Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants

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The analysis of trade-offs, as collateral sensitivity, associated with the acquisition of antibiotic resistance, is mainly based on the use of model strains. However, the possibility of exploiting these trade-offs for fighting already resistant isolates has not been addressed in depth, despite the fact that bacterial pathogens are frequently antibiotic-resistant, forming either homogeneous or heterogeneous populations. Using a set of *Pseudomonas aeruginosa*-resistant mutants, we found that ceftazidime selects pyomelanogenic tobramycin-hypersusceptible mutants presenting chromosomal deletions in the analyzed genetic backgrounds. Since pyomelanogenic resistant mutants frequently coexist with other morphotypes in patients with cystic fibrosis, we analyzed the exploitation of this trade-off to drive extinction of heterogeneous resistant populations by using tobramycin/ceftazidime alternation. Our work shows that this approach is feasible because phenotypic trade-offs associated with the use of ceftazidime are robust. The identification of conserved collateral sensitivity networks may guide the rational design of evolution-based antibiotic therapies in *P. aeruginosa* infections.

**INTRODUCTION**

Antibiotic effectiveness, currently compromised by the spread of antibiotic resistance (AR), requires not only innovation but also conservation, which may allow for improved use of current antibiotics (1). For this conservation, understanding of the trade-offs associated with AR acquisition—such as increased susceptibility to a second drug after use of the first, a phenomenon first described in the 1950s as collateral sensitivity (2)—might be particularly relevant. Various studies have been undertaken trying to exploit the evolutionary constraint imposed by collateral sensitivity patterns (3, 4), such as combinatory therapy (5, 6) or alternating collaterally susceptible drug pairs (7–9).

Despite progress in study of the collateral sensitivity phenomenon, some questions remain to be answered. In particular, there is still only limited information on the evolutionary conservation of collateral sensitivity patterns, not only between different species (10, 11) but also within different members from the same species (12–15). In the case of *Pseudomonas aeruginosa*, some studies have revealed that isolates of this pathogen obtained from patients with chronic infections and treated with distinct antibiotic classes present convergence in their collateral sensitivity phenotypes (9), while others have described major differences in the collateral sensitivity patterns associated with the acquisition of resistance to one antibiotic between replicates of the same strain of *P. aeruginosa* evolving in parallel (13). These discrepancies may lie in the degree of reproducibility of the evolutionary pathways leading to AR. In this respect, it is known that the type of resistance mutations present in a given genetic background may be restricted because of epistatic interactions (10, 16–21) and that the cumulative acquisition of AR mutations in different loci reduces the variety of pathways, leading to AR (22, 23). Since contingency may be relevant not only for AR evolution but also for the acquired collateral sensitivity phenotype (24), knowing the degree of conservation of collateral sensitivity patterns associated with the use of a specific drug in different genetic backgrounds, particularly in the case of mutants already presenting a phenotype of AR, is of special interest.

In a previous study, we observed that *P. aeruginosa* PA14 populations experimentally evolved in the presence of ceftazidime displayed a robust collateral sensitivity to amikacin (25), as a consequence of selection of large chromosomal deletions upon 1 day of adaptive laboratory evolution (ALE) (25). The chromosomal deletions included *hmgA*, which encodes an enzyme whose lack of activity leads to the hyperproduction of the brown pigment pyomelanin (26); *galU*, whose inactivation reduces ceftazidime susceptibility (27); and *mexXY*, which encodes a multidrug resistance (MDR) efflux pump that contributes to *P. aeruginosa* intrinsic aminoglycosides resistance (28). Up to 13% of patients with cystic fibrosis (CF) are infected by *P. aeruginosa* pyomelanin-producing mutants (29) that, in agreement with the phenotype of the mutants selected after ALEs, are also hypersusceptible to aminoglycosides (30). Although the fact that pyomelanin increases resistance to oxidative stress and favors bacterial persistence in chronic lung infections (26) has been considered the most likely explanation for in vivo selection of these mutants, this genetic event has also been reported in different strains of *P. aeruginosa* subjected to ALE in the presence of other β-lactams (31–34). Therefore, it remains to be established whether these deletions are selected by the antibiotic treatment or merely represent an adaptation to the lung environment of patients with CF (35), as was previously proposed (34, 35). Even further, it remains to be answered whether these deletions are selected upon ceftazidime treatment in different genetic backgrounds of *P. aeruginosa*, in particular, in mutants resistant to other antibiotics; or on the contrary, whether the evolutionary robustness of this genetic event, and of its phenotypic effects, is limited. In this study, by constructing a set of resistant mutants previously identified in different ALE experiments of *P. aeruginosa* PA14 (16, 36) in the presence of antibiotics, and by submitting them, as well as the wild-type PA14 strain, to ALE in the presence of ceftazidime, we have determined the robustness of an early event of ceftazidime resistance evolution that is associated with collateral sensitivity to tobramycin. Further, by the recreation of heterogeneous pyomelanogenic populations belonging to each genetic background and the alternation of tobramycin with ceftazidime, we found that...
driving evolution toward hypersusceptibility to the first drug is generally feasible, at least in the genetic backgrounds analyzed. This finding supports the possibility of rationally designing treatments based on collateral sensitivity convergence in *P. aeruginosa*.

**RESULTS**

As mentioned above, the conservation of collateral sensitivity to a given drug within different genetic backgrounds of a species may be contingent on the degree of conservation of the evolutionary routes toward resistance to the first drug used for selection. We and others have reported the early selection of large chromosomal deletions that contain genes encoding the intrinsic aminoglycosides resistance efflux pump MexXY (28), when ALE experiments in the presence of different β-lactams, including ceftazidime (31), piperacillin (32), or meropenem (33, 34), were performed. These data suggest that this genetic event could be one of the first evolutionary steps in the evolution of *P. aeruginosa* toward β-lactam resistance. However, since these studies were limited to a single wild-type genetic background, it remained to be analyzed whether a similar evolutionary pattern could be followed by *P. aeruginosa* PA14 strains presenting different genetic backgrounds, in particular, different antibiotic-resistant mutants. That being so, collateral sensitivity to aminoglycosides, such as tobramycin, a drug that forms part of usual therapy regimens against *P. aeruginosa* (37), would also be conserved.

**Construction and characterization of *P. aeruginosa* mutants**

Our first objective was to analyze the evolutionary conservation of ceftazidime resistance evolution in different genetic backgrounds, consisting of mutants derived from different *P. aeruginosa* PA14 ALES in the presence of antibiotics (see table S1). It has been recently suggested that AR mutations may associate with either robust or variable collateral sensitivity patterns in different genetic backgrounds, depending on whether they lead to “target” or “regulatory alterations,” respectively (10). Taking this hypothesis into account and resorting to previous different in-house ALE experiments (16, 36), we constructed a broad spectrum of strains containing single mutations that affect regulatory proteins (NfxB, ParR, or MexZ), nonregulatory proteins (NuoD or OrfN), or simultaneously containing both types of mutations. The mutant containing mutations in *nfxB, phoQ, frr*, and *pmrB* was dubbed MDR6, and the mutant containing mutations in *fusA, orfN, pmrB, mexZ, gabP, ptsP*, and *nudO* was dubbed MDR12 (see table S1 for detailed information). The susceptibility of each mutant to different antibiotics, including those of interest in this study—tobramycin and ceftazidime—is shown in Table 1.

**Evolutionary robustness of first steps of *P. aeruginosa* ceftazidime resistance evolution and collateral sensitivity to tobramycin**

To determine whether chromosomal deletions containing *mexXY* would be early selected during *P. aeruginosa* evolution in presence of ceftazidime in the set of mutants mentioned above, as it was the case in the wild-type strain PA14 (25), four biological replicates of each single (*nfxB177, parR87, mexZ43, orfN50, and nudO184*) and multiple mutants (MDR6 and MDR12), and the wild-type PA14 strain, were subjected to ALE in presence or absence of ceftazidime (a total of 64 populations) for 3 days. Upon 1 day of experimental evolution, almost every *P. aeruginosa* population challenged with antibiotic hyperproduced pyomelanin (27 of 32 populations; Fig. 1A).

This result is consistent with the presence of deletions that include *hmgA*, as those described in our previous study, because pyomelanin accumulation is due to the lack of homogentisate 1,2-dioxgenase activity provided by HmgA (26). Since we had previously determined a cause-effect relationship between the presence of chromosomal deletions containing *hmgA* and *mexXY* and the hyperproduction of pyomelanin and hypersusceptibility to aminoglycosides, respectively (25), the susceptibility of each final population to tobramycin was analyzed. All the pyomelanogenic populations obtained after the short-term evolution in presence of ceftazidime were resistant to ceftazidime and hypersusceptible to tobramycin, when compared to the parental strain from which they evolved (Fig. 1B and table S2), even when the mutants were originally less susceptible to tobramycin than the wild-type *P. aeruginosa* PA14 strain. In particular, tobramycin minimal inhibitory concentration (MIC) was reduced by up to 4-fold in PA14, 2.6-fold in *nfxB177*, 3-fold in *parR87*, 7.9-fold in *orfN50*, 6-fold in *mexZ43*, 5.3-fold in MDR6, and 10.7-fold in MDR12. Consistent with the linkage between pyomelanin production and deletion of a chromosomal region containing *mexXY*, nonpyomelanogenic populations (*parR87*, replicate 1; and *nudO184*, all replicates) were not tobramycin hypersusceptible (Fig. 1B and table S2). Further analysis of the results from the experimental evolution study has revealed that six of eight genetic backgrounds presented a significantly (*P < 0.01 in all cases) reduced MIC to tobramycin, compared to their parental strains, after the ceftazidime short-term evolution. To further analyze whether chromosomal deletions containing *mexXY* could be associated with the phenotype of hypersusceptibility, every evolved population, as well as their original parental strain, was genotyped (fig. S1A). A 163-bp polymerase chain reaction (PCR) fragment corresponding to *mexXY* was detected in every parental strain, as well as in nonpyomelanogenic populations (*parR87*, replicate 1; and *nudO184*, all replicates) and in a pyomelanogenic mixed population (PA14, replicate 2). Consistent with the observed increase in tobramycin susceptibility of most evolved populations (table S2), every pyomelanogenic tobramycin-hypersusceptible population lacked *mexXY* (fig. S1A). Overall, these results suggest that chromosomal deletions containing *mexXY* are consistently selected at first steps.

Table 1. MICs (µg/ml) of different antibiotics for the single and multiple *P. aeruginosa* PA14 mutants used in this work. MICs ≥2-fold of the MICs for the wild-type PA14 strain are highlighted in bold. TOB, tobramycin; TGC, tigecycline; CAZ, ceftazidime; CIP, ciprofloxacin; IPM, imipenem.

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*MDR6 mutant presents mutations in *nfxB, phoQ, frr*, and *pmrB*.
†MDR12 mutant presents mutations in *fusA, orfN, pmrB, mexZ, gabP, ptsP*, and *nudO*.
Fig. 1. Analysis of early steps in the evolution of *P. aeruginosa* wild-type strain and antibiotic-resistant mutants in the presence of CAZ. (A) Scheme of the resultant phenotype after the evolution of PA14, single (*nfxB*177, *parR*87, *mexZ*43, *orfN*50, and *nuoD*184) and multiple (MDR6 and MDR12) mutants, in the presence of CAZ (left part of (A)) or in the absence of antibiotic (LB; right part of (A)) for 3 days (see Materials and Methods). Pyomelanin hyperproduction was observed in 27 of 32 populations evolved in the presence of CAZ (red-colored cells). (B) Diagram showing convergence toward hypersusceptibility to TOB in the different genetic backgrounds and replicates, analyzed after short-term evolution on CAZ. In all cases, acquisition of a pyomelanogenic phenotype is associated with collateral sensitivity to TOB, irrespective of the genetic background of the evolving strain. MIC values of TOB and CAZ are included in Table S2. (C) Isolation of pyomelanogenic clones from each 27 pyomelanogenic population (left part of (A); red-colored cells) obtained in the presence of CAZ. As shown in the figure, the early steps of evolution in presence of CAZ of *P. aeruginosa*, which lead to collateral sensitivity to TOB and pyomelanin production, are conserved among the different antibiotic-resistant mutants analyzed. (D) Pyomelanogenic phenotype of *mexZ*43 mutant after 1-day evolution in the presence of CAZ or in the absence of antibiotic (LB). Pyomelanin hyperproduction was observed in 28 of 32 populations evolved on CAZ. Photo credits for (C) and (D): Inés Poveda, Centro Nacional de Biotecnología. Permission for using these images is not required.
of ceftazidime resistance evolution in P. aeruginosa, at least in the genetic backgrounds analyzed. To further test whether this observation could be conditioned by the number of replicates used during the assay for each genetic background, 32 replicates of one of the mutants (mexZ43) were subjected to evolution under the same conditions used before, in presence or absence of ceftazidime. We observed that upon 1 day of experimental evolution, a high number of mexZ43 populations challenged with antibiotic hyperproduced pyomelanin (28 of 32 populations; Fig. 1D), just 4 of 32 populations did not become pyomelanogenic (Fig. 1D). Although we are aware that there is a space for unpredictability of ceftazidime resistance evolution in the mutants analyzed, the results here described suggest that alternative genetic evolutionary trajectories in ceftazidime resistance evolution may be limited, and that phenotypic convergence toward collateral sensitivity to tobramycin is robust in P. aeruginosa, even in the case of antibiotic-resistant mutants, as those here analyzed (table S1), after ceftazidime treatment (table S2). However, we are fully aware that all mutants described here derive from P. aeruginosa PA14, and the generalization of our results to other strains will require the analysis of the effect of short-term ceftazidime evolution on a broad and diverse set of clinical strains of P. aeruginosa.

Evolution of heterogeneous pyomelanogenic populations of P. aeruginosa subjected to tobramycin/ceftazidime sequential evolution

We have shown that ceftazidime selects, in the short term, pyomelanogenic mutants presenting collateral sensitivity to tobramycin in P. aeruginosa, at least in the set of resistant mutants analyzed here (table S1). Nevertheless, there is an extensive heterogeneity within populations of P. aeruginosa in the CF lungs (38), frequently including mutants already presenting a pyomelanogenic phenotype (29, 30), which is usually associated with a reduced susceptibility to ceftazidime. Since this heterogeneity might impair the chances of exploiting the observed tobramycin collateral sensitivity associated with the use of ceftazidime in these heterogeneous populations, we tested the possibility of alternating the antibiotics, first, by using tobramycin and then second, ceftazidime, for reducing the chances of pyomelanogenic heterogeneous populations to escape from the antibiotic challenge.

The strategy would consist of three stages: A first step on tobramycin may force extinction of the ceftazidime-resistant (tobramycin-hypersusceptible) part of the populations, a second step on ceftazidime may drive evolution toward tobramycin hypersusceptibility, and then the extinction of the tobramycin-hypersusceptible cells after switching back to tobramycin (Fig. 2) would be expected. Although this strategy would be also potentially applicable to populations not resistant to ceftazidime, being reduced to a first step on ceftazidime followed by a second one on tobramycin, we decided to analyze its effectiveness in a more complex situation of clinical relevance, as it is the case of heterogeneous pyomelanogenic populations. In particular, diverse populations derived from the set of mutants were analyzed in this work (see table S1). To that end, we isolated a pyomelanogenic clone from each of the 27 pyomelanogenic populations obtained after ceftazidime short-term evolution (Fig. 1A). To note here that although the original clones from which these populations were derived belonged to a diverse set of genetic backgrounds, consisting of antibiotic-resistant mutants that contain mutations in regulatory, nonregulatory, or both types of proteins (Table 1 and table S1), all the isolated clones that hyperproduced pyomelanin (Fig. 1C) were significantly (P < 0.001 in all cases) more susceptible to tobramycin than their respective parental strain (table S3), and lacked mexXY (fig. S1B). Then, we recreated a total of 27 heterogeneous pyomelanogenic populations by mixing each of the 27 pyomelanogenic clones with its respective parental strain in a 1:1 ratio. The heterogeneous populations were dubbed PA14 +1 to +4, nfxB177 +1 to +4, parR87 +2 to +4, mexZ43 +1 to +4, orfN50 +1 to +4, MDR6 +1 to +4, and MDR12 +1 to +4. These populations were first subjected to tobramycin short-term evolution for 3 days (see Materials and Methods), and the capacity of these populations to either evolve toward tobramycin resistance or to go extinct was analyzed (Fig. 3). Since pyomelanogenic clones are less susceptible to ceftazidime (i.e., >256 µg/ml in all the parR87 clones; see “b” data in table S4) than the parental strain from which they evolved (1 µg/ml in parR87; see “a” data in table S4), ceftazidime MIC values were used to verify the extinction of the pyomelanogenic part of each population. After 3 days of evolution in the presence of tobramycin, the ceftazidime MICs for every population (i.e., 1 µg/ml in parR87 heterogeneous populations; see the “First step (TOB)” data in table S4) were close to the ceftazidime MIC for the parental strain [compare First Step (TOB) to the “a and b” MIC data in table S4]. These data suggest that the pyomelanogenic part of every population is extinct after the first step of sequential evolution. To further confirm the extinction of the pyomelanogenic clones, the phenotype (green/yellow versus brown color) of 20 clones from each of the 27 populations, a total of 540 clones, were isolated and grown in liquid medium (brown color is poorly appreciated in colonies) to detect any escape of ceftazidime-resistant cells from tobramycin treatment. As shown in Fig. 3B, none of the clones produced pyomelanin, confirming the extinction of the pyomelanogenic part of every population, a fact that was previously hinted by the ceftazidime MIC values of the resultant populations.
(see table S4). Besides that, each resultant population presented an increased tobramycin MIC, up to 48-fold, depending on the genetic background and replicate (table S4).

At this point, we specifically focused on the switch from tobramycin to ceftazidime, the second step of the sequential evolution (Fig. 2). Although we had observed conservation of tobramycin collateral sensitivity after evolution in presence of ceftazidime within the analyzed set of mutants of *P. aeruginosa* (Fig. 1 and table S2), a critical point would be to determine whether this genetic event would also be selected by ceftazidime in the tobramycin-resistant mutants obtained after the first step of evolution of the populations in the presence of tobramycin. Hence, we switched the selective pressure from tobramycin to ceftazidime (see Materials and Methods). As shown in Fig. 3, 17 of 27 populations hyperproduced pyomelanin. A second critical point emerged here: The degree of sensitization to tobramycin of highly resistant mutants to the said antibiotic was...
uncertain. To determine whether these populations presented an increased susceptibility to tobramycin, as it was observed in the wild-type PA14 background and in most of mutants analyzed in this work (Fig 1 and table S2), the tobramycin MIC was determined in all the evolved populations. Twenty-three of 27 populations presented an important increase in their sensitivity to tobramycin after switching the selective pressure from tobramycin to ceftazidime, reducing the MIC by up to 128-fold in PA14, 48-fold in nfxB177, 21-fold in parR87, 96-fold in orfN50, 43-fold in mexZ43, 4-fold in MDR6, and 11-fold in MDR12 (Fig. 4 and table S4). This analysis revealed that five of seven heterogeneous pyomelanogenic populations presented a substantially reduced MIC to tobramycin after the second step of sequential evolution on ceftazidime (table S4). These results suggest that it could be possible to exploit the tobramycin collateral sensitivity associated with the use of ceftazidime by switching back selective pressure to tobramycin, although there may be some limitations depending on the genetic background. This was the case of the multiple resistant mutants MDR6 and MDR12, which did not present a relevant reduction in tobramycin MIC after the switch to ceftazidime.

The fact that some populations were hypersusceptible to tobramycin, even without having suffered chromosomal deletions containing mexXY (6 of 27 populations; Fig. 3 and fig. S1C) or being mixed populations (4 of 27 populations; Fig. 3 and fig. S1C), indicates that reciprocal collateral sensitivity between ceftazidime and tobramycin may occur even in the absence of these deletions, a feature that remains to be explored in detail. Overall, our results indicate that this strategy could potentially be applicable from complex situations similar to the ones explored in the current work (heterogeneous pyomelanogenic-ceftazidime-resistant populations) to other ones (populations not resistant to ceftazidime).

**Cross-resistance and collateral sensitivity patterns of** *P. aeruginosa* **after tobramycin/ceftazidime sequential evolution**

We have recently described that *P. aeruginosa* replicate populations subjected to ribosome-targeting antibiotics may present common changes in the susceptibility to other antibiotics, besides those used along selection (16, 36). However, important differences have been described in the collateral sensitivity phenotype among replicate populations of the same *P. aeruginosa* strain adapted to one antibiotic (13). We have recently reported that a loss-of-function mutant of *P. aeruginosa* PA14, differing from its parental strain in the activity of just one regulator, not directly linked to AR, presents different patterns of collateral sensitivity and cross-resistance phenotypic outcomes when it acquires resistance to ribosome-targeting antibiotics (16). Since historical contingency may restrict the evolution of collateral sensitivity and cross-resistance phenotypic outcomes, we wondered whether the populations obtained after tobramycin and ceftazidime sequential evolution, besides presenting a convergent hypersusceptibility to tobramycin, may converge toward the phenotypes of cross-resistance or collateral sensitivity to antibiotics found in other structural families. To address this question, the MICs of a set of antibiotics were determined for the 27 populations. A general pattern of cross-resistance to aztreonam, imipenem, and chloramphenicol, as well as significant collateral sensitivity to fosfomycin, tobramycin, and tetracycline, was observed (*P* < 0.0001 in all cases) (Fig. 5 and table S5). Since the combination fosfomycin-tobramycin has been found to be synergistic against biofilms of CF *P. aeruginosa* strains (39) and against *P. aeruginosa* PAO1 in anaerobic environments (40), we propose that the switch back to tobramycin (Fig. 2) could also be replaced, if necessary, by the combination fosfomycin-tobramycin.

To analyze the relative efficacy of the two possible options, we...
subjected the resultant populations obtained after tobramycin/ceftazidime sequential evolution to ALE in either tobramycin or the combination fosfomycin-tobramycin, applying twice the MIC of each parental strain. We observed that 8 of 27 populations were able to escape from the switch back to tobramycin (orfN50+3, mexZ43+2 and +3, MDR6 +1 and +3, and MDR12 +2 to +4). This result agrees with the fact that although most of the populations (23 of 27) presented an important reduction in the tobramycin MIC after sequential evolution on tobramycin and ceftazidime, by up to 128-fold in PA14, 48-fold in nfxB177, 21-fold in parR87, 96-fold in orfN50, 43-fold in mexZ43, 4-fold in MDR6, and 11-fold in MDR12 (Fig. 4), tobramycin MIC values were close to those of parental strains. None of the populations survived the combination fosfomycin-tobramycin, possibly due to the synergistic effect of these antibiotics (39). We therefore propose that use of the fosfomycin-tobramycin combination is more effective than switching back to tobramycin after that treatment.

**DISCUSSION**

Bacterial evolution is known to be one of the main causes of the current AR problem; but in-depth analysis of this evolution could also help to tackle this issue through the exploitation of the evolutionary trade-offs (as collateral sensitivity) associated with AR acquisition (2, 4). However, the feasibility of this approach requires the collateral sensitivity phenotypes of different resistant mutants to be robust and reproducible (41). In this study, we describe the robustness of collateral sensitivity to tobramycin associated with the short-term use of ceftazidime in an array of *P. aeruginosa* antibiotic-resistant mutants, chosen on the basis of their differences both in resistance phenotype and in the functions affected by the mutations that they harbor. We propose that the observed evolutionary trade-offs could be exploited for treating both clonal and heterogeneous pyomelanogenic infections. Patients with CF are usually infected by heterogeneous *P. aeruginosa* populations (38) that include pyomelanogenic mutants (29, 30), which frequently present resistance to β-lactams, a feature that could compromise the use of ceftazidime. However, we have found that it is possible to drive the extinction of the pyomelanogenic mutants, first, by using tobramycin and then second, by driving the evolution of the remaining population toward tobramycin hypersusceptibility, using ceftazidime. A bottleneck for the application of this strategy would be the durability over time of tobramycin hypersusceptibility. However, it is important to highlight that the populations obtained after tobramycin/ceftazidime alternation present also collateral sensitivity to fosfomycin. We observed that it is possible to replace the switch back to tobramycin by a fosfomycin-tobramycin combination, which results in higher efficacy. These results point to the possibility of exploiting specific evolutionary trade-offs for tackling the problem of AR. However, we are aware that a detailed analysis determining the degree of conservation of the short-term ceftazidime resistance evolution in a broad and diverse set of clinical strains of *P. aeruginosa* would be required. Overall, our results and those of others, previously described (41) suggest that the analysis of phenotypic convergence and, in particular, the aspects that deal with the collateral sensitivity of *P. aeruginosa* AR mutants, is an important step forward in the rational design of therapeutic approaches capable of reducing the AR burden.

**MATERIALS AND METHODS**

**Growth conditions and antibiotic susceptibility assays**

Bacteria were grown in LB at 37°C, with shaking at 250 rpm in glass tubes. MICs of ceftazidime, aztreonam, imipenem, tobramycin, amikacin, tigecycline, tetracycline, ciprofloxacin, levofloxacin, chloramphenicol, fosfomycin, and erythromycin were determined at 37°C in Mueller-Hinton (MH) agar, using E-test strips (MIC Test Strip, Liofilchem).

**Mutant construction**

Four single mutants of *P. aeruginosa* (nfxB177, parR87, mexZ43, and nusD184) were constructed by inserting each mutant allele (table S1) by homologous recombination into the wild-type PA14, while the orfN50 single mutant was previously obtained (16). Mutant alleles were obtained by PCR from previous in-house evolved populations (16, 36), leaving approximately 500 bp upstream and downstream the corresponding single-nucleotide polymorphism, using the oligonucleotides described in table S6. PCR products containing Hind III restriction sites were cloned into the Hind III–digested and dephosphorylated pEX18Ap vector (42) and then introduced by transformation into the conjugative *Escherichia coli* S17-1 strain. Subsequently, conjugation and mutant selection were performed, as described.

**Fig. 5. Diagram showing the degree of convergence of cross-resistance and collateral sensitivity in heterogeneous pyomelanogenic populations of *P. aeruginosa* obtained after TOB/CAZ sequential evolution.** Collateral sensitivity and cross-resistance to antibiotics from different structural families were analyzed in the 27 populations obtained after sequential evolution. A population is classified as “susceptible” or “resistant” when there was an MIC change with respect to the parental strain was observed. Thickness of the triangle depends on the percentage of conservation of said phenotype. MIC values (µg/ml) are included in table S5. AMK, amikacin; ATM, aztreonam; FOF, fosfomycin; ERY, erythromycin; CHL, chloramphenicol; LEV, levofloxacin; TET, tetracycline.
elsewhere (42), using carbenicillin (350 μg/ml) and 10% sucrose. In all cases, the presence of the mutations was confirmed by Sanger sequencing. To obtain mutants containing multiple mutations, 10 independent resistant clones from end point evolved populations (16) on tobramycin or tigecycline were selected and mutations confirmed by Sanger sequencing. From them, two multiple mutants (table S1), tobramycin or tigecycline resistant, respectively, were chosen. The mutant allele of lasR was replaced by homologous recombination with that of the wild type in the two selected clones, using the above-described strategy and oligonucleotides encompassed in table S6.

**Short-term ALE in presence of ceftazidime**

Five single mutants, two multiple mutants, and PA14, four replicates of each, were subjected to short-term ALE in presence or absence of ceftazidime, resulting in a total of 64 independent bacterial populations (32 populations grown in presence of ceftazidime and 32 control populations grown without antibiotic). Cultures were grown at 37°C and 250 rpm for 3 days. Every day, the cultures were diluted (1/125), adding 8 μl of bacteria in 1 ml of fresh LB, either containing or lacking ceftazidime at the concentration that hinders the growth of each *P. aeruginosa* genetic background under these culture conditions (4 μg/ml for PA14, mexZ43, and MDR12; 5 μg/ml for nfxB177, orfN50, and MDR6; 3 μg/ml for parR87; and 2 μg/ml for nuoD184). During the 3 days, the concentration of ceftazidime 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 in populations (ceftazidime) and the MIC of tobramycin was determined at 37°C in MH agar using E-test strips.

**Sequential tobramycin/ceftazidime experimental evolution**

Pyomelanogenic clones were isolated from every individual pyomelanogenic replicate population of each genetic background previously submitted to short-term evolution in the presence of ceftazidime, resulting in a total of 27 pyomelanogenic clones (see above). Overnight bacterial cultures from each pyomelanogenic clone and its parental strain were normalized to an optical density at 600 nm of 4.0 and then mixed in a 1:1 (pyomelanogenic clone:parental strain) ratio, obtaining 27 heterogeneous populations. Cultures were grown at 37°C and 250 rpm for 6 days. Every day, during the first 3 days, the cultures were diluted (1/125) in fresh LB containing the tobramycin concentration that hinders the growth of each *P. aeruginosa* genetic background under these culture conditions (1 μg/ml for PA14; 1.5 μg/ml for nfxB177, parR87, mexZ43, and MDR6; 4 μg/ml for orfN50; and 12 μg/ml for MDR12). During the 3 days, the concentration of tobramycin was maintained. At the end of the first step of sequential experimental evolution, every replicate population was preserved at −80°C, and the MIC of ceftazidime and tobramycin was determined at 37°C in MH agar using E-test strips. The 27 populations were grown, from glycerol stocks, and every day, during the last 3 days, the cultures were diluted (1/125) in fresh LB containing ceftazidime, as described in the above-mentioned section of Material and Methods (see the “Short-term ALE in presence of ceftazidime” section). Every final population was preserved at −80°C at the end of the second step of sequential experimental evolution, and the MIC of tobramycin was determined at 37°C in MH agar using E-test strips.

**Analysis of the presence/absence of mexXY in the evolved populations**

The presence of chromosomal deletions including *mexXY* in the different genetic backgrounds and their respective evolved populations was analyzed by determining the absence of a 163-bp PCR fragment belonging to *mexXY* in 2% agarose gel. Primers used for *mexXY* genotyping are included in table S6.

**Statistical analysis**

Data were subjected to pre hoc and post hoc analyses to identify relevant differences, using either analysis of variance (ANOVA), Friedman’s, or χ² tests and Dunnett’s or Fisher’s exact test with Hochberg correction, as implemented in R.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/32/eaba5493/DC1

View/Request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**

Predicting antibiotic resistance.


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Rapid and robust evolution of collateral sensitivity in *Pseudomonas aeruginosa* antibiotic-resistant mutants

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