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Ciprofloxacin resistance rapidly declines in *nfxB* defective clinical strains of *Pseudomonas aeruginosa*

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Antibiotic-resistant bacteria could be tackled by identifying trade-offs of evolution, such as high fitness costs, which may be harnessed to force reversion to susceptibility. A decline in antimicrobial resistance can occur through compensatory mutations or by genetic reversion to the wild-type allele, which reduce fitness costs associated with resistance. We analyse here the impact of antibiotic-free environments on declining ciprofloxacin resistance in eight *nfxB* defective clinical strains of *Pseudomonas aeruginosa* spanning varied clone types and ciprofloxacin resistance levels. Ciprofloxacin resistance declines in just 100 generations, which is mainly caused by newly acquired mutations in the genes encoding the overproduced efflux pump MexCD-OprJ and not by the reversion of *nfxB* mutations of the parental strains. The rapid reversion of ciprofloxacin resistance in *P. aeruginosa* suggests the potential for reusing this essential antibiotic and underlines the need to implement evolution-based approaches against *nfxB* defective resistant mutant strains.

The efficacy of antibiotics is severely compromised by the emergence and spread of antibiotic-resistant bacterial pathogens. Among them, *Pseudomonas aeruginosa* stands out[1,2]. This opportunistic pathogen causes nosocomial and chronic infections in people with cystic fibrosis (pwCF) or chronic obstructive pulmonary disease[3–5]. These infections are frequently treated with the fluoroquinolone ciprofloxacin[6,7], but resistance is rapidly acquired by chromosomal mutations. The two principal resistance mechanisms are the acquisition of mutations in the ciprofloxacin target-encoding genes *parCE* (encoding DNA topoisomerase IV) and *gyrAB* (encoding DNA gyrase)[8–12], and the acquisition of mutations in genes encoding the regulators of the efflux pumps that extrude this drug, leading to their overproduction[6,10,13–15]. Ciprofloxacin resistance of clinical *P. aeruginosa* isolates from patients with persistent airway infections[10,16–20] is frequently due to the overproduction of the efflux pump MexCD-OprJ[21]. Overproduction of this efflux pump is mediated by the acquisition of loss-of-function mutations in the gene encoding its transcriptional repressor NfxB. In addition, *mexCD-oprJ* can be transiently overexpressed in the presence of inducer compounds, such as the antiseptic dequalinium chloride[22], which temporally inactivates NfxB. Hence, the use of either dequalinium chloride or ciprofloxacin can lead to reduced quinolones efficacy because of a transient or stable overproduction, respectively, of the MexCD-OprJ efflux pump. However, it has also been described that the temporal or stable inactivation of NfxB, in clinical and laboratory strains of *P. aeruginosa*, not only causes ciprofloxacin resistance but also transient[23] and stable[24,25] collateral sensitivity (CS) to aminoglycosides[18,20,21,23,26]. This robust trade-off, by which the acquisition of resistance to one drug leads to the susceptibility to another[25], has been described to emerge in clinical strains presenting, among others, mutations in *nfxB*[18]. In addition, it has been recently proved that the combinations ciprofloxacin-tobramycin or dequalinium chloride-tobramycin are effective in driving *P. aeruginosa* (antibiotic-resistant mutants to different drugs and varied clinical strains) to extinction[20,23,26]. However, the stability of both ciprofloxacin resistance and CS to tobramycin, associated with *nfxB*

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loss-of-function mutations, has not been determined in the absence of antibiotic selective pressure.

During periods of antibiotic restriction, it is possible that wild-type susceptible strains outcompete resistant ones and that compensatory evolution of fitness costs associated with resistance occurs[27–33]. However, restricting the use of antibiotics would be insufficient when resistance mutations do not trigger a physiological cost, since compensatory evolution is not expected to occur[34]. Furthermore, in those situations where compensatory evolution happens, it is more likely to be due to secondary mutations than to genetic reversions restoring the wild-type allele[35]. Therefore, there is uncertainty regarding the ability of periods of drug restriction to reverse antimicrobial resistance (AMR)[36–39]. However, rapid decline of resistance has been reported using Adaptive Laboratory Evolution (ALE) assays in antibiotic-free medium, with this decline being drug-specific and genetic background dependent[40,41]. The reason for the lineage-dependent decline is that fitness costs associated with a specific resistance mutation may differ in distinct genomic backgrounds[42], due to epistatic and pleiotropic interactions[42–46].

In this work, we analyse the possible decline or reversion of ciprofloxacin resistance in different clinical strains containing *nfxB* loss-of-function mutations, which are highly prevalent. We performed 15 days of ALE experiments in two different antibiotic-free environments: (I) environment absent of antibiotic (e. g. antibiotic restriction periods after the use of ciprofloxacin or ciprofloxacin-tobramycin therapies[20,26]) and (II) environment absent of antibiotic but containing dequalinium chloride (e.g., dequalinium chloride concentrations resulting from dequalinium chloride-tobramycin therapies[23]). We hypothesised that fitness costs of *nfxB* defective mutants could drive compensatory evolution in these antibiotic-free environments, causing a rapid decline of ciprofloxacin resistance (conceptual Fig. 1A). Having in mind that dequalinium chloride leads to the overproduction of the efflux pump in strains presenting a functional regulator, reversions to the wild-type allele seem less likely to be selected in its presence, although mutations in *nfxB* impeding its inhibition by dequalinium chloride could also be selected. In addition, and keeping in mind that each *nfxB* loss-of-function mutation may have a different effect on bacterial physiology -and on their associated fitness costs- depending on the starting genomic background, we hypothesised that the frequency and level of this resistance decline could be different in the eight *nfxB* defective clinical strains that were analysed.

Results

Decline of ciprofloxacin resistance in the PA14 isogenic loss-of-function *nfxB177* mutant in antibiotic-free environments

In a previous work, we induced transient *nfxB*-mediated tobramycin CS in varied clinical isolates of *P. aeruginosa* by using dequalinium chloride. In addition, we drove these clinical isolates to extinction by combining tobramycin and dequalinium chloride[23]. Importantly, dequalinium chloride did not select for ciprofloxacin resistance after 15 days of evolution (a hundred bacterial generations)[23]. Something not addressed in said article is determining if the PA14 isogenic loss-of-function *nfxB177* mutant (where the overproduction of the MexCD-OprJ efflux pump leads to ciprofloxacin resistance) may show a decline in ciprofloxacin resistance in the absence of antibiotic selective pressure, as a result of compensatory evolution. Therefore, in this work we performed ALE assays in antibiotic-free environments, with or without dequalinium chloride (see Methods; Fig. 1B). Four replicate populations of the PA14 isogenic loss-of-function *nfxB177* mutant were evolved for 15 days, with daily sequential dilutions (1%) in test tubes (64 populations). We observed that the *nfxB177* mutant reverted the ciprofloxacin MIC to the values of its parental strain PA14 after ALE in the absence of antibiotic (Wilcoxon test with FDR correction adj $p < 0.01$; Fig. 1C). We hypothesised that this loss-of-function mutation in *nfxB* could lead to a high fitness cost when grown in the absence of antibiotics and, therefore, a rapid compensatory evolution (and decline of AMR) could happen in just a hundred bacterial generations. Hence, we compared the fitness of the *nfxB177* mutant strain and that of the eight *nfxB177* populations obtained after 15 days of ALE in antibiotic-free environments (in the absence or presence of dequalinium chloride) with that of its parental strain PA14 (Fig. 1D). To do so, we estimated the fitness as the area under the growth curve recorded in antibiotic-free medium and analysed it for the *nfxB177* mutant and of each *nfxB177* population with respect to the PA14 parental strain. The *nfxB177* mutant strain presented a reduced fitness relative to its parental strain, while the evolved populations in antibiotic-free medium reverted to the fitness level of the PA14 parental strain (One-way Anova followed by Dunnett's multiple comparison test; $p < 0.0001$; Fig. 1D). Given these results, we hypothesised that the *nfxB177* ciprofloxacin-resistant populations had acquired compensatory mutations during ALE, which would be responsible for the decline of ciprofloxacin resistance observed in antibiotic-free, non-selective environments. In addition, we hypothesised that this compensatory evolution, and the associated decline of ciprofloxacin resistance could also occur in highly prevalent clinical isolates presenting mutations in this gene (see below).

Decline of ciprofloxacin resistance in *nfxB* defective clinical strains of *P. aeruginosa* in antibiotic-free environments

To analyse changes in ciprofloxacin susceptibility associated with compensatory evolution in different *nfxB* defective mutants in antibiotic-free environments, four biological replicates of 8 clinical isolates were evolved in the absence of antibiotics (Fig. 1B). Strains from the Copenhagen CF Centre, Department of Clinical Microbiology, at Rigshospitalet, belong to six different clone types (defined as lineages which differ by >10,000 SNPs), harbour different *nfxB* mutations (4 frameshift and 4 amino acid substitutions including 2 stop codons) and ciprofloxacin resistance levels (0.75–1.5 μg/mL) (strains 258, 296, 357, 76, 316, 225, 398 and 79; Table 1)[19], were evolved in the absence of antibiotic. We analysed two different antibiotic-free environments, with or without dequalinium chloride. These strains were evolved for 15 days (~100 bacterial generations), with daily sequential dilutions (1%) in test tubes (64 populations). MIC variations greater than 2-fold (determined using MIC test strips, allowing detection of small MIC changes) and with adjusted p values of <0.05 (Kruskal–Wallis test followed by each-pair Wilcoxon test with FDR correction) indicated biologically and statistically relevant variations of susceptibility, as previously described[26,41]. We observed a decline of ciprofloxacin MIC in 43 out of 64 populations (67%) evolved in antibiotic-free environments -in the absence or presence of dequalinium chloride- for 15 days (Figs. 2 and 3; Supplementary Table 1). Importantly, the MIC values of 42 of these evolved populations dropped down below the EUCAST clinical breakpoint (Fig. 3 and Supplementary Table 1). Specifically, a decline of ciprofloxacin resistance, from 3- to 16-fold, was observed in the clinical isolates 258, 296, 357, 76, 225 and 398 after ALE in the absence of dequalinium chloride (24 out of 32 populations) and in 258, 296, 76, 225 and 398 after ALE in the presence of dequalinium chloride (21 out of 32 populations). Interestingly, the highest resistance decline occurred in the clinical isolates 296 and 76 in both antibiotic-free environments. In the absence of dequalinium chloride, we observed a decline of up to 16-fold in the clinical isolate 296 and of up to 12-fold in the clinical isolate 76. In the presence of dequalinium chloride, we observed a decline of up to 16-fold in the isolate 76 and of up to 11-fold in the isolate 296. These results indicate that these two clinical isolates are the most likely to show significant decreases in ciprofloxacin resistance

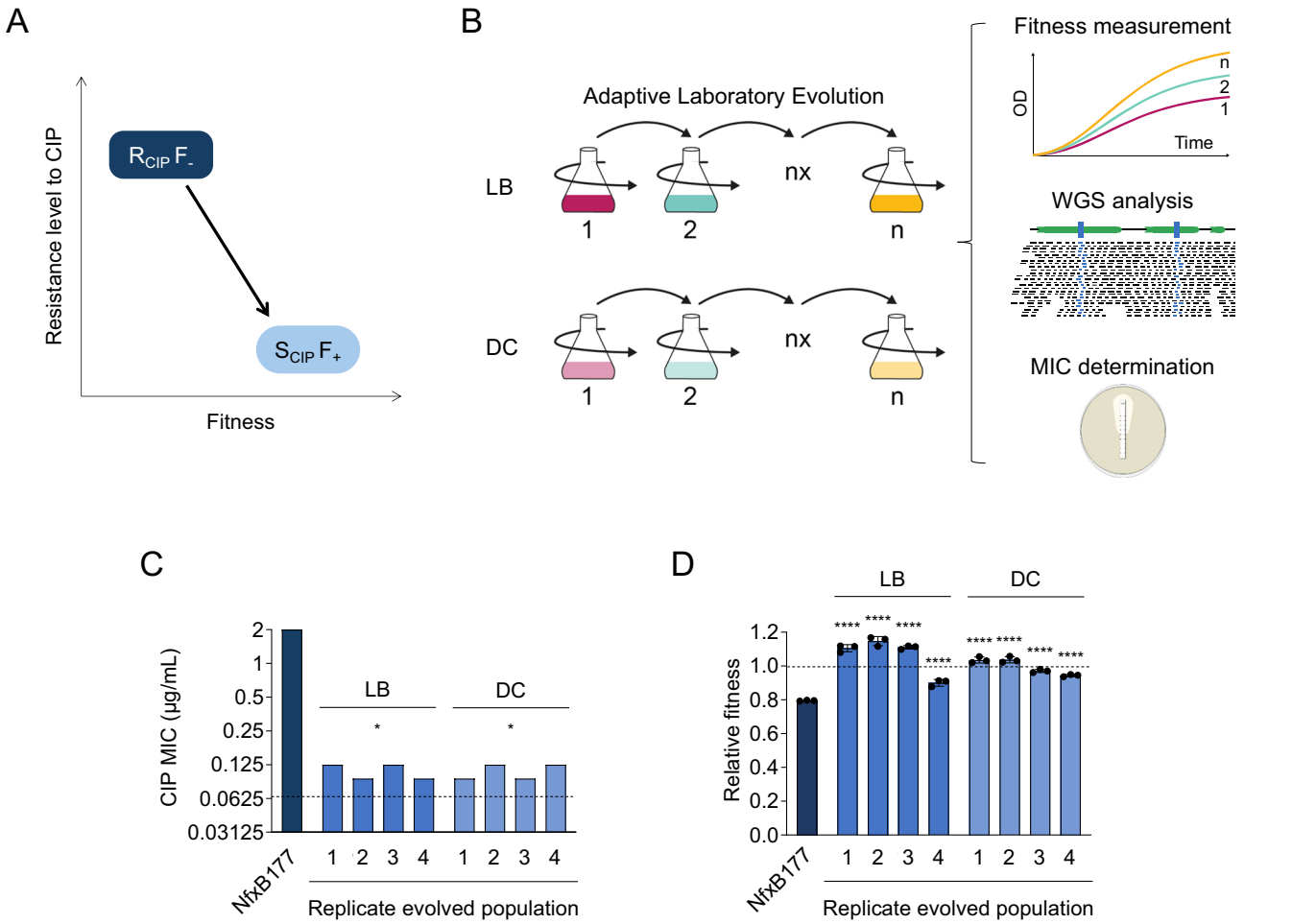

Fig. 1 | Compensatory evolution of *nfxB* mutation-related fitness costs leading to phenotypic reversion of ciprofloxacin resistance. A Compensatory evolution in antibiotic-free environments may result in a fitness (F) improvement in *nfxB* ciprofloxacin-resistant mutants (R_{CIP}) and, therefore, in the reversion or decline of ciprofloxacin resistance. This decline of resistance may occur in different antibiotic-free environments, such as those resulting from drug restriction periods (after the use of ciprofloxacin[20,26]) or those resulting from the presence of dequalinium chloride (after the use of transient CS-based therapies[23]. **B** Schematic representation of the experimental set-up of this work. **C** Ciprofloxacin (CIP) MICs (μg/mL) of populations evolved 15 days in antibiotic-free environments. CIP MICs of *nfxB177* ciprofloxacin-resistant mutant, before and after 15 days of ALE assays on rich medium (LB) or on dequalinium chloride (DC) supplemented medium (10 μg/mL). Four replicate populations for each condition are represented. CIP resistance of the *nfxB177* evolved populations declined after evolution in LB or in DC with respect to that of the *nfxB177* mutant, reaching levels close to that of the PA14 wild-type strain (0.064 μg/mL, indicated with a dashed line). Statistical significance was computed by non-parametric Kruskal-Wallis (Rank Sums) test, followed by each-pair comparison test using the Wilcoxon method where * equals $p = 0.0367$. **D** Relative fitness of populations evolved 15 days in antibiotic-free environments. Growth curves of PA14 and of the *nfxB177* ciprofloxacin-resistant mutant, before and after 15 days of ALE assays on rich medium (LB) or on DC supplemented medium (10 μg/mL), 4 replicate populations for each condition, were recorded in antibiotic-free medium. Statistical significance was computed by one-way ANOVA followed by Dunnett's multiple comparison test where **** ($p < 0.0001$). The fitness of each population was represented as the area under the growth curve. Relative fitness of the *nfxB177* mutant and of the *nfxB177* populations was calculated with respect to the PA14 wild-type strain. OD600nm vs time measurements are included in Supplementary Data 2. Relative fitness is lower in the *nfxB177* mutant than in its parental strain PA14. Fitness of the *nfxB177* evolved populations improved after evolution in LB or in DC with respect to that of the *nfxB177* mutant, presenting fitness levels close to or even higher than the PA14 strain. The mean ± SD of values from three replicates is represented.

during periods of drug restriction. However, it is relevant to remark that ciprofloxacin resistance also declined in populations from the clinical isolates 258, 357, 225, and 398 (Fig. 3 and Supplementary Table 1), with AMR decline differing depending on the parental strain. These results suggest a strong influence of the genomic background on the compensatory evolution and on AMR decline that should not be underestimated.

As hypothesised, the decline in ciprofloxacin resistance in antibiotic-free environments occurred, possibly due to compensatory evolution of fitness costs associated with ciprofloxacin resistance. In addition, the presence of dequalinium chloride, which does not select for stable ciprofloxacin resistance mutations[23], did not prevent this decline of resistance from occurring (Figs. 2 and 3, and Supplementary Table 1). Altogether, these results support that ciprofloxacin resistance of clinically relevant *nfxB* defective mutants can be rapidly compensated in the absence of selective pressure, reverting back to susceptibility (based on standard laboratory antibiotic susceptibility testing). In addition, these results support that the combinations of ciprofloxacin-tobramycin or dequalinium chloride-tobramycin that we previously proposed for treating *P. aeruginosa* infections[20,23,26], could be followed by a period of antimicrobial restriction if *nfxB* loss-of-function mutants are selected. This strategy would allow a rapid decline of resistance towards ciprofloxacin, possibly avoiding the selection of resistance to other antibiotics, something that we study below.

Table 1 | Clinical isolates used in this study

Strain	Clone type	CIP MIC (μg/mL)	*nfxB* mutations	Other relevant resistance mutations	*nfxB-mexCD-oprJ* genetic variations acquired during ALE
76	DK09	1.5	366-375 ΔGGACGCCTTC (N123fs)		*mexC* (85C > T; 132ΔT); *nfxB*-intergenic region-*mexC-mexD* Δ 7.3 kb (5159813 to 5152497)
258	DK35	0.75	59-69 ΔACCGCCCGCGA (D20fs)	MexZ (S202fs)	*mexC* (565G > T)
296	DK06	1	T77C (L26P)	MexE (A17fs), GlpT (P444S)	*mexC* (400C > T); *nfxB*-intergenic region-*mexC* Δ 1.4 kb (5155025 to 5156441)
79	DK09	0.75	C304G (R102G)	MexZ (E114fs)	-
316	DK03	1	519-521 ΔGGA (L174fs)	MexZ (V43A), NalC (P149fs),	-
225	DK32	0.75	C199T (Q67X)		*mexC* (18insC), *mexD* (3C > G; 2140-2159 ΔCTTCGGCCAGTCCTTCCAT), intergenic region (5155431 ΔGAAT; 5155462A > G)
398	DK06	1	A564C (X188C)	MexE (A17fs), GlpT (P444S)	*mexC* (207 ΔGC; 209 ΔGC)
357	DK40	0.75	101-119 ΔGCGTAAGCAAGGC-CACCCT (G34fs)	OprM (A92fs)	*mexD* (1T > G; 32C > G; 1818 ΔC; 009–1995 ΔCCGTTGATCGGTGG), intergenic region (5155458 G > C)

The clinical strains are described in ref. 19. Genetic variations acquired during ALE in each replicate evolved population are detailed in Supplementary Table 2.
CIP ciprofloxacin, X stop codon.

Evolution of cross-resistance and collateral sensitivity in antibiotic-free environments

We analysed if the populations resulting from 15 days of ALE in antibiotic-free environments could also have variations in the level of resistance to antibiotics from different structural families (tobramycin, aztreonam, fosfomycin, ceftazidime, and imipenem). To do that, we measured MICs for these antibiotics in the 64 populations and in the original parental strains (Fig. 2 and Supplementary Table 1) and evaluated the biological (fold-change ± 2) and statistical significance (adjusted p value < 0.05) of such changes. Although we observed that compensatory evolution led to a decrease of ciprofloxacin resistance in 43 out of 64 populations evolved in antibiotic-free environments, susceptibility to tobramycin reverted in just 12 populations (5 and 7 populations evolved in antibiotic-free environments with or without dequalinium chloride). An increase of fosfomycin resistance was observed in all the populations belonging to the clinical isolate 76 evolved in the absence or presence of dequalinium chloride. Moreover, an increase in aztreonam, ceftazidime, and imipenem resistance was observed in all the populations belonging to this clinical isolate. We also observed a decrease in ceftazidime resistance in three populations belonging to the clinical isolate 225 evolved in the absence of dequalinium chloride (Fig. 2 and Supplementary Table 1). Finally, a general decrease in aztreonam resistance was observed in the populations belonging to the clinical isolate 357 evolved in the absence or presence of dequalinium chloride. These results reflect the difficulty in finding a robust pattern of susceptibility that emerges in different clinical strains in the absence of selective pressure. It also highlights the necessity of identifying prevalent resistance mutations (i.e. *nfxB* defective variations) that can be easily compensated, causing a robust decline of AMR (i.e. ciprofloxacin).

Decline of ciprofloxacin resistance is due to mutations in the operon encoding the MexCD-OprJ efflux pump

The DNA of the populations from the clinical isolates 258, 296, 357, 76, 225 and 398, which showed a decline of ciprofloxacin resistance in antibiotic-free environments, was subjected to whole-genome sequencing. Sequencing reads were then compared to the genomes of their parental strains (Table 1)[19], to obtain insights into the underlying molecular causes of the decline of ciprofloxacin resistance in antibiotic-free environments, in the presence or absence of dequalinium chloride (Supplementary Table S2 and Supplementary Data 1). We identified genetic variations (SNPs and Indels) in frequencies that ranged from 100% to 10% of the reads. First, it was analysed whether the molecular reversion of resistance[47], by the restoration of the mutated allele to a wild-type one[35,40], or the acquisition of new genetic variations could explain the observed decline of ciprofloxacin resistance. Importantly, none of the original *nfxB* mutations present in the parental strains reverted back to the wild-type sequence, indicating that compensatory evolution by secondary mutations, and not genetic reversion, was causing the observed ciprofloxacin resistance decline. During the analysis, the region comprising the MexCD-OprJ efflux pump encoding genes was observed to be frequently mutated across the different genetic backgrounds (Supplementary Table S2). In particular, we detected genetic modifications (SNPs and small insertions and deletions) in the genes *mexC*, *mexD* and the intergenic region between *nfxB* and *mexCD-oprJ*, but no mutations were detected in *oprJ* (Fig. 4). Further, a deletion (1.4 kb) comprising the gene *nfxB* and most of *mexC* was detected to be acquired in populations from the clinical strain 296, both evolved in the absence or presence of dequalinium chloride (Supplementary Table S2) and a 7.3 kb deletion containing *nfxB*, *mexC* and part of the genes *mexD* and *morA* was detected in one population from the clinical strain 76. The identified mutations clearly explain the decline of ciprofloxacin resistance observed in these populations. These results indicate that the overexpression of *mexCD-oprJ* due to mutations in *nfxB*, and the associated ciprofloxacin resistance, is likely compensated in absence of antibiotics mainly by mutations disrupting the structural genes encoding the efflux pump system MexCD-OprJ.

To further confirm that mutations in genes encoding the efflux pump, such as *mexC*, are responsible for decline of ciprofloxacin resistance and compensation of fitness costs, we resort to the simplest genetic context, the PA14 isogenic loss-of-function *nfxB177* mutant. We isolated a clone from the *nfxB177* population 2 evolved in the presence of dequalinium chloride, which only acquired a single mutation in *mexC* (Supplementary Table S2 and Supplementary Data 1). We measured ciprofloxacin resistance levels in the *nfxB177mexC* mutant and in the PA14 and *nfxB177* strains. Ciprofloxacin resistance level of the double mutant declined from 2 μg/mL in *nfxB177* to 0.094 μg/mL, the level of resistance of the PA14 susceptible wild-type strain. In addition, we analysed whether this decline of ciprofloxacin resistance associated with a mutation in *mexC* would also be responsible for compensation of fitness cost. For that, we compared the fitness of *nfxB177mexC* and *nfxB177* mutants with respect to that of the PA14 wild-type (Fig. 5A). The acquisition of the *mexC* mutation restores fitness of *nfxB177* mutant to levels of the wild-type strain. These results indicate that mutations in the gene *mexC*, encoding the efflux pump, can reduce levels of resistance to ciprofloxacin and fitness cost associated with the overproduction of the efflux pump. To go further and check whether

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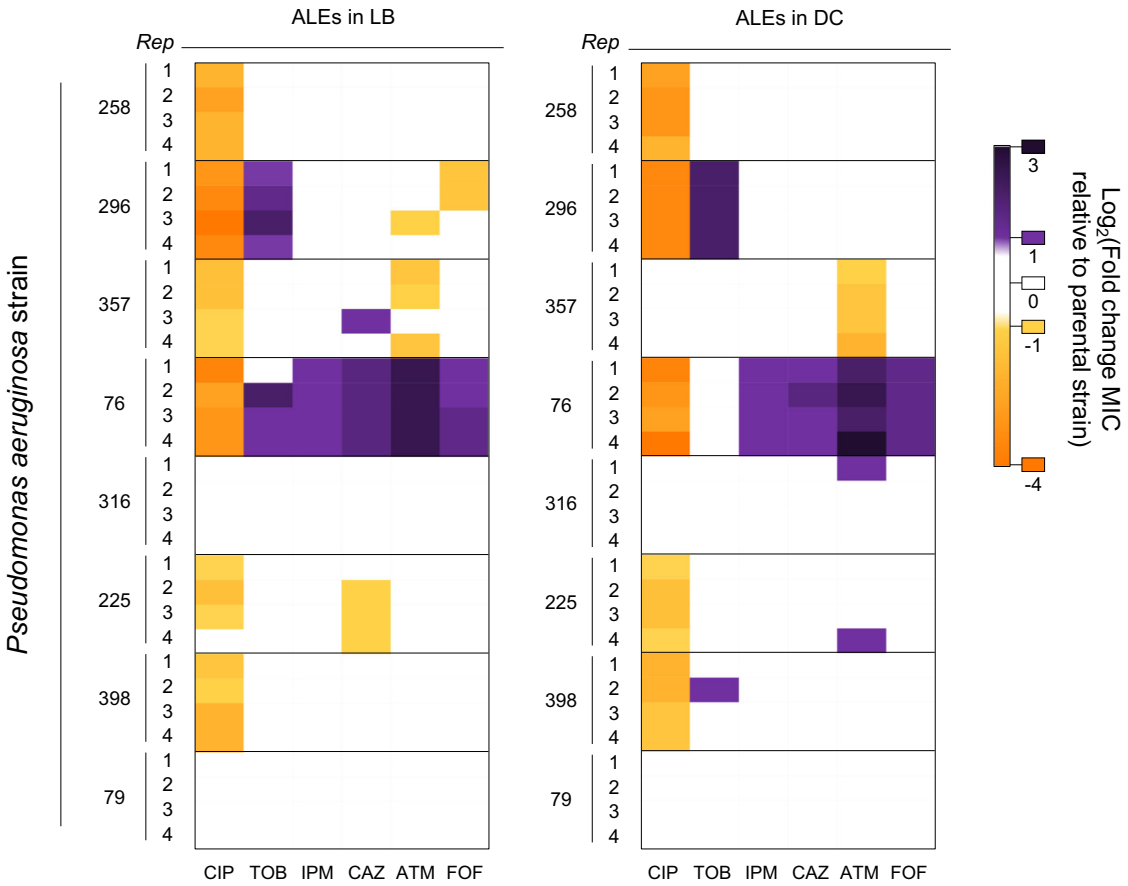

Fig. 2 | Robust decline of ciprofloxacin resistance in different *nfxB* defective clinical strains of *Pseudomonas aeruginosa* in antibiotic-free environments. Susceptibility to antibiotics from different structural families was analysed in populations (1 to 4) from 8 clinical isolates (64 populations) submitted to ALE assays in the absence of antibiotic (rich medium -LB- or rich medium supplemented with 10 μg/mL of dequalinium chloride -DC-) for 100 bacterial generations. Intensity of the colour is proportional to the log-transformed fold change of MIC of each replicate population (Rep) with respect to the MIC of the respective parental strain. Statistical significance was computed by non-parametric Kruskal–Wallis (Rank Sums) test followed by each-pair comparison test using the Wilcoxon method corrected by FDR. Only differences with FDR adjusted p-values of <0.05 were considered statistically significant. Changes of MICs above or below an increase or a decrease of 2-fold, respectively, were considered biologically relevant to classify a population as "resistant" (purple) or "susceptible" (yellow) with respect to its respective parental strain, as previously described[26]. Detailed information on the biological and statistical significance of the comparisons is presented in the Source Data. An important decline of ciprofloxacin MIC was observed in 43 out of 64 populations evolved in antibiotic-free environments (LB or DC), presenting 42 of these populations MIC values below the EUCAST clinical breakpoint (Fig. 3 and Supplementary Table 2). CIP ciprofloxacin, TOB tobramycin, IPM imipenem, CAZ ceftazidime, ATM aztreonam, FOF fosfomycin.

this double mutant can really outcompete *nfxB177* after 15 days of evolution, we performed competition assays between both strains during this period. We observed that the *nfxB177* mutant, which overproduces the efflux pump, is outcompeted by both the wild-type strain and the *nfxB177mexC* double mutant (Fig. 5B, C). Fitness compensation can occur by reversion to the wild-type allele or because of mutations in different gene(s). Here we see that the second event is the cause of fitness recovery in the evolved strains. The reason for the selection of secondary compensatory mutations in *mexC* instead of reversion of original *nfxB* mutations to the wild-type sequence may be that the second event is less frequent to occur, as expected[40,41], and/or that the *nfxB177mexC* mutant outcompetes the wild-type strain. Competition assays between both strains were performed, showing that the *nfxB177mexC* double mutant do not outcompete the wild-type strain after 15 days of evolution (Fig. 5D). Hence the cause of selecting secondary compensatory mutations in *mexC* is that the mutational space to inactivate *mexC* is larger than the one required to restore the wild-type allele in the *nfxB177* mutant.

Finally, and in addition to the mentioned mutations acquired in the region comprising the MexCD-OprJ efflux pump encoding genes, strains accumulated mutations in a large variety of genes in a clone type-specific manner (Supplementary Data 1). Genes targeted by mutations included flagella, pili, phenazines, and pyoverdine encoding genes underlining evolution to the specific ALE conditions. We did not find specific genetic variations that could clearly explain the cross-resistance and CS patterns of some of the evolved clinical strains (Supplementary Data 1). However, we cannot exclude that changes at the transcriptional level of genes encoding AMR determinants, or indirect effects caused by stress response or membrane restructuring, could be associated with susceptibility changes.

Relative fitness of *nfxB* defective clinical strain populations after ALE in antibiotic-free environments

Next, we analysed whether the observed decline of ciprofloxacin resistance, mainly associated with mutations in genes encoding the MexCD-OprJ efflux pump, was due to the compensation of fitness cost associated with *nfxB* mutations. For that, we compared the fitness of the populations from the clinical isolates 258, 296, 357 and 76, where a robust decline of ciprofloxacin resistance occurred in all the replicate populations, at least in one of the antibiotic-free environments, compared to their parental strains (Fig. 6A). The highest fitness improvement was observed in isolate 76 in both environments (One-way Anova followed by Dunnett's multiple comparison test; $p < 0.05$; Fig. 6A and Supplementary Table 3), leading to a marked decline of ciprofloxacin

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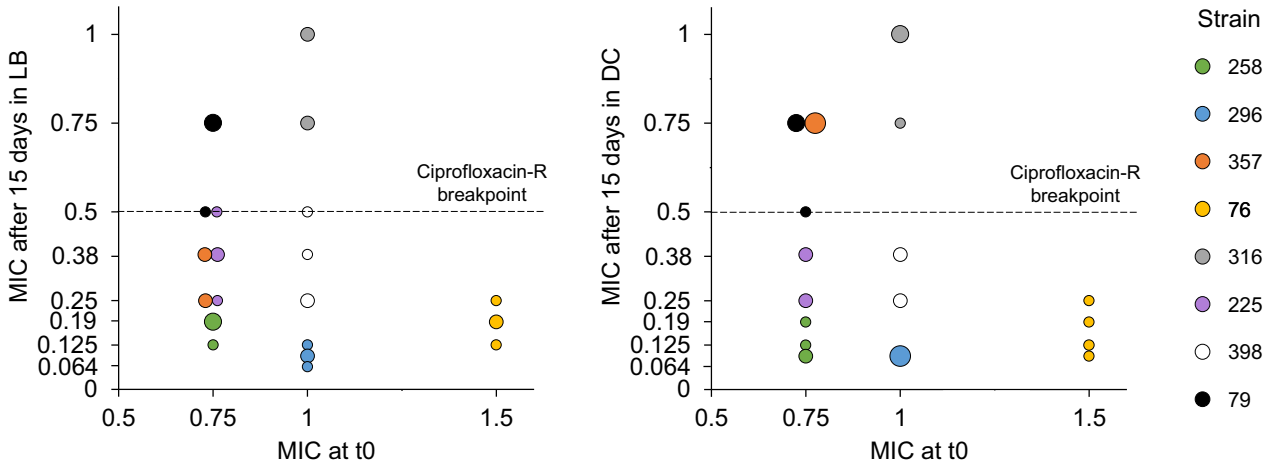

Fig. 3 | Impact of antibiotic-free environments on the decline of ciprofloxacin resistance in *nfxB* defective clinical strains of *Pseudomonas aeruginosa*. The figure shows the ciprofloxacin MIC (μg/mL) of each starting genetic background at time zero (t0) and the corresponding MIC (μg/mL) value acquired in the populations after 15 days of ALE in antibiotic-free environments: rich medium -LB- (left panel) and rich medium supplemented with 10 μg/mL of dequalinium chloride -DC- (right panel). Each of the points represents a population, and the colours indicate different clinical isolates. The size of the points is proportional to the number of populations presenting the same respective value. Raw MIC values appear in Supplementary Table 1. Points below the diagonal line indicate cases with a decrease of ciprofloxacin resistance level after compensatory evolution. Points below the horizontal line indicate cases with a MIC below the ciprofloxacin resistance clinical breakpoint (0.5 μg/mL). Most of the populations (42 out of 64) showed a decline of resistance after 15 days of ALE in the presence or absence of dequalinium chloride. Importantly, all these populations presented a ciprofloxacin MIC below the clinical breakpoint, with the degree of decline being dependent on the genetic context.

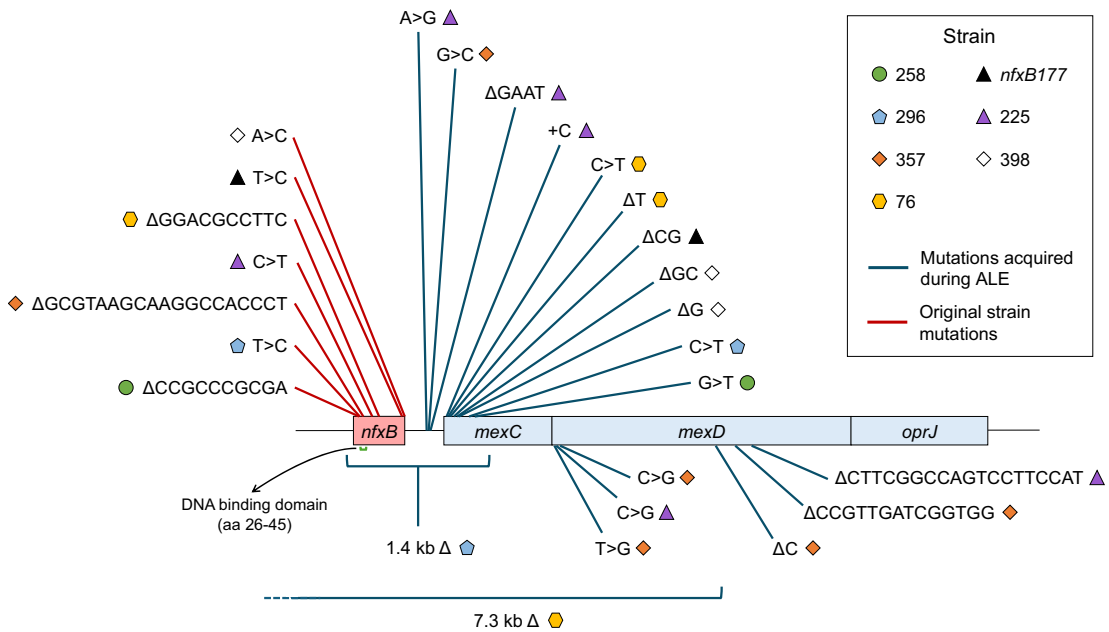

Fig. 4 | Diagram showing the genomic background dependence of the acquisition of compensatory mutations responsible for the decline of ciprofloxacin resistance in *nfxB* defective clinical strains of *Pseudomonas aeruginosa*. The position of the genetic modifications detected in the *mexCD-oprJ* operon after compensatory evolution in antibiotic-free environments with or without dequalinium chloride is indicated in the figure with blue lines. The position of the original *nfxB* mutations present in each parental clinical strain is indicated with red lines. The coloured shapes next to each genetic modification indicate the genetic context —clinical strain— in which the corresponding mutation was fixed. The genetic variations detected in each replicate evolved population during the analysis and their frequencies are indicated in Supplementary Table S2.

resistance in these populations (up to 12- and 16-fold in the absence and presence of dequalinium chloride, respectively) (Figs. 6A and 3; Supplementary Tables 1 and 3). Importantly, the clinical strain 76 is the one originally presenting the highest fitness cost (Fig. 6B and Supplementary Table 4). However, we observed that the clinical strain 296, which presented a subtle initial fitness cost (Fig. 6B and Supplementary Table 4), also had a significant decline of resistance in antibiotic-free environments (up to 16- and 11-fold in the absence and presence of dequalinium chloride, respectively) (Figs. 6A and 3; Supplementary Tables 1 and 3) but, in this case, fitness was not improved (Fig. 6A and Supplementary Tables 3). This suggests that the compensatory evolution of fitness costs associated with resistance is strongly influenced

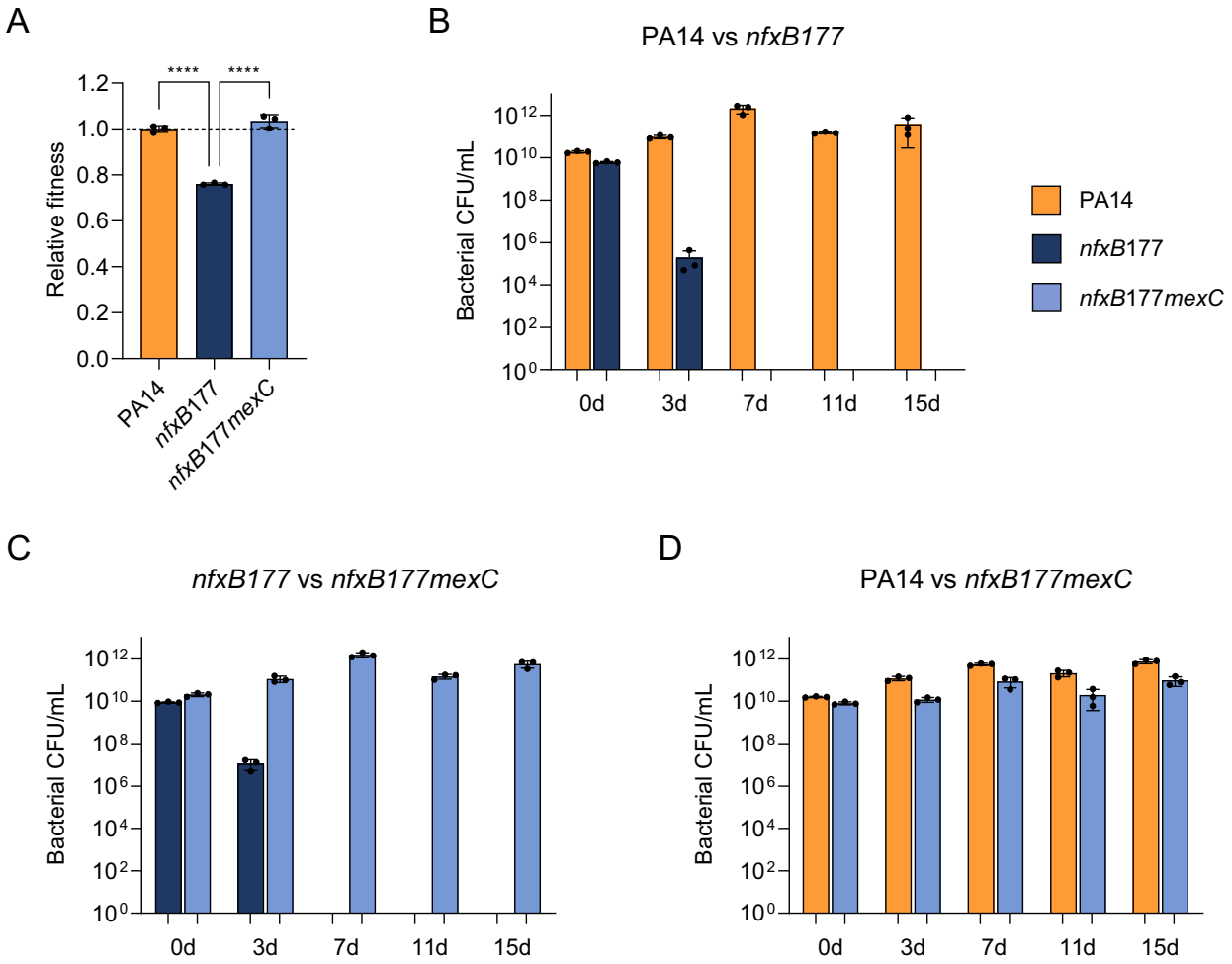

Fig. 5 | Effect of *nfxB* and compensatory *mexC* mutations in the fitness of *Pseudomonas aeruginosa*. **A** Relative fitness of PA14 (orange), *nfxB177* (dark blue), and *nfxB177mexC* (light blue) isogenic strains. The fitness of each population was measured as the area under the growth curve recorded in LB antibiotic-free medium. Relative fitness of the strains was calculated relative to the PA14 wild-type parental strain. The mean ± SD of values from three replicates is represented. Statistical significance was computed by one-way ANOVA followed by Dunnett's multiple comparison test, where **** ($p < 0.0001$). Relative fitness is lower in the *nfxB177* mutant than in its parental strain PA14, and a secondary compensatory mutation in *mexC* revert the fitness to the wild-type levels. **B**, **C**, **D** Competition among PA14 (orange), *nfxB177* (dark blue), and *nfxB177mexC* (light blue) isogenic strains. An equal amount of bacteria from strain PA14 and *nfxB177* (**B**), *nfxB177* and *nfxB177mexC* (**C**), or PA14 and *nfxB177mexC* (**D**) were cultured in LB antibiotic-free medium for 15 days with daily 1/100 dilutions in triplicates. CFUs/mL of each strain at 0, 3, 5, 7, 11, and 15 days of competition were measured and are represented as the mean ± SD of values from the three biological replicates. PA14 and the compensated *nfxB177mexC* outcompete *nfxB177*, while *nfxB177mexC* does not outcompete the PA14 wild-type strain.

by the set of mutations initially present in each parental strain. In other words, historical contingency not only restricts the evolution of resistance but also constrains compensatory evolution.

Discussion

Available information supports that reversion of resistance after periods of restriction in the use of an antibiotic is not as common as expected[36–39]. In fact, restriction on the use of an antibiotic in a specific geographic area does not always cause a reversion of resistance[48]. An important factor restricting this phenomenon is the antibiotic used to treat an infection[36,38,40,49,50], since it determines the possible resistance mechanisms selected. In this sense, the fitness cost associated with AMR mutations strongly relies on the cellular functions altered[31,51]. It also relies on the environment[52], something that may also affect compensatory evolution. In addition, CF airway environments are highly heterogeneous and show an extensive diversity, where different genotypes and resistance mechanisms may coexist[53,54]. Therefore, pre-existing resistant populations may follow different evolutionary landscapes in a CF environment, not always leading to compensatory evolution (and decline in AMR). This suggests that reversion of AMR is not a black-or-white issue and that the identification of unstable (reversible) mechanisms of resistance is of relevance for the design of evolution-based strategies to tackle AMR[55].

For AMR mutations to be unstable when selective pressure ceases, fitness costs should be high or, at least, easily compensated by the acquisition of secondary mutations that reduce resistance levels[56–58]. While many studies have focused on the effect of fitness costs in the emergence of AMR, there are limited studies analysing the stability of AMR (and of their associated trade-offs, as CS). The extent to which compensatory evolution is contingent on the starting genetic background, on initial fitness costs, or even on the environmental conditions, has not been deeply analysed. In fact, some authors raised the question of the extent to which the initial fitness cost associated with the acquisition of resistance in a mutant could affect compensatory evolution[40]. A previous work described that the decline of resistance is partly associated with compensatory evolution of fitness costs, but that there is not a perfect correlation between the degree of compensation of fitness costs and the level of resistance decline[41]. Our

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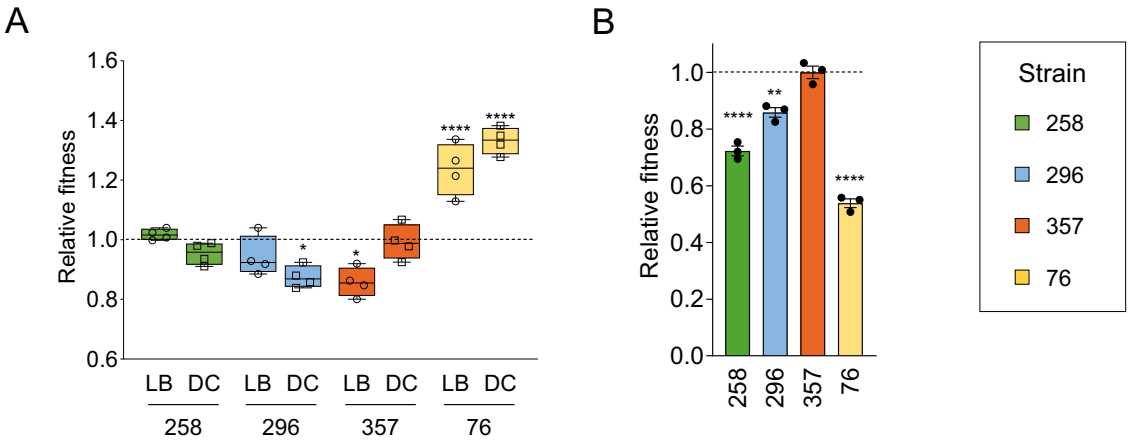

Fig. 6 | Fitness recovery of *nfxB* defective clinical strains that showed a decline of ciprofloxacin resistance in antibiotic-free environments. A Relative fitness of populations evolved after 15 days in antibiotic-free environments (rich medium -LB- or rich medium supplemented with dequalinium chloride -DC-). The fitness of each population was measured as the area under the growth curve. Relative fitness of the populations was calculated relative to their parental strains. The area below the curve of the clinical strains showing a robust decline of ciprofloxacin resistance in at least one of the environments (258, 296, 357 and 76), before and after antibiotic-free ALE, were recorded in antibiotic-free medium. Statistical significance was computed by one-way ANOVA followed by Dunnett's multiple comparison test where * ($p < 0.05$), **** ($p < 0.0001$). Each population was used as a biological replicate for statistical analysis ($n = 4$). The populations belonging to the clinical strain 76 presented an increased fitness after evolution in both antibiotic-free environments. The centre of the box plot is denoted by the median, a horizontal line dividing the box into two equal halves. The bounds of the box are defined by the lower quartile (25th percentile) and the upper quartile (75th percentile). The upper and lower boundaries of the whiskers are the maximum and minimum values of the dataset, respectively. Raw data appear in Supplementary Table 3 and OD600nm vs time measurements are included in Supplementary Data 2. **B** Relative fitness of *nfxB* defective clinical strains used in this study. Growth curves of four different clinical strains (258, 296, 357 and 76) were recorded in antibiotic-free medium. The fitness of each isolate was measured as the area under the growth curve. Relative fitness of each clinical strain was calculated with respect to the clinical strain presenting the highest fitness (strain 357). Statistical significance was computed by one-way ANOVA followed by Dunnett's multiple comparison test where ** ($p = 0.0014$), **** ($p < 0.0001$). Relative fitness is importantly lower in the isolate 76, which is one of the isolates where compensatory evolution of fitness cost clearly occurs. Raw data appears in Supplementary Table 4. The mean ± SD of values from three replicates is represented.

results show that compensatory evolution of fitness costs associated with AMR is strongly contingent on the set of mutations originally present in each parental strain and that it is more important than the initial fitness cost in constraining compensatory evolution. Crucially, although decline of resistance is dependent on historical contingency, we observed a robust decline of ciprofloxacin resistance (below the EUCAST clinical breakpoint) after just 100 bacterial generations in 66% of the populations evolved in antibiotic-free environments. Although a deeper analysis will be required to know the extent to which these results are generalisable and if it is possible to apply this therapeutic strategy, this work shows the most robust and fast decline of resistance identified to date in clinical strains presenting prevalent mutations.

As we hypothesised, decline of ciprofloxacin resistance in antibiotic-free environments, in the presence or absence of dequalinium chloride, occurred. From a genetic point of view, a decline of AMR can occur by a molecular[47] or phenotypic reversion of resistance[35,40]. In the first case, it is associated with the restoration of the wild-type allele. In the second, it is due to the acquisition of new genetic variations. Here, by whole-genome sequencing of all the populations presenting a decline of ciprofloxacin resistance, we identified compensatory mutations responsible for this phenotype. We found no reversion of original *nfxB* mutations to the wild-type sequence. In fact, compensatory mutations in the genes encoding the MexCD-OprJ efflux pump, such as *mexC*, whose expression is regulated by NfxB, seem to be responsible for the decline of ciprofloxacin resistance observed in these clinical strains. It has been previously stated that reversion to the wild-type allele occurs less frequently than compensatory mutations in secondary genes[40,41], since the mutational space to inactivate a secondary gene is larger than the one required to restore the wild-type allele in a mutant[30]. In addition, secondary compensatory mutations may also be selected because their fitness is higher than that of the wild-type. Here we show that the reason for the selection of secondary compensatory mutations in *mexC*, instead of reverting the original *nfxB* mutation to the wild-type allele, does not rely on a higher fitness of the first. Hence, our results support that mutational space that allows *mexC* inactivation is larger than that leading to the wild-type allele in the *nfxB177* mutant.

Altogether, these results suggest that ciprofloxacin resistance of clinically relevant *nfxB* defective mutants may be rapidly compensated in the absence of selective pressure, reverting back to susceptibility. This finding indicates that ciprofloxacin could potentially be reused in a short time after its restriction. However, it is important to remark that ciprofloxacin resistance is not only associated with *nfxB* loss-of-function mutations but, depending on the starting parental strain, can also be due to mutations in *mexS* and/or in *gyrA/B*[20,26]. Therefore, our results should be taken with caution, and a deeper analysis will be required to know the extent to which it is possible to apply this therapeutic strategy, based on exploiting the rapid decline of ciprofloxacin resistance, regardless of the ciprofloxacin pre-existing mutants causing the infection. This will be particularly relevant for persistent bacterial infections, because different resistant mutants can co-exist in a patient. It is also important to highlight that our results should not be generalised to other antibiotics. For that, new ALE assays would be required to detect prevalent resistance mutations that can be easily compensated, causing a robust decline of AMR during periods of drug restriction[55].

Further, CS has been explored as another trade-off potentially exploitable to tackle AMR. To be exploited, CS must be robust and emerge in different genetic backgrounds[20,26,59], since infections caused by *P. aeruginosa* in pwCF may contain different pre-existing resistant mutants[54,60]. In addition, an important requirement is that CS must be stable when antibiotic therapy ceases. The stability of CS, which has practically not been studied to date, is relevant for the implementation of evolutionary therapies based on the alternation or combination of drugs showing CS. We observed that

compensatory evolution of fitness costs associated with ciprofloxacin resistance in antibiotic-free environments did not generally alter pre-existing CS to tobramycin. Therefore, it seems reasonable to propose implementation of periods of drug restriction after the application of ciprofloxacin and a switch back to ciprofloxacin, in particular when resistance to this antibiotic is due to loss-of-function mutations in *nfxB*. However, since preservation of CS to tobramycin was not robust, it is uncertain if the combination ciprofloxacin-tobramycin, which we previously proposed for the treatment of *P. aeruginosa* infections[20,23,26], would be effective after a period of ciprofloxacin use restriction.

Finally, the application of cycling policies has been unsuccessful in some reports[61,62]. One of the reasons may be that these programs are chosen blindly, considering that any AMR mutation can be costly and assuming that AMR will disappear under exposure to the subsequent antibiotic[63]. Our purely evolutionary results allow us to inform clinicians about the possibility of using periods of restriction after the use of ciprofloxacin, especially if mutations in *nfxB* have been previously selected, since it renders decline of AMR. This may not be the case for other antibiotics, which indicates that, when deciding an antibiotic therapy for persistent bacterial infections, priority should be given to those drugs for which drug restriction periods are a real possibility. This approach could enhance the likelihood of using the same antibiotic later, even if the initial treatment fails and resistance develops, thus preserving its efficacy. We believe that the time has come to translate evolutionary knowledge into practical therapeutic strategies.

Methods

Growth conditions and antibiotic susceptibility tests

Bacteria were grown in Lysogeny Broth (LB) (Lenox, Pronadisa) at 37 °C and shaking of 250 rpm, in glass tubes. Antibiotic susceptibility was determined for ciprofloxacin, tobramycin, imipenem, ceftazidime, aztreonam, and fosfomycin at 37 °C, in Mueller-Hinton (MH) agar, using E-test strips (MIC Test Strip, Liofilchem®).

Adaptive laboratory evolution experiments

Independent bacterial populations from stock cultures of 8 different ciprofloxacin-resistant *nfxB* defective clinical strains of *P. aeruginosa* from the Copenhagen CF Centre, Department of Clinical Microbiology at Rigshospitalet, Denmark (Table 1)[19] -4 replicates of each-, were subjected to ALE in the absence of antibiotic or in the presence of 10 μg/mL of dequalinium chloride during 100 bacterial generations, resulting in 64 bacterial populations. Cultures were grown at 37 °C and 250 rpm for fifteen days in independent glass tubes to avoid cross-contamination. Every day, the cultures were diluted (1/100), adding 10 μl of bacteria in 1 mL of fresh LB, either containing or lacking 10 μg/mL of dequalinium chloride. Each condition was maintained during the entire ALE. At the end of the experimental evolution, all the replicate populations were preserved at −80 °C. In addition, MICs of the antibiotic to which the clinical isolates were initially resistant (ciprofloxacin), as well as to other and varied antibiotics, were determined at 37 °C in MH agar, using E-test strips.

Growth measurements

Growth curves were obtained by inoculating overnight cultures, in triplicate, to a final $OD_{600\ nm}$ of 0.01 in LB in a 96-well microtiter plate (Nunclon™ Delta Surface). $OD_{600\ nm}$ of the bacterial cultures was measured every 30 min during 20 h at 37 °C using a Spark 10 M Plate Reader (Tecan). Fitness (W) was determined as the area under the growth curve recorded in antibiotic-free medium. Fitness cost of each clinical strain population was calculated with respect to its parental strain as previously stated[40], using the equation: 1- ($W_{mutant}/W_{parental\ strain}$) and was expressed as percentage.

Whole-genome sequencing and analysis

The extraction of the genomic DNAs of the 8 parental strains and the 64 evolved populations, as well as DNA quality analysis was performed by the Translational Genomics Unit (Instituto Ramón y Cajal de Investigación Sanitaria-Hospital Ramón y Cajal from Madrid). Genomic DNA of each population was extracted by Chemagic™ DNA Bacterial Kit H96 (CMG-799 Chemagic™) using the equipment Chemagic™ 360/MSMI (PerkinElmer). The quantity and quality of the DNAs were evaluated using QUBIT 1x dsDNA BR 500rx (Thermofisher, Cat. Q33266) and QUBIT 1x dsDNA HS 500rx (Thermofisher, Cat. Q33231), by Qubit 2.0 Fluorometer Invitrogen (Thermofisher) and Agilent 4150 TapeStation System. Libraries construction and Whole Genome Sequencing were performed by Illumina ADN Prep Kit (Illumina). The sequencing was done using an Illumina SP300 (SP Reagent kit 2×150 nt reads) on the NovaSeq6000 system (Illumina Technology) in Healthincode (Valencia, Spain). Coverage was higher than 150x for all samples.

Sequencing reads were trimmed, and low-quality reads and potential contamination from adapters were removed using Trimmomatic (v 0.35) tool (Bolger et al., 2014). Genomic analysis was conducted by BacDist (Migle Gabrielaite and Maanmi, 2020) to identify genomic variants relative to PAO1 reference genome (NCBI: NC_002516.2) in the clinical strains or PA14 to identify genomic variants in the strain *nfxB177* (NCBI: NC_008463). BacDist filtered mutations to only retain variants with a mapping quality of at least 50, a minimum coverage of 10, and a minimum fraction of 50% of reads supporting the variant. To evaluate the presence of populations with reads below the 50% of reads thresholds, manual curation was applied after inspection using IGV. A minimum coverage of 10, and a minimum fraction of 50% of reads supporting the variant were maintained as cut-offs for the analysis. Manual inspection was only applied to *nfxB*, *mexC*, *mexD* and *oprJ* genes and the intergenic region between *nfxB* and *mexC*. The list of identified mutations is present in Supplementary Table S2.

Competition experiments

Overnight bacterial cultures from *nfxB177mexC*, *nfxB177* and the parental strain PA14 were normalised to an OD_{600nm} of 4.0 and then mixed in a 1:1 (*nfxB177mexC*: *nfxB177*, PA14: *nfxB177* and PA14: *nfxB177mexC*) ratio, in triplicate. Cultures were grown at 37 °C and 250 rpm for 15 days. Every day the cultures were diluted (1/100) in fresh LB. The determination of colony-forming units of *nfxB177mexC* and *nfxB177* in each competing population was carried out by plating dilutions of the populations containing these strains in LB and LB supplemented with 0.1 μg/mL of ciprofloxacin. The determination of colony-forming units of PA14 in each competing population was carried out by plating dilutions of the populations containing this strain in LB and LB supplemented with 0.2 μg/mL of tobramycin.

Statistical analyses

All data were checked for normality using the Shapiro-Wilk and Anderson-Darling tests. For MIC data, which did not pass the normality test, differences between strains, evolution (LB or DC) and antibiotics were computed using the non-parametric Kruskal-Wallis (Rank Sums) test, followed by each-pair comparison test using the Wilcoxon method. Data were further corrected for multiple comparisons using the FDR (False Discovery Rate) method. Data from the independently evolved populations were used as biological replicates. Only differences in MICs with adjusted p Values of <0.05 and MIC differences relative to the starting strain with a fold-change of > ±2 were considered statistically ($p < 0.05$) and biologically (fold-change > ±2) significant. Statistical analyses were conducted using JMP Pro 17 version 17.2.0. Fitness data were analysed by parametric one-way ANOVA followed by Dunnett's multiple comparison test. Three independent biological replicates were used for each experiment. Statistical analyses were conducted using GraphPad Prism 10 version 10.4.0.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
Whole genome sequencing data of the evolved populations is publicly available through the SRA number PRJNA1158267. Data needed to evaluate the conclusions of this work are present in the manuscript, Supplementary Materials and Supplementary Data. Source data are provided with this paper.

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Author contributions

SHA designed the study and performed the experimental work. PL and RLR performed the bioinformatics analysis of the whole genome sequence data. JLM, PL, RLR, HKJ and SM participated in the design of the study. All authors participated in writing the manuscript and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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