LETTER

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Evolutionary rescue from extinction is contingent on a lower rate of environmental change

Haley A. Lindsey[1], Jenna Gallie[1,2,3], Susan Taylor[1] & Benjamin Kerr[1]

The extinction rate of populations is predicted to rise under increasing rates of environmental change[1–3]. If a population experiencing increasingly stressful conditions lacks appropriate phenotypic plasticity or access to more suitable habitats, then genetic change may be the only way to avoid extinction[1]. Evolutionary rescue from extinction occurs when natural selection enriches a population for more stress-tolerant genetic variants[1,3]. Some experimental studies have shown that lower rates of environmental change lead to more adapted populations or fewer extinctions[4–9]. However, there has been little focus on the genetic changes that underlie evolutionary rescue. Here we demonstrate that some evolutionary trajectories are contingent on a lower rate of environmental change. We allowed hundreds of populations of *Escherichia coli* to evolve under variable rates of increase in concentration of the antibiotic rifampicin. We then genetically engineered all combinations of mutations from isolates evolved under lower rates of environmental change. By assessing fitness of these engineered strains across a range of drug concentrations, we show that certain genotypes are evolutionarily inaccessible under rapid environmental change. Rapidly deteriorating environments not only limit mutational opportunities by lowering population size, but they can also eliminate sets of mutations as evolutionary options. As anthropogenic activities are leading to environmental change at unprecedented rapidity[1], it is critical to understand how the rate of environmental change affects both demographic and genetic underpinnings of evolutionary rescue.

One of the first real-time evolution experiments demonstrated that evolutionary rescue from extinction is sensitive to the rate of environmental change[7]. In the 1880s, it was shown that protist lineages that had experienced a gradual increase in temperature over 7 years were able to thrive at 70 °C, whereas non-evolved populations rapidly went extinct when suddenly exposed to any temperature above 60 °C (ref. 7). Other experiments have explored the effect of the rate of environmental change on evolution in fruitflies[9], algae[6], yeast[4,5] and bacteria[8]. These pioneering studies have found that gradual change leads to lower levels of extinction or to better adapted populations. However, the precise genetic underpinnings of these evolutionary dynamics have remained unexplored.

We propagated 1,255 populations of *E. coli* via serial transfer under increasing concentrations of the antibiotic rifampicin. The three experimental treatments involved different rates of change: Sudden, Moderate and Gradual. All populations started in antibiotic-free medium and ended at a maximal concentration of rifampicin (190 μg ml^{-1}) (Fig. 1a). The Sudden populations were exposed to the maximal rifampicin concentration after their first transfer and remained at that concentration for the duration of the experiment. The Moderate populations underwent a slower increase in concentration, such that they arrived at the maximal concentration halfway through the experiment and then remained at that concentration. The Gradual populations experienced the slowest increase in antibiotic concentration, reaching the maximum on the last transfer of the experiment.

There were significant differences in the extinction rate among treatments (Pearson's chi-squared test, $\chi^2 = 709.88$, $P < 0.001$; Fig. 1b). As the rate of environmental change increased, the fraction of populations that survived the entire experiment dropped precipitously. Relative to

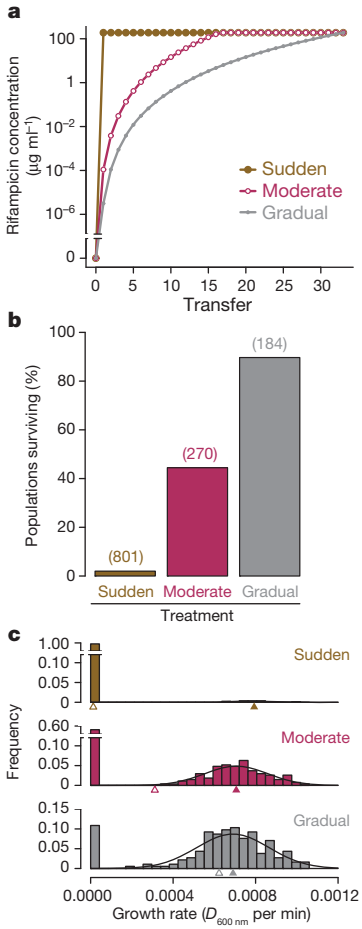

Figure 1 | Survival and growth under different rates of environmental change. **a**, The three experimental treatments. Rifampicin concentration increases from a minimum value (0 μg ml^{-1}) to a maximum value (190 μg ml^{-1}) at different rates within each treatment. **b**, The percentage of populations surviving the experiment in each treatment. The number above each bar is the corresponding number of initial populations. **c**, The growth rate of populations from the end of the experiment. Growth rate is measured in attenuance units ($D_{600\,nm}$) per minute using a spectrophotometric assay (see Methods). If a population went extinct, its growth rate was assigned to zero. Note that the full range on the ordinate scale (frequency) is different for each treatment. The open triangles give mean growth rate and the closed triangles give mean growth of surviving populations. Growth rate distributions for the surviving populations are shown with maximum likelihood normal curves.

[1]Department of Biology and BEACON Center for the Study of Evolution in Action, University of Washington, Seattle, Washington 98195, USA. [2]Department of Environmental Microbiology, Eawag, 8600 Dübendorf, Switzerland. [3]Department of Environmental Systems Science, ETH Zurich, 8092 Zurich, Switzerland.

populations in the other treatments, Gradual populations spent more time at sub-inhibitory concentrations of rifampicin where mutations conferring resistance could be selected[10]. This would give Gradual populations more opportunities for resistance mutations to occur and spread. However, does evolutionary rescue occur by the same set of mutations in all treatments? We found significant differences in growth rate between Gradual and Sudden isolates from the end of the experiment (log likelihood ratio test, $\chi^2 = 12.95$, $P = 0.0015$; Fig. 1c), which indicates that different mutations occurred in different treatments.

Mutations that confer resistance to rifampicin are known to occur in specific regions of the *rpoB* gene, which codes for the β subunit of RNA polymerase[11–13]. Figure 2a displays the *rpoB* mutations from all 13 surviving Sudden populations and a random sample of 30 Moderate and 30 Gradual populations. All mutations were non-synonymous. The Gradual and Moderate populations have significantly higher nucleotide diversity[14] than the Sudden populations (permutation tests, $P < 0.001$; Fig. 2b).

Interestingly, all isolates in the Sudden treatment possess only single mutations in *rpoB*, whereas the majority of the isolates from the other treatments contain multiple *rpoB* mutations. We identified the first mutation to arise in populations with multiple mutations by sequencing samples frozen at various points in time during the experiment. Of the 44 Gradual and Moderate isolates with multiple mutations, only a single (Moderate) isolate has exactly the same first mutation as any Sudden isolate. When confining the analysis to first mutations only, the Gradual and Moderate isolates continue to have significantly higher nucleotide diversity than the Sudden isolates (permutation tests, $P < 0.001$). This pattern is consistent with a situation in which more mutations are selectively accessible at lower antibiotic concentrations. Indeed, when we engineer the first *rpoB* mutations from a handful of isolates into a common background, we find that some mutations that are beneficial at lower antibiotic concentrations are no longer advantageous under the highest concentrations (Fig. 2c).

If the first mutations to occur in the Gradual and Moderate treatments are inviable at the maximal concentration of rifampicin (see Fig. 2c), subsequent mutations are required to salvage the lineage at the highest concentration. Such secondary mutations might only be selectively accessible after the first mutation, which might only be selectively accessible under sub-maximal antibiotic concentrations. In such a case, a mutational trajectory accessible under gradual environmental change will be unlikely to occur under rapid change.

The evolutionary trajectory of a population evolving under conditions of strong selection and weak mutation[15] can be envisioned as a series of steps between genotypes differing by a single mutation. Each step in a selectively accessible path involves an increase in genotype fitness. However, because the fitness of genotypes can change with the environment, the historical sequence of environments can qualitatively affect which paths are selectively accessible. Consider a path from one genotype to another that is selectively accessible under a sequence of distinct environments. We call such a path 'historically contingent upon some focal environment(s)' if this path is not selectively accessible in the absence of the focal environment(s) (see Fig. 3).

In the Supplementary Information, we demonstrate that if a path ending at an adaptive peak is historically contingent upon an intermediate environment, then the sign of the fitness effect of some mutations

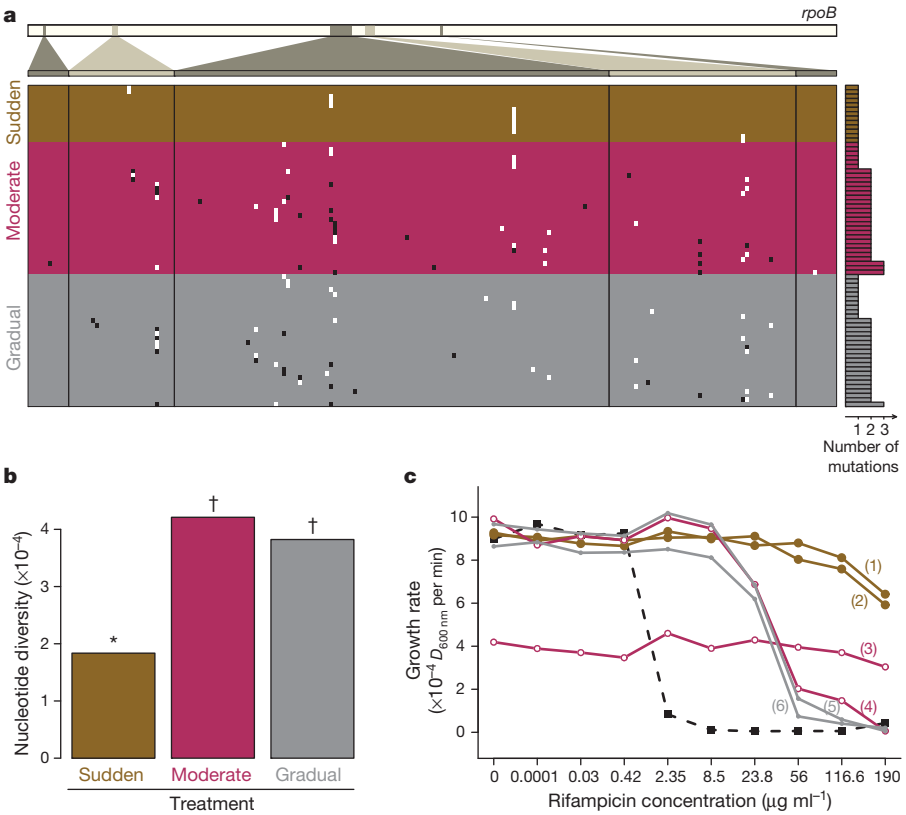

Figure 2 | Mutations and their effects. **a**, The locations of mutations in 73 isolates. The top bar represents the *rpoB* gene. Gene regions with mutations in at least one isolate are magnified for the table. Each isolate is a single row (13 Sudden, 30 Moderate and 30 Gradual). The location of the first mutation to arise in the evolutionary history of the isolate is given by a white mark and subsequent mutations are given by black marks. The number of mutations in each isolate is specified by the size of the small bar to the right of the table. **b**, The index of nucleotide diversity (π) for each treatment. Different symbols (asterisk and dagger) over the bars indicate significantly different π values by permutation tests. **c**, Mean growth rate of single mutants in different environments. Each of the first mutations to fix in six different populations was engineered into a common background (these mutations were (1) g1546t, (2) g436t, (3) t437a, (4) t1532g, (5) c1527a and (6) c1721t). The treatments have the same representation as in Fig. 1. The growth rates for the ancestral genotype are given by the squares. By comparing engineered mutants with the ancestor, we see that three of the mutations (4, 5 and 6) do not increase growth rate at the highest drug concentration.

must depend on genetic background (sign genetic epistasis[16]) and the sign of the fitness effect of some mutations must depend on environment ('sign environmental epistasis'). Environmental epistasis is a genotype by environment (G × E) interaction and can be described as plasticity in the fitness effect of a mutation[17]. Figure 3 illustrates both forms of epistasis in the context of historical contingency upon environment. Sign genetic epistasis has been found in a number of different systems[18–21], and both forms of sign epistasis have been reported in microbial systems[13,22]. For an evolutionary path ending on an adaptive peak, sign epistasis is a necessary, but not sufficient, condition to obtain historical contingency upon an intermediate environment; thus, empirical evidence for such contingency requires verification that particular configurations of epistasis exist.

To demonstrate historical contingency directly, we constructed all intermediate genotypes between an ancestor and its descendant and assessed their fitness across a spectrum of environments. We focused on two evolved isolates from different populations in the Gradual treatment, each possessing two mutations in *rpoB* (see Supplementary Information for the analysis of isolates from the Moderate and Sudden treatments). For each isolate, we genetically engineered the four relevant genotypes in a common background using an allele exchange protocol that resulted in scar-free construction of the desired genotypes (see Methods and Supplementary Information).

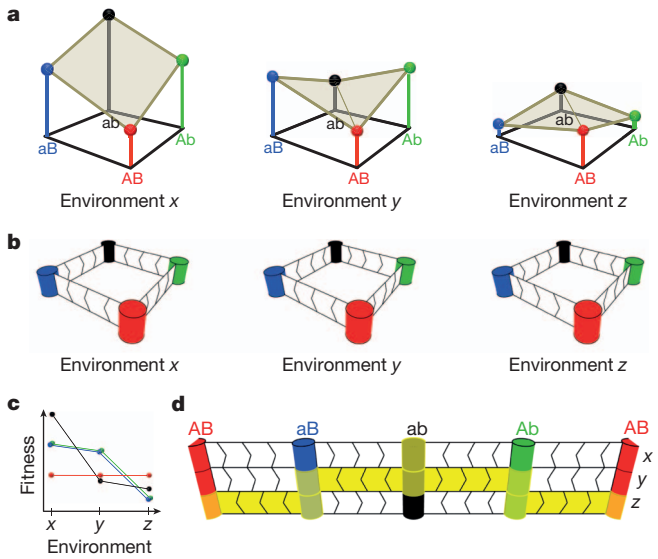

Figure 3 | A two-locus biallelic example illustrating historical contingency upon the environment. **a**, The adaptive landscapes for the four genotypes are depicted in the environments *x*, *y* and *z*. The height of each 'lollipop' gives the fitness of the genotype. When using an additive model, genetic epistasis is present when the fitness of the four genotypes are not embedded in a single plane (for example, sign genetic epistasis occurs in environments *y* and *z*, but no genetic epistasis is found in *x*). **b**, For each environment, selective accessibility between genotypes is indicated as 'flow arrows' between the neighbouring genotypes (cylinders). One genotype is selectively accessible from another in a given environment if a single mutation transforms the latter into the former and if the former has a higher fitness than the latter. **c**, A 'fitness norm of reaction' for the four genotypes is presented. When the trajectories of two mutational neighbours are not parallel, environmental epistasis is present. For example, the mutation from ab to Ab is detrimental in environment *x*, but beneficial in environment *y* (sign environmental epistasis). **d**, Imagine stacking the squares from **b**, with the *x* square on top of the *y* square and the *z* square on the bottom. Then slice the red cylinders in half and open up the stack to form the flat wall shown. Suppose the population started fixed for the ab genotype and the environments were experienced in the order $x \rightarrow y \rightarrow z$. Imagine starting on top of the black cylinders and rappelling down the wall, potentially moving horizontally to different cylinders in accord with the flow arrows. A fully descending trajectory would represent a selectively accessible path. The only two selectively accessible paths from genotype ab to genotype AB are highlighted. We note that environment *y* must be experienced: if a population in environment *x* was moved directly into environment *z*, then the genotype AB would be selectively inaccessible from ab. Thus, this is an example where an evolutionary path is historically contingent upon an environment.

The growth rate, competitive ability and viability of the engineered strains across a gradient of rifampicin concentrations are shown in Fig. 4. There is clear historical contingency upon intermediate environments for both isolates. For the lineage yielding the first Gradual isolate (Fig. 4a, b), paths from the ancestor to the double mutant through either single mutant are selectively accessible only for intermediate concentrations of rifampicin. For the lineage evolving the second Gradual isolate (Fig. 4c, d), the path actually taken (ab→aB→AB) must be completed piece-wise in different environments. For both lineages, the slow change in concentration of rifampicin was critical to the evolutionary sequence: if the environment changed from an absence of antibiotic to its maximal concentration abruptly (as in the Sudden treatment), the path taken under gradual change would not be available (Fig. 4b, d). We find similar evidence for historical contingency upon intermediate environments in the Moderate isolates (see Supplementary Information). We suggest that even if certain mutations are selectively neutral or deleterious under high levels of stress, they can nonetheless predispose the lineage to gain other mutations that allow it to escape extinction at high stress. Such priming mutations need not drift into a population; rather they can be (powerfully) selected at low levels of stress. This means that the rate of environmental deterioration can qualitatively affect evolutionary trajectories. In our system, we find that rapid environmental change closes off paths that are accessible under gradual change.

In our experiment, demographic factors certainly contribute to the higher prevalence of evolutionary rescue under a gradual increase in the level of stress. Specifically, the population growth rate of the ancestor is positive in the Gradual treatment over the initial set of transfers, whereas it is negative in the Sudden treatment. Thus, there are more chances for mutations to occur in the Gradual treatment. A fraction of isolates in the Gradual and Moderate treatments had only a single mutation in *rpoB*, some of which affected amino acid residues that also were mutated in the Sudden treatment (see Fig. 2a). Barring mutations outside of the sequenced region[23], these mutations could represent single evolutionary steps that both rescue the population under high stress and are advantageous under low stress (see mutations (1), (2) and (3) in Fig. 2c). There will be more of these mutations under lower rates of change because there will be more mutational opportunities in populations with higher growth rates. However, in this article we have also traced out another important factor that can make evolutionary rescue more likely under gradual change. If mutations selected at intermediate levels of stress predispose a lineage to receive additional epistatic mutations conferring viability under higher stress, then gradual change in the environment can 'open up' evolutionary endpoints. We propose that such historical contingency upon the environment is particularly relevant to some current problems resulting from anthropogenic activities, including the rise of antibiotic resistance and extinction in the face of climate change.

The presence of antibiotics in industrial and agricultural waste runoff[24] has the potential to select for antibiotic-resistant microbes even when the drugs are present at very low concentrations[10]. Our work suggests an additional problem with such exposure: low drug concentrations can evolutionarily 'prime' bacterial populations, by bringing a population (mutationally) closer to genotypes that would enable drug resistance at a high concentration. In a more direct medical context, rifampicin is one of the first-line drugs used to treat tuberculosis[23,25]. The mutations conferring resistance to rifampicin in our experimental *E. coli* populations are observed in similar genetic locations in *Mycobacterium tuberculosis*[23,25,26], indicating that our results may have relevance to this disease system. Specifically, we propose that the manner of change in the concentration of rifampicin within a patient could affect both the persistence and evolution of this pathogen, which has implications for disease management.

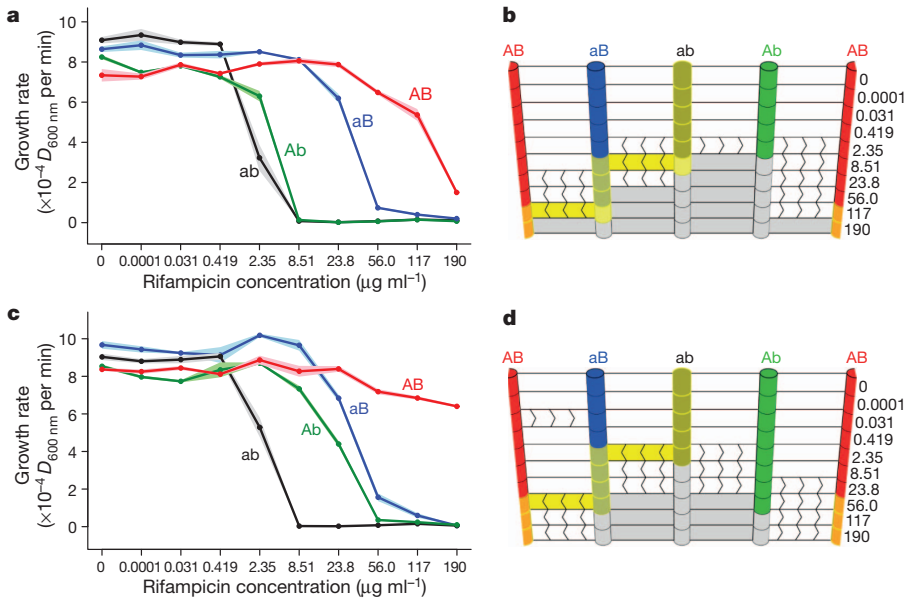

Figure 4 | Selective accessibility from the ancestral genotype to two evolved genotypes in two different Gradual populations. The evolved isolate in the first Gradual population has two mutations in *rpoB*, g428a and c1721t, yielding the amino acid substitutions R143H and S574F, respectively. The four engineered genotypes are denoted ab (bases g428 and c1721), Ab (bases a428 and c1721), aB (bases g428 and t1721) and AB (bases a428 and t1721). The evolved isolate in the second Gradual population also has two mutations in *rpoB*, a443t and c1527a, yielding the amino acid substitutions Q148L and S509R, respectively. The four engineered genotypes for the second isolate case are denoted ab (bases a443 and c1527), Ab (bases t443 and c1527), aB (bases a443 and a1527) and AB (bases t443 and a1527). **a**, The maximum population growth rate for each of the engineered genotypes corresponding to the first isolate across a gradient of rifampicin concentrations. The points are means and the shading gives the standard error. **b**, The 'accessibility wall' for the case of the first isolate. The four genotypes are cylinders of different colours and each horizontal stratum corresponds to a distinct concentration of rifampicin (labels to the right, μg ml^{-1}). As maximum growth rate is only one component of fitness, we performed pairwise competitions between all mutational neighbours across the gradient of rifampicin to determine selective accessibility (see Methods). Flow arrows indicate that the genotype from which the flow starts decreased its proportion significantly in competition with the genotype to which the flow ends (*t*-test with Bonferroni corrections for multiple comparisons, $P < 0.05$). An edge between cylinders without arrows represents a competition in which neither strain increased in proportion significantly. A grey cylinder indicates that the genotype could not form a turbid population in the relevant concentration of rifampicin (see Methods). A grey edge indicates that neither bordering genotype could form a turbid population in the relevant concentration of rifampicin starting with a population of 10–100 cells. The highlighting shows the actual evolutionary trajectory followed during the experiment. This trajectory was elucidated by sequencing the *rpoB* locus from samples frozen at various time points during the experiment. **c**, **d**, The growth rates (**c**) and selective accessibility (**d**) for the case of the second Gradual isolate.

Whereas extinction of a target population may be the preferred outcome in the case of pathogens, it is generally undesirable for populations threatened by anthropogenic climate change. There is much interest in the fate of current ecological communities under a markedly changing environment[1,2,27,28]. For logistic reasons, experimental communities in the field are sometimes subjected to a predicted amount of climate change over a greatly compressed time frame. If evolutionary rescue is sensitive to the rate of change, the estimated risk of extinction may depend on the degree of compression[28]. For instance, it was found that a more gradual increase in ambient CO_2 concentration resulted in a substantially higher species richness in a mycorrhizal fungi community, in comparison to the abrupt change typical of other CO_2 elevation experiments[29]. Such caveats aside, our study does suggest that there is genuine reason to worry about unusually high rates of environmental change. As the rate of environmental deterioration increases, there can be pronounced increases in the rate of extinction. Our work suggests that this is due not only to the lost mutational opportunities that come with a dwindling population, but also to lost potentiating mutations that would be selected under milder conditions.

METHODS SUMMARY

In total 1,255 populations of *Escherichia coli* B were initiated from a common ancestor and evolved for two months in microtitre wells with minimal glucose (MG), diluting 40-fold every 48 h into fresh media with a rifampicin concentration determined by experimental treatment (Fig. 1a). Any population completely lacking visible cloudiness after a 48-h growth period was considered extinct. From the set of populations surviving the evolution experiment, a random subset was chosen in which isolates were sequenced at the *rpoB* locus. Engineered mutants were created by site-directed mutagenesis and allelic exchange (using a variant of the pKOV vector[30]). For the growth assay, each strain or population was revived from the freezer via growth in MG over 24 h and then diluted 40-fold into fresh media with rifampicin (see Supplementary Table 1 for concentrations). Attenuance readings (over 24 h at 600 nm) were used to calculate the maximum growth rate. For the pairwise competition assay, two strains were grown separately for 24 h in MG, mixed in equal volume, and then diluted 40-fold into fresh MG with rifampicin (see Supplementary Table 1) and competed for 48 h. The initial and final densities of the competitors were determined by selective plating (one competing strain was neutrally marked with the ability to use arabinose). For the viability assay, each strain was revived from the freezer and then diluted 40-fold and 10^5-fold into MG with rifampicin (see Supplementary Table 1). Growth was determined visually after 48 h. See the extended methods for further details.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to B.K. (kerrb@uw.edu).

METHODS

Strains. *Escherichia coli* B (REL606) was used for the evolution experiment. Chemically competent DH5α-*λpir E. coli* and One Shot TOP10 *E. coli* (Invitrogen) were used for the genetic engineering protocol.

Growing and storing bacteria. Standard growth conditions: for many parts of the experiment, bacterial growth occurred in 96-well microtitre plates with a final volume of 200 µl of minimal glucose (MG) medium per well. The growth phase lasted 48 h, during which time the plates were incubated and shaken (380 r.p.m. in a microtitre plate shaker at 37 °C). Transfers to fresh media involved 40-fold dilution.

Storing bacteria: populations were pipetted in their entirety into sterile tubes with 32 µl of dimethyl sulphoxide and stored at −80 °C. Each frozen isolate was derived from a single colony that was inoculated into 5 ml of lysogeny broth (LB) medium and shaken at 205 r.p.m. overnight at 37 °C. Then 1 ml of the culture was mixed with 160 µl of 80% glycerol and stored at −80 °C.

Evolution experiment. A single culture of *E. coli* B was used to inoculate 1,255 populations in microtitre wells. All populations were serially propagated for two transfers under standard growth conditions in the absence of rifampicin (to allow for independent mutations to occur in different populations). Each population was then serially propagated under standard growth conditions in the presence of a specific concentration of rifampicin, which was dependent on treatment (Supplementary Table 1). Before each transfer, each population's well was checked carefully for cloudiness. Populations in which two independent observers did not detect any visible growth were declared 'extinct' and were not transferred. Contamination of a media control well on transfer 19 forced us to restart the evolution series from transfer 18 (which had been stored at 4 °C). All populations were frozen periodically throughout the experiment. Three Sudden populations were lost due to a transfer error and one Moderate population could not be revived from 4 °C.

Sequencing. We randomly chose 30 surviving Moderate populations and 30 surviving Gradual populations. A single colony from each of these populations and from all 13 surviving Sudden populations was selected for sequencing. PCR amplification was performed using the primers described in ref. 11. After confirmation via gel electrophoresis, the PCR product was purified using the ExoI/CIAP (calf intestinal alkaline phosphatase) method. Purified products were sequenced (Macrogen) and analysed with Sequencher (version 4.6). For isolates with multiple mutations, the order of appearance was determined by sequencing at various points in the lineage's history.

Genetic engineering. Fragments of *rpoB* containing the desired mutation(s), flanked by 500–2,000 base pairs on either side, were ligated into pCR8/GW/TOPO (Invitrogen) for storage. When the desired mutation could not be amplified alone, it was introduced into a wild-type fragment of *rpoB* in pCR8/GW/TOPO using a Phusion site-directed mutagenesis kit (NEB) and stored in DH5α-*λpir* cells. After sequence confirmation, the resulting fragments were ligated into a customized version of pKOV[30] in which bases 1117–4057 were removed and replaced with a BglII restriction site. The desired mutations were then introduced into the ancestral REL606 background using a modified version of previously described two-step allelic exchange methods[30] (see Supplementary Information). The ancestral sequence of *rpoB* was reintroduced to each engineered mutant via the same technique. These re-engineered ancestors were used in the growth and competition assays to control for the allelic replacement process.

Growth assay. Spectrophotometric growth assays were performed on all populations that survived the experiment and the engineered strains. Bacteria were revived from the freezer and then diluted 40-fold into fresh MG medium with a pre-specified concentration of rifampicin (Supplementary Table 1). The culture was grown for 24 h at 37 °C in a VersaMax spectrometer (Molecular Devices) with attenuance (D) readings taken every 30 min at 600 nm. Using a sliding window of 2 h, the rate of change in $D_{600\,\mathrm{nm}}$ at a focal time point (that is, the first derivative of the $D_{600\,\mathrm{nm}}$ curve) was approximated by the slope of a linear least-square fit to the $D_{600\,\mathrm{nm}}$ data centred on the time point of interest. The growth rate of the strain or population was the average of the top three slopes. Each strain or population was replicated four times (with position in the microtitre plate randomized) and the most deviant replicate was discarded.

Competition assay. In all competitions, one strain possessed the ability to use arabinose (Ara^+), which was a selectively neutral marker. The Ara^+ strain forms pink colonies on tetrazolium arabinose agar and grows on minimal arabinose agar, whereas the Ara^- strain forms red colonies on tetrazolium arabinose agar and does not grow on minimal arabinose agar. The two strains to be competed were revived from the freezer and equal volumes of the two stationary cultures were combined. The co-culture was then diluted 40-fold into MG medium with a pre-specified concentration of rifampicin (Supplementary Table 1) and incubated under standard growth conditions. Dilution and plating was performed on tetrazolium arabinose and minimal arabinose agar at the beginning and end of the 48 h growth phase. Let $d_i(t)$ be the bacterial density of strain i at time t. The competitive index of strain 1 relative to strain 2 is $W_{1,2} = \{d_1(48)d_2(0)\}/\{d_1(0)d_2(48)\}$, which measures how the ratio of competitors changes over a growth cycle. If $W_{1,2} > 1$, then strain 1 has increased in proportion; whereas strain 2 has proportionally increased if $W_{1,2} < 1$. Each competition was replicated four times.

Population viability assay. The engineered strain was revived from the freezer and diluted both 40-fold and 10^5-fold into fresh MG medium with a pre-specified concentration of rifampicin (Supplementary Table 1). Each diluted culture was incubated under standard growth conditions, and after 48 h, it was checked visually for cloudiness indicating growth. The strain was deemed 'viable' if at least five of six replicate cultures were cloudy and 'inviable' if at most two of six replicate cultures were cloudy (all strains fell into these two classes). A strain viable after 40-fold dilution could persist at the relevant rifampicin concentration under our experimental conditions, whereas a strain viable after 10^5-fold dilution could increase from rarity at the relevant rifampicin concentration. The ability to increase from rarity implies persistence, but the converse does not necessarily hold.

Sampling rationale. Pilot runs suggested a high extinction rate under the Sudden regime; thus, hundreds of populations were needed to estimate extinction rates and yield survivors for further analysis. Whereas isolates from all Sudden populations were sequenced, we sampled the surviving Moderate and Gradual populations with sufficient depth to estimate nucleotide diversity. Isolates from two populations from each treatment were chosen for genetic reconstruction and growth/competition/viability assays across environments (see Supplementary Information). This final stage was meant to illustrate the phenomenon of 'historical contingency upon environment.' More samples would be required to estimate its average incidence.