



Mutational background influences *P. aeruginosa* ciprofloxacin resistance evolution but preserves collateral sensitivity robustness

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Collateral sensitivity is an evolutionary trade-off whereby acquisition of the adaptive phenotype of resistance to an antibiotic leads to the nonadaptive increased susceptibility to another. The feasibility of harnessing such a trade-off to design evolutionary-based approaches for treating bacterial infections has been studied using model strains. However, clinical application of collateral sensitivity requires its conservation among strains presenting different mutational backgrounds. Particularly relevant is studying collateral sensitivity robustness of already-antibiotic-resistant mutants when challenged with a new antimicrobial, a common situation in clinics that has hardly been addressed. We submitted a set of diverse *Pseudomonas aeruginosa* antibiotic-resistant mutants to short-term evolution in the presence of different antimicrobials. Ciprofloxacin selects different clinically relevant resistance mutations in the preexisting resistant mutants, which gave rise to the same, robust, collateral sensitivity to aztreonam and tobramycin. We then experimentally determined that alternation of ciprofloxacin with aztreonam is more efficient than ciprofloxacin–tobramycin alternation in driving the extinction of the analyzed antibiotic-resistant mutants. Also, we show that the combinations ciprofloxacin–aztreonam or ciprofloxacin–tobramycin are the most effective strategies for eliminating the tested *P. aeruginosa* antibiotic-resistant mutants. These findings support that the identification of conserved collateral sensitivity patterns may guide the design of evolution-based strategies to treat bacterial infections, including those due to antibiotic-resistant mutants. Besides, this is an example of phenotypic convergence in the absence of parallel evolution that, beyond the antibiotic-resistance field, could facilitate the understanding of evolution processes, where the selective forces giving rise to new, not clearly adaptive phenotypes remain unclear.

collateral sensitivity | antibiotic resistance | convergent evolution | *Pseudomonas aeruginosa* | phenotypic convergence

Besides its relevance for human health, antibiotic resistance (AR) is one of the few evolutionary processes that can be experimentally addressed. Consequently, the study of the evolution processes involved in the acquisition of resistance is relevant not just in the AR field, but also in that of evolution in general. Since AR is the result of bacterial evolution, evolution-based approaches aiming to find the Achilles' heel associated with AR acquisition could be useful to tackle this relevant health problem (1). In this regard, evolutionary therapeutic strategies aiming to improve the efficacy of available antibiotics and to reduce the probability of selection of resistance are particularly interesting (2). One of the most promising trade-offs associated with the acquisition of resistance that could be exploited for implementing such novel therapeutic approaches is collateral sensitivity (CS), by which the acquisition of resistance to one drug renders an increased susceptibility to another (3).

Several studies, based on the alternation (4–7) or the combination of pairs of drugs (8–10), have tried to exploit CS (11, 12). However, for this exploitation to occur, a conserved CS phenotype must emerge when different strains become resistant to a particular drug. Unfortunately, although a few cases of robust CS have been described in model strains (5, 13, 14), this is not a common trait. Indeed, CS phenotypes are rarely conserved when different strains are compared; they differ not just among different isolates of the same species (15, 16), but also when replicated populations of the same strain and evolving in the presence of the same antibiotic are compared (17–20). While in the first case, epistasis and pleiotropy may shape the fitness effects associated with AR acquisition, restricting the type of mutations that can be selected in each genomic background (15, 21–23) and, therefore, the associated CS; in the second situation, genetic drift and population bottlenecks (24) might be responsible for the observed

Significance

Bacterial adaptation to the presence of an antibiotic often involves evolutionary trade-offs, such as increased susceptibility to other drugs (collateral sensitivity). Its exploitation to design improved therapeutic strategies is only feasible if collateral sensitivity is robust, reproducible, and emerges in resistant mutants; these issues are rarely addressed in available publications. We describe a robust collateral sensitivity phenotype that emerges in different antibiotic-resistance mutational backgrounds, due to different genetic events, and propose therapeutic strategies effective for treating infections caused by *Pseudomonas aeruginosa* antibiotic-resistant mutants. Since conserved collateral sensitivity phenotypes do not confer adaptation to the presence of antibiotics, our results are also relevant for understanding convergent evolution processes in which the force selecting the emerging phenotype remains unclear.

The authors declare no competing interest.

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lack of conservation. This lack of conservation when different strains and replicated populations that present the same genetic background are analyzed would be a major drawback for implementing therapies based on the exploitation of CS networks since the emergence of this phenotype will be unpredictable if it is different for each isolate.

As mentioned above, most works in the field have focused on the evolution of model antibiotic-susceptible strains, despite the fact that antibiotic-resistant mutants are frequently encountered in clinics. When an isolate is resistant to one antibiotic, a different one is used for treating the infection that it produces—a common situation in clinics—with a possibility that resistance to this second drug could be selected. If this novel resistance is associated with a robust CS phenotype, this information may lead to prioritization of the use of a third antimicrobial, to which the newly selected resistant strain becomes hypersusceptible. Therefore, the identification of robust CS patterns, conserved in different antibiotic-resistant mutants, is a prerequisite for using this evolutionary trade-off for tackling AR. In the current work, we intend to fill this gap by analyzing the robustness of the CS phenotypes that may emerge in different *Pseudomonas aeruginosa* antibiotic-resistant mutants, previously identified in adaptive laboratory evolution (ALE) experiments of *P. aeruginosa* PA14 (13, 21), when they are submitted to short-term ALE experiments in the presence of antimicrobials belonging to different structural and functional categories.

The acquisition of robust CS patterns in different genomic backgrounds—understood as isolates with different genomes—and genetic mutational backgrounds—here understood as strains with the same genome, but presenting genetic changes that might impact AR evolution (21)—can be considered an example of convergent evolution, understood as the acquisition of the same phenotype by different organisms when confronted with similar selection pressures (25). Particularly relevant is the fact that any conserved phenotype emerging in different organisms, from bacteria to humans, as a result of convergent evolution is generally considered to be selected because it improves the adaptation of the evolved organism to the applied selective force (26, 27). Opposite to this situation, CS is not adaptive to the presence of antibiotics; it is a trade-off associated with the selection of the primary adaptive phenotype, which is resistance to the selective antimicrobial. One of the possible reasons behind convergent evolution is parallel evolution, defined as a situation in which the same genetic event responsible for the convergent phenotype (in our case, CS) is selected, irrespective of the genetic background (28). However, although one example of parallel evolution leading to robust CS has been recently reported (29), the genetic events behind CS robustness are not always conserved (30, 31).

P. aeruginosa is a relevant opportunistic pathogen with high prevalence at hospitals and the main cause of chronic infections in cystic fibrosis (CF) patients. It is included in the critical priority list of antibiotic-resistant pathogens (32), as well as in the group of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter spp.*) that comprises six of the organisms for which the development of novel antibiotics/therapeutic options is an urgent need (33). The identification of robust CS networks associated with the use of drugs commonly used to treat infections caused by *P. aeruginosa*, such as fluoroquinolones, may then be useful.

The fluoroquinolone ciprofloxacin is extensively used to treat a wide range of infections caused by *P. aeruginosa* (34, 35). The two principal mechanisms of acquisition of ciprofloxacin

resistance in this bacterium include mutations in the ciprofloxacin target-encoding genes *gyrAB* (encoding DNA gyrase) and *parCE* (encoding DNA topoisomerase IV) (36–40) and the acquisition of mutations leading to overexpression of *mexEF-oprN*, *mexA-B-oprM*, *mexXY*, and *mexCD-oprJ* (34, 38, 41–43), which encode efflux pumps able to extrude ciprofloxacin. Antibiotic-resistant *P. aeruginosa* clinical isolates often contain multiple ciprofloxacin-resistance mutations in *gyrA*, *gyrB*, *mexA*, *mexB*, *nfxB*, *mexZ*, or *mexS* (5, 43, 44). Importantly, ciprofloxacin-resistant clinical isolates of *P. aeruginosa* presenting mutations in *gyrA*, *gyrB*, *mexB*, and *nfxB* seem to display CS to aminoglycosides (5), which also form part of usual therapies against *P. aeruginosa* (45). Although the authors suggested that *nfxB* mutations might be responsible for the observed CS to aminoglycosides, an example of parallel evolution leading to phenotypic convergence, it remains to be firmly established to what extent different ciprofloxacin-resistance mutations, individually or jointly acquired in different mutational backgrounds, as preexisting antibiotic-resistant mutants, may be shaping the robustness of CS to aminoglycosides.

In a previous work, we determined that different antibiotic-resistant mutants of *P. aeruginosa* PA14 submitted to ALE in the presence of ceftazidime displayed a robust pattern of CS to the aminoglycoside tobramycin. This phenotype is associated with the deletion of large chromosomal regions—also found in isolates from CF patients (46)—containing *mexXY*, which encodes an intrinsic aminoglycosides-resistance efflux pump (29), hence being an example of parallel evolution. In this work, we go one step further and analyze the robustness of CS associated with short-term ALE assays performed by using preexisting antibiotic-resistant mutants in the presence of ciprofloxacin, tobramycin, or aztreonam, antibiotics widely used for treating *P. aeruginosa* infections. We found that the different preexisting resistant mutants that evolved under ciprofloxacin challenge present conserved, robust CS to tobramycin and aztreonam. However, unlike the situation in which ceftazidime was the selective force (29), the genetic events selected by ciprofloxacin were different depending on the AR mutational background. The conservation of an emerging CS phenotype in these different antibiotic-resistant mutants provides an example of phenotypic convergence in the absence of parallel evolution that can be exploited to tackle AR. Indeed, based on this information, we rationally designed and experimentally validated in vitro that evolution-based approaches can drive extinction of preexisting antibiotic-resistant mutants of *P. aeruginosa*, supporting the potential of exploiting CS convergence for tackling *P. aeruginosa* infections, including those due to antibiotic-resistant mutants.

Results

Evolution of Resistance and CS Associated with Short-Term ALE in the Presence of Tobramycin, Aztreonam, or Ciprofloxacin.

In order to find out robust CS phenotypes, ALE experiments in the presence of antibiotics should be performed, using different antibiotics and in different genetic backgrounds. Indeed, it has been described that the loss-of-function of a single gene, not directly related with AR, modifies the evolutionary trajectories followed by *P. aeruginosa* PA14 in the presence of antibiotics and their patterns of CS (21), a feature that might compromise CS exploitation. Further, to have a situation closer to that found at clinics, strains to be tested should include antibiotic-resistant mutants, not only a model susceptible strain—an experimental approach that has been rarely addressed. Consequently, we analyzed the evolutionary conservation of CS associated with the short-term

use of three different antibiotics—tobramycin, aztreonam, and ciprofloxacin—in antibiotic-resistant *P. aeruginosa* mutants presenting different AR mutational backgrounds. To such goal, we used a set of well-defined antibiotic-resistant mutants (29), derived from *P. aeruginosa* PA14 and containing single (*nfxB*, *parR*, *orfN*, and *mexZ*) and multiple (MDR6 and MDR12) mutations in genes encoding both, regulatory and nonregulatory proteins (Table 1). These different types of mutations were specifically chosen because, besides the fact that they are regularly found in clinical isolates, it has been previously described that AR mutations may lead to either robust or variable CS patterns in different genomic backgrounds, depending on whether they produce “target” or “regulatory” alterations, respectively (16).

Four biological replicates of each single (*nfxB177*, *parR87*, *mexZ43*, and *orfN50*) and multiple antibiotic-resistant mutant (MDR6 and MDR12) strain and the wild-type PA14 strain were submitted to ALE in the presence of tobramycin, aztreonam, or ciprofloxacin, or in the absence of antibiotics (control populations) for 3 d (112 populations in total). As expected, a decrease in the susceptibility to each of the three drugs—tobramycin, aztreonam, and ciprofloxacin—was observed in all the populations evolved in their presence, respectively (Fig. 1 and *SI Appendix*, Tables S1–S3). Statistical analysis was performed by using both nonparametric tests on nontransformed data and parametric tests of $\log_2(\text{FC})$, where FC is fold change, as described in *Materials and Methods*. Nonparametric tests were used to assert significance in the differences between each combination of strain treatment with its respective control populations grown in the absence of any drug (*SI Appendix*, Table S4). Since statistically significant changes may be detected with a magnitude not associated with a relevant change in resistance, in the present study, we have settled for a threshold (above or below 2- or 0.5-fold, respectively) as the indicator of the potential biological significance. Differences surpassing this threshold were statistically validated by using $\log_2(\text{FC})$ -based parametric tests. In all cases, both types of statistical analyses (parametric and nonparametric) were consistent in detecting significant differences ($P < 0.05$).

In the populations submitted to ALE in the presence of tobramycin, the tobramycin minimal inhibitory concentration

(MIC) increased up to 4-fold in PA14, 16-fold in *parR87*, 8-fold in *orfN50*, 8-fold in *nfxB177*, 12-fold in *mexZ43*, 5.3-fold in MDR6, and 3-fold in MDR12 ($P < 0.05$ in all cases and using all approaches). Under aztreonam exposure, aztreonam MIC increased up to 8-fold in PA14; 2.7-fold in *parR87*; 4-fold in *orfN50* and MDR12; 24-fold in *nfxB177*; 12-fold in *mexZ43*; and 12-fold in MDR6 ($P < 0.005$ in all cases). Finally, ALE challenge with ciprofloxacin increased the MIC to this drug up to 46.9-fold in PA14, 128-fold in *parR87*, 15.8-fold in *orfN50*, 16-fold in *nfxB177*, 48-fold in *mexZ43*, 24-fold in MDR6, and 10.5-fold in MDR12 ($P < 0.005$ in all cases). It is worth noting that the greatest increase of AR occurred in *parR87* in the presence of tobramycin and ciprofloxacin and *nfxB177* in the presence of aztreonam, indicating that some mutational backgrounds are more prone to evolve toward higher levels of resistance than others. The question remains whether these differences are due to the acquisition of different AR mutations or whether the same AR mutations have a different effect depending on the mutational background where they are acquired. Control populations that evolved in the absence of antibiotics generally did not present variations in their susceptibility to antibiotics with respect to the MIC of parental strains, and, in the few cases in which these changes occurred, they were minor, not statistically significant, changes (*SI Appendix*, Table S4). This feature supports that the changes in MICs (above or below 2- or 0.5-fold, respectively) observed in populations challenged with antibiotics were due to antibiotic selection, not to a nonspecific adaptation to the growth medium.

In order to ascertain the evolutionary conservation of CS associated with the short-term use of tobramycin, aztreonam, or ciprofloxacin in the different mutational backgrounds, MICs to antibiotics from different structural families were determined for each of the final populations and their parental strains, making a total of 714 MICs (*SI Appendix*, Tables S1–S4). Short-term ALE in the presence of tobramycin or aztreonam resulted in cross-resistance to ceftazidime, ciprofloxacin, and aztreonam, at least in some of the analyzed mutational backgrounds ($P < 0.05$). The tested populations did not present a clear pattern of CS to other drugs, except for the case of fosfomycin (Fig. 1 and *SI Appendix*, Tables S1 and S2). In particular, CS to fosfomycin was observed in replicate populations from PA14 and four out of six mutational backgrounds (*orfN50*, *nfxB177*, *mexZ43*, and MDR12) submitted to short-term ALE in the presence of tobramycin ($P < 0.05$). The possibility of alternating or combining these two antibiotics has been proposed (13, 29, 31, 47, 48) and hence will not be discussed here. Interestingly, no cross-resistance to other drugs was observed in populations submitted to short-term ALE in the presence of ciprofloxacin, while CS to all the analyzed drugs was observed in replicate populations from at least three different mutational backgrounds. It is worth noting the robustness of CS toward two antibiotics (tobramycin and aztreonam), which was detected in replicate populations from PA14 and from six and five out of six mutational backgrounds analyzed, respectively (Fig. 1 and *SI Appendix*, Table S3). In particular, tobramycin MIC was reduced up to 2-fold in *nfxB177*, *mexZ43*, and MDR12; 2.6-fold in PA14; 3-fold in *parR87* and MDR6; and 6-fold in *orfN50* ($P < 0.05$ in all cases except in *mexZ43*). In the case of aztreonam, MIC was reduced up to 2-fold in PA14, *nfxB177*, and MDR6; 3-fold in *parR87* and MDR12; and 6-fold in *orfN50* ($P < 0.05$ in all cases except in MDR12). It is relevant to highlight that the observed CS was not reciprocal; while populations evolved in the presence of

Table 1. Original genetic events of the *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12 mutational backgrounds

Mutational background	Gene	Type of genetic modification	Amino acid change
<i>parR87</i>	<i>parR</i>	SNP	Glu87Lys
<i>orfN50</i>	<i>orfN</i>	Deletion	Val50fs
<i>nfxB177</i>	<i>nfxB</i>	SNP	Phe177Ser
<i>mexZ43</i>	<i>mexZ</i>	SNP	Val43Gly
MDR6	<i>mexC</i>	SNP	Thr267Ala
	<i>pmrB</i>	SNP	Leu87Gln
	<i>frr</i>	SNP	Ile98Ser
MDR12	<i>phoQ</i>	SNP	Val260Gly
	<i>fusA</i>	SNP	Tyr552Cys
	<i>fusA</i>	SNP	Tyr683Cys
	<i>orfN</i>	Deletion	Val50fs
	<i>pmrB</i>	SNP	Met46Ile
	<i>mexZ</i>	SNP	Val43Gly
	<i>gabP</i>	SNP	Ser267Phe
	<i>ptsP</i>	SNP	Leu537Pro
	<i>nuoC</i>	SNP	Gln184*

Resistant or susceptible to

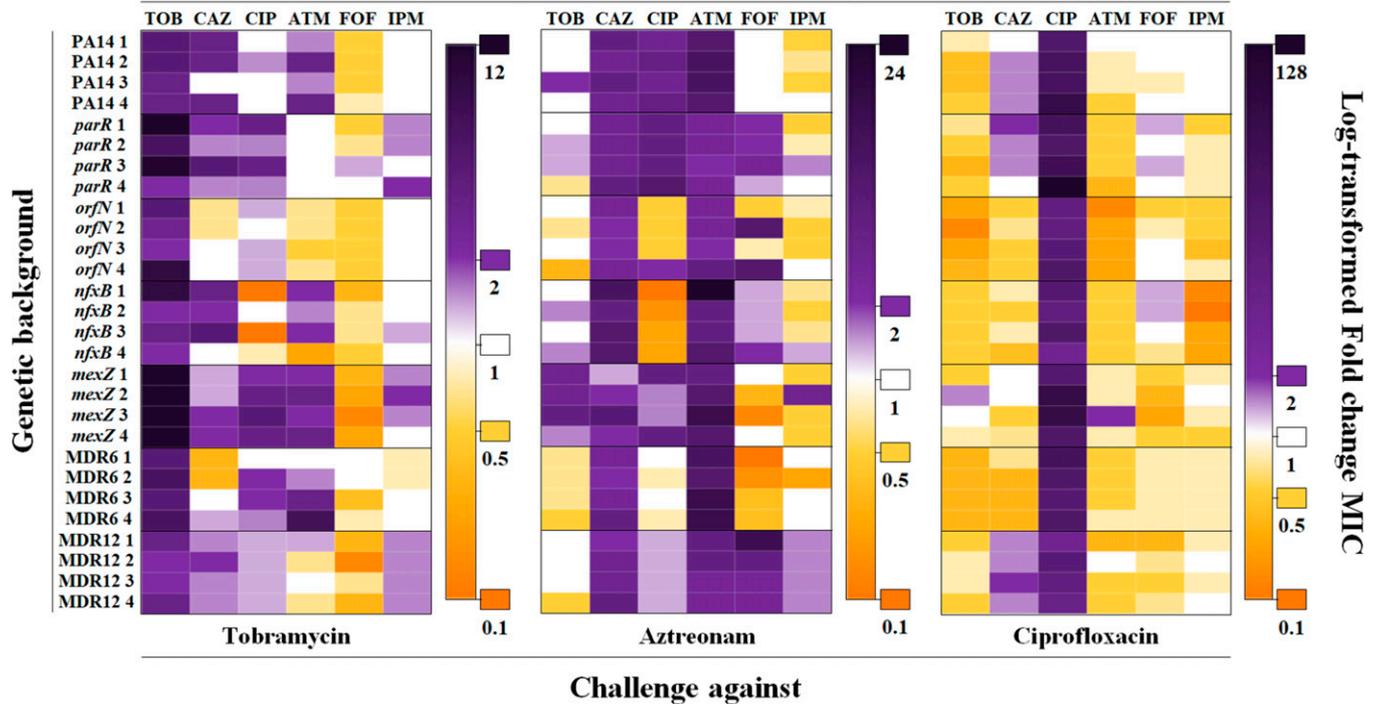


Fig. 1. Diagram showing robustness of CS to tobramycin and aztreonam in PA14 and in different mutational backgrounds submitted to short-term ALE on ciprofloxacin. Cross-resistance and CS to antibiotics from different structural families were analyzed in PA14 and the mutational backgrounds *parR87*, *orfN50*, *nfxB177*, *mexZ43*, *MDR6*, and *MDR12* (four replicate populations for each) submitted to ALE in the presence of tobramycin, aztreonam, or ciprofloxacin for 3 d. Intensity of the color is proportional to the log-transformed FC regarding the MIC of the respective parental strain. Since control populations evolved in the absence of antibiotics may present, on rare occasions, subtle changes (below or above 2- or 0.5-fold, respectively) in their susceptibility to antibiotics with respect to the MIC of parental strains, changes in MICs above or below 2- or 0.5-fold, respectively, were considered physiologically relevant to classify a population as “resistant” (purple) or “susceptible” (orange). MIC values ($\mu\text{g/mL}$) of populations evolved in the presence of tobramycin, aztreonam, or ciprofloxacin are included in *SI Appendix, Tables S1–S3*, respectively. MIC values ($\mu\text{g/mL}$) of control populations evolved in the absence of drugs are included in *SI Appendix, Table S4*. ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; FOF, fosfomycin; IPM, imipenem; TOB, tobramycin.

ciprofloxacin presented a robust CS to aztreonam and tobramycin, populations challenged with either aztreonam or tobramycin did not present an equivalent CS to ciprofloxacin. Further, while ciprofloxacin-resistant populations did not present cross-resistance to other antibiotics, cross-resistance was frequently observed in aztreonam- and tobramycin-resistant populations. This means that the order in which antibiotics are used might be fundamental for implementing evolution-based strategies to deal with infections and AR.

Evolutionary Strategies Based on Robustness of CS to Drive Extinction of Preexisting *P. aeruginosa*-Resistant Mutants.

CS to aminoglycosides has been described in clinical strains of *P. aeruginosa* from CF patients treated with ciprofloxacin (5), and CS to tobramycin and aztreonam has been observed in PAO1 during ALE experiments in the presence of ciprofloxacin (5). Here, we have observed that ciprofloxacin rapidly selects mutants presenting CS to tobramycin and aztreonam in the *P. aeruginosa* PA14 genomic background. Since this strain presents genomic and physiological differences with PAO1 (49, 50), the conservation of the same CS pattern between them supports its robustness among strains presenting different genomic backgrounds. In addition, we found that the same robust CS phenotype was found when different mutants, already presenting AR mutations, were submitted to ciprofloxacin-selective pressure. Altogether, these findings support the robustness of the observed CS in different strains, including former antibiotic-resistant mutants. Therefore, we tested the possibility of alternating ciprofloxacin with these antibiotics. This strategy consisted of two stages: a first step on ciprofloxacin, which would drive evolution

toward CS to tobramycin and aztreonam; and a second step on tobramycin or aztreonam, which would drive extinction of tobramycin–aztreonam-susceptible cells (Fig. 2A). Between treatments, cells were stocked in glycerol. This two-step design of the experiment allowed us to decouple CS (the aim of this work) from potential hysteresis situations (51) that might compromise the interpretation of the results.

We started with the 28 ciprofloxacin-resistant populations (*SI Appendix, Table S3*) belonging to PA14 and to six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, *MDR6*, and *MDR12*), four replicate populations of each, previously submitted to short-term ALE on ciprofloxacin, and 28 control populations (previously unchallenged with ciprofloxacin). As mentioned above, these ciprofloxacin-resistant populations became resistant to ciprofloxacin, being all MICs above the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint ($0.5 \mu\text{g/mL}$) (*SI Appendix, Table S3*), and presented CS to aztreonam and tobramycin, being MICs below the EUCAST clinical breakpoint (16 and $2 \mu\text{g/mL}$, respectively), with the exception of tobramycin ones in *MDR12* (*SI Appendix, Table S3*). At this point, we focused on the switch from ciprofloxacin to tobramycin or aztreonam (Fig. 2A). Even though we had observed conservation of CS to tobramycin and aztreonam after evolution in the presence of ciprofloxacin within the analyzed set of mutational backgrounds (Fig. 1 and *SI Appendix, Table S3*), a critical point would be determining if the proposed strategies could be effective to eliminate cell viability in most of them. Hence, we switched the selective pressure from ciprofloxacin to tobramycin (28 ciprofloxacin-resistant populations and 28 control

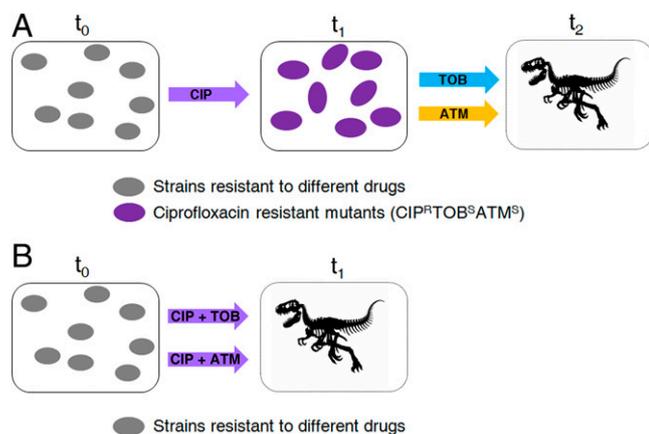


Fig. 2. General model illustrating evolution of antibiotic-resistant mutants of *P. aeruginosa* submitted to the alternation of ciprofloxacin with tobramycin or aztreonam or the combination of ciprofloxacin with tobramycin or aztreonam. (A) Evolution starts when different antibiotic-resistant mutants are treated with ciprofloxacin at time 0 (t_0). Then, there is evolution toward ciprofloxacin resistance and CS to tobramycin and aztreonam (purple cells), rendering ciprofloxacin ineffective (t_1). Subsequently, treatment is switched to tobramycin (TOB) or aztreonam (AZT) that may result in the elimination of cells susceptible to tobramycin and aztreonam (t_2). (B) Evolution starts when different antibiotic-resistant mutants are treated with a ciprofloxacin–tobramycin or a ciprofloxacin–aztreonam combination at time 0 (t_0). Since ciprofloxacin-resistance acquisition leads to CS to tobramycin and aztreonam, it may be expected that drug combinations result in a reduced rate of adaptation or the elimination of cells (t_1).

populations) or aztreonam (28 ciprofloxacin-resistant populations and 28 control populations) (*Materials and Methods*). As shown in Fig. 3A, 11 out of 28 ciprofloxacin-resistant populations submitted to short-term ALE in the presence of aztreonam became extinct, whereas this only occurred in 4 out of 28 ciprofloxacin-resistant populations submitted to short-term ALE on tobramycin. In both cases, the extinction differences were statistically significant ($P < 0.05$). These results suggest that exploiting the aztreonam CS associated with the use of ciprofloxacin by switching selective pressure from ciprofloxacin to aztreonam, although a feasible approach, could be ineffective in driving extinction of the model strain PA14 and of some mutational backgrounds, such as MDR12, at least at the concentrations tested.

It has been suggested that CS may not only improve treatment when drugs are applied sequentially, but it may also serve to optimize combinatory therapy, as it has been described for the combinations ciprofloxacin–aminoglycosides and ciprofloxacin– β -lactams (8). Thus, we decided to analyze the antibiotic-combination efficacy of the ciprofloxacin–tobramycin and ciprofloxacin–aztreonam pairs of drugs in our set of preexisting antibiotic-resistant mutants. We submitted the populations belonging to PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12), four replicate populations of each, to the drug combinations ciprofloxacin–tobramycin (28 populations) or ciprofloxacin–aztreonam (28 populations) at the concentrations used for previous ALE assays in the presence of ciprofloxacin, aztreonam, or tobramycin (Fig. 1 and *SI Appendix, Tables S1–S3*) (*Materials and Methods*). As shown in Fig. 3B, 23 and 25 out of 28 populations submitted to short-term ALE in the presence of either ciprofloxacin–tobramycin or ciprofloxacin–aztreonam became extinct. In both cases, the extinction differences were statistically significant ($P < 0.05$). To further analyze if the high efficiency of these combinatory therapies could be influenced by a synergistic antibiotic interaction between the antibiotics used, we performed 14 checkerboard analyses (*Materials and Methods*) for

PA14 and the six mutational backgrounds here analyzed and the two pairs of drugs tested. We did not observe synergy (neither antagonism) between ciprofloxacin and tobramycin or between ciprofloxacin and aztreonam in any of the genetic backgrounds analyzed (fraction inhibitory concentration [FIC] index ≥ 0.5 and ≤ 4 in all cases) (*SI Appendix, Table S5*). This observation, in addition to the above-mentioned results (Fig. 3B), reinforces that CS to tobramycin and aztreonam associated with the use of ciprofloxacin optimizes the efficiency of the ciprofloxacin–tobramycin and ciprofloxacin–aztreonam pairs of drugs. These results suggest that exploiting the CS associated with the use of ciprofloxacin is a possibility that should be considered, particularly in the case of pre-existing antibiotic-resistant mutants, a feature in agreement with previous data indicating that quinolone-resistant subpopulations isolated from CF patients may be eradicated using aminoglycoside and β -lactam drugs (5).

Genetic Variations Associated with both Ciprofloxacin Resistance and CS in *P. aeruginosa* PA14 and in Different Antibiotic-Resistant Mutants.

To gain insights into the genetic causes of the acquisition of ciprofloxacin resistance and the robust CS pattern observed in the populations submitted to short-term ALE in the presence of ciprofloxacin, the 28 populations independently evolved during 3 d and their parental strains (PA14, *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) were subjected to whole-genome sequencing. Control populations evolved in the absence of drugs were not whole-genome-sequenced since they did not present any relevant change in the MICs of the tested antibiotics. The genome of the 28 ciprofloxacin-resistant populations was compared to the ones of their respective parental strains in order to determine newly genetic events acquired during evolution. A total of 50 genetic events were identified (Table 2): 8 in PA14, 8 in *parR87*, 5 in *orfN50*, 4 in *nfxB177*, 11 in *mexZ43*, 9 in MDR6, and 5 in MDR12. All detected gene variants were located just within five different genes, and their acquisition was dependent on the genetic background (Table 2): *mexS* variants were acquired in PA14, *parR87*, *mexZ43*, and MDR6; *nfxB* variants were acquired in *parR87*, *orfN50*, *mexZ43*, and MDR12; *gyrAB* variants were acquired in *nfxB177* and MDR12; and an *orfN* variant was acquired in *mexZ43*.

Despite *orfN* mutations having been described in ALE assays in the presence of ciprofloxacin (52), only a single replicate of *mexZ43* presented a mutation in this gene. Instead, the most prevalent mutations were found in *gyrAB*, encoding the quinolones' target (36–40), in *mexS*, encoding a regulator of the expression of *mexEF–oprN*, and in *nfxB*, encoding a regulator of the expression of *mexCD–oprJ*, which may lead to overexpression of these efflux pumps (34, 38, 41–43) (Table 2 and Fig. 4). Importantly, *P. aeruginosa* isolates from chronically infected CF patients treated with ciprofloxacin frequently present mutations in these genes (5, 43, 44). In particular, we identified 15 different variants of *mexS*, 5 different variants of *nfxB*, 3 different *gyrA* variants, and a single *gyrB* variant. Notably, variant NfxBHis21Pro was acquired in three out of the four mutational backgrounds presenting mutations in this gene, suggesting an important phenotypic impact, and variants NfxBThr39Pro, GyrAThr83Ile, GyrAAsp87Gly, and GyrBSer466Phe have already been described as clinically relevant (34, 36, 40, 43), further supporting our experimental approach.

Variants in which an isoleucine replaces the threonine at position 83 of GyrA are among the most frequent alterations associated with ciprofloxacin resistance in *P. aeruginosa* occurring in clinical and in vitro-selected resistant mutants (34, 36, 38–40, 53–55). The second most frequent variant in GyrA is

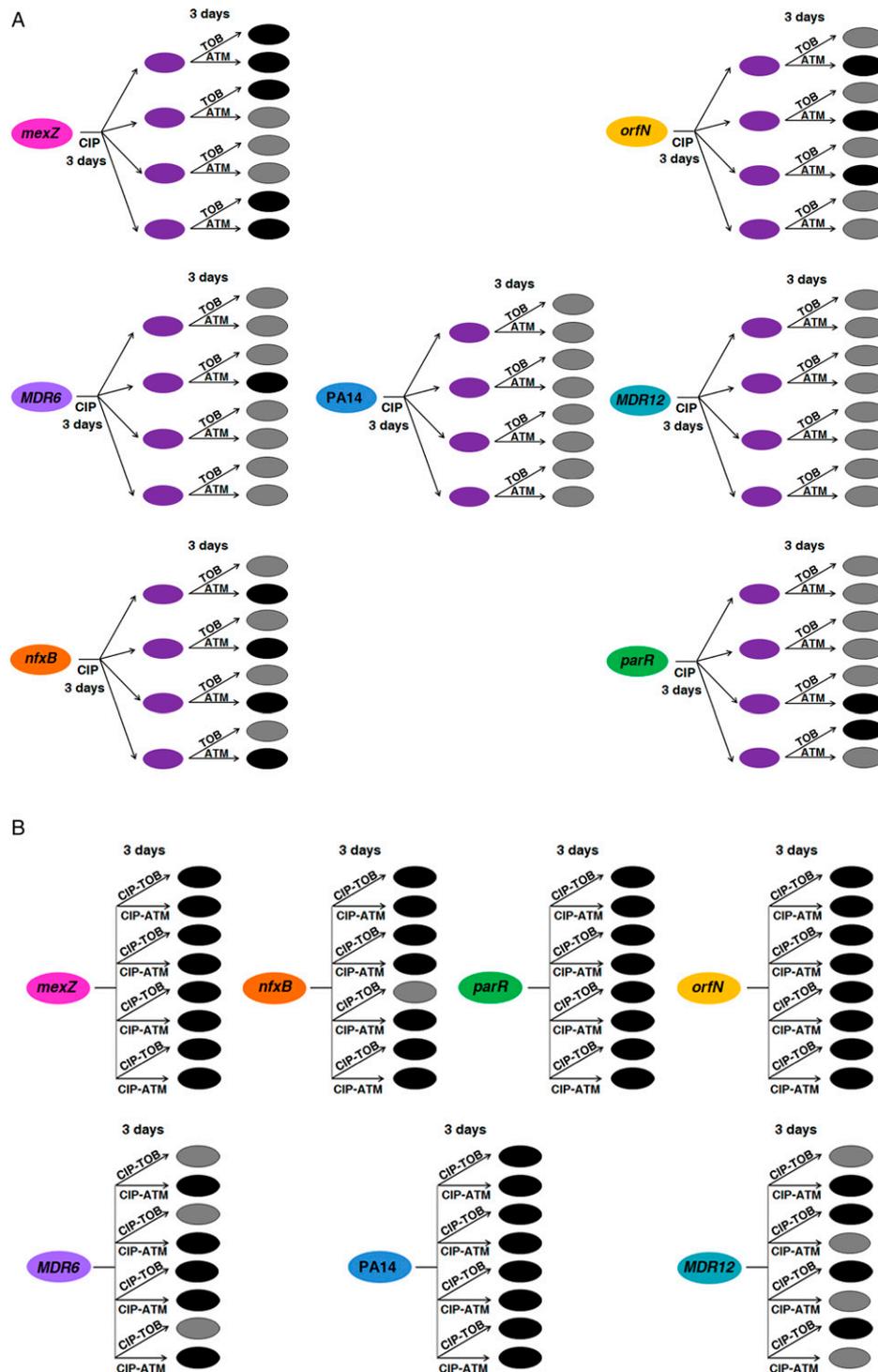


Fig. 3. Diagram showing the efficacy of the alternation of ciprofloxacin with tobramycin or aztreonam and the combination of ciprofloxacin with tobramycin or aztreonam for driving extinction of *P. aeruginosa* PA14 and different antibiotic-resistant mutants. (A) Short-term evolution of PA14 and six mutational backgrounds (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, *MDR6*, or *MDR12*), four replicate populations of each parental strain, was performed during 6 d: 3 d in the presence of ciprofloxacin (CIP) or the absence of antibiotic (control populations), leading to ciprofloxacin-resistant populations (purple cells), and 3 d in the presence of tobramycin (TOB) or aztreonam (AZT). CS to tobramycin and aztreonam was observed in 21 and 19 out of 28 populations after a first step on ciprofloxacin (SI Appendix, Table S3). Populations that were extinct at the end of the experiment are represented in black, while surviving populations are colored in gray. Most of the populations (24 out of 28) submitted to short-term ALE in the presence of tobramycin grew after 3 d. However, short-term ALE in the presence of aztreonam led to extinction of 11 out of 28 populations. This evolutionary strategy was efficient in driving extinction of ciprofloxacin-resistant mutants belonging to *nfxB177*, *parR87*, *mexZ43*, *orfN50*, and *MDR6*, but ineffective in driving extinction of PA14 and *MDR12*. (B) Short-term evolution of PA14 and six mutational backgrounds (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, *MDR6*, or *MDR12*), four replicate populations of each parental strain, was performed during 3 d in the presence of the ciprofloxacin-tobramycin (CIP-TOB) or the ciprofloxacin-aztreonam (CIP-ATM) combination. Growth of the 84 control populations was confirmed in the three drugs independently used at the concentrations present in the drugs combinations. A total of 25 out of 28 populations submitted to short-term ALE in the presence of the ciprofloxacin-aztreonam combination were extinct, while it occurred in 23 out of 28 populations submitted to short-term ALE in the presence of the ciprofloxacin-tobramycin combination. These results indicate that CS may not only improve treatment when drugs are applied sequentially, but it may also serve to optimize combinatory therapy.

Table 2. Newly acquired genetic events after ciprofloxacin ALE by replicates of PA14 and of *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12 mutational backgrounds

Mutational background	Gene	Position	Genetic change	Nucleotide location*	Amino acid change	Replicate	Coverage,% [†]
PA14	<i>mexS</i>	2820352	SNP	379T > G	Tyr127Asp	1, 2, 3, 4	43, 28, 23, 50
	<i>mexS</i>	2820074	SNP	101G > C	Arg34Pro	1, 2, 3	20, 10, 14
	<i>mexS</i>	2820118	SNP	145C > T	Gln49*	3	17
<i>parR87</i>	<i>mexS</i>	2820695	SNP	722T > A	Val241Glu	1, 2	17
	<i>mexS</i>	2820327	Deletion	355delA	Thr119fs	2, 3, 4	86, 10, 23
	<i>mexS</i>	2820695	SNP	722T > G	Val241Gly	2	23
<i>orfN50</i>	<i>nfxB</i>	5428144	SNP	115A > C	Thr39Pro	1, 3	39, 18
	<i>nfxB</i>	5428448	Deletion	421delG	Ala141fs	4	80
	<i>nfxB</i>	5428228	SNP	199C > T	Gln67*	1, 2, 4	16, 37, 87
<i>nfxB177</i>	<i>nfxB</i>	5428148	SNP	119T > G	Leu40Arg	2	49
	<i>nfxB</i>	5428091	SNP	62A > C	His21Pro	3	100
	<i>gyrB</i>	5671	SNP	1397C > T	Ser466Phe	1, 2	99, 95
<i>mexZ43</i>	<i>gyrA</i>	2015001	SNP	248C > T	Thr83Ile	3	84
	<i>gyrA</i>	2015289	SNP	536C > T	Ala179Val	4	93
	<i>mexS</i>	2820463	SNP	490C > T	Gln164*	1	36
	<i>mexS</i>	2820619	SNP	646A > C	Asn216His	2, 3	10, 31
	<i>mexS</i>	2820782	SNP	809T > G	Leu270Arg	2, 4	10, 23
	<i>mexS</i>	2820380	SNP	407G > T	Gly136Val	2, 3, 4	10, 11, 21
	<i>mexS</i>	2820020	SNP	47T > C	Leu16Pro	4	15
MDR6	<i>orfN</i>	2040286	Insertion	148insG	Val50fs	2	79
	<i>nfxB</i>	5428091	SNP	62A > C	His21Pro	2	26
	<i>mexS</i>	2820797	SNP	824G > C	Gly275Ala	1, 2, 3, 4	38, 28, 11, 36
MDR12	<i>mexS</i>	2820239	SNP	266T > C	Phe89Ser	1, 2, 4	30, 29, 28
	<i>mexS</i>	2820529	SNP	556C > T	Leu186Phe	3	22
	<i>mexS</i>	2820758	SNP	785A > T	His262Leu	3	20
MDR12	<i>gyrA</i>	2015013	SNP	260A > G	Asp87Gly	1, 2, 3, 4	18, 100, 100, 100
	<i>nfxB</i>	5428091	SNP	62A > C	His21Pro	1	31

Del, deletion; fs, frameshift; ins, insertion.

*Nucleotide location of the mutations referred to the specific gene in which they are located and their associated amino acid changes.

[†]The coverage indicates the percentage of reads of each mutant allele in the total number of reads, corresponding to the same region of the genome, within each population at the end of the ALE assay.

the replacement of aspartate at position 87 with asparagine, glycine, or tyrosine residues (34, 36, 38–40, 43, 55). It is important to notice that, while new mutations were acquired in *nfxB*, a gene encoding a regulator of *mexCD-oprJ* expression, in *parR87*, *orfN50*, *mexZ43*, and MDR12, target mutations in *gyrAB* were the only ones acquired in *nfxB177*, and, with the exception of MDR12, no mutations were found in genes encoding the quinolone targets in the other mutational backgrounds (Table 2 and Fig. 4). It is remarkable noting that MDR12 originally presented a mutation in *mexZ*, which encodes the negative regulator of the expression of the *mexXY* efflux pump encoding genes (56). Altogether, these results suggest that mutations in regulators of the expression of efflux pumps may precede target mutations in the acquisition of quinolones' resistance, something that has already been reported in clinical strains of patients treated with ciprofloxacin, in which mutations in *nfxB* were generally acquired early in the lineage evolution and were less persistent than mutations in *gyrAB* (57). According to what is found in clinical strains presenting mutations in both target-encoding genes and genes encoding negative regulators of efflux pumps (40, 58), the ciprofloxacin resistance level of the ciprofloxacin-resistant *nfxB177* populations is higher than the ones of ciprofloxacin-resistant populations selected from other mutational backgrounds (SI Appendix, Table S3).

In this work, we describe a conserved CS to tobramycin in the populations submitted to short-term ALE on ciprofloxacin (Fig. 1).

This phenotypic convergence toward CS to aminoglycosides has been described in ciprofloxacin-resistant clinical isolates of *P. aeruginosa* presenting mutations in *gyrA*, *gyrB*, *mexB*, and *nfxB* (5). In agreement with the findings of this work, our results indicate that mutations in *nfxB* are clearly associated with CS to tobramycin. This is easily deduced from the ciprofloxacin-resistant *orfN50* populations, which only acquired mutations in *nfxB* and whose tobramycin MIC was reduced up to sixfold, with respect to the parental strain *orfN50*. To further analyze these genotype–phenotype relationships, individual clones, with each one presenting single mutations in either *nfxB*, *mexS*, *gyrA*, or *gyrB*, were isolated from the ciprofloxacin-resistant populations as described in *Materials and Methods*, and their susceptibility to antibiotics was determined. In particular, a NfxBHis21Pro ciprofloxacin-resistant mutant was isolated from an *orfN50* population (replicate 3). Confirming our hypothesis, the mutant presents a reduction of tobramycin MIC, from 3 to 0.75 µg/mL. Moreover, a ciprofloxacin-resistant mutant containing the single-mutation NfxBAIa141fs was isolated from a *parR87* population (replicate 4), presenting a reduction of tobramycin MIC, from 1.5 to 0.5 µg/mL. These results indicate that *nfxB* mutations cause CS to tobramycin (at least the ones selected in *orfN50* and *parR87*). Further, mutations in *mexS* have been associated with an increased susceptibility to aminoglycosides and β-lactams (59). Taking into consideration those observations, mutations in *mexS* are

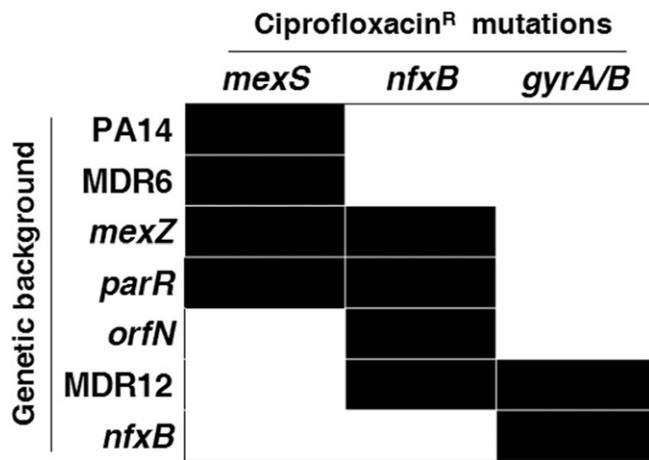


Fig. 4. Diagram showing genetic causes of ciprofloxacin-resistance acquisition and mutational background dependence. Classical mutations regularly found in clinical isolates from patients treated with ciprofloxacin, within *gyrAB*, *nfxB*, and *mexS*, were acquired (black boxes) during ALE in the presence of ciprofloxacin for 3 d in 28 populations belonging to PA14 and 6 different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12). We identified 15, 5, 3, and 1 different variants of *mexS*, *nfxB*, *gyrA*, and *gyrB*, respectively. As observed, early steps of ciprofloxacin-resistance evolution are dependent on the mutational background (Table 2).

likely responsible for the CS to tobramycin observed in PA14 and MDR6. This conclusion is further supported by the fact that the ciprofloxacin-resistant populations from these mutational backgrounds only acquired mutations in *mexS* and showed a 2.6-fold and 3-fold reduction of tobramycin MIC, respectively. To further analyze this possibility, a ciprofloxacin-resistant mutant containing the single-mutation MexSThr119fs was isolated from the *parR87* replicate population 4. The mutant presents a reduction of tobramycin MIC, from 1.5 to 0.75, confirming the role of the *mexS* mutation in tobramycin CS. In the case of ciprofloxacin-resistant populations derived from *nfxB177*, which only acquired mutations in *gyrAB*, their tobramycin MICs were reduced in twofold, with respect to the parental strain, indicating that mutations in *gyrAB* may as well be responsible for tobramycin CS in this mutational background. Two different ciprofloxacin-resistant mutants containing the single-mutations GyrAThr83Ile or GyrBSer466Phe were isolated from two different *nfxB177* populations (replicate 3 and 1, respectively), presenting a reduction of tobramycin MIC from 1 to 0.5. This denotes that phenotypic convergence toward CS to aminoglycosides in clinical isolates from patients treated with ciprofloxacin may be associated with the selection of mutations in different genes that lead to an increased efflux of antibiotics or to alterations in drug targets, although the strength of SC to aminoglycosides associated with *nfxB* mutations is considerably higher than that of mutations in the other genes. Besides tobramycin CS, we observed that CS to aztreonam is also associated with the acquisition of resistance to ciprofloxacin in the different mutational backgrounds tested. In this regard, the contribution of *mexS*, *nfxB*, *gyrA*, or *gyrB* mutations in CS to aztreonam is observed in the mutants MexSThr119fs, NfxBA141fs, NfxBHis21Pro, GyrAThr83Ile, and GyrBSer466Phe isolated from ciprofloxacin-resistant populations derived from *parR87*, *orfN50*, and *nfxB177*. The NfxBHis21Pro ciprofloxacin-resistant mutant isolated from the *orfN50* population presented a reduction of aztreonam MIC from 6 to 1.5 $\mu\text{g}/\text{mL}$, and the NfxBA141fs ciprofloxacin-resistant mutant isolated from the *parR87* population presented a reduction of aztreonam MIC from 3 to 0.5 $\mu\text{g}/\text{mL}$, indicating

that *nfxB* mutations cause CS to aztreonam (at least the ones of *orfN50* and *parR87*). The ciprofloxacin-resistant mutant isolated from the *parR87* population containing MexSThr119fs as a single mutation presented a reduction of aztreonam MIC from 3 to 1 $\mu\text{g}/\text{mL}$, indicating that in this mutational background—at least—this mutation leads to CS to this antibiotic. Finally, the GyrAThr83Ile and GyrBSer466Phe ciprofloxacin-resistant mutants isolated from two different *nfxB177* populations presented a reduction of aztreonam MIC from 2 to 1 $\mu\text{g}/\text{mL}$, indicating that *gyrA* and *gyrB* mutations cause CS to aztreonam (at least the ones selected in *nfxB177*), but that the strength of this phenotype is considerably lower than the ones associated with *mexS* and *nfxB* mutations.

It is worth emphasizing that the mutations that have been selected during our ALE experiments in the presence of ciprofloxacin are the most frequently found in patients infected by *P. aeruginosa* and treated with ciprofloxacin (34, 36, 38–40, 53–55, 57) and that mutations in *nfxB*, the ones that present a higher CS to tobramycin and aztreonam, are generally early acquired during ciprofloxacin treatment (57), supporting the potential impact of the results reported in the present work, regarding robustness of CS in clinical settings.

Discussion

Phenotypic convergence is a common process in evolution that may be the consequence of parallel evolution: The same genetic events are selected when organisms confront the same selective force (60). In this regard, we have recently described that parallel evolution underlies the acquisition of a robust pattern of CS to certain antibiotics, associated with the short-term ALE in the presence of ceftazidime in different mutational backgrounds of *P. aeruginosa* (29). However, besides being the result of parallel evolution, phenotypic convergence can also emerge, even when different genetic events, giving up to the same phenotype, are selected during evolution (61). In this case, it is usually argued that the emergent, conserved phenotype is selected because it is adaptive to the selective pressure, despite different genetic solutions being taken to deal with the challenge. However, it has been discussed that, on occasion, the observed convergent phenotypes do not provide direct adaptation to the selective force (62), and CS, a phenotype that is maladaptive to the presence of antibiotics, is an example of this situation. We have identified a robust, convergent CS pattern in different antibiotic-resistant mutants, associated with the use of ciprofloxacin, which is not caused by parallel evolution. In particular, we observed the selection of mutations in different genes driven by the same selective force (ciprofloxacin) in different mutational backgrounds, leading to a functionally equivalent change in susceptibility to second drugs (tobramycin and aztreonam). The current study describes a robust pattern of CS associated with the use of a specific drug that is not caused by parallel evolution in different genetic backgrounds, including preexisting antibiotic-resistant mutants.

Parallel evolution leading to the emergence of a conserved maladaptive phenotype was proposed by Haldane (63), in order to explain the high prevalence of some inherited diseases. The hypothesis, which was later experimentally validated (64), is that the selective force behind the unexpectedly high prevalence of those inherited diseases was infection; the selected mutations protect from infection, and the inherited disease was just the associated, maladaptive, trade-off. However, when parallel evolution is not the cause of convergent evolution, phenotypic convergence is usually explained as a mechanism of adaptation

to the selective force (26, 27); a maladaptive, convergent phenotype is not expected to be selected. Differing from this situation, CS is maladaptive to antibiotic challenge; it is an evolutionary trade-off, whose emergence is hardly inferred, even when the selective force underlying evolution is known. Our results hence provide an example of phenotypic convergence (CS) unlinked to parallel evolution and not providing direct adaptation to the antibiotic-selective force. Besides the relevance concerning AR, our findings have consequences for understanding processes of phenotypic convergence without a clear link between selection and adaptation (62).

As stated above, the exploitation of the information concerning CS requires this phenotype to be conserved among different strains; otherwise, the phenotype will be unpredictable and with no use in clinics. Despite efforts in the field, few cases of robust CS have been reported, and, with the exception of one recent study from our laboratory (29), none of them has been performed using well-defined antibiotic-resistant mutants as a starting point. We believe that this aspect is relevant because infections by antibiotic-resistant organisms are common, and they need to be treated with another different drug, which can lead to the selection of additional resistance phenotypes. Knowing if the novel resistance concurs with a robust CS phenotype, shared in different resistant mutants, is needed to implement evolution-based strategies to fight multidrug resistance. Herein, we found a robust CS phenotype to aztreonam and tobramycin, associated with the acquisition of ciprofloxacin resistance by different *P. aeruginosa* mutants, formerly resistant to antibiotics, which constitutes an important step forward in this endeavor.

The exploitation of in vitro-generated knowledge regarding AR mutations requires that these mutations are also present in clinical isolates. Notably, mutations in the same genes reported in the present article to be involved in ciprofloxacin resistance, such as mutations in target-encoding genes *gyrAB* or in efflux pumps' regulators encoding genes *mexS* and *nfxB*, have been ascertained in *P. aeruginosa* clinical isolates from patients treated with ciprofloxacin (5, 43, 44). Further, variants of clinical relevance, such as NfxBThr39Pro, GyrAThr83Ile, GyrAAsp87Gly, or GyrBSer466-Phe (34, 36, 40, 43), were acquired in the populations submitted to short-term ALE in the presence of ciprofloxacin during this study, indicating that our results are not only close to reality, in terms of clinical relevance, but that these clinically relevant mutations might be associated with CS to tobramycin and aztreonam in clinical isolates, a feature that has not been addressed yet. This led us to consider the alternation of ciprofloxacin with tobramycin or aztreonam and the use of a ciprofloxacin–tobramycin or a ciprofloxacin–aztreonam combination. We observed that 25 out of 28 populations (belonging to PA14 and 6 different preexisting antibiotic-resistant mutants) challenged with the ciprofloxacin–aztreonam combination and 23 out of 28 populations challenged with the ciprofloxacin–tobramycin combination were extinct after 3 d of short-term ALE. This is in agreement with a previous work performed with the PA14 model strain of *P. aeruginosa* that pointed out the efficacy of the combination of drug pairs containing ciprofloxacin and aminoglycosides or β -lactams (8). In addition, we observed that 11 out of 28 populations were also extinct after the alternation of ciprofloxacin with aztreonam. Therefore, our results support that it might be possible to exploit CS associated with ciprofloxacin-resistance acquisition to design evolution-based approaches to tackle *P. aeruginosa* infections containing preexisting antibiotic-resistant mutants. In this regard, it is worth mentioning that blind—not evolutionary-based—clinical trials of antibiotic combinations for fighting *P. aeruginosa* infections are

regularly performed (65), while rational evolutionary-based information, as the results presented here, supporting these trials are generally absent. However, we are aware that testing the proposed treatments in different infection models, as well as confirming the robustness of CS in clinical strains of *P. aeruginosa*, presenting different genomic backgrounds, not just different mutational backgrounds, would also be needed for the translation of the results into clinical practice. Besides, the fact that the acquisition of quinolone resistance, when due to overexpression of MDR efflux pumps, can be associated with cross-resistance to other drugs (66) and that, on occasion, growing in biofilms may hide the CS phenotype (67) should also be taken into consideration.

We conclude that robust CS patterns may result not just from parallel evolution, as we recently described (29), but also upon the selection of different genetic mechanisms, as we describe here. We therefore propose that the search for robust CS patterns in different genetic backgrounds (including antibiotic-resistant isolates) of a species could pave the way for the design of new evolutionary strategies to promote the extinction of bacteria causing infections. The finding of these robust CS networks is particularly relevant in the case of already-resistant mutants challenged with another antibiotic, a situation rarely explored until now, which is explored here and that is rather common and highly relevant in clinical settings.

Materials and Methods

Growth Conditions and Antibiotic-Susceptibility Assays. Bacteria were grown in glass tubes in Luria-Bertani (LB) broth at 37 °C with shaking at 250 rpm. MICs of ceftazidime, aztreonam, imipenem, tobramycin, ciprofloxacin, and fosfomycin were determined at 37 °C in Mueller Hinton (MH) agar using E-test strips (MIC Test Strip, Liofilchem).

Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam. Four single mutants, two multiple mutants, and PA14 were subjected to short-term ALE—four replicates of each—in the presence of ciprofloxacin, tobramycin, or aztreonam or the absence of antibiotic (control populations), resulting in a total of 112 independent bacterial populations. Cultures were grown at 37 °C and 250 rpm for 3 d in independent glass tubes to avoid cross-contamination. Every day, the cultures were diluted (1/100), adding 10 μ L of bacteria in 1 mL of fresh LB containing the concentration of antibiotic (close to MIC) that hinders the growth of *P. aeruginosa* PA14 and each mutational background under these culture conditions (ciprofloxacin: 0.1 μ g/mL for MDR6; 0.2 μ g/mL for MDR12; 0.3 μ g/mL for PA14, *orfN50*, *nfxB177*, and *mexZ43*; and 0.4 μ g/mL for *parR87*; tobramycin: 0.75 μ g/mL for PA14 and *nfxB177*; 1.5 μ g/mL for MDR6 and *mexZ43*; 2 μ g/mL for *orfN50*; 2.5 μ g/mL for *parR87*; and 12 μ g/mL for MDR12; aztreonam: 1 μ g/mL for MDR12; 4 μ g/mL for PA14, *nfxB177*, and MDR6; 6 μ g/mL for *parR87*; 7 μ g/mL for *orfN50*; and 8 μ g/mL for *mexZ43*) or without antibiotics (control populations). During the 3 d, the concentration of ciprofloxacin, tobramycin, or aztreonam was maintained. Every replicate population was preserved at -80 °C at the end of the experimental evolution. In addition, the MIC of the antibiotic used for selection and of antibiotics from other structural families was determined at 37 °C in MH agar using E-test strips.

Alternation of Ciprofloxacin with Tobramycin or Aztreonam Using Short-Term ALE Experiments. Short-term ALE experiments in the presence of tobramycin or aztreonam were performed for 3 d at 37 °C and 250 rpm using the 28 populations previously challenged with ciprofloxacin for 3 d and belonging to PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12). The 28 ciprofloxacin-resistant populations and the 28 control populations (not challenged with ciprofloxacin) were grown from glycerol stocks, and every day, during 3 d, the cultures were diluted (1/100) in fresh LB containing tobramycin or aztreonam at the concentration that hinders—but allows—the growth of each *P. aeruginosa* mutational background under these culture conditions, which are described in *Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam*. Extinction of the populations

was determined by measuring the absorbance OD_{600nm} of 100 µL of bacterial cultures the last day of ALE in a 96-well microtiter plate (NUNC) in a Tecan Infinite 200 plate reader and by plating out final cultures on LB to look for viable cells.

Combination of Ciprofloxacin with Tobramycin or Aztreonam Using Short-Term ALE Experiments. Four replicate populations from PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) were grown from glycerol stocks. Every day, during 3 d, the cultures were diluted (1/100) in fresh LB medium containing a combination of ciprofloxacin-tobramycin (28 populations), ciprofloxacin-aztreonam (28 populations), or each single drug (84 control populations). Each antibiotic was added at the concentration that hinders—but allows—the growth of each *P. aeruginosa* mutational background under these culture conditions, which are described in *Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam*. Extinction of the populations was determined by measuring the absorbance OD_{600nm} of 100 µL of bacterial cultures the last day of ALE in a 96-well microtiter plate (NUNC) in a Tecan Infinite 200 plate reader and by plating out final cultures on LB to look for viable cells.

Whole-Genome Sequencing and Analysis of Genetic Changes. The genomic DNA of each ciprofloxacin-resistant population and parental strain was extracted with the Gnome DNA kit (MP Biomedicals). The assay of DNA quality, libraries' construction, and whole-genome sequencing was performed by Macrogen. Pair-end libraries (2 × 150) were constructed with Truseq DNA PCR-free and sequenced by using an Illumina NovaSeq6000 system. Coverage was greater than 300x for all samples. Genome sequence, gene coordinates, and annotations were obtained from the nucleotide database GenBank. The quality of Illumina short reads was verified by using FASTQC (68). RNA-STAR was used to align reads against *P. aeruginosa* genome UCBPP-PA14 (NC_008463.1) (69). Optical and PCR duplicates were detected by using the MarkDuplicates (Picard) function of The Genome Analysis Toolkit (70). SAMtools was used to index alignment files in BAM format (71). Single-nucleotide polymorphism (SNPs) and small insertions and deletions (INDELS) were detected by using freebayes (72). The impact of SNPs and INDELS was evaluated by using SnpEff (73), and annotated results were saved in the VCF format. Genetic variants were detected by using SnpEff viewer (74) and the IGV browser (75).

Isolation of Ciprofloxacin-Resistant Mutants. Clones presenting either of the single mutations NfxBA141fs, NfxBHis21Pro, MexSThr119fs, GyrAThr83Ile, or GyrBSer466Phe were isolated from the ciprofloxacin-resistant populations *parR87.4*, *orfN50.3*, *parR87.4*, *nfxB177.3*, or *nfxB177.1*, respectively. The presence or absence of mutations in the quinolone-resistance-determining regions of *gyrA* and *gyrB* in *mexS* or in *nfxB* were searched by PCR in each individual clone by using the oligonucleotides described in *SI Appendix, Table S5* and Sanger sequencing. Four pairs of primers, which amplify 378 bp of *gyrA*, 511 bp of *gyrB*, 627 bp of *nfxB*, and 1,076 bp of *mexS*, were used (*SI Appendix, Table S6*). After PCR, the amplicons were purified by using the QIAquick PCR purification kit (QIAGEN) and Sanger sequenced at Macrogen.

Synergy Assessed by Checkerboard Analysis. Standard checkerboard broth microdilution assays were performed in PA14 and in six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) using 10 serially diluted concentrations of ciprofloxacin, seven of aztreonam or

tobramycin, and a no-drug control, a total of 14 96-U-well plates. First, 90 µL of MH medium with ciprofloxacin (0.025 to 12.8 µg/mL), aztreonam (1 to 64 µg/mL), or tobramycin (0.125 to 8 µg/mL) was added to each well of two different 96-U-well plates for the analysis of each mutational background, with the exception of MDR12, for which tobramycin concentrations ranged between 4 and 256 µg/mL. Ten microliters of cells were inoculated into each well to a final OD₆₀₀ of 0.01. Bacteria were grown at 37 °C for 48 h without shaking. The FIC of ciprofloxacin, aztreonam, and tobramycin was calculated as the MIC of the combination of ciprofloxacin with each of the other drugs divided by the MIC of each of the drugs alone. The FIC index was calculated as the addition of the FICs of both drugs. An FIC index value of <0.5 was considered to indicate synergy, and an FIC index of >4 was considered to indicate antagonism (76).

Statistical Analysis. Bidirectional nonparametric tests on the raw data and parametric bidirectional and unidirectional tests on log₂-transformed data were performed.

Data were first subjected to Shapiro-Wilk and Levene's tests to assert normality and homocedasticity. Subsequently, each combination of strain and treatment was compared against its respective control. Nonparametric Kruskal-Wallis, Dwass-Steel-Critchlow-Fligner, and Mann-Whitney *U* tests were applied to the untransformed data to assert significance of the difference. Additionally, FC was calculated, and the significance of the FCs observed was asserted by using ANOVA and bidirectional *t* tests on log₂-transformed data (log₂ FC). The significance of the variation in the number of extinct populations in alternated and combined evolution experiments was estimated by using a likelihood-ratio test. All statistical analyses were performed by using the R package (<https://www.R-project.org/>).

Data Availability. All study data are included in the article and/or supporting information.

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