in collateral effects upon adaptation to one drug. In several cases, we even observed contrasting patterns of either collateral sensitivity or resistance. For example, CEF-adapted populations exposed to GEN produced three cases of cross-resistance, five cases of collateral sensitivity, and two neutral effects (fig. 3*A*; supplementary fig. S4, Supplementary Material online). Similar variation in collateral effects are also found for all other antibiotic treatments, with the exception of IMI, most likely due to low sample size (i.e., only two populations could be included in the analysis). Moreover, such divergence in collateral effects upon antibiotic resistance evolution has not yet been reported for other bacterial species. These findings strongly suggest that our replicate *P. aeruginosa* populations from a particular antibiotic treatment achieved resistance through different molecular mechanisms, which in turn had opposite effects on the interaction with a second antibiotic.

Cross-Resistance May Result from the Chemical Similarity between Drugs

Recent studies have followed Szybalski and Bryson's hypothesis that the chemical relatedness between different drugs could explain the prevalence of cross-resistance, finding a partial correlation between them in *E. coli* (Lázár et al. 2014). However, the reported relationship was strongly biased by the aminoglycosides, since no cross-resistance to such drugs was observed and these possess a chemical structure that is fundamentally different to that of the other drugs. In the case of *P. aeruginosa*, we often observed cross-resistance to the aminoglycosides, thus allowing us to further explore the range of relationships between drug similarities and the frequency of cross-resistances. For such an analysis, we first inferred the chemical similarity among all antibiotics by calculating the Jaccard's index obtained from the pairwise comparison of their chemical fingerprints (supplementary fig. S6, Supplementary Material online). We then related these similarities to the frequencies of collateral resistances (FCR) for all drug pairs (see Materials and Methods, and Lázár et al. 2014). Our analysis revealed a significant correlation between these two parameters (fig. 4). Importantly, drugs targeting the same cellular process are not necessarily chemically similar (e.g., IMI and PIT or DOR and CAR) and in these cases we do not find high levels of cross-resistance. Altogether, chemical similarity

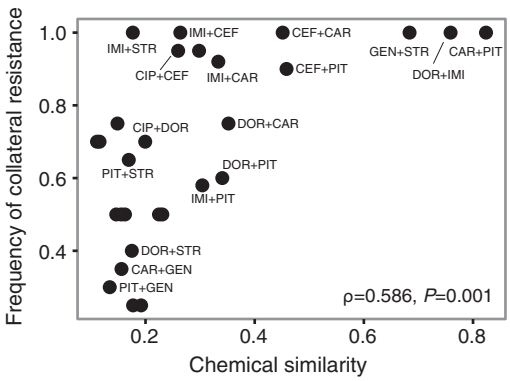

Fig. 4. Chemical similarity correlates with frequency of collateral resistance. Pairwise Jaccard's similarity indexes were calculated based on the chemical fingerprints of each antibiotic. The frequency of collateral resistance (FCR) was then determined as $FCR = (R_{A\to B} + R_{B\to A})/L_{AB}$, where $R_{A\to B}$ is the number of populations resistant to drug A with cross-resistance to drug *B* (and *vice versa* for $R_{B\to A}$), and L_{AB} is the total number of populations adapted to *A* and *B*. A significant correlation was then found between the chemical similarity and the FCR (Spearman's rank correlation). Each point corresponds to a chemical comparison between any two given drugs. Labels are shown for some, but not all, of these pairwise comparisons.

appears in most cases to be a key determinant of the probability of cross-resistance.

Distinct Mutations Underlie the Evolution of High Antibiotic Resistance

To better understand the genetic mechanisms selected during the evolution of high antibiotic resistance, we obtained whole genome sequences for 81 evolved populations: 71 antibiotic adapted populations and 10 controls. We characterized genomic variations using a previously established analysis pipeline (see Materials and Methods; Jansen et al. 2015). An important step of the pipeline is to remove substitutions which occurred in the control populations, as these may result from adaptation to general experimental conditions and could thus obscure the signals relevant for adaptation to the antibiotics (see supplementary table S4, Supplementary Material online for a list of mutations found in the evolved control populations). We further annotated the genes with mutations in coding regions using DAVID, the *Pseudomonas* Database (available online at: http://pseudomonas.com; last accessed May 19, 2017), and published information to group them by function and their likely involvement in antibiotic resistance. In general, we observed an average of 10.5 genes affected per antibiotic environment (fig. 5*A*), but with different degrees of mutational diversity (supplementary fig. S7, Supplementary Material online). Most mutations were nonsilent and may thus have contributed to adaptation (fig. 5*B* and *C*): 89% of the mutations were observed in coding regions (Intergenic vs. all other mutations, χ^2 test, $\chi^2 = 50.94$, df = 1, $P < 0.0001$); 83% of the variants found in coding regions (e.g., different types of nonsynonymous, insertions/deletions [indels], or frameshift mutations) led to partial or complete loss of function (Intergenic and Synonymous SNPs vs. all other type of variants, χ^2 test, $\chi^2 = 39.15$, df = 1, $P < 0.0001$), and more than half of the variants observed were fixed at levels above 40% (Number of variants <40%

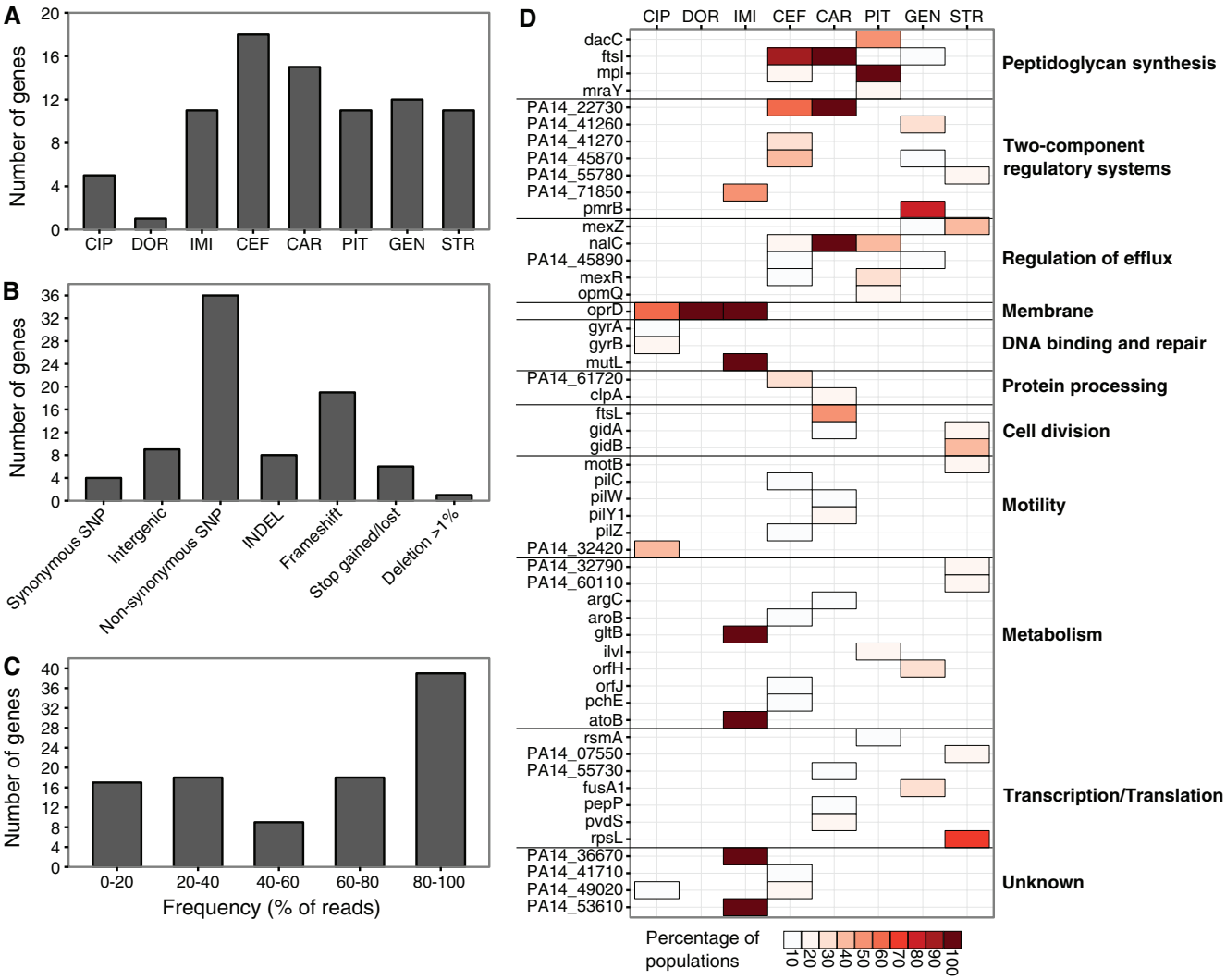

Fig. 5. Genomics of adaptation. Distribution of the number of genes with substitutions per (*A*) antibiotic used in the evolution experiment, (*B*) type of mutational change, and (*C*) average frequency class within the replicate populations. (*D*) Functional effect of mutations found in coding regions of the listed genes (vertical axis, left side) across evolution experiments with different antibiotics (horizontal axis). Functional information (right side) is inferred from a combined analysis using DAVID, the *Pseudomonas* database and publications. Different shades of red indicate the percentage of affected populations per evolution experiment with a particular antibiotic.

fixed vs. variants ≥40% fixed, χ^2 test, $\chi^2 = 50.94$, df = 1, $P = 0.003$).

For subsequent analysis, we focused on genes having mutations in their coding regions only (fig. 5D). Interestingly, the only two surviving populations adapted to IMI showed mutations in *mutL*, a gene coding for a DNA repair enzyme generally associated with hypermutator phenotypes (Oliver et al. 2002; Montanari et al. 2007; Ciofu et al. 2010); these populations accumulated as many mutations in half the number of generations as the others during the entire experiment (supplementary table S5, Supplementary Material online). The various DOR adapted populations produced a unique SNP in a single gene, *oprD*: a membrane protein (fig. 5D). The populations adapted to all other antibiotics showed a larger number of genomic changes across a variety of genes (fig. 5D).

The functional annotation revealed changes in three main functions across evolved populations (fig. 5D): The first group of affected genes is related to direct targets of the β-lactam antibiotics and was primarily identified in populations adapted to CEF, CAR, and PIT. This group included genes associated with peptidoglycan synthesis (supplementary table S5, Supplementary Material online): the penicillin-binding protein 3 (PBP3) gene *ftsI*, and also the UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase gene *mpl*. Nonsilent mutations in these genes were previously demonstrated to increase resistance against β-lactams, either by limiting the interaction between antibiotics and the products of these genes, or by indirectly inducing SOS responses or enhanced efflux (Miller et al. 2004; Tsutsumi et al. 2013).

The second group of genes is related to seven different two-component regulatory systems (fig. 5D). This group was generally affected in response to adaptation to β-lactam and aminoglycoside antibiotics, whereby variation in mutated systems seems to depend on the antibiotic class. Two-component systems are essential for bacteria to recognize different environmental stimuli and coordinate a fine-tuned response via a complex phosphorylation signal transduction system (Hoch 2000; Robinson et al. 2000; Ramos et al. 2005). *P. aeruginosa* in particular possesses a large number of such regulatory systems, which it uses to control cellular division, development, stress responses, and pathogenicity (Rodrigue et al. 2000).

The third main group of mutated genes was found in β-lactam and aminoglycoside adapted populations, and included different efflux regulatory systems (fig. 5D). These genes modulate the activity of distinct efflux pumps which can lead to single or multi-drug resistance (Hancock and Speert 2000; Li et al. 2000; Poole 2001; Piddock 2006). Importantly, two of them (*mexZ* and *nalC*) are closely related to those regulating the *tet* efflux pumps (TetR-like repressors), which were previously demonstrated to contribute to collateral sensitivity against aminoglycosides in tetracycline resistant *E. coli* (reviewed in Baym et al. 2016b). These genes were further proposed as a pivotal group to be exploited in sequential treatment strategies (Baym et al. 2016b).

We additionally identified changes in several other known or at least suggested antibiotic resistance genes (fig. 5D), including the membrane protein main component gene *oprD*, the DNA gyrase subunit genes *gyrA* and *gyrB*, as well as other genes involved in cellular processes such as cell division and motility (Macfarlane et al. 2000; Drenkard and Ausubel 2002; Livermore 2002; Amin et al. 2005). Our analysis further revealed changes in genes with currently unknown function and/or no previous implication in antibiotic resistance, and/or mutations in noncoding regions, which may still influence resistance if regulatory regions are affected (e.g., possibly relevant for DOR-adapted populations). Some of these changes occurred at lower frequency within the replicate populations, but their exact contribution to the observed evolutionary pattern is uncertain and may be interesting to address in future studies. Furthermore, among the PIT-adapted populations we also found substantial variation in the sets of mutated genes, even though they almost all consistently affected the same two functional categories: peptidoglycan synthesis and efflux regulatory systems. This variation could explain the different levels of resistance observed for these populations, whereby different groups of mutated genes lead to higher or lower levels of resistance.

Specific Resistance Mechanism Associate with High Growth Costs

The accumulation of multiple mutations during adaptation to antibiotics could translate into stronger reductions in fitness under drug-free conditions. In fact, in a similar study with *E. coli*, populations accumulating a large number of mutations or deletions had also very low fitness in drug-free environments (Lázár et al. 2014). Similarly, in *P. aeruginosa* we found that the populations with the strongest decrease in growth rate have a significantly larger number of mutations in their genomes (supplementary fig. S8A, Supplementary Material online). However, none of the other measured fitness parameters correlated with the number of mutations, suggesting that this alone is not enough to explain the observed variation in fitness. Moreover, when we looked for overrepresented genes in populations with the most extreme growth costs, we observed some functions to be more prevalent than others (supplementary fig. S8B–D, Supplementary Material online). In particular, genes involved in peptidoglycan synthesis (*mpl*, *dacC*, or *ftsI*), regulation of efflux (*nalC*) or part of two-component regulatory systems (*pmrB*) were more frequently found in populations with lower relative fitness across the measured growth parameters. Interestingly, multiple genes affecting these cellular processes were found within the same populations having strong fitness reductions (*nalC* together with *mpl* and *dacC* were often found in PIT resistant populations). Altogether, the costs of adaptation seem to be dependent on both the number of mutations accumulated and the specific mutated resistance mechanism.

Parallel Patterns of Collateral Resistance across Treatments Is Not Linked to Similar Mutational Profiles

Next, we assessed whether parallel mutational changes could explain the observed patterns of collateral resistance across

different treatments in *P. aeruginosa* (Fig. 3*B*). In contrast to the findings for *E. coli* (Lázár et al. 2014), we found comparatively little mutational overlap between the populations from different antibiotic treatments (supplementary fig. S9*A*, Supplementary Material online). Interestingly, in the few cases where similar mutational profiles were observed among populations resistant to different drugs, the two compared antibiotics appear to be chemically related, and the populations showed cross-resistance (supplementary fig. S9*B* and *C*). However, these correlations were not statistically significant, because our data set also included several populations with high cross-resistance against chemically similar drugs, but with completely distinct mutational profiles (supplementary fig. S9*B* and *C*). Therefore, it seems that the parallel patterns of cross-resistance across treatments are based on different sets of mutations.

This is also true for populations from different treatments that adapted to drugs of the same class. For instance, the populations that adapted to the two aminoglycosides showed high levels of cross-resistance among them, but adaptation to GEN was more often accompanied by mutations in the two-component regulatory gene *pmrB*, whereas those adapted to STR predominantly showed mutations in genes involved in cellular division (i.e., *gidA* or *gidB*), or in efflux regulation (i.e., *mexZ*; fig. 5*D* and supplementary table S5, Supplementary Material online).

Contrasting Collateral Effects Have Unique Genomic Profiles

We identified substantial variation in the incidence of collateral sensitivity and cross-resistance within particular evolution experiments. For instance, five of the CEF adapted populations showed collateral sensitivity against GEN and STR, whereas the rest suffered cross-resistance or neutral effects against the same drugs (fig. 3*B*). Similarly, three of the GEN adapted populations displayed collateral sensitivity against CAR and PIT, whereas the others were resistant (fig. 3*B*). Such variation could be the result of distinct resistance mechanisms selected during adaptation to each drug, which in turn led to contrasting sensitivities against other antibiotics. To explore this possibility, we focused on four exemplary cases, for each of which we repeatedly found contrasting variation in collateral effects to other antibiotics. These included the populations adapted to the two aminoglycosides (GEN, STR), which both showed such variation towards PIT and CAR (fig. 3*B*). We also considered the reverse two cases, for which populations that had adapted to either PIT or CAR produced contrasting collateral patterns towards GEN and STR (fig. 3*B*). For these four cases, we used hierarchical cluster analysis (based on Ward's criterion method and bootstrapping to assess cluster stability [Murtagh and Legendre 2014]) to relate populations with varying collateral effects to corresponding genomic variation. In particular, we first assessed whether populations adapted to a given drug would cluster together based on their sensitivity against the two other antibiotics (fig. 6*A*, *C*, *E*, and *G*). Then, we asked whether the same populations would also cluster together based on their sets of mutated genes (fig. 6*B*, *D*, *F*, and *H*).

We identified clusters that consistently link the contrasting patterns of collateral sensitivity to particular genomic variations. Intriguingly, the two-component regulatory system and TetR-like repressors appear to play a pivotal role in determining these patterns. For the GEN-adapted populations, sensitivity to PIT and CAR was associated with mutations in *pmrB* (fig. 6*A* and *B*), a sensor kinase implicated in resistance against cationic antimicrobial peptides, polymyxins, and aminoglycosides in *Salmonella* and *P. aeruginosa* (McPhee et al. 2003). Aminoglycoside resistance mediated by *pmrB* is hierarchically controlled by two regulatory systems (PhoP-PhoQ and PmrB-PmrA) that ultimately remodel lipid A in the outer membrane resulting in a reduction of the membrane's negative charge. Such alterations were shown to enhance sensitivity to β-lactams in aminoglycoside resistant *E. coli* (Lázár et al. 2013; Baym et al. 2016b). Altogether, this suggests that *pmrB* could be the main driver of β-lactam sensitivity in GEN resistant populations.

Collateral sensitivity against penicillins in STR-adapted populations is linked to mutations in the gene *mexZ* (fig. 6*C* and *D*), a TetR-like repressor modulating the expression of the MexXY-OprM efflux system (Aires et al. 1999). This gene was found to mediate aminoglycoside resistance in *P. aeruginosa* PAO1 and clinical isolates from cystic fibrosis patients by increasing cellular drug efflux, through an upregulation of the *mexXY* genes (Westbrock-Wadman et al. 1999). Interestingly, in *P. aeruginosa*, extrusion of the aminoglycosides appears to be exclusively mediated by MexXY-OprM, which, when overexpressed, is also able to extrude most penicillins (except for carbenicillin and sulbenicillin), some cephalosporins, and meropenem (Masuda et al. 2000). However, to the best of our knowledge, its possible role in mediating collateral sensitivity against penicillins has not been reported before. In addition, the expression of collateral sensitivity could be further influenced by other mutations. For instance, a mutation in *motB*, a gene required for flagellar motility in *P. aeruginosa* (Doyle et al. 2004), is present in addition to mutated *mexZ* in population ten, which shows collateral sensitivity to CAR but not PIT (fig. 6*C* and *D*).

In the case of the penicillin-adapted populations, collateral sensitivity against aminoglycosides appears to be associated—among others—with mutations in the gene *nalC*, an efflux regulator of the MexAB-OprM system known to confer intermediate to high levels of resistance to multiple drugs (Cao et al. 2004). Populations adapted to CAR and PIT with mutations in this gene consistently showed a more sensitive phenotype against both aminoglycosides (fig. 6*E*–*H*). Interestingly, *tet* efflux regulators, which are related in their function to *nalC*, were previously shown to mediate collateral sensitivity against aminoglycosides in tetracycline resistant *E. coli* (reviewed in Baym et al. 2016b), thus suggesting a pivotal role of this type of resistance mechanism in determining antibiotic susceptibility trade-offs.

It is noteworthy that the exact distribution of the variation in sensitivity against aminoglycosides in CAR-adapted populations is not well captured by our approach. All the identified clusters show genomic changes that affect the same three functions, yet the exact mutations and their frequencies

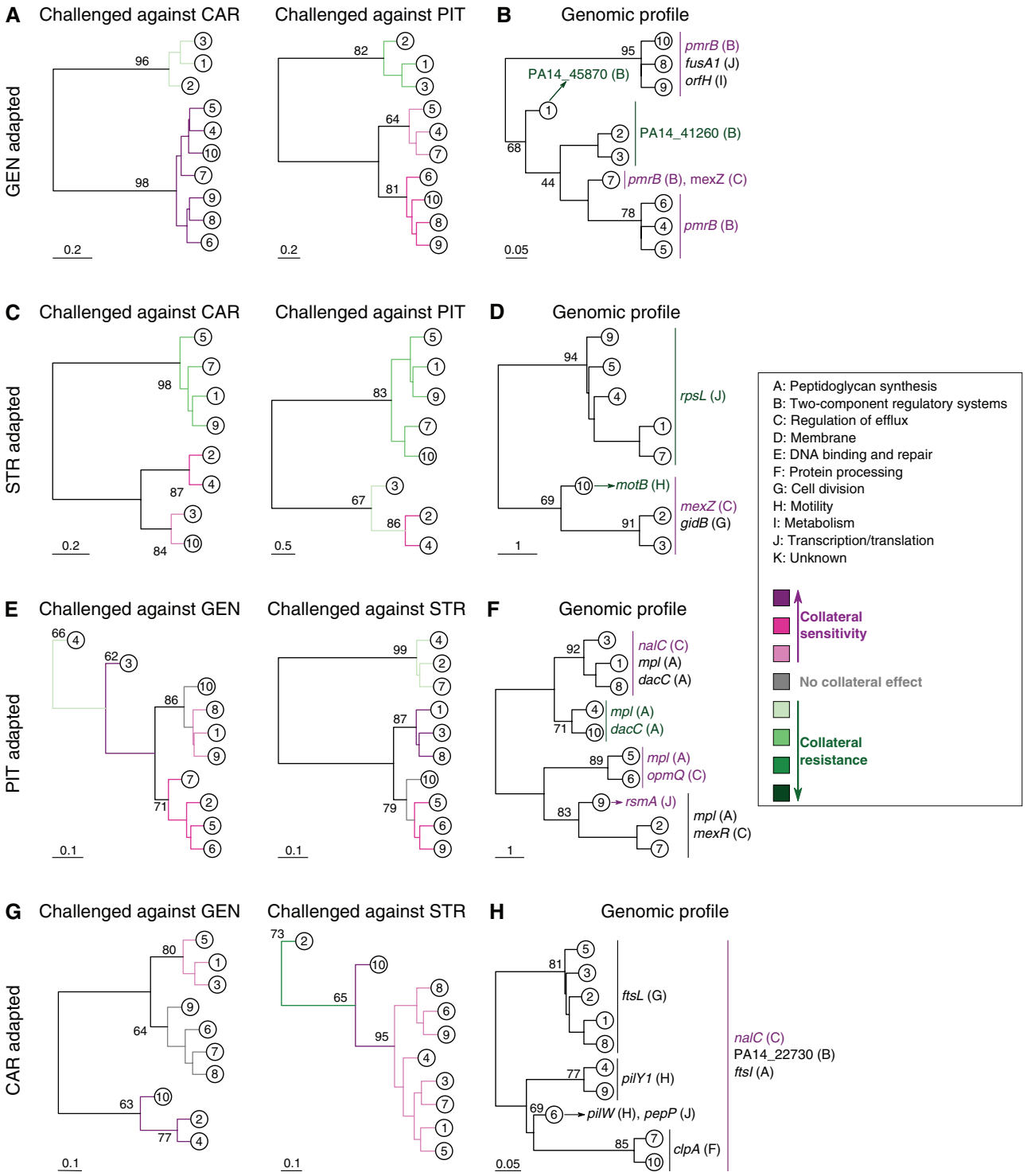

Fig. 6. Genomic determinants of collateral sensitivity. We employed a hierarchical clustering analysis using the Ward's criterion method and bootstrapping to identify the genomic determinants of variation in collateral profiles in four treatments. In two of these treatments, replicate populations that had adapted to either GEN (*A*, *B*) or STR (*C*, *D*) produced variation in collateral profile to PIT and CAR. In the other two treatments, replicate populations that had adapted to either PIT (*E*, *F*) or CAR (*G*, *H*) showed such variation towards GEN and STR. We first evaluated the clustering of populations adapted to GEN (*A*), STR (*C*), PIT (*E*), and CAR (*G*) based on the strength of collateral effects to the other two drugs (fig. 3*B*), highlighting clusters of those with collateral sensitivity (lines in different shades of purple, see legend to the right) versus those with collateral resistance (lines in different shades of green). The circled numbers always indicate the same replicate populations from a particular treatment across the related panels. Populations clustering together based on their collateral effects also often clustered together based on their genomic profile (*B*, *D*, *F*, *H*). The mutated genes present in the various clusters are given to the right of the dendrograms, followed by letters for their functional annotation in brackets (see legend to the right for the annotation categories). If a specific cluster mainly included populations associated with collateral sensitivity, then the gene names are given in purple. In cases, where clusters mainly included populations associated with collateral resistance, the gene name is given in green.

within the populations differ (fig. 6G and H, supplementary fig. S10 and table S5, Supplementary Material online). The observed phenotypic variation may then be caused by only some of the variable genes, by specific mutations only, by the combination of specific allelic variants, or by the frequency difference of certain mutations. Therefore, the observed collateral effects are likely influenced by additional factors, which could not be identified directly by the cluster analysis.

Mutations in *nalC*, *mexZ*, and *pmrB* Can Cause Collateral Sensitivity

Different regulatory systems were generally associated with collateral sensitivities in the evaluated populations. To further evaluate such a role, we assessed antibiotic sensitivity in genetically modified ancestral PA14, in which we reintroduced a selection of four of the identified mutational changes (Materials and methods), namely the inferred ~500 bp deletion in *nalC*, a SNP in *mexZ* leading to an early stop codon (Q95stop), and two nonsynonymous mutations in *pmrB* causing a P254L and a V136E amino acid substitution. In all cases, the constructed mutants showed almost the exact same response as the evolved populations against at least two of the considered antibiotics (fig. 7 and supplementary table S6, Supplementary Material online). In particular, the SNPs introduced in *pmrB* and *mexZ*, originally observed in the GEN- and STR-adapted populations, caused high resistance to these aminoglycosides, and led to significantly increased sensitivity to the penicillins PIT and CAR (fig. 7; supplementary table S6, Supplementary Material online). Similarly, the introduced deletion in *nalC*, originally observed in CAR-adapted populations, caused resistance to the tested penicillins while significantly increasing sensitivity to both GEN and STR (fig. 7; supplementary table S6, Supplementary Material online). Thus, two-component regulatory systems and efflux

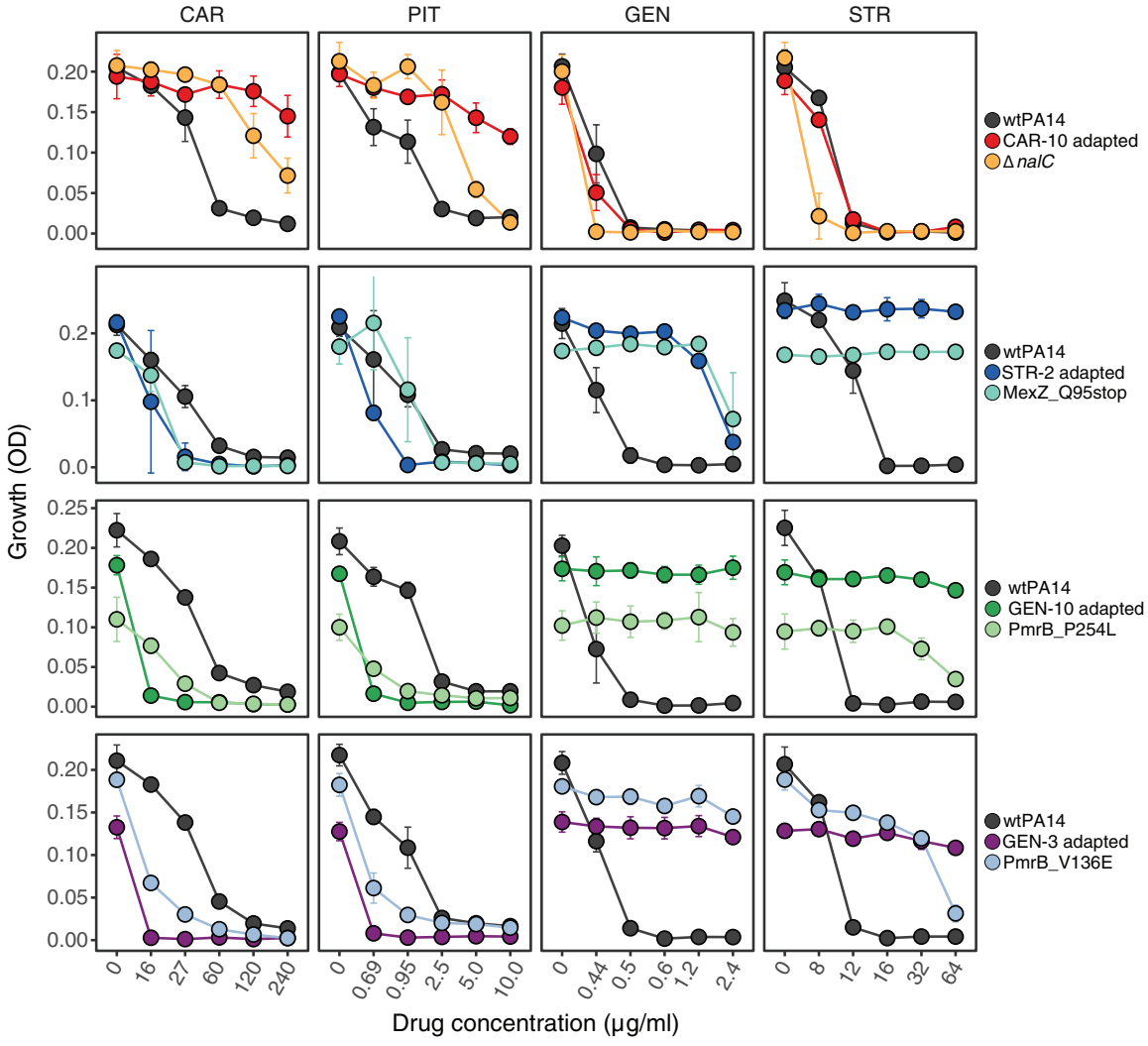

Fig. 7. Functional analysis of different regulatory genes. We focused on four specific mutations identified in the evolved populations to associate with collateral sensitivity and introduced these into the ancestral PA14 strain. The resulting mutants were then tested against various concentrations of CAR, PIT, GEN, and STR (from left to right). In all cases, the ancestral PA14 (always in black) and the adapted population (always in darker colors), from which the particular mutation was extracted, were tested simultaneously with the corresponding constructed mutants. Points and error bars show the mean OD ± SD of five technical replicates per antibiotic concentration. For each set of bacterial populations challenged against a particular drug, we performed a GLM followed by Tukey's honest significant difference (HSD) test. For a summary of the statistical results see supplementary table S6, Supplementary Material online.

regulators can be responsible for the observed collateral sensitivity in *P. aeruginosa*.

The tested genes *nalC*, *mexZ*, and *pmrB* are involved in the regulation of efflux pumps or alteration of the outer membrane, and can thus influence antibiotic resistance (Westbrock-Wadman et al. 1999; McPhee et al. 2003; Cao et al. 2004; Daigle et al. 2007; Lázár et al. 2013; Baym et al. 2016b). To the best of our knowledge, the results of our functional genetic analysis demonstrated for the first time in *P. aeruginosa* that such genes can also differentially lead to collateral sensitivity against aminoglycosides (*nalC*) and penicillins (*mexZ* and *pmrB*). Importantly, two distinct aminoglycoside resistance mechanisms independently led to penicillin hypersensitivity: a loss of function (LOF) mutation in *mexZ*, and two different nonsynonymous mutations in the two-component regulatory system gene *pmrB*. Intriguingly, contrasting collateral effects emerged from alterations in functionally related genes: both *mexZ* and *nalC* are from the TetR-family of repressors, and LOF mutations in each of them led to completely opposite susceptibilities against penicillins and aminoglycosides. Such contrasting results could be due to an impairment of one of the efflux pumps upon upregulation of the other, a phenomenon previously seen in other efflux systems such as MexCD and MexAB, or MexEF and MexAB (Gotoh et al. 1998; Maseda et al. 2000; Jeannot et al. 2008), but not between MexXY and MexAB.

Conclusions

We here demonstrated that *P. aeruginosa* adapts rapidly to high-level antibiotic stress and that such adaptation may influence resistance against other antibiotics. Cross-resistance correlates well with chemical similarity of the antibiotics, in agreement with previous work. Collateral sensitivity was identified in several cases, yet different in direction to those cases previously reported for other bacteria, such as *E. coli*. Surprisingly, adaptation to a particular antibiotic produced both cross-resistance and collateral sensitivity across the replicate populations. Our genomic analysis suggests that alternative mechanisms were favored during resistance evolution, which then resulted in these contrasting cross-resistance patterns. Intriguingly, regulatory systems appear to play a key role in mediating the observed collateral effects. Functional genetic analysis revealed that four of the identified mutations in three regulatory genes (*nalC*, *mexZ*, and *pmrB*) can indeed cause collateral sensitivities in *P. aeruginosa*. Overall, we expect our results to help the development of novel antibiotic therapy that exploits fitness trade-offs during drug resistance evolution.

Materials and Methods

Bacteria and Media

All experiments were conducted with *Pseudomonas aeruginosa* PA14. Cells were grown at 37 °C in sterile M9 minimal medium supplemented with 0.2% glucose and 0.1% casamino acids. All antibiotics were prepared according to manufacturer's instructions (table 1). All experiments were carried out in 96-well plates shaken and incubated at 37 °C for 12 h and treatments being randomized across each plate. After 12 h of growth, optical density (OD) measurements were taken in BioTek plate readers. Randomization schemes of plates for each experiment were different from each other.

Selection of Highly Resistant Mutants

We challenged 20 isogenic populations of PA14 against increasing concentrations of each of the studied antibiotics (160 populations in total; fig. 1A). As a control for the adaptation to growth in medium without antibiotics, we also included 20 populations growing in M9 only, resulting in a total of 180 populations. Experiments were initiated with half the antibiotic concentration that causes growth inhibition of at least 90% (IC90) for each drug in a final volume of 100 µl per well. Every 12 h 50% volume was transferred into a freshly prepared plate. Every fourth transfer (four generations), OD was measured and the antibiotic concentration was increased 1.5 times. Experiments were concluded when reaching ~40 times the IC90 (48 transfers) or when ten or less of the 20 populations had reached extinction. Whenever the antibiotic concentration was increased, the preceding plates were frozen at −80 °C in 1:4 (v/v) of 86% glycerol.

Fitness Measurements

All evolved populations were grown overnight (ON) at 37 °C and 180 rpm in M9 media with the corresponding antibiotic they were adapted to. Cultures were then centrifuged, washed with fresh M9 media and diluted in 100 µl of M9 without any drug to a starting OD of 0.08 (~10^5 CFU/ml). For each population, four technical replicates were considered, and then incubated inside a plate reader at 37 °C for 24 h. Within the plate reader, OD measurements were taken at regular intervals of 15 min, for a total of 96 measurements per population and replicate. The collected growth data was then analyzed using the R package "grofit" to obtain three growth parameters: growth rate, length of lag time, and maximum yield. We standardized the fitness of each population relative to the mean of the evolved controls (adapted to M9 media only).

Collateral Sensitivity and Resistance Assays

We measured collateral effects for the experimentally evolved populations with high levels of resistance. We tested these populations and also the ancestral PA14 against ten different concentrations of a given antibiotic in randomized order (each concentration was replicated three times; total of 21,120 concentration and population combinations). To quantify the change in resistance or sensitivity, we first considered growth relative to the no-drug environment in order to account for differences in fitness defects among populations originated after adaptation. We then calculated the area under the curve using a spline approximation of the ancestral PA14 and subtracted the one obtained for each population. Positive values reflect a higher cumulative growth at increasing concentrations in the population compared with the ancestor, thus indicating cross-resistance; conversely, negative values represent collateral sensitivity. To derive significant sensitivity or resistance, we performed a Wilcox on rank

test for all populations adapted to a given environment when challenged against a given antibiotic, comparing significant differences from 0. *P* values were adjusted for multiple testing using the false discovery rate, FDR (supplementary fig. S5, Supplementary Material online). We subsequently asked how many cases of direct or indirect adaptation had occurred for each antibiotic. Direct adaptation considers how often adaptation to a given drug leads to resistance (or sensitivity) against other drugs, whilst indirect adaptation reflects how often adaptation to other drugs lead to resistance (or sensitivity) against a particular antibiotic. For example, we asked how many cases of resistance against all other drugs were observed upon adaptation to CIP (direct adaptation); as well as how many cases of resistance against CIP were found upon adaptation to any other drug (indirect adaptation).

Chemical Similarity

We inferred chemical relatedness as previously described by using the Jaccard's similarity index contrasting the chemical fingerprints of all antimicrobial compounds used here (Lázár et al. 2014). We then correlated these pairwise comparisons to the frequency of cross-resistance calculated by:

$$FCR = (R_{A\rightarrow B} + R_{B\rightarrow A})/L_{AB},$$

where $R_{A\rightarrow B}$ is the number of populations resistant to drug *A* with cross-resistance to drug *B*, $R_{B\rightarrow A}$ is the number of populations resistant to drug *B* with cross-resistance to *A*, and L_{AB} is the total number of populations adapted to *A* and *B*.

DNA Extraction

To identify the genetic changes leading to high-level resistance evolution, we sequenced full genomes for whole populations of the ancestral *P. aeruginosa* PA14, ten evolved controls and 71 populations adapted to different drugs. Frozen material from all populations was thawed, and 10 µl of each were transferred into 15 ml of M9 minimal medium with the corresponding antibiotic. All populations were shaken and incubated at 37 °C O/N. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations for Gram-negative bacteria. Eighty-one populations in total were sequenced at the Institute for Clinical Microbiology, Kiel University Hospital, using the Illumina HiSeq paired-end technology (Bentley et al. 2008) with an insert size of 150 bp and 300× coverage.

Genomic Analysis

We employed an established pipeline encoded in serial bash and Perl scripts used previously for the genomic analysis of *P. aeruginosa* PA14 (Jansen et al. 2015). Briefly, reads with unreliable quality were removed using Skewer (Jiang et al. 2014). Samples were then mapped to the published *P. aeruginosa*_UCBPP_PA14_NC008463 reference genome available at (http://pseudomonas.com/strain/download; last accessed May 19, 2017). Mapping was performed using bwa and samtools (Li et al. 2009; Li and Durbin 2010) and then visually inspected for low-quality areas using IGV (Integrated genome viewer, Broad Institute; www.broadinstitute.org/software/igv/; last accessed May 19, 2017).

Duplicated regions were removed for single nucleotide polymorphisms and structural variant calling (SNPs and SV) using MarkDuplicates in Picardtools (https://braod.institute.github.io/picard/; last accessed May 19, 2017). To call SNPs and small indels above a threshold frequency of 0.1 and base quality above 20, we employed both frequentist and heuristic methods using respectively SNVer and VarScan (Wei et al. 2011; Koboldt et al. 2012). To identify larger indels and other SV, we used Pindel and CNVnator (Ye et al. 2009; Abyzov et al. 2011). The resulting output files were filtered for duplicates, ancestral variants, and variants found in the evolved controls. We used a combination of sources to identify and annotate the variants using snpEff (Cingolani et al. 2012), DAVID, the *Pseudomonas* database (available online at: http://pseudomonas.com; last accessed May 19, 2017) and information from published work. Further count statistics, analysis and visualizations were done in the R platform (R Core Team).

Mutational diversity was calculated as in (Chevereau et al. 2015). Briefly, we calculated the entropy $H = -\sum[p_j(\log_2 p_j + (1-p_j)\log_2(1-p_j)]$, where p_j is the probability that a given locus *j* is mutated in a random population. *H* then measures the diversity of mutated loci in the populations adapted to a given drug. Standard error was obtained from jackknife resampling in the R platform.

In order to link the observed collateral effects to the underlying genetic changes we performed a hierarchical clustering analysis. For this, we focused on four treatments, which repeatedly produced contrasting patterns of collateral effects. These included populations adapted to either GEN or STR (the two aminoglycosides), which produced variation in their collateral profiles towards PIT and CAR. We also considered the reverse two cases, for which replicated populations that had adapted to PIT and CAR showed contrasting patterns of collateral effects towards GEN and STR. For these four cases, we first obtained the Euclidean similarity of the sensitivities of evolved populations against the considered drugs. Then we used hierarchical clustering based on Ward's minimum variance method, including the Ward's criterion, which aims at finding compact, spherical clusters, and combined it with bootstrapping to asses cluster stability (Murtagh and Legendre 2014). The same process was then used to infer clusters based on the genomic profiles of the same populations, including only genes with mutations within their coding regions. For each antibiotic, we then built dendograms for the clustering results and assessed to what extent given genomic clusters coincided with clusters having collateral resistance or sensitivity phenotypes.

The obtained genome sequences are available from NCBI SRA database under the BioProject number: PRJNA355367.

P. aeruginosa PA14 Genome Editing

Deletion of *nalC* and single nucleotide mutations in *pmrB* and *mexZ* were introduced in *P. aeruginosa* PA14 based on a two-step recombination method previously described (Trebosc et al. 2016). DNA fragments corresponding to 700-bp up-

Table 2. Oligonucleotides Used.

Oligo Name	Sequence (5′–3′)
oVT8	GTTTTCCCAGTCACGACGC
oVT464	AGAATTGAGGCCTCTCGAGGAATTCTTAGAGGT CCCAGGCATTG
oVT465	TGAGGAACAGGGTTTGCTGAGAGCGTTTC
oVT466	TCAGCAAACCCTGTTCCTCAAGGCCCTC
oVT467	CCGCAAGCTTCCTGCAGGCTCTAGACTGATGGAA ACCTTTGCC
oVT468	AGAATTGAGGCCTCTCGAGGAATTCTCGATCTCG ACGAACTGC
oVT469	CCGCAAGCTTCCTGCAGGCTCTAGACAACGACAG CTCGATGTC
oVT470	AGAATTGAGGCCTCTCGAGGAATTCCTCTCGCTGA AGCAGGTG
oVT471	CCGCAAGCTTCCTGCAGGCTCTAGAATCATCTTCG GCGTCAGTC
oVT472	AGAATTGAGGCCTCTCGAGGAATTCTCCTGGCCT TCCTCGTAC
oVT473	CCGCAAGCTTCCTGCAGGCTCTAGAAGGTAGGC GGAGAAAACG
oVT474	TCTGCGCGGATTCTGATAGC
oVT475	TCCCTGGAAATGCAGTGAGC
oVT476	GACGACTACCTGACCAAGCC
oVT477	CCTTCAGCCACAGGTCGATG
oVT478	CGAGGTCCATGTCCATCACC
oVT479	TCGTTCTCGTTGTAGTGGCG
oVT480	CCTTGATCAGGTCGGCGTAG
oVT481	AAGCTACCGTGACAGAACCC

and downstream of the *nalC* region to be deleted (position 1,391,565–1,390,977 on PA14 genome, GenBank CP000438.1) were amplified by PCR using primers oVT464/oVT465 and oVT466/oVT467, respectively. The resulting DNA fragments were introduced into pVT77 previously digested with EcoRI/XbaI using NEBuilder HiFi DNA assembly (New England Biolabs). For the allelic replacement of wildtype *pmrB* and *mexZ*, 1.4-kb DNA fragments were amplified by PCR using primers oVT468/469, oVT470/471 and oVT472/473 on the evolved populations GEN-3, GEN-10, and STR-2, which respectively contained the PmrB_V136E, PmrB_P254L and MexZ_Q95stop mutations. The resulting DNA fragments were cloned into pVT77, digested with EcoRI/XbaI, using NEBuilder HiFi DNA assembly.

The obtained plasmids were transformed into *E. coli* conjugative strains MFD*pir* or S17-1 and transferred into *P. aeruginosa* PA14 as described previously (Trebosc et al. 2016). After conjugation, genomic plasmid integration was tested on LB agar plates containing 100 μg/ml sodium tellurite—when *E. coli* S17-1 was used additional 30 μg/ml Chloramphenicol were added to selection plates to eradicate the *E. coli* cells. Plasmid integration into the PA14 genome was confirmed by PCR with primers oVT8 and oVT474, oVT476, oVT478, and oVT480 for *nalC* deletion, PmrB V136E, PmrB P254L, and MexZ Q95stop mutations, respectively. PA14 clones were transferred to LB agar plates containing 1 mM isopropyl-β-D-1-thiogalactopyranoside and 200 μg/ml 3′-azido-3′-deoxythymidine to select for plasmid removal from the genome. Clones were screened by PCR using primers oVT474/oVT475, oVT476/oVT477, oVT478/oVT479, and oVT480/oVT481 for *nalC* deletion, PmrB V136E, PmrB P254L, and MexZ Q95stop mutations, respectively. The genomic deletion and mutations were finally confirmed by DNA sequencing (Microsynth AG, Balgach, Switzerland) (table 2).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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