Antibiotic collateral sensitivity is contingent on the repeatability of evolution

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Antibiotic resistance represents a growing health crisis that necessitates the immediate discovery of novel treatment strategies. One such strategy is the identification of collateral sensitivities, wherein evolution under a first drug induces susceptibility to a second. Here, we report that sequential drug regimens derived from in vitro evolution experiments may have overstated therapeutic benefit, predicting a collaterally sensitive response where cross-resistance ultimately occurs. We quantify the likelihood of this phenomenon by use of a mathematical model parametrised with combinatorially complete fitness landscapes for \textit{Escherichia coli}. Through experimental evolution we then verify that a second drug can indeed stochastically exhibit either increased susceptibility or increased resistance when following a first. Genetic divergence is confirmed as the driver of this differential response through targeted and whole genome sequencing. Taken together, these results highlight that the success of evolutionarily-informed therapies is predicated on a rigorous probabilistic understanding of the contingencies that arise during the evolution of drug resistance.

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The emergence of drug resistance is governed by Darwinian dynamics, wherein resistant mutants arise stochastically in a population and expand under the selective pressure of therapy. These evolutionary principles underpin resistance to the presently most effective therapies for bacterial infections, cancers, viral infections and disparate problems such as the management of invasive species and agricultural pests. Biological mechanisms of drug resistance often carry a fitness cost in the absence of the drug and further, different resistance mechanisms can interact with one another to produce non-additive fitness effects, a phenomenon known as epistasis. These trade-offs can induce rugged fitness landscapes, potentially restricting the number of accessible evolutionary trajectories to high fitness or rendering evolution irreversible.

Identifying evolutionary trade-offs forms the basis of an emerging strategy for combating drug resistance; prescribing sequences of drugs wherein the evolution of resistance to the first induces susceptibility to the next. Where this occurs, the first drug is said to induce collateral sensitivity in the second. Conversely, where the first drug induces increased resistance in the second, collateral (or cross) resistance has occurred. Recently, in vitro evolution experiments have been performed, in both bacteria and cancers, to identify drug pairs or sequences exhibiting collateral sensitivity. One common protocol for these experiments proceeds by culturing a population in increasing concentrations of a drug to induce resistance, and then assaying the susceptibility of the resultant population to a panel of potential second-line therapies. From these experiments, sequences or cycles of drugs in which each induces collateral sensitivity in the next have been suggested as potential therapeutic strategies to extend the therapeutic efficacy of a limited pool of drugs. For some cancer therapies, which often have severe side-effects and high toxicity, such sequential therapies may be the only way to combine the use of multiple drugs.

Drug pairs that are identified as collaterally sensitive in a small number of in vitro evolutionary replicates may not in fact induce collateral sensitivity each time they are applied. This hypothesis arises from the observation that evolution is not necessarily repeatable; resistance to a drug can arise through multiple different mechanisms, as has been observed in cancers and bacteria. Further, one mechanism may confer resistance to a second drug, whilst another may induce increased susceptibility, as was recently demonstrated in a drug screen of over 3000 strains of Staphylococcus aureus. In previous experimental evolution studies to identify collateral sensitivity this phenomenon has been directly observed. For example, Barbosa et al. observed contrasting collateral response in evolutionary replicates of Pseudomonas aeruginosa. Oz et al. observed the same phenomenon in E. coli wherein a pair of evolutionary replicates was performed under exposure to the ribosomal (30S) inhibitor tobramycin, resulting in one exhibiting increased sensitivity to chloramphenicol and one exhibiting increased resistance. Similar effects are evident in cancer studies. Zhao et al. observed that the sensitivity of a BCR-ABL leukaemia cell line to cabozantinib can both increase and decrease following exposure to bosutinib, and identified a single-nucleotide variation responsible for this differential collateral response.

The extent of the impact of differential collateral response on the design of sequential drug therapies is not yet fully understood. Here, we provide a clear evolutionary explanation for differential patterns of collateral response through a combination of mathematical modelling and experimental evolution. Through mathematical modelling we demonstrate the extent to which the existence of multiple evolutionary trajectories to drug resistance can render collateral sensitivities stochastic, and discuss the implications for in vitro experimental evolution. We next empirically demonstrate the existence of multiple trajectories in the evolution of E. coli through in vitro experimental evolution. Previous studies have explored the collateral response by considering all pairs from a pool of antibiotics, each with a small number of evolutionary replicates. We instead perform 60 parallel evolutionary replicates of E. coli under ceftoloxime to demonstrate the extent of heterogeneity in second-line drug sensitivity. Through genomic sequencing we confirm that different mutations (i.e., different evolutionary trajectories) are responsible for this heterogeneity. Critically, we find that collateral sensitivity is never universal, and is in fact rare. Finally, we derive collateral sensitivity likelihoods which we argue are critical statistical benchmarks for the clinical translation of sequential drug therapies.

Results
Mathematical modelling of evolution. The potential impact of divergent evolution can be conceptualised in the classical fitness landscape model of Wright, wherein genotypes are projected onto the two dimensional x-y plane and fitness represented as the height above this plane. Evolution can be viewed as a stochastic ‘up-hill’ walk in this landscape wherein divergence can occur at a saddle. Figure 1 shows such a schematic fitness landscape annotated to demonstrate the capacity for divergent evolution and the potential effects on collateral sensitivity.

Previous studies have attempted to empirically determine the structure of the fitness landscape for a number of organisms and under different drugs. In these studies, a small number of mutations associated with resistance are first identified. Strains are engineered corresponding to all possible combinations of presence and absence of these mutations and the fitness of each strain is measured by a proxy value, for example minimum inhibitory concentration (MIC) of a drug or average growth rate under a specific dose. These measurements are combined with the known genotypes to form a fitness landscape. However, to derive fitness landscapes through this method, the number of strains that must be engineered grows exponentially with the number of mutations of interest. Thus only small, combinatorially complete, portions of the true fitness landscape can be measured, for example, consisting of 2–5 alleles. Nevertheless, these restricted fitness landscapes can provide valuable insight into the evolution of drug resistance.

Mira et al. derived fitness landscapes for E. coli with all combinations of four fitness conferring mutations (M69L, E104K, G238S and N276D) in the TEM gene and measured fitness under 15 different β-lactam antibiotics (See Supplementary Fig. 1, Supplementary Table 1), using the average growth rate (over 12 replicates) as a proxy of fitness. Of these 15 landscapes, 14 were identified as having multiple local optima of fitness, indicating the potential for the divergence of evolutionary trajectories. We utilised these landscapes, coupled with mathematical modelling (see Methods), to estimate the likelihood of the different evolutionary trajectories from a wild-type genotype (denoted 0000) to each of the fitness optima. Using this model, we performed in silico assays for collateral sensitivity, mirroring the approach taken by Imamovic and Sommer (Fig. 2). For each drug, we first stochastically simulated an evolutionary trajectory from the wild-type genotype to a local fitness optimum genotype and then, for all other landscapes, compared the fitness of this local optimum genotype to that of the wild-type. A schematic of this simulation is shown in Fig. 2a. Figure 2b shows an example of two evolutionary trajectories that can arise stochastically in this model under the fitness landscape for ampicillin. We exhaustively enumerated all tables of collateral response that can arise under this model (see Supplementary Figs. 2–10 for...
Further details). Figure 2c shows the best case (most susceptible following evolution), worst case (highest resistance following evolution) and mostly likely collateral response tables that arise in this analysis, along with the mean collateral response table (expectation of collateral response for each pair). This analysis suggests that there is remarkable variation in collateral response arising solely from the stochastic nature of mutation that ultimately drives evolution under a first drug. Indeed, we find a total of 82,944 unique tables can arise, of which the most likely occurs with probability 0.0023. Amongst the 225 ordered drug pairs, only 28 show a guaranteed pattern of collateral sensitivity, whilst a further 94 show a pattern of guaranteed cross-resistance. For 88 pairs, the first drug can induce either collateral sensitivity or cross-resistance in the second as a result of divergent evolution under the first drug. Critically, if a collateral response table is chosen at random from this table, then the expected probability that the first of these two drugs will induce cross-resistance in the second is 0.513 (determined from 10^6 simulations of this process).

**Experimental evolution induces heterogeneous collateral response.** The mathematical model used above represents a simplification of biological reality as the assumption of a monomorphic population need not hold and the parametrisation is made using incomplete fitness landscapes. To experimentally validate our predictions, we verified the existence of divergent collateral response through experimental evolution. Mirroring previous experimental approaches, we performed in vitro evolution of *E. coli* (strain DH10B carrying phagemid pBC SK(−) 198, expressing the β-lactamase gene SHV-1) in the presence of the β-lactam antibiotic cefotaxime. Bacterial populations were grown using the gradient plate method with concentrations of cefotaxime varying between approximately 0.1 and 1000 μg ml⁻¹ and 256 μg ml⁻¹ over a course of 10 passages lasting 24 h (see Fig. 3a and Methods for details). In total, 60 replicates of experimental evolution were performed. We denote the resulting populations by X1−X60. For replicates X1−X12, aliquots were taken following each second passage and the MIC to a panel of second-line drugs assayed. A time-series for the MIC of X1−X12 replicates under cefotaxime is shown in Fig. 3b.

As expected, the replicates exhibit increased resistance to cefotaxime over the ten passages, although with varying magnitude and different trajectories.

For each of a panel of eight second-line antibiotics (Table 1), the MIC for the replicates X1−X60 was determined following passage ten, in addition to the MIC for the parental strain (Supplementary Dataset 1, Methods). Figure 4 shows how the MICs of X1−X60 differ from the parental line. As predicted, we find that the collateral change in sensitivity is highly heterogeneous, and show that both collateral sensitivity and cross-resistance can arise to the antibiotics piperacillin (PIP), ticarcillin/clavulanate (TCC) and ampicillin/subbactam (AMS).

**Genomic profiling reveals divergent evolution.** Differential patterns of drug resistance could be driven by the different replicates having experienced different numbers of sequential mutations along a single trajectory wherein each induces a shift in response (temporal collateral sensitivity), by evolutionary divergence at a branching point in the landscape or by non-genetic mechanisms of resistance. To elucidate the underlying mechanisms, we first performed targeted sequencing of the SHV gene for each of the 10 passage time points for 12 evolutionary replicates (X1−X12) (Fig. 3b). Through this analysis we identified five variants of SHV-1 amongst the 12 replicates. X1, X5, X7−X9 and X11 all possess wild-type SHV-1, X2 possesses the substitution G242S, X3 possesses G238C, X4 and X6 both possess G238A, and X10 and X12 both possess G238S. This analysis revealed no evidence of double substitutions in SHV, indicating a minimum of four fitness conferring substitutions that can occur in SHV-1 during exposure to cefotaxime, and confirming the existence of a multi-dimensional evolutionary branching point in the fitness landscape. Further, the sensitivity of the population to a second drug appears to be (at least partially) dependent on which of these substitutions occurs (Fig. 3, 4). For example, replicate X3 (harbouring G238C) exhibits a significant increase in susceptibility to TIC, PIP and SAM, whilst those replicates found to harbour wild-type SHV-1, or the other SNVs, exhibit either cross-resistance or no significant change in susceptibility to these drugs.

Through targeted sequencing of SHV alone we cannot not exclude the possibility that mutations in other genes, or large scale genomic alterations such as insertions or deletions, drive...
Further divergence in collateral response. To explore whether additional background mutations arose during selection, we produced draft genome sequences for the replicates X1–X12 after passage 10 and looked for evidence of additional mutations. This genomic data confirmed the SHV-1 mutations found by Miri et al.29 represented as a graph of genotypes. Arrows indicate fitness conferring mutations between genotypes represented as nodes. Blue nodes indicate genotypes from which there is only a single ARMS with collateral sensitivity (blue) or cross-resistance (red) in each case. CS - collaterally sensitive, CR - cross resistant indicate the follow-up drug under which the fold-change from wild-type susceptibility is calculated. Bar charts indicate, for each labelled mutation site, the fold-change from wild-type susceptibility. Squares indicate local optima of fitness of this resultant genotype is compared to the wild-type genotype for each of the panel of antibiotics. This genomic data con.

We conclude that mutations in SHV-1 are the primary drivers of cefotaxime resistance as they are associated with the most substantial increases in MIC. In other replicates do not exhibit collateral sensitivity, the resistant cells could face a fitness cost in the absence of drug. Similar patterns of fitness trade-off have been exploited in cancer treatments by using dose-modulation (adaptive) therapies that extend survival by inducing competition between sensitive and resistant cells.35–37

We conclude that mutations in SHV-1 are the primary drivers of cefotaxime resistance as they are associated with the most substantial increases in MIC. For example, for replicate X12, which exhibits the highest endpoint MIC, no additional
**Table 1 Antibiotic drugs used in this study**

| Antibiotic                  | Abbreviation | Antibiotic group     | Notes                                      |
|-----------------------------|--------------|----------------------|--------------------------------------------|
| Cefotaxime                  | CTX          | Cephalosporin        |                                            |
| Ciprofloxacin               | CIP          | Fluoroquinolone      |                                            |
| Ampicillin/sulbactam        | SAM          | β-lactam combination | 2:1 ratio of ampicillin to sulbactam       |
| Gentamicin                  | GNT          | Aminoglycoside       |                                            |
| Ticarcillin/clavulanate     | TIC          | β-lactam combination |                                            |
| Phosphomycin                | PMC          | Phosphomycin         |                                            |
| Ceftolozane/tazobactam      | CFT          | β-lactam combination |                                            |
| Piperacillin                | PIP          | Penicillin           |                                            |
| Cefazolin                   | CFZ          | Phosphomycin         |                                            |

**Fig. 3** Experimental evolution reveals divergent collateral response. **a** A schematic of the evolutionary experiment. *E. coli* were grown using the gradient plate method and passaged every 24 h for a total of 10 passages. Sixty replicates of experimental evolution were performed. **b** The MIC for 12 replicates (X1–X12) under cefotaxime exposure was measured following passages 0, 2, 4, 6, 8 and 10. These values are plotted, revealing heterogeneity in the degree of resistance evolved to cefotaxime. Targeted sequencing of the SHV gene was performed following each passage revealing four different SNVs between the replicates marked by geometric shapes (triangle—G242S, hexagon—G238C, square—G238A and pentagon—G238S). Mutations are marked at the earliest time point they were detected in each replicate.
mutations were detected. In contrast, X1, X5, X8, X9 and X11 all had genomic mutations, lacked SHV-1 variants and had the lowest final cefotaxime MIC. We excluded the possibility of amplifications of SHV-1 by consideration of read depth ratios. The ratio of reads mapped to the gene and reads mapped to the plasmid backbone was very similar across all samples. The ratio of plasmid reads to chromosomal reads did differ across samples, but the fraction of plasmid-derived reads did not correlate with the MIC for cefotaxime (Supplementary Dataset 2) and is more likely due to variation in extraction efficiency for chromosomal versus plasmid DNA. We excluded the possibility of amplifications or deletions in chromosomal genes by consideration of read depth ratios (Supplementary Fig. 11).

We note that X7 exhibits an increase in resistance to cefotaxime without any associated genomic alterations. Similarly X1, X5, X9 and X12 exhibit mutations, but none that are known to be associated with antibiotic resistance. Thus, we can infer that physiological adaptation or epigenetic adaptation may also be driving resistance to cefotaxime.

**Collateral sensitivity likelihoods.** Our experimental results demonstrate that the evolution of antibiotic resistance is non-repeatable, and that the efficacy of a second-line drug can depend on the specific evolutionary trajectory that occurs under a first. As such, where a pair of drugs exhibit collateral sensitivity in a small number of experimental replicates, it need not be the case that collateral sensitivity always occurs. Rather than give up entirely on the concept of collateral sensitivity between drugs, we propose that collateral sensitivity likelihoods (CSLs) should be derived. By deriving the likelihood of collateral sensitivity between drugs,
we can quantify the risk associated with different drug sequences. Figure 5a shows an example table of collateral similarity likelihoods derived from the in silico evolution model. We note that whilst there exist 28 drug pairs exhibiting guaranteed collateral similarity ($P = 1.0$, right), there also 16 others with likelihood $1.0 > P > 0.75$ of collateral sensitivity. Where collateral sensitivity is assayed from a small number of experimental evolution replicates, these drug pairs may appear to exhibit universal collateral sensitivity and could thus unexpectedly fail stochastically. Conversely, if no universally collaterally sensitive drugs were known, drug pairs exhibiting a high likelihood of collateral sensitivity might represent the best option available.

Figure 5b shows the experimentally derived CSLs for antibiotics administered following cefotaxime. We find that collateral sensitivity is rare, with $P = \frac{1}{30}$ for TIC being the most likely. If we also consider the likelihood that sensitivity of the second-line drug is unchanged, then it is clear that PIP or gentamicin (GNT) are the best second-line drugs following cefotaxime (amongst those we have assayed). Conversely, cross-resistance is near universal in cefazolin and cefetolozane/tazobactam. For puromycin and ampicillin/sulbactam (SAM), we estimate that cross-resistance occurs with probability $P > 0.5$, but that the probability of no change or collateral sensitivity is still high ($P > 0.3$ in both cases). Drugs such as these highlight the importance of deriving collateral sensitivity likelihoods by means of multiple evolutionary replicates, as a single evolutionary replicate may identify unchanged sensitivity where cross-resistance is likely.

**Discussion**

We have demonstrated the existence of an evolutionary branching point in the fitness landscape of *E. coli* under cefotaxime that can induce divergent evolution and differential collateral response to second-line antibiotics. By means of 60 replicates of experimental evolution, we have estimated the likelihood of collateral sensitivity in each of eight second-line therapies. Critically, we find that collateral sensitivity is never universal, and is in fact rare. Furthermore, by consideration of a mathematical model of evolution parameterised by small, combinatorially complete fitness landscapes, we have highlighted the extent and importance of evolutionary divergence. This modelling highlights that divergent collateral response is likely common (occurring in 14/15 drugs for which empirical landscapes were derived) and further, that even where collateral sensitivity is reported from a small number of evolutionary replicates, cross-resistance can still occur with high likelihood.

Table 2 Mutations identified through whole-genome sequencing

| Replicate | SHV-1 SNVs | Chromosomal SNVs | Deletions (ranges) | IS1D insertions |
|-----------|------------|------------------|--------------------|----------------|
| Parental  |            |                  |                    |                |
| X1p10     |            |                  |                    |                |
| X2p10     |            |                  |                    |                |
| X3p10     |            |                  |                    |                |
| X4p10     |            |                  |                    |                |
| X5p10     |            |                  |                    |                |
| X6p10     |            |                  |                    |                |
| X7p10     |            |                  |                    |                |
| X8p10     |            |                  |                    |                |
| X9p10     |            |                  |                    |                |
| X10p10    | G238S      | 3630620 C > A (envZ R339L); 771931 C > T (speF L115L) | IS1D at 2401329 (upstream of omcP) | IS1D at 2401801 (upstream of omcP) |
| X11p10    | G238S      | 3630620 C > A (envZ R339L) | IS1D at 2906979 interrupts gshA; 12 kb deletion adjacent | IS1D at 2906979 interrupts gshA; 12 kb deletion adjacent |
| X12p10    | G238S      |                  |                    |                |

The single-nucleotide variants (SNVs), insertions and deletions identified through whole-genome sequencing of the replicates X1-X12 following passage 10 are listed.

Taken together, our results indicate that we must take care when interpreting collateral sensitivity arising in low-throughput evolution experiments. To this end, we propose that collateral sensitivity likelihoods should be evaluated by use of multiple parallel evolutionary replicates to better capture the inherent stochasticity of evolution. The high-throughput experimental evolution necessary to accurately evaluate CSLs between many drug pairs could be facilitated by automated cell culture systems, such as the morbidostat developed by Toprak et al., which incorporates automated optical density measurements and drug delivery to track and manipulate evolution.

It should be noted that although the evolution of pathological bacteria within the clinic is most likely stochastic, it is unclear whether the gradient plate system model used in the present study, and others, correctly captures this stochasticity. The gradient plate method proceeds by serial replating of bacterial populations that induces population bottlenecks and strong selection. This mode of population dynamics clearly differs from that with *E. coli* experience naturally. We note that our experimental results are derived only for the gradient plate method and that other protocols without serial passing have also been explored. Such experimental designs may exhibit less stochastic dynamics and thus permit the derivation of collateral sensitivity likelihoods with fewer replicates. Alternatively, it may be the case that additional stochasticity is introduced as evolutionary phenomena such as clonal interference, wherein multiple fitter clones compete, do not occur. To empirically determine collateral sensitivity likelihoods it may be the case that we must employ novel in vitro experimental techniques to more closely match in vivo dynamics. Here too, automated culture systems such as the morbidostat could help, as automated changes to the drug concentration can prevent the bacterial population expanding too rapidly, mitigating the need for serial replating and permitting high-throughput experiments.

The mathematical model we have presented does not capture the full complexity of evolution. For example, we do not account for deletions, insertions or duplications of genes such as SHV. Nevertheless, this model still proves useful in providing intuition about the extent to which stochasticity can drive differential collateral response. We can expect the introduction of additional mutational complexity to introduce further stochasticity. An immediate improvement to our modelling would be to extend the model to account for alternative population dynamics; for example, permitting heterogeneous populations, variable population sizes or drug pharmacodynamics. A further complication
Collateral sensitivity likelihoods. **a** (Left) The table of collateral sensitivity likelihoods (CSLs) derived from the mathematical model. Each entry indicates the likelihood that the first drug (columns) induces increased sensitivity in the second (rows). (Right) The CSL table thresholded for drugs with $P = 1.0$ (top) and $P > 0.75$ (bottom) probability of inducing collateral sensitivity. **b** The estimated likelihoods for collateral sensitivity, cross-resistance or no change in sensitivity derived from the 60 replicates of experimental evolution is that drug resistance can arise by physiological adaptions in addition to genetic mutation, which our mathematical modelling does not take into account. We see evidence for physiological adaption in the evolution of the replicate X7 which exhibits increased resistance to cefotaxime without associated mutations. Further, changes in sensitivity arising from such phenotypic plasticity may be reversible over short time scales. Ultimately, by the use of extended mathematical models we may be able to better simulate in vitro experiments in order to understand how generalisable they are to in situ evolutionary dynamics.

As an alternative to high-throughput evolutionary experiments, we note that drug sequences are frequently prescribed in the clinic. Thus, the distributed collection of matched pre- and post-therapy drug sensitivity assays, potentially coupled with genomic sequencing where this is feasible, could provide sufficient data to determine CSLs. This approach is particularly appealing as the CSLs derived would not be subject to the caveats regarding experimentally derived measures of collateral sensitivities outlined above. Further, clinically derived CSLs would readily account for non-genetic adaptions and inter-patient variabilities in physiology that may impact drug sensitivities. A similar approach has already been employed in the treatment of HIV to monitor the evolution of drug resistance.

Regardless of the approach taken to derive CSLs, what is clear is that we must move beyond the present methodology of designing drug sequences through low-replicate-number experimental evolution, and towards an evolutionarily informed strategy that explicitly accounts for the inherent stochasticity of evolution.

**Methods**

**Mathematical modelling of evolution.** The probabilities for evolutionary trajectories through the empirically derived fitness landscapes were calculated from a previously published mathematical model. Briefly, the population is assumed to be isogenic and subject to strong selection weak mutation evolutionary dynamics. Thus, the population genotype (taken from domain $\{0, 1\}^k$) is modelled as periodically replaced by a fitter (as determined by the landscape) neighbouring genotype (defined as any genotype whose Hamming distance from the population genotype is equal to one). This process is stochastic and the likelihood of a genotype, $j$, replacing the present population genotype, $i$, is given by

$$P(i \rightarrow j) = \begin{cases} \left(\frac{2^{r-1}(1-r)}{r^k}\right) & \text{if } f(j) > f(i) \text{ and } \text{Ham}(i,j) = 1 \\
0 & \text{otherwise.}
\end{cases}$$

(1)

Where no such fitter neighbour exists, the process is terminated. The value of $r$ determines the extent to which the fitness benefit of a mutation biases the
Collateral sensitivity analysis and significance testing. To determine collateral sensitivity (or cross-resistance) we determined which evolutionary replicates exhibited a significantly different MIC from the parental line via a likelihood ratio test. In total, 60 comparisons were performed for each of the 9 drugs, yielding a total of 540 comparisons. A Bonferroni correction was used to account for multiple hypothesis testing. For those replicates exhibiting a significant ($p < 0.05/540$) change in MIC, the collateral sensitivity was determined.

$$CR = m_{i} - m_{0}$$  \hspace{1cm} (5)

Otherwise, we set $CR = 0$.

Targeted sequencing of SHV. Plasmid DNA was isolated using the Wizard Plus Miniprep DNA purification systems (Promega). Sequencing of the SHV gene was performed using M13 primers (MClab, Harbor Way, CA).

Whole-genome sequencing. For genome sequencing, total DNA was prepared using MasterPure Complete DNA Purification Kit (Epicentre; Madison, Wisconsin). NexteraXT libraries were prepared and sequenced on an Illumina NextSeq 500 at the Genomics Core at Case Western Reserve University. Paired sequence reads were mapped using bwa-mem to the DH10B genome (accession CP000948.1), the pBC SK− plasmid (https://www.novopeprolabs.com/vector/V12548), and the SHV-1 gene (accession JX268741.1). Reads were also assembled into contigs using velvet. Three approaches were used to identify de novo mutations. First, SNVs were called using the mapped reads using the Genome Analysis Toolkit (GATK). Second, large deletions were identified using a combination of detection of low-coverage regions of the reference based on read mapping results and BLAST searches between the DH10B reference sequence and the contigs. Insertion sequence (IS) elements present in the DH10B genome were identified using ISFinder and locations for IS elements were mapped in the contigs using ISSeeker.

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

Data availability. All MIC measurements are available in Supplementary Dataset 1. All sequencing data are deposited to the NCBI sequence read archive under accession code PRJNA515080. The Python code used in the mathematical modelling and statistical analyses are available at: https://github.com/Daniel-Nichol/CollateralSensitivityRepeatability.

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Author contributions
D.N., R.B., and J.S. conceived of the experiment which was performed by J.R., C.B., and P.J. J.R. and C.B. analysed the experimental data. D.N., R.B., and J.S. conceived of the mathematical model which was implemented by D.N. Mathematical results were analysed by D.N., P.J., A.A., and J.S. All participated in writing the manuscript.

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