Bypass of genetic constraints during mutator evolution to antibiotic resistance

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Genetic constraints can block many mutational pathways to optimal genotypes in real fitness landscapes, yet the extent to which this can limit evolution remains to be determined. Interestingly, mutator bacteria elevate only specific types of mutations, and therefore could be very sensitive to genetic constraints. Testing this possibility is not only clinically relevant, but can also inform about the general impact of genetic constraints in adaptation. Here, we evolved 576 populations of two mutator and one wild-type Escherichia coli to doubling concentrations of the antibiotic cefotaxime. All strains carried TEM-1, a β-lactamase enzyme well known by its low availability of mutational pathways. Crucially, one of the mutators does not elevate any of the relevant first-step mutations known to improve cefotaxime activity. Despite this, both mutators displayed a similar ability to evolve more than 1000-fold resistance. Initial adaptation proceeded in parallel through general multi-drug resistance mechanisms. High-level resistance, in contrast, was achieved through divergent paths; with the a priori inferior mutator exploiting alternative mutational pathways in PBP3, the target of the antibiotic. These results have implications for mutator management in clinical infections and, more generally, illustrate that limits to natural selection in real organisms are alleviated by the existence of multiple loci contributing to fitness.

1. Introduction

A striking insight gained over the past decade of research is that epistatic interactions are common in experimental fitness landscapes [1]. Of particular interest is the observation of a special type of epistasis termed ‘sign epistasis’ [2], according to which mutations are beneficial or deleterious depending on the presence or absence of others [3–8]. Under sign epistasis, neighbouring mutation combinations display contrasting fitness values, introducing ‘ruggedness’ in the surface of the fitness landscape. This feature can have at least two important evolutionary consequences. First, it generates mutational pathways whose intermediate steps are no longer arranged in ascending order. Therefore, the number of pathways accessible by natural selection decreases [2] and successful adaptation becomes contingent upon the identity of first-step mutations [7]. A second, more drastic consequence is the emergence of multiple fitness peaks, which requires a particularly extreme form of epistasis known as reciprocal sign epistasis [9]. Overall, these observations led to the conjecture that natural selection may be limited by pervasive genetic constraints, contributing to explain the surprising degree of repeatability observed in many microbial evolution experiments [10,11].

One case presumably well suited to investigate the influence of genetic constraints on adaptation is that of bacterial mutators. Bacteria with a high mutation rate are frequently isolated in clinical settings [12,13] and in laboratory evolution experiments [14,15]. Their emergence is an eventual consequence of the genetic structure of asexuals, which allows mutator alleles to hitchhike with beneficial mutations occurring in the same genome [16,17]. Mutators pose a serious concern in clinical infections because they can readily evolve further adaptations, such as those promoting evasion of the immune system [18], increasing resistance to antibiotics [19] or alleviating the resistance fitness cost [20].
Different mutators, however, display a characteristic tendency to elevate only some types of transitions, transversions or frameshifts. This is because mutator phenotypes arise from alterations in specific mutation-avoidance mechanisms, whose malfunctioning permits the accumulation of particular mutational classes [21].

Here, we hypothesized that such mutational idiosyncrasy could render mutators particularly sensitive to genetic constraints. Each mutator genotype is expected to heighten the occurrence rate of only an arbitrary subset of beneficial first-step mutations [22]. If such a subset does not include the best possible mutations, mutator populations could be compelled to follow suboptimal paths—either leading to the same or a different fitness peak. Furthermore, in cases where the initial steps were drastically limited, mutators failing to raise the relevant first-step mutations may not be more able to adapt than their wild-type counterpart. Such stringent circumstances could happen when adaptation requires very specific multiple amino acid substitutions, as is observed in some cases of antibiotic resistance [8,23].

To study these possibilities, we characterized the evolution of wild-type and mutator strains of *Escherichia coli* towards increased resistance to the third-generation β-lactam antibiotic cefotaxime (CTX). Our experimental design sought to track mutations in the gene coding for the β-lactamase enzyme TEM-1 (*blaTEM-1*). The original allele, well adapted to hydrolyse early β-lactams, diversified naturally through the accumulation of point mutations into hundreds of variants with greater activity against later compounds of this family [24]. This natural evolution has been extensively emulated in a variety of laboratory experiments, yielding TEM-1 as one of the best-characterized models for the study of molecular evolution [7,25–27].

In the specific case of mutations conferring CTX resistance, there are several dozens of substitutions known to increase the resistance phenotype [26]. Despite the potentially huge number of combinations among these, only a small fraction are observed repeatedly both in nature and in laboratory evolution experiments—a signature of epistatic constraints [7,24]. Interestingly, we realized that the most frequent CTX resistance substitutions observed in clinical isolates arise from G:C → A:T transitions, one of the two dominant nucleotide changes in the spectrum of mismatch repair-deficient strains, the most prevalent type of strong bacterial mutators [28]. These substitutions include the top-ranked G238S and E104K, which appear combined in many notorious naturally occurring TEM alleles, and substitutions R164H and A237T [24,26]. In addition, the other transition characteristic of these mutators (A:T → G:C) also generates clinically relevant substitutions, such as M182T, H153R and I173V [24,26]. We next examined the case of MutT-deficient mutators, the second most prevalent type of strong mutator bacteria [28]. In sharp contrast, the elevation of A:T → C:G transversions peculiar to this background does not match any of the common CTX resistance mutations found in natural isolates [26]. Substitutions of intermediate to low effect, nonetheless, have been described (S268R, E240A, E104A and F72V), yet they have exclusively been observed in laboratory settings [26].

We reasoned that CTX resistance evolution could be a suitable model to examine the extent to which limited availability of mutational pathways can hinder the evolution of bacterial mutators. Studying this possibility is doubly relevant; not only because of the clinical importance of mutators, but because it can also inform about the more general question of the impact of genetic constraints in evolution. We thus serially propagated 192 independent populations of a *ΔmutT* mutator, a mismatch repair-deficient *ΔmutH* mutator and wild-type *E. coli* in the presence of periodically increasing concentrations of CTX. Crucially, we expected that the performance of the *ΔmutT* mutator would depend on its ability to exploit rare mutational pathways compatible with its narrow mutational spectrum. We also conjectured that any spectrum-dependent differences would be levelled out by a general increase in the supply of beneficial mutations. The rationale for this is that such an increase will favour that the best mutations are available for selection, irrespective of the genetic background. To test this, in the experimental set-up, half of the populations for each strain carry a single chromosomal copy of *blaTEM-1*, whereas the remaining half contain approximately 18 plasmidic copies of this allele [29]. Overall, we established six experimental settings (three mutational spectra with two gene dosages), for a total of 576 experimental populations.

2. Results

(a) Dynamics of adaptation to increasing antibiotic concentrations

To test whether different mutational spectra confer disparate ability to evolve high-level, multi-step CTX resistance, we serially passaged mutator and wild-type bacteria to doubling concentrations of the drug; starting from 1/4 to 1024 times the minimal inhibitory concentration (MIC) of the ancestral strain. In general terms, the results show that an increase in mutational supply, either through a mutator phenotype or by multiple plasmidic copies of *blaTEM-1*, translates into a greater fraction of replicates surviving at the end of the experiment (figure 1). More quantitatively, we found a strong linear correlation between the final number of survivors and the estimated product of mutation rate and *blaTEM-1* copy number (Pearson’s *r* = 0.87, *p* = 0.026; electronic supplementary material, figure S1). This indicates that the *ΔmutT* genotype, despite not elevating mutagenesis to the typical CTX resistance *blaTEM-1* mutations, is not broadly hindered to evolve high levels of resistance.

In the *blaTEM-1* single-copy setting, the anticipated advantage of the *ΔmutH* over the *ΔmutT* genotype was not observed (figure 1a). Indeed, the final number of survivors was greater in the *ΔmutT* than in the *ΔmutH* background (9/96 versus 3/96), although the values are not statistically different (Fisher’s exact test, *p* = 0.1332). It is worth noting that 18 *ΔmutH* populations went prematurely extinct at 2 mg l⁻¹ of CTX, a significant drop in the survival curve (log-rank test, *p* ≈ 0.001) which may reflect an early evolutionary dead-end associated with its mutational spectrum. Regarding the *blaTEM-1* multiple-copy setting, we found no discrepancies between the two mutator backgrounds (figure 1b), neither in terms of final number of survivors (Fisher’s exact test, *p* = 0.5354) nor in survival curves (log-rank test, *p* = 0.808).

As pointed out in the Introduction, the most common *blaTEM-1* CTX resistance substitutions arise mainly from transitions, which are only elevated in the *ΔmutH* genotype [21]. Taking this into account, it is revealing to compare the performance of the plasmid-harbouring wild-type (figure 1b) with that of both mutator populations carrying a single-copy *blaTEM-1* (figure 1a). An increased copy number confers a capacity to
translates into a higher number of final survivors. Note that the anticipated advantage of the only one elevating mutagenesis to the most-beneficial known substitutions in TEM-1 (see text for details).

Figure 1. Experimental evolution of wild-type and mutator E. coli to CTX resistance. The figure shows the number of surviving lineages against antibiotic concentration, which was doubled every 2 days. The different curves correspond to wild-type (black squares), \( \Delta \text{mutH} \) (red circles) and \( \Delta \text{mutT} \) (blue triangles) lineages; carrying either one \((a)\) or multiple \((b)\) copies of the gene coding for the \( \beta \)-lactamase TEM-1. Overall, an increase either in mutation rate or in \( \text{bla}_{\text{TEM-1}} \) copy number translates into a higher number of final survivors. Note that the anticipated advantage of the \( \Delta \text{mutH} \) over the \( \Delta \text{mutT} \) mutator was not realized, despite being the only one elevating mutagenesis to the most-beneficial known substitutions in TEM-1 (see text for details).

reach high resistance similar to hypermutable (7/96 versus 9/96 and 3/96 final survivors; Fisher’s exact test, \( p > 0.3 \)), although the wild-type’s survival curve is notably unique (log-rank test, \( p < 0.0001 \)). This suggests that the evolutionary paths followed by the different strains are not simply the result of the well-known \( \text{bla}_{\text{TEM-1}} \) CTX resistance substitutions. If that were the case, the wild-type would be expected to perform better than the \( \Delta \text{mutT} \) strain, because the latter is effectively non-mutator for these typical substitutions whereas the former should produce them at an approximately 18-fold higher rate \([29]\). In addition, the \( \Delta \text{mutH} \) genotype should enjoy the best outcome, because it elevates approximately 100- to 300-fold the required transitions \([30,31]\).

At least two non-mutually exclusive possibilities might explain these results. First, both \( \Delta \text{mutT} \) and the wild-type strains could be substituting non-canonical \( \text{bla}_{\text{TEM-1}} \) CTX resistance mutations. The wild-type may thus profit from the fact that an increase in copy number elevates not only the two transitions, but also all the six possible base substitutions. Although the effect on each mutation type is moderate, the overall elevation in mutational supply becomes comparable to that of the \( \Delta \text{mutH} \) background. Additionally, the greater allelic diversity generated may be fuelling evolution through homologous interplasmid recombination \([32]\) (although the electronic supplementary material, figure S1 gives no clear support for this hypothesis). In turn, the \( \Delta \text{mutT} \) strain elevates only one type of base substitution (\( A \rightarrow T \rightarrow C \rightarrow G \) transversions), but it does so by approximately 500- to 10 000-fold \([30,33]\); a huge increment that may allow this genotype to accumulate rare \( \text{bla}_{\text{TEM-1}} \) CTX resistance substitutions.

A second possibility is that other chromosomal loci played a substantial role in the acquisition of high-resistance phenotypes. Expanding the mutational target size should, in principle, mitigate spectrum-dependent differences, which could explain the lack of impairment observed in the chromosomally \( \text{bla}_{\text{TEM-1}} \)-encoding \( \Delta \text{mutT} \) genotype. Candidate loci include genes controlling multi-drug resistance determinants, such as efflux pumps and porin channels; and those coding for the penicillin-binding proteins, the target of \( \beta \)-lactam antibiotics \([34]\).

(b) Phenotypic characterization of evolved lineages

To gain insights into putative non-\( \beta \)-lactamase-mediated resistance mechanisms, we assembled a collection of 160 clonal isolates and tested them against a panel of chemicals, antibiotics and bacteriophage viruses. The largest part of the collection consisted in endpoint isolates from: (i) lineages that survived at the end of the experiment \((n = 50)\), and (ii) the highest resistant lineages within each setting that went extinct before the end of the experiment \((n = 57)\). To compensate that most mutator populations went extinct very late, the collection was completed with 43 clones from random lineages isolated at low CTX concentrations (less than or equal to 0.5 mg\,l\(^{-1}\)). The battery of assays (see Material and methods) was designed to examine the status of AcrAB, the main efflux pump involved in \( \beta \)-lactam export \([35]\), and the outer membrane porins OmpF and OmpC, the primary routes of entry of these drugs \([36]\).

To assess the validity of the tests, the loci \( \text{acrA}, \text{acrB}, \text{acrR}, \text{envZ}, \text{ompR}, \text{ompF} \) and \( \text{ompC} \) were sequenced in two independent strains positive for efflux and porin alterations. Both strains confirmed non-synonymous point mutations in several of these genes: \( \text{acrB} \) and \( \text{acrR} \) in both cases, together with \( \text{ompC} \) in one case and \( \text{ompR} \) in the other (see the electronic supplementary material, table S1).

The collection exhibited a high frequency of altered porin phenotypes (79%) and, to a lesser extent, increased efflux phenotypes (43%; figure 2)—a combination usually observed in multi-drug-resistant isolates \([37]\). Intriguingly, we found that 20% of the strains exhibit hypersusceptibility to erythromycin, a well-known substrate of AcrAB \([38]\). This phenotype could be a by-product of mutations improving the specificity of AcrAB for CTX, at the cost of reducing erythromycin extrusion \([39]\). Alternatively, it could emerge from mutations in the peptidoglycan synthesizing apparatus (including but not necessarily limited to PBP3, the molecular target of CTX), with pleiotropic effects in the organization of the outer membrane that result in enhanced passive diffusion of hydrophobic agents such as erythromycin \([40]\). Whatever the mechanism, we reported this phenotype owing to its prevalence, which might well be an underestimation given the high frequency of increased efflux phenotypes observed.
Figure 2. Phenotypic profiles of representative evolved clones. Bars represent the frequency of isolates displaying alterations in permeability (black), efflux (dark grey) and erythromycin hypersusceptibility (light grey). White bars correspond to isolates without alterations. (a) Phenotypic profiles of wild-type and mutator strains. Only differences in erythromycin hypersusceptibility were found to be statistically significant (see main text). (b) Phenotypic profiles according to the antibiotic concentration from which the clones were isolated. No statistically significant differences between early and late clones were observed. These results suggest that general multi-drug resistance evolved early and in parallel across the different backgrounds, and therefore is not sufficient to explain the final survival patterns.

Figure 3. Substitutions in TEM-1 and PBP3 generated by wild-type and mutator strains. The histogram shows abundance of different amino acid substitutions among 70 isolates of wild-type (black), ΔmutH (red) and ΔmutT (blue) populations. (a) Substitutions in TEM-1. (b) Substitutions in PBP3, grouped by matching of the underlying nucleotide change with the mutational spectrum of ΔmutT (A: T → C : G, left), ΔmutH (G : C → A : T and A : T → G : C, centre) and wild-type (others, right) backgrounds. Note the contrasting levels of molecular idiosyncrasy observed in both proteins, indicating that mutational pathways are more constrained in TEM-1 than in PBP3.

Only 11% of the isolates showed no apparent alteration in any of these phenotypes.

No significant profile differences were detected among the three genotypes (Fisher’s exact test, p > 0.15; figure 2a). The only exception is erythromycin hypersusceptibility, at least 10 times more frequent in either mutator than in the wild-type background (Fisher’s exact test, p = 0.00014); suggesting that this phenotype may be arising only from a few, very specific point mutations. We also found no significant differences among clones isolated from early and late CTX concentrations (less than or equal to 0.5 versus more than or equal to 16 mg l⁻¹, Fisher’s exact test, p > 0.3; figure 2b). Taken together, the results indicate that efflux and permeability adaptations were acquired early and in parallel across the different genotypes, and therefore are not sufficient to explain the final survival patterns.

(c) Genotypic characterization of evolved lineages

Given the evidence provided above, we reasoned that high-level resistance may presumably be the result of either non-canonical blaTEM-1 substitutions or the combination of blaTEM-1 substitutions with alterations in ftsH, the gene coding for the main target of CTX (the transpeptidase PBP3). To explore these possibilities, we sequenced both loci from a subset of 70 strains from the collection of isolates used for the phenotypic assays (see the electronic supplementary material, table S2).

We found 21 out of 70 strains to be positive for non-synonymous blaTEM-1 substitutions, all of which belonged to the multiple-copy setting (n = 44; see the electronic supplementary material, table S2). Loss or insufficiency of expression of the chromosomal bla gene in the single-copy setting (n = 26) was discarded because all the analysed isolates retained inhibitor-susceptible resistance to ampicillin (data not shown). In total, we identified 27 point mutations corresponding to just five amino acid changes (E104K, H153R, R164S, M182T and G238S; figure 3a). These substitutions gave rise to five different alleles, including the double-mutant TEM15 and TEM112 alleles, and the triple-mutant TEM52 allele (see the electronic supplementary material, tables S2 and S3). All these substitutions and alleles have already been described both in clinical and experimental studies, and details about them can be read elsewhere [24]. The most frequent amino acid change in our dataset was G238S (20/27), in agreement with its prevalence in previous reports [7,41]. As anticipated, most of the mutations arose from either G : C → A : T or A : T → G : C transitions. The only exception was R164S, arising from a C : G → A : T transversion, detected just in one wild-type isolate. In consequence, it is not surprising that ΔmutH was the background where the greatest portion of substitutions were observed (16/27; figure 3a), and also that exhibited the highest...
average number of substitutions per sequence (1.25; electronic supplementary material, figure S2).

Examination of the ftsl locus revealed 54 out of 70 strains displaying non-synonymous substitutions, with a remarkably high degree of polymorphism. We identified 92 point mutations (34 unique), affecting 31 positions, accounting for a total of 39 different alleles (figure Sb and electronic supplementary material, tables S2 and S3). Some of these variants have been already associated with resistance to β-lactam antibiotics, either in laboratory strains of *E. coli* (A257V and N361S) [42,43] or in related species such as *Salmonella enterica* (P311S) [44] and *Haemophilus influenzae* (L369F) [45]. All the substitutions but one mapped within the transpeptidase module (D237–V577) of the PBP3 protein, clustered in the surroundings of the three catalytic motifs (STVK310, SSN361 and KTG496) [46]. The sole mutation observed in the non-catalytic module (G57–D237), R167C, affected the first position of the conserved motif RYYPSG172. This module is thought to function as an intramolecular chaperone, and thus mutations in it could contribute to resistance by influencing the folding or stability of the whole protein [46]. Mutations in this module, indeed, have been reported in *E. coli* to confer resistance to ceftazidime, a β-lactam antibiotic structurally very close to CTX [47].

In contrast to what was observed in the bla locus, both mutators accumulated highly idiosyncratic substitutions in ftsl, including double- and triple-mutant alleles where all mutations correspond to each specific mutational spectrum (figure 3 and electronic supplementary material, tables S2 and S3). Regarding to this, only 7 out of 83 substitutions found in both mutators did not arise from spectrum-specific base changes, suggesting that these are mutations with strong beneficial effects. Interestingly, the ΔmutT background was the one where the greatest portion of substitutions were observed (52/92), exhibiting the highest average number of substitutions per isolate (2.08; electronic supplementary material, figure S2) and the highest total number of triple mutant alleles (eight in total, six unique; see the electronic supplementary material, tables S2 and S3).

Two lines of evidence indicate that these observed polymorphisms in the loci bla and ftsl are likely to explain high-level resistance patterns. On the one hand, all sequenced strains contained substitutions in at least one of these two genes, with the unsurprising exception of five clones from the wild-type background isolated at low concentrations (less than or equal to 0.5 mg l⁻¹; electronic supplementary material, table S2). On the other hand, although some isolates exhibited alterations in both TEM and PBP3 (12/70), there was a statistically significant tendency to lack changes in PBP3 when TEM substitutions are already present (Fisher’s exact test, *p* < 0.0001). Actually, whenever a triple-mutant allele was observed at either loci, alterations at the other locus were completely absent (electronic supplementary material, table S2).

### 3. Discussion

This work was motivated by the question of whether limits to natural selection posed by genetic constraints could become particularly apparent in mutator bacteria. To gain insights into this issue, we monitored the evolution of two distinct mutators in an experimental system where only one of them elevates known beneficial mutations. Under such conditions, the performance of the a priori inferior mutator would depend on its ability to exploit secondary mutational pathways, if available. In the extreme case, we hypothesized that its performance could be as limited as that of the wild-type.

We found that both mutators were indistinguishable in terms of their ability to evolve high-level CTX resistance. In view of the above considerations, a reasonable explanation for these results relies on the seemingly large size of the global mutational target for CTX resistance evolution. Such large size provides multiple alternative evolutionary routes and, consequently, increases the likelihood that a particular mutational spectrum raises fitting beneficial mutations. The causes for this large mutational target size are at least twofold. First, adaptation can proceed via changes at loci other than bla, most notably at ftsl. Second, the mutational target within the ftsl locus is apparently very broad in itself. In this respect, it is worth mentioning the high proportion of PBP3 substitutions observed only once (22/34; electronic supplementary material, table S2), which points at the existence of many other possible mutations that just went unsampled [48]. This is consistent with the fact that more than 30 PBP3 substitutions not detected here have been described elsewhere to confer resistance to a variety of β-lactams, including CTX [42–45,49].

A recent study with *Pseudomonas aeruginosa* reported minor differences between hypermutability and stress-induced mutagenesis in promoting adaptation, a result akin to ours in that both experiments involved different mutational spectra [50]. However, the negligible effect on evolvability observed here is particularly revealing, because our experimental design purposely involved both a narrow-spectrum mutator and a well-characterized model of constrained molecular evolution. A comparison with the literature lead us to believe that this result is probably not peculiar to our specific conditions, but rather it could be fairly general. While our system, in fact, exhibited substantial flexibility in evolutionary trajectories, this flexibility is similar to what is typically observed in many bacterial evolution experiments [51]: hard adaptive constraints are rarely observed at the nucleotide level [52], and even in the examples that reported the highest degree of molecular convergence, adaptation involved more than one loci and featured a considerable amount of intralocus diversity [53–55]. Therefore, given that mutational target sizes seem not strictly limited, we expect spectrum effects on evolvability to be modest at best in most cases.

In a broader evolutionary context, this work contributes to the question to what extent the ruggedness of fitness landscapes can hinder natural selection. Several reports have documented both inter- [4,5,56] and intralocus [27,57,58] sign epistasis in different model systems. These observations promoted the idea that genetic constraints could be prevalent and hence adaptation could proceed through very few mutational paths to optimal genotypes [8,11]. This possibility implies that evolution may be largely repeatable and, perhaps, even predictable [11]. It has been argued, however, that adaptive limitations can be particular to low-dimensional fitness landscapes, which to date have been the only ones amenable to empirical examination [11]. The rationale for this is that, because increasing dimensionality implies that genotypes have more neighbours, what might look like an isolated fitness peak at some scale can actually be connected at higher dimensions [59]. An example along these lines can be found in the case of substitution G238S in TEM-1 β-lactamase. *In vitro* experiments showed that this substitution is the first-step...
mutation that confers the highest resistance to CTX [26]. Most interestingly, they revealed that once it becomes fixed, epistatic interactions strongly restrict which other mutations can be substituted next [7]. In contrast to these limitations, our results uncovered the existence of multiple alternative pathways emerging from the combination of G238S with various substitutions in PBP3 (electronic supplementary material, table S2); illustrating how high dimensionality facilitates the bypass of local adaptive constraints in real fitness landscapes.

Apart from evolvability, there are other possible long-term consequences to mutational spectrum differences. Mutational biases forcibly impose a high level of divergence at the molecular level. This idiosyncratic variability, although perhaps equivalent in fitness under the selective conditions, might confer distinct properties in other environments. In our experiment, each mutator exhibited a marked preference for accumulating amino acid changes either in TEM or in PBP3 (figure 4). It is easy to imagine different long-term advantages and disadvantages for both resistance mechanisms. Some of the observed TEM alleles, for instance, are able to hydrolyse β-lactams not primarily targeting PBP3, thus conferring resistance to a broader range of compounds [60]. In addition, given that these enzymes are commonly plasmid-encoded in natural isolates, they could recombine at a higher rate and also be more readily transmissible through horizontal-gene transfer [61]. In turn, being an essential gene, PBP3 alleles are likely to entail fitness costs in other conditions. Such costs have already been reported in some cases, including reduced growth at high temperatures [42] and impaired biofilm formation [62]. Alterations in the cell wall structure, however, could also have an adaptive value: a recent report provided by Dr Roy Kishony [67]. The multiple-copy blaTEM-1 strains were obtained by transducing the galK::xpf::fp, blatem cassette from an E. coli MC4100 kindly provided by Dr Roy Kishony [67]. The multiple-copy blaTEM-1 strains were obtained by transformation with plasmid pBRAC1; a pBR322 derivative which, in an attempt to reduce the fitness burden of carrying the plasmid [68], was digested with enzymes NcoI and PstI to excise the tetracycline resistance cassette. Note that these strains do not carry the blaTEM-1 chromosomal copy. All genotypes were confirmed by PCR and gel electrophoresis. Bacteria were cultured in a modified version of M9 minimal media (1 ml Deep Well, Axygen), covered with loose-fitting plastic lids to allow aeration. To minimize cross-contamination, populations were grown without agitation at 37 °C in 96-well microtiter plates (1.1 ml Deep Well, Axygen), covered with loose-fitting plastic lids to allow aeration. To minimize cross-contamination, populations were arranged in a chessboard pattern across each plate, such that half of the wells contained sterile medium [67]. The fraction of these control wells that got contaminated on a daily basis was below 0.5%. Every 24 h, 16 µl of each culture was transferred into fresh 800 µl of medium, allowing a minimum of approximately 5.7 generations per day (note that this estimate represents a lower limit, owing to drops in final population density along the course of the experiment). Serial passage was conducted

4. Material and methods

(a) Strains and media

All strains are derivative of E. coli AB1157, obtained from the laboratory collection of Dr Bruce R. Levin. This strain naturally carries the gene coding for the β-lactamase AmpC, which was deleted to prevent interference with the experimental system. Knockouts of the genes ampc, mutH and mutT were done by P1 transduction and subsequent pCP20-mediated removal of the kanamycin resistance cassette from the appropriate Keio clone [65,66]. The single-copy blaTEM-1 strains were generated by transducing the galK::xpf::fp, blatem cassette from an E. coli MC4100 kindly provided by Dr Roy Kishony [67]. The multiple-copy blaTEM-1 strains were obtained by transformation with plasmid pBRAC1; a pBR322 derivative which, in an attempt to reduce the fitness burden of carrying the plasmid [68], was digested with enzymes NcoI and PstI to excise the tetracycline resistance cassette. Note that these strains do not carry the blaTEM-1 chromosomal copy. All genotypes were confirmed by PCR and gel electrophoresis. Bacteria were cultured in a modified version of M9 minimal medium, optimized to support maximal growth in the experimental conditions [66]. The modification consists of supplementing M9 salts with 1% glucose (G8270, Sigma-Aldrich) and 1% acid hydrolysate of casein (casamino acids, Becton Dickinson).

(b) Evolution experiment

For each of the six genotypes used here, a total of 96 parallel lineages were serially propagated in the presence of increasing concentrations of CTX (Claforan, Sanofi-Aventis). The populations were grown without agitation at 37 °C in 96-well microtiter plates (1.1 ml Deep Well, Axygen), covered with loose-fitting plastic lids to allow aeration. To minimize cross-contamination, populations were arranged in a chessboard pattern across each plate, such that half of the wells contained sterile medium [67]. The fraction of these control wells that got contaminated on a daily basis was below 0.5%. Every 24 h, 16 µl of each culture was transferred into fresh 800 µl of medium, allowing a minimum of approximately 5.7 generations per day (note that this estimate represents a lower limit, owing to drops in final population density along the course of the experiment). Serial passage was conducted

Figure 4. Mutational spectrum effects on divergence of mutational pathways. Bars represent number of isolates with different combinations of substitutions in TEM-1 and PBP3. From plasmid-carrying lineages surviving at the end of the experiment (MIC more than or equal to 64 mg l⁻¹). (a) ΔmutH background. (b) ΔmutT background. To aid visualization, shades of grey are proportional to the number of isolates. Note how the mutational spectrum directs populations through divergent adaptive paths; each mutator preferentially accumulating substitutions either in TEM-1 or PBP3.
during 28 days. The minimal inhibitory concentration of cefotaxime (MIC) in the experimental medium for all parental strains was determined to be 0.064 mg L\(^{-1}\) by the microdilution method (CLSI, 2006), irrespective of plasmid carriage. The first two passages were done in the absence of antibiotic. At day three, the populations were exposed to 0.016 mg L\(^{-1}\) CTX (1/4 × MIC). The antibiotic concentration was subsequently doubled every 48 h, until a final concentration of 64 mg L\(^{-1}\) CTX (1024 × MIC) was reached. Throughout the course of the experiment, the number of surviving populations was estimated by visual examination, and the plates were regularly stored at −80 °C.

(c) Phenotypic characterization

After completion of the evolution experiment, single clones from 160 representative lineages were isolated by streaking onto Luria broth (LB) agar plates. These isolates were transferred to 96-well microtiter plates, incubated overnight and stored at −80 °C for later analysis. All incubations were conducted in LB broth at 37 °C without agitation. The phenotypic characterization measured susceptibility to a collection of chemicals, antibiotics and bacteriophage viruses. Assays were performed in triplicate after overnight growth of a replica of the frozen collection plates. These overnight cultures were 1:100 diluted and re-grown for 4 h. A 96 pin replicator was then used to transfer aliquots to square Petri dishes containing LB agar supplemented with varying concentrations of each specific agent. To examine the status of the AcrAB pump, the major pump extruding β-lactams, we estimated the MIC of three well-known substrates such as acriflavine, tetracycline and erythromycin (all purchased from Sigma-Aldrich) [35]. Altered-efflux mutants were identified using a conservative criterion: either the MIC of one them was elevated more than or equal to fourfold, or the MIC of at least two rose more than equal to twofold. To study alterations in porins OmpF and OmpC, the major route of entry of the antibiotic into the cell [36], we assessed resistance to the bacteriophages Tu1a and Tu1b, which use these porins, respectively, as their attachment site [69]. The resistance criterion was growth on a multiplicity of infection sufficient to prevent growth of the ancestor strain. As positive controls, Keio-derived knockout mutants of the genes acrR, ompF and ompC were routinely included in the aforementioned assays.

(d) Genetic characterization

PCR amplification and Sanger sequencing of the chromosomal bla gene was performed with oligonucleotides 5’-TGAAAC TT CCGA AATGCC C-3’ and 5’-CCCTCG TTCAC GTC TTC C-3’. Regarding the plasmid bla gene, pBRAC1 extraction and purification were performed using the Qiagen plasmid mini kit, and sequencing was carried out with oligonucleotides 5’-GCCAG GACTTG TTCAC ACAATGATG-3’ and 5’-CCAGCA GAGATGTC ACCA AACC-3’. The internal oligonucleotide 5’-CATGTC GCCCTA ACAACA ACC-3’ was also employed for sequencing, owing to the large size of the gene (approx. 1.8 kb). Sequencing services were provided by Secugen (www.secugen.es), sequence analysis were carried out with RIM (traceedit) and sequence alignments were performed with MAFFT v. 6 (mafft.cbrc.jp/align/ software).

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Correction to ‘Bypass of genetic constraints during mutator evolution to antibiotic resistance’

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The funding statement presented in [1] is incomplete. The correction is as follows:

‘This work was supported by predoctoral fellowship FI05/00569 to A.C. and by Plan Nacional de I+D+i and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015)—co-financed by European Development Regional Fund ‘A way to achieve Europe’ ERDF and grant FIS PI13/00063’.

Reference