Potential for Adaptation Overrides Cost of Resistance

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ABSTRACT Aim: To investigate the cost of antibiotic resistance versus the potential for resistant clones to adapt in maintaining polymorphism for resistance. **Materials & methods:** Experimental evolution of *Escherichia coli* carrying different resistance alleles was performed under an environment devoid of antibiotics and evolutionary parameters estimated from their frequencies along time. **Results & conclusion:** Costly resistance mutations were found to coexist with lower cost resistances for hundreds of generations, contrary to the hypothesis that the cost of a resistance dictates its extinction. Estimated evolutionary parameters for the different resistance backgrounds suggest a higher adaptive potential of clones with costly antibiotic resistance mutations, overriding their initial cost of resistance and allowing their maintenance in the absence of drugs.

KEYWORDS: Evolvability; Antibiotic Resistance; Experimental Evolution; Epistasis; Clonal Interference;

INTRODUCTION

Bacterial populations can acquire antibiotic resistance (AR) as a result of transfer and acquisition of new genetic material between individuals of the same or different species but also by chromosomal DNA mutations, which alter existing bacterial proteins. One landmark

example of this second process is provided by *Mycobacterium tuberculosis* (the etiologic agent of tuberculosis in humans), which albeit incapable of horizontal gene transfer can display "total drug resistance" [1]. This kind of resistance is typically acquired by the sequential accumulation of mutations that alter the cellular target for the drug action. During this process extensive clonal competition has also been observed [2]. Understanding how these AR determinants disseminate and are maintained in bacterial populations is therefore of paramount importance.

Mutations that confer spontaneous AR can occur at relatively high rates [3]. For instance, rifampicin (Rif) resistance occurs spontaneously at frequencies that can be higher than 10^{-8} in wild isolates of *E. coli* [4]. In fact, the levels of resistance in pathogenic populations continue to rise at an alarming rate [5,6] having reached the same significance as any other virulence factor [7]. However, in the absence of the drug, AR mutations typically bear a cost [8-10]. This cost depends on the specific resistance allele [11], on the environment [12,13] and on the genomic background where the mutation happens to arise [14,15]. Nevertheless, suppressive mutations that mitigate this cost can occur either in the presence or absence of antibiotics. In the absence of drugs, one can expect that sensitive bacteria, will sweep through the population, driving the AR mutant to extinction. However, more often than not, resistant strains have been observed to acquire additional beneficial mutations that reduce the costs of resistance without loss of resistance, thus preventing the elimination of resistance alleles [16]. These compensatory mutations are common in clinical isolates [17,18] and hinder the possibility of reverting the resistance mutation, due to their epistatic nature [19]. Since the probability of a compensatory mutation tends to be much higher than that of reversion for several resistance mutations [10,20-23], it is likely that resistance alleles may take long periods of time to be eliminated from populations.

Many mutations that confer AR occur in essential genes and are likely to result in pleiotropic effects altering several bacterial traits. Important examples include resistance to Rif and streptomycin (Str) [24,25]. Rif is one of the frontline anti-tuberculosis drugs [26]. The co-occurrence of resistance to this antibiotic and isoniazid typically classifies *M. tuberculosis* as multi-drug-resistant. These are the two most commonly used and effective drugs for the treatment of tuberculosis. The main genetic target for Rif resistance is *rpoB,* which codes for the β subunit of the RNA polymerase. Besides typically decreasing the rate of transcription and consequently the growth rate, *rpoB* resistance mutations are probably some of the most pleiotropic among AR mutations. Their effects can range from regulation of competence, sporulation and germination in *Bacillus subtilis* [27], temperature and phage sensitivity in *E. coli* [28], growth advantage in stationary phase in *E. coli* and *Salmonella enterica* [29], increased antibiotic resistance in *Staphylococcus aureus* [30,31], amongst others (see [26] for a review). *rpoA* and *rpoC*, as well as additional mutations in *rpoB,* have been pointed as targets for compensatory mutation relieving the deleterious effects of *rpoB* Rif resistance mutations in *M. tuberculosis*, *S. enterica* and *E. coli* [17,32-34]. Another frequent resistance is Str, occurring through mutations in the *rpsL* gene, which codes for the S12 subunit of the ribosome compromising translational speed and accuracy. Compensatory mutations for Str resistance have been observed in *Salmonella thyphimurium*, and some of the target genes include *rpsL*, *rpsD, rpsE* and *rplS* [12,35,36], which encode the ribosomal subunits S12, S4, S5 and L19. Recent analysis of 161 genomes of *M. tuberculosis* with a broad range of resistance profiles, revealed seven possible additional targets for compensatory mutations to Str resistance [37].

Given such broad targets for increasing the fitness of AR mutants we may expect that these resistances change the evolvability of bacteria, through altering the range of beneficial mutations that can be accessed by a given resistance genotype. Here we use the term

74 evolvability as “the genome’s ability to produce adaptive variants” [38]. In this sense, a costly
75 AR mutant may be able to effectively compete with a less costly resistance allele (*i.e.*, with a
76 higher selective coefficient when in direct competition) if the evolvability of the former is
77 higher. The conditions for this to happen will depend on the distribution of effects of beneficial
78 mutations (DEBM), as well as on the rate of beneficial mutations available to each genotype
79 [22,39,40].
80 Most studies addressing the competitive fitness effects associated with AR, consider resistant
81 strains competing with the ancestral sensitive strain. However, in many natural environments
82 the frequency of resistance can be very high and competition between different resistant alleles
83 may be a common event [2,37,41-45]. For instance in [2], a monoclonal *Mycobacterium*
84 *tuberculosis* infection was followed and both clonal sweeps and the coexistence of different
85 resistant mutants were observed in the dynamics of the population. Competition between
86 different resistant strains is also likely to occur whenever there is spatial heterogeneity, with
87 different areas posing different selective pressures [2,46,47]. Furthermore, bacteria with
88 multiple resistance alleles are also commonly segregating in natural populations [48-50]. This
89 competitive context might therefore play a crucial role in the maintenance of antibiotic
90 resistance.
91 Here we study the process of fitness recovery mimicking an environment with different
92 resistance backgrounds competing at high frequency. We use an experimental evolution
93 approach [51] to test the ability of clones with costly AR alleles to coexist or even outcompete
94 clones with less costly resistances. In a drug free environment, the differences in the fitness
95 costs of resistance alleles should determine their probability of extinction. Contrary to this
96 simple expectation, we observe the maintenance of costly resistance alleles over hundreds of
97 generations even when their fitness impairment should predict fast extinction. We infer that

differences in adaptive potential for each AR mutant exist and suggest that these can explain the observed outcomes in the evolution of resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All bacterial strains used in this study were derived from *Escherichia coli* K12 MG1655 and have in common the following genotype: *galK::yfp/cfp* cm^R (pKD3), Δ*lacIZYA*. The yellow (*yfp*) and cyan (*cfp*) alleles were integrated at the *galK* locus under the control of the *lac* promoter and were constructed by P1 transduction[52] of *yfp/cfp* inserts from previously constructed strains [53]. In these strains the fluorescence marker is constitutively expressed. In this common backbone different antibiotic resistance mutations were introduced by P1 transduction. The donor strains were spontaneous antibiotic resistant mutants previously obtained by plating the sensitive bacteria in Luria-Bertani (LB) supplemented with agar and 100 mg/mL of either streptomycin or rifampicin [14]. A total of eight strains were constructed: *K43T*^{Str}-YFP, *S531F*^{Rif}-YFP, *H526Y*^{Rif}-YFP, *H526D*^{Rif}-YFP and *K43T*^{Str}-CFP, *S531F*^{Rif}-CFP, *H526Y*^{Rif}-CFP, *H526D*^{Rif}-CFP, such that the same resistance allele was introduced in the two fluorescence backgrounds. *K43T*^{Str} confers resistance to streptomycin and all the other aminoacid changes confer resistance to rifampicin. In order to confirm the identity of the mutations transferred by P1 transduction, the antibiotic resistance target gene (*rpoB* for rifampicin or *rpsL* for streptomycin) was amplified and sequenced using the following primers: for the relevant fragment of the *rpoB* gene, 5'-CGTCGTATCCGTTCCGTTGG-3' and 5'-TTCACCCGGATAACATCTCGTC-3' and for the *rpsL* gene, 5'-ATGATGGCGGGATCGTTG-3' and 5'-CTTCCAGTTCAGATTTACC-3'. Each resistant

clone was grown from a single colony in LB medium supplemented with the respective antibiotic at 37ºC with aeration and stored in 15% glycerol at - 80°C.

Fitness assays and test for frequency dependent selection

The fitness costs of antibiotic resistance mutations were first measured in competition against a sensitive reference strain (Table S1). The reference strain carried a *yfp* allele if the resistant strain carried the *cfp* allele (and vice versa). Competitions were done after acclimatization, where each bacterial strain was grown in the same environment of the competition: in a 96 well plate with 150µL of LB per well at 37°C with aeration. Acclimatization consisted of two consecutive passages where 5µL from the first 24 hour grown culture were used to inoculate a new plate for another 24h. Competitions were performed by inoculating ~10^5 cells of both competitor and reference strain in LB medium and allowed to grow for 24 hours (~9 generations). The initial and final ratios of both strains were determined by Flow Cytometry. Fitness effects of the resistance mutations were estimated as the slope between 0 and 24h of the $\ln(f(N_R)/(1-f(N_R)))$, where $f(N_R)$ is the frequency of resistant bacteria in the population or one of the reference resistance backgrounds, in the case of the competitions of the evolved clones. *H526Y*^{Rif} and *H526D*^{Rif} strains were tested for negative frequency dependent selection in the same conditions as described above, and the number of cells was measured using the Flow Cytometer BD LSR Fortessa (BD Biosciences), at different initial ratios of the two strains: 100:1, 10:1, 1:1, 1:10 and 1:100 (*H526Y*^{Rif}:*H526D*^{Rif}).

Long-term propagation of resistant populations

Prior to the start of the long-term competitions, acclimatization of the bacterial strains was performed in plates with a checkered arrangement (one plate for YFP strains, another for CFP

strains). Each well was inoculated with an independent starting sample, from a frozen culture, into 150µl of LB medium, to maximize the probability of sampling a large pool of beneficial mutations. We note that it is possible that beneficial mutations occur during the acclimatization period, since the rate of mutations which compensate for the cost of resistance can be very high [22]. After 24 hours, 5µl of the grown cultures where inoculated into fresh LB media and after 48h the numbers of bacteria were measured. Appropriate dilutions were done to achieve the required initial ratio (1:1) of YFP and CFP strains for the long-term evolution of competing clones. This evolution was performed in the same conditions as the fitness assays, with daily passages of about ~10^5 bacteria for ~280 generations (30 days). Samples of the evolving populations were frozen every day, from which the relative abundance of each resistance was followed by measuring the frequencies of their linked fluorescent alleles by Flow Cytometry. The following three pairs of mutants were studied: *K43T*^{Str} *vs S531F*^{Rif}, *H526Y*^{Rif} *vs H526D*^{Rif} and *H526Y*^{Rif} *vs S531F*^{Rif}. For each pairwise competition of mutants, 16 replicas were performed: half where one of the resistances, say R1, was linked to the *yfp* background and the other half where it was linked with the *cfp* background. Pairwise combinations of mutants (R1-YPF vs R2-CFP and R1-CFP vs R2-YFP) were settled in a checkerboard arrangement (Figure 1), where half of the wells were filled solely with LB to control for external contamination.

Estimation of relative parameters of beneficial mutations

The dynamics of a given costly resistance allele (R1) when competing with another resistant clone (R2), with a different fitness cost, were analyzed under a simple model of positive selection, that assumes the occurrence of new beneficial alleles which are sweeping towards fixation. We first fitted the simplest possible model (Model 1), which can allow for a costly resistance allele to be maintained for hundreds of generations despite its initial cost. In this

model we assume an initial population composed of two distinct genotypes, with different resistances and initial fitnesses, w_{R1} and w_{R2}. The initial frequency of the more costly genotype, $R2_0$, is taken from a uniform distribution within the interval $R2_{Experimental0} \pm 0.1$, while that of the other genotype is $R1_0=1-R2_0$. At generation T_N a new genotype, with fitness w_N, is assumed to have arisen and reached a frequency $N_0=0.001$. From that time onwards it is assumed to change in frequency deterministically towards fixation. Therefore, we modeled selection deterministically in discrete time with two genotypes for $t<T_N$, and three for $t>T_N$. We estimated, by maximum likelihood, the parameters $R2_0$, T_N and w_N that best fit the observed values of ln(M/(1-M)), where M is the measured frequency of a fluorescent marker, at several time points, assuming a normal distribution for measurement error (with average 0 and standard deviation 0.2). The search for the set of parameters that maximize the likelihood of the data in the space of the possible parameters was performed using the Nelder-Mead Method, as implemented in Mathematica 8.0 (http://mathworld.wolfram.com/Nelder-MeadMethod.html), with 100 iterations and repeated for 100 realizations with different initial starting combinations of parameter values. While Model 1 could provide a reasonable fit for some of the replicate experimental lines, for others it did not. We therefore fitted the next simplest model (Model 2) which assumes that two beneficial mutants (N1 and N2), one for each background and fluorescent marker (with fitnesses W_{N1} and W_{N2}), emerged at times (T_{N1} and T_{N2}). We selected the model with the lower AIC (Akaike Information Criteria) to test if the different resistances would have distinct evolvabilities. Specifically, for each pair of competing resistances and each experimental evolved population, we asked if the times of appearance of beneficial mutations or the effects of accumulated beneficial mutations were significantly different. If the genetic target for acquiring beneficial mutations is larger for resistance background R2 than for R1, but the effects of the compensatory mutations are similar, then we expect the time of appearance of

mutations to be smaller in R2 than in R1. If the target is similar but the effects depend on the background we expect to observe a significant difference between the effects accumulated in one resistance background versus the other. The method used here is a discrete adaptation of existing methodologies to infer evolutionary parameters [54], with the added difference of allowing for different initial fitness values in competing genetic backgrounds. Code for these simulations is available upon request. The inference process and summary relative effects are shown for a single line, as an example, in Figure S1.

Whole genome sequencing of H526Y^{Rif} *and S531F*^{Rif} *evolved clones*

A single clone of each resistance (*H526Y*^{Rif} and *S531F*^{Rif}) from each evolved population was isolated in LB agar plates with rifampicin at the end of the evolution experiment. In six populations the frequency of clones from the *S531F*^{Rif} background was very low and no resistant clone was sampled. DNA was extracted from each sampled clone, and then pooled according to resistance background. Paired-end sequencing using Illumina MiSeq Benchtop Sequencer, with mean coverage of 207x was performed. The resulting reads were trimmed at a Phred quality score of 99.9%, and were then aligned using *Escherichia coli* K12 MG1655 (NC_000913.2) as the reference genome. Mutation prediction was done using version 0.23 of BRESEQ pipeline (http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing), with polymorphism detection on. Other settings were used as default except for: a) requirement of a minimum coverage of 3 reads on each strand per polymorphism; b) polymorphism predictions occurring in homopolymers of length greater than 3 were discarded; c) polymorphism predictions with significant (p-value<0.05) strand or base quality score bias were discarded.

RESULTS

Pairwise competitions between different AR alleles were performed in an antibiotic free environment for around 280 generations. Each strain carries a neutral fluorescence marker. Three pairs of resistance mutations were studied: 2 pairs involving 3 different Rif resistance alleles, and one pair involving a Str and a Rif resistance allele (Figure 1). The costs of each resistance allele measured against a sensitive reference strain of *E. coli* are shown in Table S1. The cost of resistance in relation to the sensitive is higher for the $S531F^{Rif}$ mutation (0.1 ± 0.01) and the $K43T^{Rif}$ mutation (0.09 ± 0.03), followed by $H526Y^{Rif}$ (0.07 ± 0.01) and $H526D^{Rif}$ (0.06 ± 0.02) mutations. We then inferred the fitness cost of the same resistance mutations but this time in the presence of another resistance. Whether a resistance allele was more or less costly was estimated from the change in frequency of the fluorescent markers, linked to the resistances, during the first three days of evolution where the pairs of AR mutants were competing (Figure 2, 3 and 4, and also Figure S2 for each of the replicates identified by its neutral marker). We assume that during this short period new beneficial mutations have not yet arisen, or are at a frequency too low to interfere with the relative fitness differences that both clones may have.

For each pairwise evolution experiment we query whether different AR mutants show distinct adaptive potential. For that, we inferred the fitness effects of new beneficial mutations that could explain the observed long-term frequency dynamics of the AR alleles. We allowed for one or two beneficial mutations of different effects to emerge at different times, in either of the backgrounds (see Methods). We sought to infer the combination of parameters (time of emergence, effect of the beneficial mutation, and the initial frequency of one of the resistance alleles) that provided the best fit to the observed evolutionary dynamics. We started by fitting

a model where a single new beneficial mutation (with effect S_N, at time T_N) escapes stochastic loss in the background with lower initial fitness (Model 1). We estimated the value of S_N and T_N that best fits the dynamics of each resistance, under this model. We then used a model assuming that two beneficial mutations had increased in frequency, one in each background, and fitted the parameters of this second model (T_{N1}, S_{N1}, and T_{N2}, S_{N2}) to the dynamics observed. The lines shown in Figure S3 represent the model that best fits the data, and the inferred parameters are presented in Table 1, 2 and 3.

Potential for adaptation causes the maintenance of high cost resistance

We first studied the fate of two AR mutations, conferring resistance to the same antibiotic – Rif – and altering the same aminoacid in the β subunit of RNA polymerase: *H526Y*^{Rif} and *H526D*^{Rif}. While the costs of these resistances are not statistically different when measured against a sensitive strain, their relative fitness cost differs when they compete. From the initial generations of competition between these two strains, a fitness difference of 0.074 was inferred, with strain *H526Y*^{Rif} being highly detrimental in this competitive environment (Figure 2A, black solid line). The cost carried by strain *H562Y*^{Rif} leads to the rapid decline in its frequency, detected in the first tens of generations. With such a relative fitness cost, it should go extinct in 100 generations (as indicated by the black line). Strikingly though, *H526Y*^{Rif} is clearly able to resist extinction in the majority of the replicate lines that were evolved. Four of the lines show a frequency too low to be reliably detected by Flow Cytometry. However, plating these populations at generation 280 allows observing the presence of fluorescent colonies of the *H526Y*^{Rif} background at extremely low frequencies. This lack of extinction can be expected under two different scenarios: negative frequency-dependent selection and/or an ability to

265 access higher effect beneficial mutations. We therefore tested for a possible signature of
266 negative frequency-dependent selection, i.e., advantage from rarity of the *H526Y*^{Rif} mutation
267 when competing against *H526D*^{Rif}. In competitions between these two backgrounds, starting
268 with different frequencies of the *H526Y*^{Rif} allele, no advantage from rarity can be detected
269 (Figure S4), so frequency-dependent selection is unlikely to be responsible for the observed
270 lack of extinction of this mutation. On the other hand, analysis of the long-term frequency
271 dynamics revealed a significant difference between the fitness effects of the mutations inferred
272 to have emerged, with its median difference (s*H526Y*^{Rif} – s*H526D*^{Rif}) deviating from 0 (P<0.001,
273 Wilcoxon Signed Rank Test, Figure 2B). The mean difference in fitness effects between these
274 two genetic backgrounds was 0.07 (Table 1, Figure 2B), with *H526Y*^{Rif} background
275 accumulating stronger effect mutations. Regarding the times of appearance of new beneficial
276 mutations, no significant difference was detected between the backgrounds (P=0.41, Wilcoxon
277 Signed Rank Test, Figure 2C). When considering these results together, there is a strong
278 indication that the mean effect of beneficial or compensatory mutations for *H526Y*^{Rif} is higher,
279 thus qualifying the strain with the *H526Y*^{Rif} mutation as more evolvable than the strain with the
280 *H526D*^{Rif} mutation. Such higher evolvability can explain the avoidance of extinction of
281 background *H526Y*^{Rif}, contrary to the *a priori* expectation based on its lower initial fitness.
282
283 *Higher evolvability of H526Y*^{Rif} *upheld in competition with an alternative resistance*
284
285 We then studied the ability of the *H562Y*^{Rif} background to outcompete a mutant bearing a
286 resistance mutation in a different aminoacid, *S531F*^{Rif}. Resistance to the antibiotic rifampicin
287 is now provided by mutations causing different aminoacid substitutions in the β subunit of RNA
288 polymerase. . Relative to the sensitive strain, *H526Y*^{Rif} is estimated to impose a smaller mean

fitness cost than *S531F*^{Rif} (see Table S1), although the difference is not significant. The relative fitness difference between strains *H562Y*^{Rif} and *S531F*^{Rif} inferred from their direct competition is 0.015 (Figure 3A, black solid line). All lines initially decrease in frequency, in accordance with a cost of *H526Y*^{Rif}, but in all replicates this tendency is inverted. After generation 30, the frequency of each of the resistance background stabilizes at 50% in all populations. After generation 125, additional changes in frequency can be detected. This is expected if other arising beneficial mutations increase in frequency in one or both backgrounds. In some replicate lines one of the resistance alleles starts to increase in frequency but later decreases. This is likely the result of clonal interference [55], with multiple beneficial mutations competing amongst them. There were more replicates in which *H526Y*^{Rif} increased in frequency than expected, even under the assumption that the initial fitness difference between the backgrounds would be negligible. At generation 280, 14 out of 16 lines have a frequency of this allele above that expected by chance ($p=0.004$, Binomial two-sided test), which is even more striking considering that its initial frequency is below 50% in most of the lines. This deviation suggests again differences in the adaptive potential between the resistance backgrounds.

We tested for possible differences in the adaptive potential of each strain by inferring the time of appearance and fitness effects of new beneficial mutations that could explain the changes in frequency of the neutral markers (Figures 3B and 3C). The mean value for differences in fitness between the two genetic backgrounds was 0.034 (see Figure 3B and Table 2). The median of the distribution of relative effects deviates significantly from 0 ($P<0.001$, Wilcoxon Signed Rank test, Figure 3B). The times of appearance of the new inferred beneficial mutations, however, did not appear to be significantly different between the genetic backgrounds ($P=0.3$, Wilcoxon Signed Rank test, Figure 3C). The overrepresentation of the *H526Y*^{Rif} resistance background in the evolving populations, together with this analysis, indicates that *H526Y*^{Rif} has

313 a higher evolvability across different competitive contexts and therefore can be easily
314 maintained.
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316 *Potential for adaptation drives the fate of resistances to different antibiotics*
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318 Differences in evolvability are expected to be larger amongst resistances affecting different
319 genes than between alleles from the same gene. This is so because the target for beneficial
320 mutations is expected to be more similar between mutations affecting the same function than
321 between mutations impairing different traits. To query if the costs or the evolvabilities are
322 determinant to the competition of resistances to distinct drugs, we studied the fate of the Str
323 resistance allele ($K43T^{Str}$) when in competition with the Rif resistance allele ($S531F^{Rif}$). $K43T^{Str}$
324 is estimated to impose a cost to the sensitive strain of ~0.09, which is not statistically different
325 from the cost imposed by $S531F^{Rif}$. However, upon competition between these two strains,
326 $K43T^{Str}$ shows a disadvantage of around 0.03 relative to $S531F^{Rif}$ (Figure 4A, black solid line).
327 This is observed in the initial 25 generations, where $K43T^{Str}$ decreases in frequency in most
328 replicate competitions. The long-term evolutionary dynamics, however, depart from those
329 observed in the previous studied cases. In Figure 4A, a higher variation in the outcome of which
330 resistance wins the competition emerges in this pair of competing resistances. In the vast
331 majority of the replicate lines the $K43T^{Str}$ mutation survived extinction for the duration of the
332 experiment, contrary to what would have been expected from its initial relative fitness cost. In
333 most of the lines, $K43T^{Str}$ is kept at a stable frequency of around 30% and in three lines it rises
334 in frequency, sweeping to majority status (above 99% frequency) by generation 280. Contrary
335 to expectations, extinction (frequency below 1%) of the more costly allele was only observed
336 in two of the populations. Most of the lines where the $K43T^{Str}$ resistance was kept at a stable

but low frequency at the middle time points had different outcomes, with some rising and others decreasing in frequency. Overall, at the end of the 280 generations, the background with the *K43T*^{Str} allele reached a frequency higher than 50% in 6 out of 15 evolved replicate lines. The inference of evolutionary parameters performed for the long-term dynamics indicates that there is a significant difference in the strength of mutations acquired by each background (P=0.014, Wilcoxon Signed Rank Test, Figure 4B). *K43T*^{Str} acquires beneficial mutations of a stronger effect than does the background *S531F*^{Rif} (mean difference 0.03, Table 3). No significant difference was detected for the times of appearance of beneficial mutations between both backgrounds (P=0.6, Wilcoxon Signed Rank Test, Figure 4C). The differences inferred from the dynamics in Figure 4A therefore depart from the expectation of a non-epistatic model of beneficial mutations, where both backgrounds would access mutations of similar effect. Extinction might have been avoided for this Str resistance mutation by its ability to accumulate stronger effect mutations when competing with the Rif resistant strain.

Genetic characterization and fitness determination of evolved clones isolated from the long-term competition between H526Y^{Rif} *and S531F*^{Rif}

In the three cases of long-term competitions studied, the differences in evolvability inferred resulted from differences in the effects of the beneficial mutations acquired. We inferred that *H526Y*^{Rif} could acquire higher effect mutations, relative to those emerging in *S531F*^{Rif} clones, even though these two different alleles cause very similar fitness costs. To gain further insight into the mutations acquired by the *H526Y*^{Rif} background in this competitive context, we performed whole genome sequencing (see Methods for a description of the procedure) of a sample of *H526Y*^{Rif} and of *S531F*^{Rif} clones, after these two lineages had evolved in competition

(Figure 3). Table 4 shows the mutations identified in each of the evolved backgrounds. We could identify 12 (in 10 distinct genes or intergenic regions) for possible beneficial (and compensatory) mutations in *H526Y*^{Rif} (all single point mutations), and 10 (all in different genes/intergenic regions) in *S531F*^{Rif} (seven of them were single point mutations , the other three involved transpositions of insertion sequence elements). Mutations in the *H526Y*^{Rif} background were observed at a higher frequency compared to the ones observed in *S531F*^{Rif}. This suggests that the mutations appearing in the *H526Y*^{Rif} background have stronger effects, since they are acquired by different replicate evolving populations, pointing to a rapid fixation due to their beneficial effect. To enquire if this is indeed the case we performed competition assays between each individual clone and the ancestral strains. Since the whole genome sequencing was performed with a mixture of clones (see Methods) we targeted sequencing each individual clone to gain access to the haplotypic composition of each clone. For the clones carrying the *H526Y*^{Rif} mutation, we targeted the most prevalent mutations detected (see Table 4). Fifteen out of the 16 *H526Y*^{Rif} clones carried at least one mutation in the target genes (Supplementary Table 2) and one clone was found to have two mutations, one single point mutation in *rpoA* and another in *waaZ*. The aminoacid change T196I in *rpoA* was present in 8 independently evolved clones. Since all of them have the same genetic background (*H526Y*^{Rif} YFP), this suggests that this mutation could already have been present prior to the long-term evolution experiment. All *S531F*^{Rif} clones, except one, carried one of the previously identified mutations (Supplementary Table 2) and *rpoC* was also a target for beneficial mutations in this Rif^{R} background. We then measured the competitive fitness of each of these evolved clones to directly assay their fitness advantage. In order to increase in frequency during the long-term propagation, these mutants had to outcompete their ancestral and also the competitor with a different resistance background. Figure 5 shows that when competing either against their

respective ancestral or the other resistance clone, the selective effects of the evolved *H526Y*^{Rif} clones are stronger than the selective effects of the mutations acquired by the *S531F*^{Rif} background. For the latter background, the mutations are advantageous against their ancestral, supporting their increase to detectable frequencies, but most are neutral or even deleterious versus the opposite *H526Y*^{Rif} background. Remarkably, mutations in *rpoA* and *rpoC* provided the strongest competitive fitness advantages. Overall, these results therefore provide further support for the previously identified higher evolvability of the *H526Y*^{Rif} background and strongly suggest that the *H526Y*^{Rif} resistance may be easily maintained in populations.

DISCUSSION

In order to understand the relative role of cost versus evolvability in the maintenance of AR mutations we studied how subpopulations carrying different resistant alleles compete. This system mimics the composition of a population after drug exposure, where the different AR mutants co-exist at relatively high frequencies. This scenario has rarely been studied, though it possibly occurs in natural contexts, as has been observed in strains sampled from the same patient [2,45]. Here we address this situation by following the fate of pairwise combinations of different alleles conferring resistance to the same or different drugs. We observe that the cost of resistance measured in competition against the sensitive bacterium is not always a good predictor of the difference in costs between two resistance mutations, and transitivity between these two fitness measures is not always observed. Therefore, to understand why some resistant alleles are rarely segregating while others are pervasive, it is important to also measure their selective coefficients when in coexistence in addition to measure their costs against the sensitive strain.

In this work we studied two sets of mutations whose fitness costs were barely distinguishable when compared with the sensitive strain. The first set comprised 3 alleles of the same gene (*H526Y*^{Rif}, *H526D*^{Rif} and *S531F*^{Rif}), thus conferring resistance to the same drug; the second group comprised alleles of different genes, (*S531F*^{Rif} and *K43T*^{Str}), thus conferring resistance to different antibiotics. In the first set we found that *H526Y*^{Rif} has a very significant cost (0.074) when competing with mutation *H526D*^{Rif} and it is less costly when competing with *S531F* (0.015), implying that, all else being equal, *H526Y*^{Rif} should rapidly go extinct when competing with *H526D*^{Rif}, but do so at a slower pace when in competition with *S531F*^{Rif}. The long-term outcome of these competitions indicated that this does not always occur and the *H526Y*^{Rif} allele can be maintained in both cases. The evolvability analysis undertaken suggests that the difference between *H526Y*^{Rif} and *H526D*^{Rif} could be attributed to a higher mean effect of beneficial mutations accessible to *H526Y*^{Rif}. This result was unexpected, because this pair of mutations involves the same aminoacid replacement and the different single point mutations cause a fitness defect of similar magnitude. Given this, one could expect similar evolvabilities between the resistance backgrounds [56]. The outcome of the competition between *H526Y*^{Rif} and *S531F*^{Rif} (mutations in different aminoacids but again in the same gene) shows a tendency for the former to increase in frequency, and this could also be due to access of the former background to higher effect mutations, when in comparison with the latter. This observation is further supported by the different competitions between *H526Y*^{Rif} and *S531F*^{Rif} clones, where competitive fitness assays showed stronger effects of the mutations acquired by *H526Y*^{Rif}, as predicted by our theoretical analysis. The two cases of competition between different AR mutations both show a long-term advantage of the *H526Y*^{Rif} allele, through its higher mean effect of mutations. Interestingly, *H526Y*^{Rif} has been reported to be the second most frequent mutation, in a variety of clinical settings, among the ones that confer Rif resistance in *M.*

tuberculosis, even though it was also estimated that this mutation was the third mutation more costly in the same set [57,58]. This could be explained by the particularly high evolvability of this mutation. In the case of the pair of AR mutants composed of *K43T*^{Str} and *S531F*^{Rif}, differences in evolvability were expected *a priori* since these mutations alter genes responsible for different cellular traits. In fact, evolution was less reproducible in this situation, with different replicates following different dynamics. Our results indicate that *K43T*^{Str} acquired beneficial mutations of stronger effect than *S531F*^{Rif}, suggesting that their DEBM and hence their evolvability are different.

The ability to access specific subsets of beneficial mutations determines how evolvable an organism is. If there were no constraints, then all genotypes, regardless of their composition or competitive context, would be able to adapt in a predictable sequence of mutational events. However, pleiotropy and epistasis may limit the access to new beneficial mutations [14,59,60], imposing different evolutionary outcomes in different genetic backgrounds, environmental conditions and/or competitive contexts. This is particularly relevant in the context of AR, as emergence of resistance mutants is fairly common. The resistant clones would presumably be driven to extinction when competing against less costly strains, in the absence of the drugs. Here we show that it is very likely that such extinction events will not occur and, instead, mutations that buffer the effects of resistance alleles will accumulate [36,49,61,62], allowing their maintenance. Our results indicate that different adaptive abilities and the access to strong effect beneficial mutations depends on the genetic background and the competitive context, determining the long-term fate of a given resistance allele.

Antibiotic based treatments focus on immediate clinical results, but the adaptive potential of resistant bacteria is subtle [63,64] and we suggest it can affect their long-term fate within a host

or between hosts. An interesting recent observation suggests that the effects of mutations conferring resistance to streptomycin tend to be smaller for genotypes that are well adapted to a given environment, relative to genotypes not adapted at all [15]. This observation, along with the one we have made here, indicates that it is relevant to determine the relative roles of cost versus evolvability in other environments of special clinical relevance [65], in order to be better able to predict the evolution of pathogens carrying resistance alleles.

CONCLUSIONS

The frequency of antibiotic resistance constitutes an alarming concern for public health. A key factor determining the extinction or maintenance of resistance alleles is the fitness costs they may entail. High cost resistance alleles are expected to rapidly go extinct. However, this may not be an inescapable fate. If the availability of beneficial mutations is dependent on the genetic background, clones with less fit resistance alleles may also have higher evolvability, *i.e.* a higher potential for adaptation. If so, this will lead to the maintenance of resistances with a higher initial cost in populations. Here we perform competitions between strains of *Escherichia coli*, which carry resistance alleles of different costs, and estimate the relative differences in their adaptive potential. We demonstrate that costly resistance alleles can coexist with resistance alleles of lower cost for hundreds of generations, suggesting that their adaptive potential can override the initial relative cost of resistance.

FUTURE PERSPECTIVE

Antibiotic resistance poses an ever-increasing danger to public health, and its maintenance is the result of a multitude of processes, which require increasing evaluation. How the costs of resistance depend both on the specific resistant alleles, the environment where the bacteria grow

and the ecological context to which they are exposed should lead to a better understanding on how resistance can be reduced or avoided. The ability of resistant bacteria for acquiring compensatory mutations and revert to sensitive state across environments should also be evaluated with the help of increasing powerful genomic technics. This is especially important for bacteria carrying multiple resistances as these are becoming more and more common. Assaying the ability of resistance alleles to emerge and thrive in ecologically relevant contexts, which are very likely to include several resistances competing simultaneously and multiple biotic factors will become crucial for our proper understanding of their long-term pathogenicity.

EXECUTIVE SUMMARY

Long-term evolution of polymorphic antibiotic resistance populations

- In all three pairwise competitions studied, the more costly resistance avoided extinction with high probability.
- In the majority of populations the costly resistance did not sweep to fixation.
- The second most frequent rifampicin resistance mutation to segregate in natural pathogen populations ($H526Y^{Rif}$) shows a high cost but also higher resistance to extinction, across multiple competitive contexts.

Estimation of evolutionary parameters

- The distribution of effects for the beneficial mutations depends on the resistance background, with more costly resistance backgrounds acquiring mutations of stronger effects.
- Differences in rate of acquisition of beneficial mutations were not detected between the resistance backgrounds.

Sequencing and competitive fitness of evolved populations

- Whole genome sequencing of the evolving replicate populations with the *H526Y*^{Rif} and *S531F*^{Rif} competing clones revealed that the *H526Y*^{Rif} background acquired more mutations at higher frequencies pointing towards its increased adaptive potential.
- The number of targets identified for beneficial mutations was not significantly different between the resistance backgrounds, supporting the results from the theoretical analysis.
- Competitive fitness assays showed that the mean effects of beneficial mutations is different between these two resistant backgrounds (*H526Y*^{Rif} and *S531F*^{Rif}), as inferred from theoretical modeling.

Conclusions

- The initial relative difference in fitness costs between resistances is not predictive of their long tern evolution.
- The long-term outcome of competitions between pairs of distinct antibiotic resistance alleles is polymorphism for resistance.
- The results indicate that it is crucial to understand the ecological contexts and the adaptive potential of antibiotic resistance mutations in order to make informed clinical decisions regarding the treatment of bacterial infections.

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DATA ACCESSIBILITY

The whole genome sequencing data will be available through the NCBI Sequence Read Archive (SRA) database.

AUTHOR CONTRIBUTIONS

JMS, AS and IG designed the research, analyzed and wrote the paper; JMS, AS and CB performed the experiments.

FIGURE LEGENDS

Figure 1. Testing the evolvability of different antibiotic resistances. First, polymorphic populations are created by mixing, in a 1:1 ratio, clones with two different antibiotic resistance mutations. All clones are isogenic, except for this different resistance mutation and a neutral fluorescent marker. 16 replicate populations are followed for each competition, where half of these replicates have one of the resistances linked with one of the neutral markers, while the other to the other marker. 3 different competitive scenarios were studied, between Rifampicin mutations (circles) and between Rifampicin and Streptomycin (squares). Next, populations are passaged in rich media in 96 well plates, organized in a checkered layout and separated by control wells (with media but without bacteria). Every 24 hours, all populations are passaged serially into a new 96 well plate with fresh media, during 30 days. The frequencies of the neutral markers (and hence the resistance alleles) are analyzed to unravel possible differences in the evolutionary parameters according to the resistance background.

Figure 2. High evolvability of costly *H526Y* allows its long-term maintenance A) Long-term dynamics, for 30 days (280 generations) of evolution in 15 replicates of a population composed of resistance strain H562Y and resistance strain *H526D*. Shown are the dynamics for the *H526Y* background. The slope of the black line represents the initial difference in fitness between the resistances. **B)** Whisker-box shows the relative fitness differences inferred for new beneficial mutations between the two resistant backgrounds, with *H526Y* background as a reference. **C)** Whisker-box shows the relative differences in time of appearance inferred for new beneficial mutations between the two resistance backgrounds.

Figure 3. Different evolvabilities between Rifampicin resistance alleles A) Long-term dynamics, for 30 days (280 generations) of evolution in 16 replicates of a population composed of resistance strain *H562Y* and resistance strain *S531F*. Shown are the dynamics for the *H526Y* background. The slope of the black line represents the initial difference in fitness between the resistances. **B)** Whisker-box shows the relative fitness differences inferred for new beneficial mutations between the two resistant backgrounds, with *H526Y* background as a reference. **C)** Whisker-box shows the relative differences in time of appearance inferred for new beneficial mutations between the two resistance backgrounds.

Figure 4. Differences in evolvability between Rifampicin and Streptomycin resistance alleles A) Long-term dynamics, for 30 days (280 generations) of evolution in 14 replicates of a population composed of resistance strain *K43T* and resistance strain *S531F*. Shown are the

dynamics for the *K43T* background. The slope of the black line represents the initial difference in fitness between the resistances. **B)** Whisker-box shows the relative fitness differences inferred for new beneficial mutations between the two resistant backgrounds, with *K43T* background as a reference. **C)** Whisker-box shows the relative differences in time of appearance inferred for new beneficial mutations between the two resistance backgrounds.

Figure 5. Differences in selective effects of evolved clones from the competition between *H526Y*^{Rif} and *S531F*^{Rif}. A) Fitness effects of selected clones evolved from the *H526Y*^{Rif} background, when in competition with their ancestor (light bars) or against their other resistant competitor (S531F^{Rif}, dark bars). **B)** Fitness effects of selected clones evolved from the *S531F*^{Rif} background, when in competition with their ancestor (light bars) or against their other resistant competitor (H526Y^{Rif}, dark bars).

Figure S1. Example of the fitting process for a simulated experimental population. Experimental data, shown as blue circles, was simulated with a given set of parameters. Normally distributed noise (with mean 0 and standard deviation 0.2, shown also as error bars) was added to the experimental points. Red lines show the inferred trajectories given by simple model (one mutations) and the model assuming two mutations, with the fitness of the mutant (WN) and its time of appearance (TN) as parameters. Dotted arrows show the time at which sweeping beneficial mutants effectively change the dynamics of the resistance allele. Shown are also the calculations to obtain the relative parameters used in the distributions of relative effects and time. A) Model 1, with a single mutation. B) Model 2, with a mutation in each background, which has a lower AIC and is, therefore, chosen as the best model to explain the data.

Figure S2. Long-term dynamics with the identification of the fluorescent backgrounds. Dynamics are shown as in Figure 2, but here the color of each line corresponds to the fluorescent marker of the mutation whose logarithm of the ratio is being plotted. **A)** Competitions between resistances *H526Y* and *H526D*. **B)** Competitions between resistances *H526Y* and *S531F*. **C)** Competitions between resistances *K43T* and *S531F*.

Figure S3. Long-term dynamics from the inferred parameters for each replicate population. Inferred dynamics are shown as solid lines and experimental data is shown as full circles. **A)** Competitions between resistances *H526Y* and *H526D*. **B)** Competitions between resistances *H526Y* and *S531F*. **C)** Competitions between resistances *K43T* and *S531F*.

Figure S4. Test for negative frequency dependence selection in competitions between resistance strains *H526Y* and *H526D*. X-axis shows the initial frequency of the *H526Y* strain and Y-axis shows the fitness effect inferred from the slope of the dynamics for the first 24 hours of the competition.

TABLE LEGENDS

Table 1. Evolutionary Parameters estimated for the competition between strains *H526Y* and *H526D*. W stands for the fitness of the emerging haplotype and T its time of appearance. Initial Freq stands for the inferred initial frequency of the *H526Y* background. In the cases

where only one of the backgrounds has acquired a mutation, the inferred parameters are in bold for the background where no mutation was inferred.

Table 2. Evolutionary Parameters estimated for the competition between strains *H526Y* and *S531F*. The meaning of the parameters is as in Table 1.

Table 3. Evolutionary Parameters estimated for the competition between strains *K43T* and *S531F*. The meaning of the parameters is as in Table 1.

Table 4. Potential compensatory mutations identified in the genomes of the clones evolved in the competition between resistances H526Y and S531F

Mutations were identified in the genomes of the evolved clones in comparison with a reference genome. Shown are the Single Nucleotide Polymorphisms (SNPs) or Insertion Sequence (IS) events identified in either of the backgrounds, and the frequencies at which they were detected. Mutations that occurred between genes (intergenic) are identified as such, otherwise all remaining mutations occurred within the gene indicated. The mutations previously identified to be compensatory are identified with a grey shaded cell.

Table S1. Fitness costs imposed by the antibiotic resistance alleles (*K43T*, *S531F*, *H526Y*, *H526D*) measured in competition against the sensitive reference strain.

Table S2. Genotypes of the evolved clones from competition between $H526Y^{Rif}$ and $S531F^{Rif}$