1 **Chromosomal barcoding of *E. coli* populations reveals lineage** 2 **diversity dynamics at high resolution**

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15 Evolutionary dynamics in large asexual populations is strongly influenced by multiple
16 competing beneficial lineages, most of which segregate at very low frequencies. However,
17 technical barriers to tracking a large number of these rare lineages in bacterial populations
18 have so far prevented a detailed elucidation of evolutionary dynamics. Here, we overcome
19 this hurdle by developing a chromosomal-barcoding technique that allows simultaneous
20 tracking of ~450,000 distinct lineages in *E. coli*, which we use to test the effect of sub-
21 inhibitory concentrations of common antibiotics on the evolutionary dynamics of low-
22 frequency lineages. We find that populations lose lineage diversity at distinct rates
23 corresponding to their antibiotic regimen. We also determine that some lineages have
24 similar fates across independent experiments. By analyzing the trajectory dynamics, we
25 attribute the reproducible fates of these lineages to the presence of pre-existing beneficial
26 mutations, and we demonstrate how the relative contribution of pre-existing and de novo
27 mutations varies across drug regimens. Finally, we reproduce the observed lineage
28 dynamics by simulations. Altogether, our results provide both a valuable methodology for
29 studying bacterial evolution as well as insights into evolution under sub-inhibitory
30 antibiotic levels.

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Sequencing advances have generated tremendous breakthroughs in the identification of beneficial mutations arising in laboratory evolution experiments, as well as mutations contributing to the emergence of anti-cancer or anti-bacterial drug resistance in clinical environments¹⁻⁴. However, measuring the dynamics of evolutionary processes remains a major challenge, particularly in large asexual populations where many low-frequency, small-effect mutations are known to spread simultaneously⁵⁻⁸. A complete quantitative description of evolutionary dynamics therefore requires tracking a large number of individual lineages, most of which occur at extremely low frequencies (10^{-6} – 10^{-5}), in parallel and over multiple generations.

Whole-genome sequencing (WGS) techniques currently fall short of fulfilling this requirement at typical read depths, as they are usually unable to detect mutations at frequencies below $\sim 0.1\%$ ^{9,10}. Various alternative solutions have been applied to reconstruct population dynamics from trajectories of individual lineages at much higher resolution than accessed by WGS¹¹⁻¹³. One method that dramatically increases the frequency resolution of individual lineages is based on tagging chromosomes of individual cells with a genetic “barcode” that can be identified by deep sequencing¹⁴. Previous work implemented this approach in *S. cerevisiae*, where chromosomal insertion of $\sim 500,000$ random barcodes using the *Cre-loxP* recombination system allowed a quantitative description of evolutionary dynamics of yeast populations ($\sim 10^8$ cells)^{5,15,16}. In bacteria, however, technical barriers have limited the number of unique chromosomal barcodes to ~ 100 – 500 ¹⁷⁻¹⁹. Such low levels of barcode diversity preclude us from addressing important problems in bacterial evolution, where high-resolution lineage tracking is essential. For example, one such scenario is evolution in the presence of sub-inhibitory amounts of antibiotics, where the dynamics are driven by multiple mutations of low frequency and small fitness effects²⁰.

Here we present a method based on the Tn7 transposon to generate *E. coli* populations of $> 10^7$ cells carrying 10^5 – 10^6 unique chromosomal barcodes. To demonstrate the utility of this technique, we evolved barcoded populations by serial passaging in the presence of sub-inhibitory concentrations of two common antibiotics, chloramphenicol and trimethoprim. While previous studies on high-resolution microbial barcoding focused primarily on inferring the distribution of selection coefficients on new mutations¹⁶, we pursued a complementary, ecology-inspired approach that focuses on

tracking the diversity of lineages over time and across populations. This approach is amenable to a wider variety of conditions, such as time-varying environments and initial populations that already contain substantial genetic diversity. We found that different drug regimens elicited distinct and highly-reproducible dynamics of lineage diversity. In particular, low amounts of trimethoprim unexpectedly slowed the rate of lineage diversity loss even beyond conditions without any antibiotic, hinting the possibility that in this regime the antibiotic could be primarily functioning as a signaling molecule^{21,22}. We also quantified the extent to which individual lineages had similar fates across replicate populations evolving in the same condition. Analysis of the dynamics of these lineages' frequency trajectories revealed the relative contributions of pre-existing mutations (standing genetic variation that arose prior to the experiment) versus de novo mutations (acquired during the experiment). In general, stronger selection pressure generated faster loss of lineage diversity and more reproducible dynamics at the level of individual lineages due to pre-existing beneficial mutations, while weaker selection produced slower diversity loss and less reproducible dynamics due to a greater role of de novo mutations.

Results

Efficient chromosomal barcoding of *E. coli* cells. Several robust genome-editing methods available for *E. coli* have long made this organism a flagship of genetic manipulations²³. A high-resolution chromosomal-labeling technique, however, has not been available, making it difficult to analyze the evolutionary dynamics of large bacterial populations segregating at low frequencies. To address this problem, we harnessed the well-established site-specific recombination machinery of the Tn7 transposon^{24,25}. We placed the *tnsABCD* genes, which encode the transposase biochemical machinery, under the control of an arabinose-inducible pBAD promoter in a temperature-sensitive “helper” plasmid (**Fig. 1A**), while the Tn7 arms (Tn7L, Tn7R), which target the genetic cargo at a neutral *attnTn7* attachment site, are on a separate “integration” plasmid (**Fig. 1B**). We placed the barcode cassette carrying a 15-nucleotide random sequence (the “barcode”) and the adjacent marker of selection between the Tn7 arms on the integration plasmid (**Fig. 1B,C**). To minimize the preparation of barcode libraries to two consecutive PCR reactions, we added sequences flanking the barcode cassette that are complementary to Illumina adapter primers (**Fig. 1C**). We used these sequences both to PCR-amplify barcodes directly

on cell cultures as well as to anchor i5/i7 Illumina indices to the amplified barcodes (Methods). Using this Tn7 transposon system, we integrated barcodes into a fixed location on the *E. coli* chromosome in two steps (Fig. 1D). First, we transformed cells with the Tn7 helper plasmid and pre-conditioned them by inducing the transposase machinery. Second, we transformed the pre-conditioned cells with the barcoded integration plasmid. We simultaneously selected for chromosomal barcode integration and removal of the helper plasmid by plating on selective media and incubating the plates at 37°C (Methods).

To assess the quality of the library preparation, we sequenced the barcode library at three points during the process: 1) the raw DNA library, as synthesized by the manufacturer and prior to incorporation of barcode cassettes into the integration plasmid (Methods); 2) the integration plasmid library, before incorporation into the chromosome; and 3) the library of barcodes successfully incorporated into the *E. coli* chromosomes. We identified $\sim 1.3 \times 10^6$ unique barcodes in the raw library (Supplementary Table 1), with a fairly narrow distribution of frequencies: all barcodes but one had frequencies between 3×10^{-7} and 3×10^{-5} (Extended Data Fig. 1A). The nucleotide composition of these barcodes was also very close to random, as quantified by the entropy of nucleotides per position (Extended Data Fig. 1B). Incorporating the barcodes onto plasmids and then onto chromosomes reduced this diversity ($\sim 8.4 \times 10^5$ unique barcodes on plasmids and $\sim 4.6 \times 10^5$ on chromosomes; Supplementary Table 1). The process also introduced more redundancy into the distribution of frequencies, especially the chromosomal incorporation step, with some barcodes reaching frequencies of $\sim 10^{-3}$ (Extended Data Fig. 1A). These increases in redundancy also led to a minor decrease in nucleotide entropy (Extended Data Fig. 1B). However, the presence of some barcodes with high initial frequencies did not appear to play a major role in the resulting lineage dynamics during evolution, as we show below.

We also compared frequencies of individual barcodes between the different initial libraries, which showed a weak but significant correlation of frequencies between steps (Extended Data Fig. 1C,D); that is, barcodes that started at high frequencies tended to remain at high frequencies during the preparation process, but with fluctuations due to the incorporation procedure. To test the effect of noise from the PCR and sequencing processes, we also performed four independent sequencing replicates on the chromosomal library (Supplementary Table 1). The frequencies of individual barcodes across these

replicates are strongly correlated (**Supplementary Fig. 1**), indicating that our sequencing and barcode identification methods are reproducible.

Laboratory evolution of the barcoded population. Bacteria are often exposed to antibiotic concentrations far below the minimal inhibitory concentration (MIC), both in natural environments and in patients receiving antimicrobial therapy^{26,27}. Previous studies have shown that, compared to a lethal dosage, sub-MIC concentrations greatly expand the mutational space by allowing a large number of small-effect mutations to enter a population simultaneously²¹. The importance of low-frequency lineages in sub-MIC concentrations make these conditions a perfect setting to employ our barcoded population of *E. coli*. To this end, we chose two common antibiotics with distinct modes of actions: chloramphenicol (CMP), which inhibits protein synthesis via inactivation of peptidyl transferase activity of the bacterial ribosome²⁸; and trimethoprim (TMP), which functions as a competitive inhibitor of the essential protein dihydrofolate reductase²⁹. Previous work demonstrated that sub-inhibitory concentrations of antibiotics as low as 1% MIC can still select for resistant mutants over the wild type³⁰. Thus, we aimed for antibiotic concentrations around 1% MIC in our evolution experiments. Specifically, we identified concentrations that reduced the total number of cells by no more than 30% at the end of a single propagation cycle (~10 hours), compared to the untreated culture (**Methods**). These concentrations were 1 µg/mL CMP (6.25% MIC) and 0.1 µg/mL TMP (1% MIC) (**Supplementary Fig. 2**). To reduce the selection pressure even further below the minimal selective concentration, we also chose ultra-sub-MIC concentrations that were 10% of the aforementioned concentrations.

Altogether, we conducted laboratory evolution via serial passaging in five conditions: “low CMP” (6.25% MIC), “ultra-low CMP” (0.625% MIC), “low TMP” (1% MIC), “ultra-low TMP” (0.1% MIC), and a control without any drug (**Extended Data Fig. 2A,B**). We evolved 14 independent replicate populations in each of these five conditions (**Extended Data Fig. 2C,D and Supplementary Table 2**). We diluted batch cultures (500 µL each) grown in 96 well plates by 1:100 every ~6 generations (that is, passaging twice daily; **Extended Data Fig. 2E,F**) with a bottleneck population size of $\sim 3 \times 10^7$ cells (**Extended Data Fig. 2C,D**). To sustain the selection pressure during the evolutionary experiment (~420 generations), we gradually increased antibiotics in some of the

conditions (**Extended Data Fig. 2A,B** and **Supplementary Fig. 3; Methods**). This helped to keep the number of cells at the end of each passage roughly constant along the entire evolutionary experiment for each population (**Extended Data Fig. 2C,D**). We randomly chose three replicate populations from each condition for all further analysis (sequencing and growth measurements), resulting in 15 total populations to consider.

We expected two major components of selection in the experiment: selection for traits that are beneficial to the specific type and concentration of antibiotic, and selection for traits that are beneficial in the general laboratory growth conditions (growth medium, aeration, *etc.*). It is possible that there are tradeoffs between these two types of traits, i.e., costs associated with the acquisition of drug resistance^{31,32}. To assess the contributions of these factors, we measured two quantitative properties of each evolving population at several time points (**Supplementary Table 3**): the IC₅₀ (antibiotic concentration inhibiting 50% of growth; **Supplementary Fig. 4** and **Methods**), which provides an overall measure of the population's ability to grow in a specific antibiotic; and the growth rate in the absence of drug, which measures the adaptation of the populations to the general laboratory conditions and also measures possible costs of resistance. For populations evolving in low CMP and low TMP conditions, the IC₅₀ measurements revealed a moderate increase in antibiotic resistance over the evolution experiment (**Extended Data Fig. 3A,B**). In contrast, both ultra-low conditions, as well as the control without drug, produced no measurable improvement in IC₅₀ measured at concentrations ≥ 1 $\mu\text{g/mL}$ for CMP and ≥ 0.5 $\mu\text{g/mL}$ for TMP (**Extended Data Fig. 3A,B**). Furthermore, the increases in drug resistance evolved in the low CMP and TMP conditions were accompanied by a growth rate reduction in the absence of antibiotics (**Extended Data Fig. 3C,D**). In particular, the population that evolved to resist the highest levels of CMP (low CMP replicate 1, **Extended Data Fig. 3A**) showed the strongest growth rate reduction among the three replicate populations in the same condition (**Extended Data Fig. 3C**). However, we observed no growth rate cost in the populations evolved under ultra-low CMP. Instead, the growth rate trajectories for these populations were generally similar to those for the populations that evolved in the absence of antibiotics (**Extended Data Fig. 3C**). Surprisingly, in the populations evolved under ultra-low TMP, the improvement in growth rate lagged behind that of the populations evolved under no antibiotics (**Extended Data Fig. 3D**), suggesting that the rate of adaptation to growth conditions at 0.1% MIC of TMP

was diminished, despite the fact that we observed no improvement in TMP IC50 (Extended Data Fig. 3B).

Barcodes allow high-resolution monitoring of lineage trajectories. To elucidate the evolutionary dynamics of these populations at the level of individual lineages, we sequenced the barcodes of the same 15 populations at 16 time points (Supplementary Table 1). From the sequenced barcodes, we assembled frequency trajectories for all $\sim 4.5 \times 10^5$ initial lineages over the course of the experiment (Fig. 2 and Extended Data Fig. 4). Plots of these trajectories immediately suggest several important qualitative insights. First, just one or two lineages dominated each population ($>70\%$) by the end of the experiment (see also Supplementary Table 2). Second, some lineages rose to high frequency in multiple independent populations (lineage colors match across panels in Fig. 2 and Extended Data Fig. 4). Third, there is evidence of widespread clonal interference: some lineages that initially increased in frequency due to positive selection later decreased due to competition from higher-fitness lineages. Fourth, Fig. 2 suggests that the rate of lineage diversity loss was reproducible between replicate populations in the same condition, while systematically distinct across different conditions. This implies that condition-specific selection, rather than selection for general laboratory conditions or neutral dynamics, dominated the lineage dynamics at the whole-population level. Indeed, although genetic drift causes the loss of many individual low-frequency lineages, approximately $2N$ generations (where N is the effective population size) are required for a single lineage to fix by genetic drift alone³³. Since the effective population size in our experiments was of order 10^7 (Extended Data Fig. 2C,D), neutral dynamics cannot be responsible for the overall loss of lineage diversity we observe, which includes near-fixation in several populations.

Quantifying lineage diversity dynamics across conditions. To quantify the dynamics of lineage diversity, we adopted a measure of diversity widely used in ecology^{34,35}:

$$\text{Diversity index } {}^qD = \left(\sum_{\text{lineage } k} x_k^q \right)^{1/(1-q)}, \quad (1)$$

where x_k is the frequency of the k th barcoded lineage and q is the “order” of the diversity index, which determines the sensitivity of diversity to abundant versus rare barcodes

(**Methods**). In general, we can interpret the diversity index as the effective number of lineages present in the population. When $q = 0$, the diversity index simply counts the number of unique barcoded lineages, irrespective of their frequencies. This case is equivalent to measuring diversity as “species richness” in ecological contexts³⁴. When $q = 1$, the diversity index weighs all barcoded lineages by their frequencies, which is sometimes known as the Shannon diversity because it is equivalent to the exponential of the Shannon entropy of the frequencies. In the limit of $q \rightarrow \infty$, the diversity index equals the reciprocal of the maximum lineage frequency, meaning that it depends only on the most abundant lineage and no others. Thus, by comparing the lineage diversity index across different q values, we can estimate the relative contributions of rare and abundant lineages to that diversity. Note that if all lineages have equal frequencies, then the diversity index equals the actual number of lineages for any value of q . We emphasize that “diversity” here refers to the diversity of lineages descending from the initial population, meaning it must decrease monotonically over time, unlike the genetic diversity of the population which can increase due to new mutations.

We first tested the diversity index on the initial barcode libraries. For each of the initial barcode libraries (raw DNA library, plasmid library, and chromosomal library; **Extended Data Fig. 1**), we calculated the diversity index in **Eq. 1** for $q = 0$, $q = 1$, and $q = \infty$ (**Supplementary Fig. 5A**). As expected, we see that the diversity decreased at each step of the library preparation (except for ${}^{\infty}D$ in going from the raw library to the plasmid library, which suppressed the frequency of the most-frequent barcode; cf. **Extended Data Fig. 1A**). We also see that the diversity index decreases as the order q increases, since increasing the weight of higher-frequency lineages reduces their effective number. Indeed, the total number of unique chromosomal barcodes is ${}^0D \approx 4.5 \times 10^5$ (**Supplementary Fig. 5A, left panel**), but the effective number of lineages, using $q = 1$ to weigh by their unequal frequencies (**Extended Data Fig. 1A,B**), is approximately 10-fold lower, ${}^1D \approx 4.6 \times 10^4$ (**Supplementary Fig. 5A, center panel**). To test the robustness of the diversity index to noise in the PCR and sequencing process, we also calculated diversities of the four replicate samples of the initial chromosomal library (**Supplementary Fig. 5A**). Although there is noise at the level of individual low-frequency barcodes across these replicates (**Supplementary Fig. 1**), the diversity indices are highly reproducible, with all replicates falling within 10% of each other for all q values (within 2% for $q = 1$).

Furthermore, while pooling these replicate samples increases the total number of unique barcodes 0D by 70-80% (**Supplementary Fig. 5A, left panel**), it only increases the $q = 1$ by 5-6% (**Supplementary Fig. 5A, center panel**). Thus, while sampling noise in the sequencing process can have an important effect on estimates of the absolute number of barcodes, the effective number is relatively robust at the sampling and sequencing depths we are using.

We now turn to assessing the diversity of the populations in the evolution experiment. **Figure 3A** shows the effective number of lineages for each population over the time of the experiment, using three different values of q . Low CMP produced the fastest collapse of lineage diversity, extinguishing over 90% of unique barcodes in less than 50 generations. This behavior was displayed at all q values, indicating that rare and frequent barcodes contributed equally to these dynamics. In contrast, populations in low TMP lost 0D diversity more rapidly compared to ultra-low CMP and no drug conditions, but it lost 1D and ${}^\infty D$ diversities at approximately the same rate; this indicates that the dynamics of abundant lineages were similar in these three conditions, but that low-frequency lineages disappeared more quickly in low TMP. More surprising was the fact that populations under ultra-low TMP lost diversity even more slowly than did populations under no drug. Indeed, the diversity of populations under ultra-low TMP maintained 40-50% of the initial effective diversity ($q = 1$) up to generation ~ 120 , whereas populations without drug had only $\sim 1\%$ of their initial diversity by that time point. This difference in the rates of lineage diversity deterioration between ultra-low TMP and no drug conditions is consistent with our measurements of growth rate reduction in the absence of drug, wherein the rate of adaptation under ultra-low TMP lagged behind that of no drug populations (**Extended Data Fig. 3D**). Interestingly, 0D diversity under ultra-low TMP was similar to the other populations by generation ~ 250 , but its diversity at larger q remained higher until the very end of the experiment. Towards the end of the experiment, we saw that the effective diversity of all populations is 1-5 lineages (1D), consistent with the observations from **Fig. 2**. However, we note that despite the drop in lineage diversity, there was still an ample number of surviving barcode lineages: each population retained a few thousand barcodes by the end of the experiment, as seen in 0D (**Fig. 3A**).

Reproducibility of individual lineage dynamics. Not only did different antibiotic regimens produce distinct patterns of lineage diversity loss, but we also observed that these patterns are consistent across replicate populations. In **Fig. 3B** we show the Pearson correlation coefficients between the 1D diversity trajectories from all pairs of populations. While all trajectories are somewhat correlated since they must monotonically decrease, we saw stronger similarity among trajectories from the same condition. To further dissect whether individual lineages have similar fates across populations, we must quantify the similarity between lineage frequencies in different populations.

To compare the lineage composition of two or more populations at a single time point, we used a definition of diversity dissimilarity from ecology (**Methods**). Suppose we have M populations whose lineage compositions we want to compare. We first calculate the diversity index (**Eq. 1**) for all M populations pooled together, ${}^qD_{\text{pooled}}$. We then calculate the diversity index for each population alone and determine the mean across all populations, ${}^qD_{\text{mean}}$. The ratio of these two quantities, shifted and rescaled, measures the dissimilarity among lineage compositions^{34,35}:

$$\text{Dissimilarity of lineages} = \frac{{}^qD_{\text{pooled}} / {}^qD_{\text{mean}} - 1}{M - 1}. \quad (2)$$

If the lineage compositions of all populations are identical, then the pooled population has diversity equal to the mean diversity, and so the dissimilarity equals zero. In contrast, if the lineage compositions of M populations have no overlap, then the pooled population has diversity M times greater than that of the mean single population, and so the dissimilarity index equals 1. As with the diversity index, the parameter q allows us to vary the importance of low- and high-frequency lineages in the overall lineage dissimilarity. For $q = 0$, lineage dissimilarity measures how many lineages multiple populations have in common, regardless of their frequencies, while for $q \rightarrow \infty$, it compares only the highest-frequency lineages (**Methods**).

We first tested the measure of lineage dissimilarity by calculating it between all pairs of initial barcode libraries (**Supplementary Fig. 5B**). We see very low dissimilarity among the sequencing replicates of the chromosomal library; for example, the $q = 1$ dissimilarity among the chromosomal replicates is $\sim 3\%$ (**Supplementary Fig. 5B, center panel**). As with the diversity indices themselves, we see little effect of pooling samples to increase read depth, indicating that our read depth is sufficient. This analysis furthermore

confirms that the largest change in barcode frequencies occurs during incorporation of the plasmid library onto the chromosomes, as the raw library and plasmid library are more similar to each other than to the chromosomal libraries (**Supplementary Fig. 5B**; cf. **Extended Data Fig. 1C,D**).

We then calculated the dissimilarity across all evolved populations at each time point. In **Fig. 4A (left panel)**, we show the $q = 1$ dissimilarity between all pairs of populations at generation 120. At the beginning of the experiment, the populations were identical and so the dissimilarity between all pairs of populations was zero. By generation 120, we see that many pairs of populations from different conditions have already diverged (dissimilarity close to the maximum value of 1), while pairs of populations from the same conditions remained more similar (except for low TMP). Furthermore, we see some similarity even between conditions: populations under the weakest antibiotic pressures (ultra-low CMP, ultra-low TMP, and no drug) all maintained similarity between conditions comparable to their similarity between replicates. However, by the end of the experiment at generation 420 (**Fig. 4A, right panel**), most of the similarity between populations disappeared. The main exception was low CMP, where the three replicate populations maintained strong similarity. There was also a small amount of residual similarity among the populations in low CMP, ultra-low CMP (two out of three replicates), and low TMP (two out of three replicates). In contrast, replicate populations under ultra-low TMP and no drug showed no similarity among each other by the end of the experiment. There was also strong similarity between replicate 3 in ultra-low CMP and replicate 2 of ultra-low TMP.

We can further quantify the reproducibility of lineage dynamics by calculating diversity dissimilarity among all replicate populations in each condition over time (**Fig. 4B** and **Supplementary Fig. 6**). For $q = 0$, we see the dynamics of within-condition dissimilarity were similar to the dynamics of the diversity indices themselves in **Fig. 3A**. That is, populations under low CMP diverged from each other most rapidly, followed by low TMP, then by ultra-low CMP and no drug concurrently, and finally by ultra-low TMP diverging last. Interestingly, all conditions settled at an intermediate amount of ~ 0.8 dissimilarity for $q = 0$ by the end of the experiment; this value corresponds to having about 20% of their lineages in common (**Methods**). The $q = 1$ lineage dissimilarity, which accounts for heterogeneity in lineage frequencies, shows some differences with the $q = 0$ case that simply counts barcodes. With $q = 1$, low CMP populations actually diverged

more slowly from each other than did populations in the other conditions. Moreover, the low CMP populations actually reached some maximum level of $q = 1$ dissimilarity around generation 150, and then began converging toward more similar lineage compositions. At the other extreme, ultra-low TMP and no drug populations reached maximum dissimilarity by the end of the experiment, while low TMP and ultra-low CMP populations had an intermediate value of dissimilarity. The dissimilarity index with $q = \infty$ shows a similar pattern of rise and fall for low CMP populations; since this case depends only on the most frequent lineage, it implies that the high level of similarity between low CMP populations by the end of the experiment was due to them sharing the same dominant lineage.

Altogether, this analysis shows that lineage dynamics under identical conditions are highly reproducible, even at the level of individual lineages. Specifically, it suggests that some lineages repeatedly rose to high frequency over multiple experiments. To demonstrate this more explicitly, in **Fig. 5A,B** we compare the frequencies of all barcoded lineages at the end of the experiment for three replicate populations in low CMP. Furthermore, in **Fig. 5C** we show the overlap of the top 10 barcodes (ranked by mean frequency over time) between all three replicate populations in low CMP: four of the top 10 in each population are shared among all replicates; with three more barcodes shared between two replicates (see also **Supplementary Table 4**). Indeed, the most frequent lineage in replicates 2 and 3 is the exact same lineage (green trajectories in **Fig. 2**); this lineage is also the second-most frequent lineage in replicate 1. In contrast, **Fig. 5D,E,F** shows these same plots for the no drug populations, which have little similarity among their most frequent lineages. In particular, the only overlap between their top 10 barcodes is one barcode shared between replicates 2 and 3 (**Fig. 5F** and **Supplementary Table 4**). In **Supplementary Figs. 7 and 8** we show direct comparisons of lineage frequencies between all pairs of populations.

One possible explanation for the repeated dominance of certain lineages is that those lineages may have started at unusually high frequencies (**Extended Data Fig. 1A**). However, several lines of evidence refute this possibility. In **Supplementary Fig. 9** we compare the initial and final frequencies for all lineages in each population. In general, the final frequencies are poorly correlated with the initial frequencies; in particular, the initial frequencies of the lineages that dominate at the end of the experiment are often below or very near the average initial frequency (gray vertical bars in **Supplementary Fig. 9**). We

also calculate the lineage dissimilarity of each population at each time point compared to the initial population (**Supplementary Fig. 10**); this also shows that the evolved populations are almost maximally dissimilar from the initial populations, indicating that the initial frequencies of lineages are largely independent of their final values.

Individual lineage trajectories reveal the relative contributions of pre-existing and de novo mutations. To further probe the striking amount of similarity among some populations at the level of individual lineages, we first note that many lineages within populations appear to follow similar trajectories (**Extended Data Fig. 4**). We therefore performed hierarchical clustering of a subset of high-frequency lineage trajectories in each population, based on the correlation coefficients between trajectories (**Supplementary Figs. S11 and S12**; see also **Methods**). The trajectories indeed formed well-defined clusters with distinct dynamics (**Supplementary Fig. 13**); in particular, similar clusters appeared in replicate populations from the same condition, while clusters in populations from different conditions appeared to be more dissimilar. A few clustered trajectories also shared barcodes with very similar sequences, suggesting these lineages are actually the same (i.e., the apparently distinct barcodes arose from sequencing errors; **Supplementary Fig. 14**); however, the vast majority of clustered trajectories involved unrelated barcode sequences, suggesting they are truly distinct lineages with highly-correlated dynamics.

The clustering analysis revealed two key types of adaptive trajectories. Trajectories of the first type increased immediately at the beginning of the experiment, before later getting outcompeted by other lineages (**Fig. 6A, left panel**). The initial increase suggests that these lineages carried pre-existing beneficial mutations at the beginning of the experiment; these mutations were presumably acquired during the ~30 generations of growth that occurred during preparation of the barcode library (**Fig. 1D**). Trajectories of the second type were initially flat and only started to increase later in the experiment (**Fig. 6A, right panel**); these lineages presumably acquired beneficial mutations de novo during the experiment. To test these interpretations, we identified trajectories of both types in each population (**Supplementary Fig. 13**) and compared the lineages in each type across populations; lineages with pre-existing mutations should show the same behavior across multiple populations, while lineages with de novo mutations should not. Indeed, we see significant overlap in lineages with putative pre-existing mutations across populations (**Fig.**

6B, left panel), while almost no overlap can be observed in lineages with putative de novo mutations (**Fig. 6B, right panel**). We also compared the initial frequencies of trajectories in both types of clusters; a set of lineages carrying pre-existing mutations with similar selection coefficients (and which therefore cluster together) should have more similar initial frequencies due to their similar growth dynamics during library preparation, while lineages that only acquire de novo mutations later should have unrelated, more variable initial frequencies. We find that trajectories with putative pre-existing mutations do in fact have somewhat less variable initial frequencies on average (**Supplementary Fig. 15**; Welch's t-test, p -value ≈ 0.03).

To assess the overall contributions of pre-existing and de novo beneficial mutations to the evolutionary dynamics, we summed all trajectories of each type in each population (**Fig. 6C**). In low CMP, the lineages with pre-existing beneficial mutations dominated from the beginning of the experiment (**Fig. 6C, left panel**); in fact, we observed no trajectories with the signature of de novo mutations in this condition (**Supplementary Fig. 13**). This does not mean that no de novo mutations occurred in this condition, but rather that they apparently occurred on the background of lineages with pre-existing beneficial mutations. For example, the dominant purple lineage in replicate 1 (**Fig. 2**) appears to have acquired at least a second strongly beneficial mutation around generation ~ 324 . The existence of strongly beneficial pre-existing mutations in low CMP explains why these populations demonstrated such strong convergence in lineage composition (**Figs. 4 and 5A,B,C**). In contrast, most other populations show a balance between lineages with pre-existing mutations and lineages with de novo mutations, with pre-existing mutations driving the initial dynamics and de novo mutations dominating later. For example, the populations evolving in no drug also have lineages with apparent pre-existing beneficial mutations (**Fig. 6C, left panel**), but they rapidly lost frequency after generation ~ 120 , when the lineages with de novo mutations took over (**Fig. 6C, right panel**). This is also consistent with the low level of convergence we see in these populations (**Figs. 4 and 5D,E,F**). Finally, we note that the populations in ultra-low TMP and in no drug conditions appear to have similar selection on their trajectories with pre-existing mutations (**Fig. 6C, left panel**), even though populations in ultra-low TMP lost their diversity much more slowly than did the populations in no drug (**Fig. 3A**). The difference arose on the de novo

444 mutations, which appeared to be under much weaker selection in ultra-low TMP than in no
445 drug (**Fig. 6C, right panel**).

446
447 **Lineage dynamics in constant drug conditions.** In the aforementioned evolution
448 experiment, we increased the concentrations of drugs in some conditions to maintain
449 approximately constant selection pressure as the populations adapted (**Extended Data Fig.**
450 **2**). To verify the robustness of those results, we repeated the evolution experiments in
451 conditions with constant rather than changing drug concentrations, and with consistent
452 bottlenecking every 12 hours (**Methods**). We also sequenced the barcodes at several earlier
453 time points to better capture the initial dynamics (**Extended Data Fig. 5** and
454 **Supplementary Fig. 16**).

455 Evolution with constant concentrations recapitulated all the major conclusions of
456 the original experiment in increasing concentrations (**Extended Data Fig. 6-8** and
457 **Supplementary Figs. 17 and 18**). In particular, constant low CMP again produced the
458 fastest loss of lineage diversity (**Extended Data Fig. 6A**; cf. **Fig. 3A**). The lineage
459 dissimilarity of these populations also diverged more quickly with $q = 0$ but more slowly
460 with $q = 1$ compared to the other populations (**Extended Data Fig. 7B**; cf. **Fig. 4B**),
461 indicating the dominance of pre-existing beneficial mutations, which were again identified
462 by clustering the lineage trajectories (**Extended Data Fig. 8B**; cf. **Fig. 6C**). We also
463 recovered the effect of TMP on slowing diversity loss compared to no drug; however, in
464 the second experiment this occurred in the “low TMP” condition rather than in “ultra-low
465 TMP” (**Extended Data Fig. 6A**; cf. **Fig. 3A**). This mismatch between the experiments is
466 due to the difficulty of accurately reproducing such low concentrations of TMP in separate
467 dilutions (TMP concentrations are tenfold lower than CMP concentrations; see **Methods**):
468 “low TMP” in the second experiment turned out to be closer to “ultra-low TMP” in the first
469 experiment, while “ultra-low TMP” in the second experiment appeared to be similar to no
470 drug at all. This difference, however, is not essential as we nevertheless observe that some
471 low concentration of TMP produces the same effect in both experiments.

472 Other comparisons of lineage dynamics between populations in the same
473 conditions, and between different conditions, were generally similar in the experiment with
474 constant concentrations, albeit noisier because the weaker selection pressures and shorter
475 time scale of the second experiment produced a smaller range of lineage dynamics. For

example, the rates of lineage diversity loss were once again similar across replicates in the same conditions (**Extended Data Fig. 6B**; cf. **Fig. 3B**), except for distortions in some comparisons (e.g., low TMP replicate 2, ultra-low TMP replicate 1, and no drug replicate 2) due to noise in the lineage diversity estimates at single time points (**Extended Data Fig. 6A**). The smaller dynamic range of trajectories also meant that trajectory clustering was less robust (**Supplementary Figs. 17 and 18**), but this analysis nevertheless recovered similar patterns of pre-existing and de novo beneficial mutations (**Extended Data Fig. 8**).

Simulations reproduce observed lineage dynamics. To further test our interpretations of the experimental results, we simulated evolutionary dynamics of a barcoded population under a serial dilution scheme similar to the experiment (see **Methods** for complete details). We focused on varying the supply of pre-existing mutations, which accumulate during the preparation of the barcode library, and the supply of de novo mutations, which accumulate during the evolution experiment. In general, we found that having both types of variation was necessary to qualitatively reproduce trajectories similar to the observed experimental data (**Extended Data Fig. 9**; cf. **Fig. 2** and **Extended Data Fig. 4**).

While we explored a range of parameter values (mutation rates and distributions of selection coefficients of mutations) in the simulations, for simplicity we present two contrasting cases (**Methods**). Both cases have pre-existing as well as de novo mutations, but pre-existing mutations dominate in the first case (intended to mimic low CMP conditions), while de novo mutations dominate in the second case (intended to mimic no drug conditions). We simulated two replicate populations in each case. We first checked whether pre-existing and de novo mutations in the simulations led to trajectories similar to what we observed from the clustering analysis of the real data. Indeed, the simulated trajectories for these two types of lineages qualitatively match the behavior of the observed trajectories (**Supplementary Fig. 19**; cf. **Fig. 6A** and **Supplementary Fig. 13**).

Next, we analyzed the lineage diversity of the simulated populations. We calculated the effective number of lineages (**Eq. 1**) in each of the populations over time (**Extended Data Fig. 10A**). As in the experiment, the simulated replicates show a high degree of reproducibility in their diversity dynamics, and the dominance of pre-existing beneficial mutations clearly accelerates the decay of diversity. This supports our interpretation of the experimental data regarding the increased role of pre-existing

mutations in low CMP compared to other conditions. We also simulated populations under purely neutral dynamics, and confirmed that neutral processes alone cannot reproduce the observed amount of diversity loss (cf. **Fig. 3A**). Moreover, we tested the effect of finite sampling of barcodes on the estimated diversity. That is, we subsampled 10% of each simulated population three times and calculated the lineage diversities based on these subsamples (**Supplementary Fig. 20**). We found that the effect of subsampling was negligible, especially for $q > 0$ where lineages are weighed by their frequencies.

Finally, we calculated the lineage dissimilarity (**Eq. 2**) across replicates in each of these conditions (**Extended Data Fig. 10B**). This further demonstrates how our two contrasting simulation conditions mimic the experimental data: the populations dominated by pre-existing mutations initially diverged from each other but then later converged, as seen in the low CMP population, while the populations dominated by de novo mutations simply diverged monotonically. Again, the simulated neutral populations diverged much more slowly than what we observed in the experiment (cf. **Fig. 4B**), and the effect of subsampling on the estimated dissimilarity was negligible (**Supplementary Fig. 21**). This analysis also supports our conclusion that the fates of individual lineages are not strongly determined by the initial frequency distribution, since the distribution used in our simulations was the same as the one measured for the real chromosomal library (**Extended Data Fig. 1A**). If this distribution played a dominant role in determining which lineages fixed, then we would see a high level of convergence across replicates in all cases; however, that does not happen without strongly-selected pre-existing mutations.

Discussion

We have demonstrated a method that generates *E. coli* populations carrying 10^5 - 10^6 unique chromosomal barcodes. We tested the utility of the method by evolving the barcoded population in the presence of sub-inhibitory concentrations of two common antibiotics. Fitness phenotyping, especially of spontaneous single mutations, has been the major focus of previous applications of high-resolution microbial barcoding¹⁶. However, this approach is applicable only under a strict set of conditions: environments must be constant, the population must start with minimal genetic diversity, and the population can be meaningfully tracked only over a short amount of time before any lineages rise to high frequency. Here we have pursued a complementary, ecology-inspired approach to high-

540 resolution lineage tracking which focuses on measuring lineage diversity over time and
541 between populations. Because this approach is more phenomenological, it can be applied
542 to a wider variety of conditions, such as time-varying environments and initial populations
543 that already contain substantial genetic diversity. While lineage diversity has been used in
544 some previous studies^{5,19}, here we have provided a comprehensive investigation of how it
545 can be used to analyze lineage-tracking data from DNA barcodes in bacterial populations.
546 We believe our methodology provides a valuable guide for the design and analysis of future
547 barcoding applications in bacteria, especially for experiments involving fluctuating
548 environments and comparisons across large numbers of environments and replicates.

549 We found that each drug regimen in our experiment prompted a distinct and
550 reproducible rate of lineage diversity loss (**Fig. 3**), with the exception of ultra-low CMP
551 and no drug conditions, which exhibited similar diversity loss dynamics (although they
552 differed in other ways). Since the effect of genetic drift on the loss of lineage diversity at
553 the population scale was limited, owing to the large size of the evolving populations ($>10^7$)
554 and the short time scale (~ 420 generations), we can interpret the rate of lineage diversity
555 loss as a proxy for the rate of adaptation in the population. Curiously, the diversity
556 dynamics were reproducible regardless of the rate itself; for example, both the low CMP
557 condition, which induced the fastest rate of adaptation, and ultra-low TMP condition,
558 which induced the slowest adaptation dynamics, exhibited high reproducibility between
559 evolutionary replicates. This observation implies a surprisingly deterministic dynamics at
560 the level of lineage diversity, despite differences in the distributions of fitness effects across
561 conditions in our experiment.

562 One of the most surprising features of these results is that there is some range of
563 low TMP concentrations that reduce the rate of lineage diversity loss even beyond the rate
564 under no drug at all. While it is difficult to determine the precise concentration at which
565 this effect occurs, owing to the low magnitude of these concentrations, we have reproduced
566 it across replicate populations in the same experiment (**Fig. 3A**) as well as across
567 populations in different evolution experiments (**Extended Data Fig. 6A**) to demonstrate
568 that such conditions exist. Since the growth dynamics of these populations indicate they
569 experience a similar number of generations per passage as the other populations (**Extended**
570 **Data Fig. 2E,F**), we hypothesize that the observed delay in adaptation in TMP is due to a
571 reduction in selection coefficients on beneficial mutations, compared to both higher

concentrations of TMP and the no drug condition. A previous study estimated the distribution of selection coefficients from spontaneous mutations in *E. coli* by measuring growth rates of single-gene knockouts as a proxy³⁶. This indeed also showed that the distribution of selection coefficients under TMP was narrower than the distribution under no drug. Other previous studies have suggested that, at a low dosage, antibiotics might operate not as a weapon, but rather as signaling molecules that trigger transcriptional activation of multiple genes, including genes involved in the biosynthesis of amino acids, ribosomal proteins, purines, and pyrimidines²¹. If TMP, which is known to affect transcription³⁷, indeed induces a new metabolic state in bacterial cells at ultra-low concentration, it can potentially lead to a shift in the distribution of beneficial mutations. We expect future work will elucidate the detailed mechanism underlying the delayed adaptation in low concentrations of TMP.

An important problem in evolutionary biology is reliably quantifying the reproducibility of evolutionary processes^{38,39}. Adaptation in near-lethal drug concentrations often appears to be nearly deterministic due to the existence of a handful of strongly-beneficial mutations, which repeatedly accumulate in similar orders across replicate populations⁴⁰. However, it is more challenging to quantify the reproducibility of evolutionary dynamics when they are driven by many small-effect mutations at the whole-population level. Our chromosomal barcoding system in *E. coli* allowed us to directly address this problem by quantitatively comparing lineage dynamics across independent replicate populations subjected to identical antibiotic regimes. We found not only that the rate of lineage diversity loss was highly reproducible for each selection condition (**Fig. 3**), but also that the dynamics were deterministic at the level of individual lineages in some cases (**Fig. 4**), with a few lineages rising to high frequency in multiple independently evolving populations (**Fig. 5**).

Populations can adapt to new environments using two sources of variation: pre-existing mutations (*i.e.*, standing genetic variation) and de novo mutations arising after the environmental change⁴¹. Previous work has shown both the enrichment of pre-existing mutations^{30,42} and the emergence of new resistance-conferring mutations^{30,43} under sub-MIC concentrations of antibiotics. However, the relative contributions of these two sources of variation to evolutionary dynamics has remained unknown. Here, we developed a quantitative approach to address this problem by measuring lineage composition

dissimilarity^{34,35} between replicate populations (**Fig. 4**) and analysis of individual lineage trajectories (**Fig. 6**). These results suggest that lineages present at very low frequencies at the initial population, but nonetheless independently reaching high frequency in the replicate populations, are repetitively selected because they carry pre-existing beneficial mutations. Thus, the extent of similarity in lineage composition between evolutionary replicates at a particular time point quantitatively reports on the contribution of standing genetic variation to the observed dynamics. As expected, the rapid adaptation under low CMP was accompanied by the lowest lineage dissimilarity among selection regimes (**Fig. 4**), indicating a substantial contribution of pre-existing mutations. Conversely, pre-existing variation plays a less important role in ultra-low TMP and no drug conditions (**Fig. 5C**), where the evolutionary dynamics was rather driven mostly by de novo mutations.

Overall, our results demonstrate significant evolutionary insights gleaned from high-resolution lineage tracking using chromosomal barcodes in *E. coli*. Our experimental barcoding protocol based on the Tn7 transposon machinery is straightforward to implement and can be readily be reproduced in a variety of systems. We have furthermore shown how to obtain a robust quantitative analysis of the resulting lineage data using ecological diversity indices and trajectory clustering. Altogether, we envision that this tool will find wide applicability in addressing diverse questions in bacterial population and evolutionary dynamics.

Methods

Design of the chromosomal barcode integration system in *E. coli*. The design of the chromosomal integration system of the barcode library is based on the pGRG25 plasmid, which carries all the components of the Tn7 transposon site-specific recombination machinery^{25,44,45}. We separated the Tn7 transposase machinery (*tnsABCDE*) from the Tn7 arms (Tn7L, Tn7R) onto two independent plasmids: the temperature-sensitive *helper* plasmid (pSC101 temperature-sensitive origin of replication) carrying the *tnsABCD* genes under the control of an arabinose-inducible pBAD promoter (**Fig. 1A**), and the *integration* plasmid (R6K gamma *pir*+ dependent origin of replication) with the Tn7 arms flanking the barcode-carrying cassette and spectinomycin resistance gene (**Fig. 1B,C**). The separation of the recombination machinery *tnsABCD* from the integration segment flanked by the Tn7 arms achieved two major goals. First, the introduction of the helper plasmid into the cells prior to the integration plasmid allowed us to precondition the cells for chromosomal recombination by inducing the expression of the transposase complex, which we expect to increase the integration efficiency. Second, the transient nature of the integration plasmid eliminated any possibility of barcodes lingering outside of their designated chromosomal location due to unsuccessfully-cured plasmids. This is especially important for barcoding temperature-sensitive strains that cannot be grown at non-permissive temperatures.

The barcode-carrying cassette (**Fig. 1C**) is designed to minimizing the number of library preparation steps required for Illumina deep-sequencing technology. The only variable region in the cassette is the barcode sequence of 15 random nucleotides. Thus, the barcode library has a maximum possible diversity of 4^{15} ($\sim 10^9$) unique barcodes. The barcodes are asymmetrically located 9 nt downstream of the 5' end of the cassette. This short stretch of 9 nt is sufficient to locate the barcodes in the raw sequencing data files and to reduce the sequence redundancy in the Illumina flow cell. The cassette is flanked by the sequences complementary to the Illumina adapter primers used to anchor library specific indices recognized by the Illumina sequencing platforms (MiSeq, HiSeq, or Nextseq) (**Fig. 1C**). The Integrated DNA Technology (IDT) gBlocks service synthesized the barcode cassettes (allowing the incorporation of 15 consecutive and variable nucleotides), which we then cloned into the Tn7 integration plasmid (**Fig. 1B**). We characterized the resulting library of barcodes by deep sequencing prior to integration into the genome (**Extended Data Fig. 1**). We found that the actual diversity of this initial barcode library was three

orders of magnitude lower than the maximal theoretical diversity (1.3×10^6 unique barcodes) (**Supplementary Table 1**). Although the distribution of barcode frequencies was fairly narrow (**Extended Data Fig. 1A**), sampling from a relatively small number of unique barcodes could have been a factor that contributed to the frequency distribution bias observed in the plasmid integration library (see below). Thus, to reduce the bias, a different approach for generating the initial barcode diversity, such as primers with diversified positions synthesized using manual mixing, can be used in the future library preparations.

Design of the barcode-carrying cassette. The barcodes consist of 15 random nucleotides each, which we placed 9 nt downstream of the 5' end of the 288-nt long cassette:

```
gtcgcgcccggNNNNNNNNNNNNNNNNtattctcggtagtgggatacgacgataccgaagaca
gctcatgttatatcccgcggttaaccaccatcaaacaggattttcgcctgctggggcaaa
ccagcgtggaccgcttgctgcaactctctcagggccaggcgggtgaagggcaatcagctgt
tgcccgtctcactggtgaaaagaaaaaccaccctggcgcccaatacgcaaaccgcctctc
cccgcgcggttgccgattcattaatgcagctggcacgacagggtttccc
```

We placed the barcode-carrying cassette between sequences complementary to Illumina adaptor primers (forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and, finally, flanked by sequences complementary to the integration site in the Tn7 integration plasmid:

```
gatatcggatcctagtaagccacgttttaattaatcagatccctcaatagccacaacaac
tggggggcaaacagtcggttgctgattggtcgctcggcagcgtcagatgtgtataagagaca
gtcgcgcccggNNNNNNNNNNNNNNNNtattctcggtagtgggatacgacgataccgaagaca
gctcatgttatatcccgcggttaaccaccatcaaacaggattttcgcctgctggggcaaa
ccagcgtggaccgcttgctgcaactctctcagggccaggcgggtgaagggcaatcagctgt
tgcccgtctcactggtgaaaagaaaaaccaccctggcgcccaatacgcaaaccgcctctc
cccgcgcggttgccgattcattaatgcagctggcacgacagggtttcccctgtctcttata
cacatctccgagcccacgagacgccactcgagttatttgccgactaccttggtgatctcg
cctttcacgtag
```

Integrated DNA Technology (IDT) (<https://www.idtdna.com/pages/products/genes-and-gene-fragments/gblocks-gene-fragments>) synthesized the resulting 492 nt-long sequence as double-stranded gBlocks with randomly mixed bases (at the 15 consecutive positions indicated by N).

Generation of plasmid barcode library. We digested the empty Tn7 integration plasmids with NotI, purified them by ethanol precipitation, and then mixed the plasmids with gBlocks in a 1:3 molar ratio in the presence of NEBbuilder HiFi DNA (New England Biolabs) assembly mix, according to the manufacturer's instructions. We used a control without NEBbuilder HiFi DNA assembly mix to determine the background level (Tn7 integration plasmid lacking the cassette). We concentrated the reactions by ethanol precipitation and transformed them into 100 μ L of TransforMax™ EC100D™ *pir*⁺ cells (Lucigen). We resuspended the transformed cells in 1 mL of SOC medium, regenerated them for 1 hour at 37 °C with shaking followed by overnight incubation on the bench, and then plated the cells on 35 μ g/ml kanamycin LB agar plates (100 μ L of transformed cells per plate). We plated several microliters of diluted cultures separately to determine the number of colony-forming cells. We incubated plates overnight at 37 °C. Cells transformed with DNA but without the assembly mix produced no colonies. Transformation of DNA with the assembly mix generated a total of $\sim 2.4 \times 10^6$ colonies (**Fig. 1D**). We scraped all the colonies from the plates, pooled them together, and thoroughly mixed them. Finally, we extracted plasmids from the pooled cells with a Qiagen midi kit.

Chromosomal integration of the barcode library into *E. coli* cells. This is a two-step process, the first step being the transformation of the recipient *E. coli* MG1655 cells with the Tn7 helper plasmid and induction of the transposase integration machinery. The second step is the transformation of the Tn7 integration plasmid library, which integrates the barcodes into the chromosome (**Fig. 1D**). We grew cells transformed with the Tn7 helper plasmid overnight until saturation in LB supplemented with 100 μ g/mL ampicillin at 30 °C. We then diluted these cells 1:100 and grew them under the same condition for 45 min. We added 0.2% arabinose and diluted cells to OD_{600nm} of 0.5. We then harvested the cells, washed them 3 times with ice-cold water, and transformed them with the Tn7 integration

720 plasmid library using electroporation. We resuspended the transformed cells in 1 mL of
721 SOC medium, regenerated them for 1 hour at 30 °C with shaking followed by overnight
722 incubation on the bench, and then plated them on 100 µg/mL spectinomycin LB agar plates
723 (100 µL of transformed cells per plate). We plated several microliters of diluted culture
724 separately to determine the number of colony-forming cells. We incubated plates overnight
725 at 37 °C and produced $\sim 2 \times 10^6$ colonies in total. All randomly-picked colonies (over 50)
726 failed to grow on ampicillin, suggesting that overnight incubation at 37 °C is sufficient to
727 cure the majority of cells of the Tn7 helper plasmids. Similarly, we observed no growth
728 on kanamycin, showing that the Tn7 integration plasmids were no longer present. We
729 further validated the chromosomal incorporation of the barcode-carrying cassettes by
730 colony PCR with a pair of primers directed to the Tn7 integration site. All randomly-
731 picked colonies (over 50) were positive for the chromosomal integration. We scraped all
732 the colonies from the plates, pooled them together, thoroughly mixed them, aliquoted them
733 with 15% glycerol, and stored them at -80 °C.

734 As shown in **Extended Data Fig. 1A**, chromosomal incorporation of the plasmid
735 library reduced the number of unique barcodes and increased the barcode frequency bias.
736 We believe the major source of this perturbation is the extensive variation in the size of
737 individual colonies on agar plates, which translates into orders of magnitude differences in
738 the number of individual cells. Indeed, each individual grown colony can consist of 10^6
739 to 10^9 individual cells⁴⁶. In principal, we can substantially reduce such variation by
740 shortening the incubation time of the chromosomal library on the agar plates from 16 to
741 only 6-7 hours. This approach produces micro-colonies with much smaller variation in the
742 colony sizes, as was previously demonstrated⁴⁷.

743
744 **Deep sequencing of the gBlocks library, the Tn7 integration plasmid library, and the**
745 **naïve barcoded *E. coli* population.** Sample preparation for deep sequencing involved
746 four steps. First, we amplified the barcode-carrying cassettes with Illumina adaptor primers
747 (forward adaptor: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse
748 adaptor: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). We used gBlocks
749 and the Tn7 integration plasmid library directly as DNA templates in the PCR reaction. In
750 the case of the naïve barcoded *E. coli* population, we performed the PCR reaction either
751 directly on the cells or, alternatively, on the genomic DNA extracted from the barcoded

population with Nucleospin Microbial DNA prep kit (Machary-Nagel), using the manufacturer's instructions. The PCR reaction mix was prepared as follows:

- 1 µl (250 U) PrimeSTAR GXL DNA polymerase (TaKaRa)
- 1 µl 10 ng/µl Adaptor_forward primer
- 1 µl 10 ng/µl Adaptor_reverse primer
- 10 µl 5x reaction buffer
- 4 µl 2.5mM dNTPs
- 50 ng genomic DNA or 3 µl bacterial culture
- Bring to 50 µl with water

The PCR amplification conditions were:

- x1 cycle of
 - 94°C 5min
- x30 cycles of
 - 95°C 10 sec
 - 55°C 15 sec
 - 68°C 45 sec
- x1 cycle of
 - 68°C 5 min

In the second step, we separated the PCR products on 1% agarose gel, then excised and purified it with NucleoSpin Gel extraction kit (Machary-Nagel) following the manufacturer's instructions. Third, we subjected the gel-purified product of the first PCR reaction to a second PCR reaction using a unique pair of index primers (i5 and i7) from Nextera XT DNA library preparation kit (Illumina), using the following conditions:

- 1 µl (250 U) PrimeSTAR GXL DNA polymerase (TaKaRa)
- 5 µl Nextera XT index1 i7 primer
- 5 µl Nextera XT index2 i5 primer
- 10 µl 5x reaction buffer
- 4 µl 2.5mM dNTPs
- 55 ng DNA
- Bring to 50 µl with water

The PCR amplification conditions were:

- x1 cycle of
 - 94°C 5min
- x12 cycles of
 - 95°C 10 sec

- 55°C 15 sec
- 68°C 45 sec
- x1 cycle of
- 68°C 5 min

In the fourth and final step, we purified the product of the second PCR reaction with Agencourt AMPure XP PCR purification kit (Beckman Coulter). We performed sequencing on MiSeq or NextSeq platforms (Illumina).

Laboratory evolution. We subjected the naïve barcoded *E. coli* population to laboratory evolution via serial passaging under five distinct growth conditions: low chloramphenicol (CMP) (1-3 µg/mL), ultra-low CMP (0.1 µg/mL), low trimethoprim (TMP) (0.1-1.2 µg/mL), ultra-low TMP (0.01-0.1 µg/ml), and no drug (**Extended Data Fig. 2A,B**). We gradually increased the concentrations of the antibiotics throughout the evolution experiment to sustain a relatively constant level of cell density for low CMP and low TMP conditions. To this end, upon completion of each growth cycle, we measured cell density of the evolving populations by absorbance at 600 nm. Once the cell density of the populations grown under low CMP and low TMP conditions increased beyond ~80% of that of the populations in no drug, we increased the concentration of the corresponding antibiotic by ~30-50% (**Supplementary Fig. 3**).

We grew cells at 37 °C in a 96-well microtiter plate (500 µL per well) in supplemented M9 medium (0.2% glucose, 1mM MgSO₄, 0.1% casamino acids, 0.5 mg/ml thiamin). Between passages, we grew cultures for 8-9 hours (during the day) or 10-12 hours (during the night). We used saturated culture from a previous passage to inoculate a fresh plate by 1:100 dilution (5 µL of saturated cultured into 500 µL of fresh medium). Under these conditions, cultures reached saturation within ~6 hours of growth. Thus, during the alternating growth cycles of 8-9 and 10-12 hours, cultures spent at a stationary phase in the incubator under the growth conditions between 2-3 and 4-6 hours, respectively. Overall, we performed 70 passages. We measured optical density (OD) of the cultures at 600 nm at the end of each passage (**Supplementary Table 2**). We convert the raw OD measurements to an estimated number of cells (**Extended Data Fig. 2C,D**) as follows: we first multiply by 10 to correct for dilution of the measured culture, then multiply by 2 to standardize the OD to 1 cm path length, then multiply by cell density 10⁸ cells/mL/OD,

and finally multiply by 0.2 mL as the volume of the well. We stored every second passage at -80 °C after addition of 15% glycerol.

We also repeated the evolution experiment with all drug concentrations held constant: low CMP at 1 µg/mL, ultra-low CMP at 0.1 µg/mL, low TMP at 0.1 µg/mL, ultra-low TMP at 0.01 µg/ml, as well a condition with no drug. As before we propagated 14 identical cultures per condition. We grew cells at 37 °C in a 96-well microtiter plate (500 µL per well) in supplemented M9 medium (0.2% glucose, 1 mM MgSO₄, 0.1% casamino acids, 0.5 mg/ml thiamin). For these experiments, we performed serial passaging of the cultures consistently every 12 hours. To this end, we used the saturated culture from a previous passage to inoculate a fresh plate by 1:100 dilution (5 µL of saturated cultured into 500 µL of fresh medium). Overall, we performed 30 passages and stored every second passage at -80 °C after addition of 15% glycerol.

Growth measurements of the evolving barcoded *E. coli* populations. We estimated resistance of the evolving populations by calculating the change in IC₅₀ of CMP or TMP, following the methodology in ref⁴⁸. We sampled cells from three populations each in low CMP, ultra-low CMP, and no drug at passages 0, 8, 10, 12, 20, 30, and 70. We diluted these samples 1:100 into growth medium supplemented with 0, 1, 2, 4, 8, or 16 µg/mL of chloramphenicol, and followed their growth by OD measurements at 600 nm (**Supplementary Fig. 4A, Supplementary Table 3**). We calculated the area under these growth curves over the time of growth (**Supplementary Fig. 4B**) and normalized the area values so that they equaled 1 at zero antibiotic (**Supplementary Fig. 4C**). We determined the IC₅₀ by calculating the concentration of antibiotic at which growth (defined as normalized area under the growth curve) was reduced by 50% relative to zero antibiotic (**Supplementary Fig. 4C**). We inferred the IC₅₀ concentration by interpolating the area versus drug concentration curves. We similarly obtained IC₅₀ values for three populations each in low TMP, ultra-low TMP, and no drug at the same time points, but with TMP concentrations 0, 0.5, 1, 2, 5, 10, and 20 µg/mL (**Supplementary Table 3**).

Deep sequencing of the evolving barcoded *E. coli* populations. We sequenced barcodes at 16 time points over the evolution experiment for the same 15 independent populations used for the growth measurements. We first amplified these 240 bacterial cultures with

Illumina adaptor primers (15 μ L of defrosted cells from each culture). We performed the second PCR in two groups, each with 96 unique Nextera XT primers. We then pooled together 96 PCR reactions from each group, spiked them with 30% of PhiX DNA, and sequenced them on a Nextseq High Output 75 platform. The sequencing protocol commenced with 9 dark cycles to account for the sequence redundancy preceding the barcode area. For one sample (ultra-low CMP, replicate 2, passage 54) all PCR reactions failed, and thus we excluded this sample from all further analysis.

Analysis of sequencing data. First, we exclude sequencing samples that report fewer than 10^6 reads; this affects six time points from the evolving populations (**Supplementary Table 1**). All remaining samples have between 1.3×10^6 and 1.4×10^7 reads. Next, we exclude all reads with minimum base quality score less than 10 (Phred scale), which affects 0.02-0.16% of reads (**Supplementary Table 1**). To identify barcodes, we first align each read to the reference sequence for the barcode cassette. We allow up to three mismatches or one indel with respect to the reference; we also require that the read overlap the barcode by at least 10 nt. With these criteria we identify barcodes on more than 95% of reads in all but 17 samples from the original experiment (**Supplementary Table 1**). For each read with a valid alignment, we extract a barcode as the sequence aligning to the variable region in the reference.

To correct for sequencing errors in the raw barcodes, we use the bartender package⁴⁹ on default settings to cluster together barcodes with similar sequences. In general, this method assumes that a low-frequency barcode differing at only one or two bases from a high-frequency barcode is the result of a sequencing error, so that the low-frequency barcode is merged into the high-frequency one. This produces a set of putative true barcodes for the sample.

To ensure that we identify true barcodes consistently across samples, we first pool raw barcodes and perform clustering on these pooled samples. We pool barcodes both across time points for each population (to build trajectories of barcodes over time in each population) as well as across populations for each time point (to compare barcodes between populations). After clustering, we disaggregate the true barcodes from the pooled data back into the individual samples, where we normalize them by the total number of reads in

that sample. This yields a set of lineage frequencies $\{x_k(t)\}$ (where the index k runs over all barcodes) for each population at each time point t .

Quantifying lineage diversity. The simplest way to quantify the diversity of barcoded lineages in a population is to count the number of unique barcodes observed at a particular time point (**Fig. 3A**). However, if lineages differ widely in frequency, then this measure may not be very informative and will suffer from significant sampling bias (since very low-frequency barcodes will be under-sampled). A more general approach is to define the effective number of lineages using the diversity index qD from ecology³⁴. We construct this definition in analogy with the case where all lineages are at equal frequency, so that the number of lineages is simply the reciprocal of this frequency:

$$\text{number of lineages} = \frac{1}{\text{frequency of each lineage}}. \quad (\text{S1})$$

When lineages are not at equal frequencies, we replace the frequency in the denominator by a mean frequency over all lineages. Define the generalized mean (also known as the power mean) of a quantity h_k , with normalized weights p_k ($\sum_k p_k = 1$) and parameter q :

$$\langle h_k | p_k, q \rangle = \left(\sum_k p_k h_k^{q-1} \right)^{1/(q-1)}. \quad (\text{S2})$$

The parameter q controls how strongly the mean depends on very small or very large values of h_k : lower values of q allow relatively more weight from low values of h_k , while higher values of q more strongly weight high values of h_k . When $q = 2$, **Eq. S2** reduces to the ordinary arithmetic mean; when $q = 1$, it is equivalent to the geometric mean; and when $q = 0$, it is the harmonic mean.

We therefore define the effective number of lineages as the reciprocal of the generalized mean frequency over all lineages, weighing each frequency by itself^{34,35}:

$$\begin{aligned} {}^qD &= \frac{1}{\langle x_k | x_k, q \rangle} \\ &= \left(\sum_{\text{lineage } k} x_k^q \right)^{1/(1-q)}. \end{aligned} \quad (\text{S3})$$

The diversity index is mathematically equivalent to the exponential of the Renyi entropy in physics⁵⁰. Special values of the parameter q correspond to common ecological measures of diversity:

$$q = 0: \quad {}^qD = \text{number of lineages with nonzero frequency (species richness)} \quad (\text{S4})$$

$$q = 1: \quad {}^qD = \exp\left(-\sum_{\text{lineage } k} x_k \log x_k\right) \text{ (Shannon diversity)} \quad (\text{S5})$$

$$q = 2: \quad {}^qD = \left(\sum_{\text{lineage } k} x_k^2\right)^{-1} \text{ (reciprocal mean frequency, also known as Simpson concentration)} \quad (\text{S6})$$

$$q = \infty: \quad {}^qD = \frac{1}{\max_{\text{lineage } k} x_k}. \quad (\text{S7})$$

909 Note that the effective number of lineages according to **Eq. S3** equals the actual number of
 910 lineages for any q if all lineages have equal frequency. **Figure 3** shows the diversity
 911 indices for $q = 0$, $q = 1$, and $q = \infty$ for each population over the course of the evolution
 912 experiment.

913

914 **Quantifying dissimilarity of lineage composition between populations.** We can also use
 915 diversity indices to quantitatively compare the lineage compositions of two or more
 916 populations. Let M be the number of populations we are comparing ($M \geq 2$), and let $x_k^{(p)}$
 917 be the frequency of lineage k in population p ($= 1, 2, \dots, M$) at a particular time point. If
 918 we pool together all M populations, the frequency of lineage k is

$$919 \quad \bar{x}_k = \frac{1}{M} \sum_{\text{population } p} x_k^{(p)}. \quad (\text{S8})$$

920 The total diversity of the pooled population (“gamma diversity”) is^{34,35}:

$$\begin{aligned} 921 \quad {}^qD_{\text{pooled}} &= \frac{1}{\langle \bar{x}_k | \bar{x}_k, q \rangle} \\ &= \left(\sum_k \bar{x}_k^q \right)^{1/(1-q)}. \end{aligned} \quad (\text{S9})$$

922 We can decompose this total diversity into two factors:

$$923 \quad {}^qD_{\text{pooled}} = {}^qD_{\text{mean}} {}^qM_{\text{eff}}. \quad (\text{10})$$

924 The first factor on the right-hand side of **Eq. S10** is the mean diversity across all
 925 populations (“alpha diversity”):

$${}^qD_{\text{mean}} = \frac{1}{\left\langle \frac{1}{{}^qD^{(p)}} \middle| \frac{1}{M}, q \right\rangle} \quad (S11)$$

$$= \left(\frac{1}{M} \sum_{\text{population } p} [{}^qD^{(p)}(t)]^{1-q} \right)^{\frac{1}{1-q}},$$

where ${}^qD^{(p)}$ is the diversity of population p alone (**Eq. S3**). The second factor on the right-hand side of **Eq. S10** is the effective number of distinct populations (“beta diversity”):

$${}^qM_{\text{eff}} = \frac{{}^qD_{\text{pooled}}}{{}^qD_{\text{mean}}} \quad (S12)$$

$$= \left(\frac{\sum_{\text{lineage } k} \bar{x}_k^q}{\frac{1}{M} \sum_{\text{population } p} \sum_{\text{lineage } k} [x_k^{(p)}]^q} \right)^{1/(1-q)}.$$

This quantity has a minimum value of 1 if the populations have identical lineages at identical frequencies, and a maximum value of M if none of the populations have any lineages in common. To simplify the interpretation of this quantity across cases where we may be comparing different numbers of populations, we shift and rescale ${}^qM_{\text{eff}}$ to obtain a measure of dissimilarity between populations that ranges from 0 to 1:

$$\text{Dissimilarity between populations} = \frac{{}^qM_{\text{eff}} - 1}{M - 1}. \quad (S13)$$

We plot this normalized quantity in all figures (**Fig. 4, Extended Data Fig. 4, Extended Data Fig. 10, and Supplementary Figs. 5, 6, 10, and 21**).

In the case of $q = 0$, ${}^0M_{\text{eff}}$ simply measures how many lineages with nonzero frequency are in common between the populations under comparison. Let $B^{(p)}$ be the set of lineages with nonzero frequencies in population p , and let $|B^{(p)}|$ denote the number of lineages in this set. Then the effective number of distinct populations is

$${}^0M_{\text{eff}} = \frac{|\cup_{\text{population } p} B^{(p)}|}{\frac{1}{M} \sum_{\text{population } p} |B^{(p)}|}. \quad (S14)$$

For two populations ($M = 2$), we can rewrite this as

$$\begin{aligned}
{}^0M_{\text{eff}} &= \frac{|B^{(1)} \cup B^{(2)}|}{\frac{1}{M}(|B^{(1)}| + |B^{(2)}|)} \\
&= 2 - \frac{1}{\frac{1}{2}\left(\frac{|B^{(1)}|}{|B^{(1)} \cap B^{(2)}|} + \frac{|B^{(2)}|}{|B^{(1)} \cap B^{(2)}|}\right)},
\end{aligned} \tag{S15}$$

where we have invoked the inclusion-exclusion principle for sets: $|B^{(1)} \cup B^{(2)}| = |B^{(1)}| + |B^{(2)}| - |B^{(1)} \cap B^{(2)}|$. That is, ${}^0M_{\text{eff}}$ equals two minus the harmonic mean of the fractions of overlapping lineages between the populations. For example, ${}^qM_{\text{eff}} = 1.8$ means that the two populations have 20% of their lineages in common.

In the case of $q = 1$, the effective number of lineages is the Shannon diversity (**Eq. S5**). Therefore the effective number of distinct populations is equivalent to the exponential of the Jensen-Shannon divergence between the populations:

$${}^1M_{\text{eff}} = \exp \left[\frac{1}{M} \sum_{\text{population } p} \sum_{\text{lineage } k} x_k^{(p)} \log \left(\frac{x_k^{(p)}}{\bar{x}_k} \right) \right]. \tag{S16}$$

This is also equivalent to the weighted sum of the Kullback-Leibler divergences between each population and the pooled population.

In the case of $q = \infty$, the effective number of distinct populations depends only on the most abundant lineage in the pooled population and across all populations. That is,

$${}^\infty D_{\text{pooled}} = \frac{1}{\max_{\text{lineage } k} \bar{x}_k}, \tag{S17}$$

and

$${}^\infty D_{\text{mean}} = \frac{1}{\max_{\text{population } p} \max_{\text{lineage } k} x_k^{(p)}}, \tag{S18}$$

and so

$${}^\infty M_{\text{eff}} = \frac{\max_{\text{population } p} \max_{\text{lineage } k} x_k^{(p)}}{\max_{\text{lineage } k} \bar{x}_k}. \tag{S19}$$

Clustering lineage frequency trajectories. For each population from the original experiment, we exclude barcoded lineages that have zero detected frequency at more time points than a minimum number calculated as

$$\lfloor 0.5(\text{total number of time points} + 11) \rfloor, \quad (\text{S20})$$

where $\lfloor \cdot \rfloor$ is the floor function that rounds the argument down to the nearest integer. This ensures that all pairs of remaining lineages have at least 10 time points at which they both have nonzero frequency. This leaves between 177 and 969 lineages for each population. For the evolution experiment with constant drug conditions, we take the top 500 lineages ranked by mean frequency over time. In both cases we cluster the frequency trajectories $\{x_k(t)\}$ for these lineages using the hierarchical clustering routine in SciPy⁵¹. The distance metric between two lineages k_1 and k_2 is

$$\Delta(x_{k_1}(t), x_{k_2}(t)) = 1 - \rho(\log x_{k_1}(t), \log x_{k_2}(t)), \quad (\text{S21})$$

where $\rho(\log x_{k_1}(t), \log x_{k_2}(t))$ is the Pearson correlation coefficient between the logarithms of both frequency trajectories (excluding time points where either frequency is zero); **Supplementary Fig. 11** shows matrices of all pairwise trajectory correlations, while **Supplementary Fig. 12** shows this data in histogram form. **Supplementary Fig. 17** shows the same data but for the evolution experiment with constant drug conditions. We use the “average” linkage method (equivalent to unweighted pair group method with arithmetic mean, or UPGMA), which calculates the distance between two clusters as the arithmetic mean of the distances between all trajectories in both clusters. Other linkage methods produce qualitatively similar results. The hierarchical clustering results in dendrograms as shown in **Supplementary Figs. 11** and **17**. Finally, we form flat clusters by setting thresholds on the dendrograms, which we manually choose for each population; these thresholds are shown on the dendrograms in **Supplementary Fig. 11** and range from 0.15 to 0.65, which roughly mean that the minimum correlations between trajectories within clusters ranges from 0.35 to 0.85. For the evolution experiment with constant drug conditions, we set these thresholds to be 0.06 for all populations (**Supplementary Fig. 17**), since the smaller dynamic range of the trajectories creates a greater risk of over-clustering.

Simulations. We performed all simulations with SodaPop⁵² using a multiplicative fitness function. We first accounted for the evolutionary dynamics during the barcode library preparation. We evolved 10^7 cells, all initially clonal, for 30 generations, which corresponds to the approximate number of generations accrued during preparation of the barcode library on the *E. coli* chromosomes (**Fig. 1D**). We set the rate of arising mutations to $\mu = 10^{-5}$ per genome per generation, reflecting estimates of beneficial mutation rates

999 in bacteria^{53,54}. We assumed that mutations arising during this period were neutral during
1000 library preparation but contained a cryptic selection coefficient effect s that is only realized
1001 during evolution under drug conditions. We randomly drew these cryptic selection
1002 coefficients from an exponential distribution. At the end of each of the simulated library
1003 preparation, we randomized the cell indices and sequentially assigned barcodes to the cells,
1004 using the experimentally-measured distribution of chromosomal barcode frequencies
1005 (**Extended Data Fig. 1A**). From these initial populations, we then simulated the evolution
1006 experiment itself for 400 generations.

1007 While we tested a range of parameter values for these simulations, here we focus
1008 on just two cases that qualitatively reproduce the range of phenomena we observe in the
1009 experiments, along with a control condition of purely neutral population dynamics. We
1010 call the first case “pre-existing dominated,” since the evolutionary dynamics are primarily
1011 driven by beneficial mutations already present at the beginning of the evolution
1012 experiment, rather than de novo mutations acquired during the experiment. In this case,
1013 we use $s = 0.05$ as the mean selection coefficient for pre-existing mutations arising during
1014 library preparation. De novo mutations occur during the evolution experiment at rate $\mu =$
1015 2.5×10^{-4} and are chosen to be deleterious with probability 0.9 and beneficial with
1016 probability 0.1; for either type of mutation, we draw the selection coefficient from an
1017 exponential distribution with mean $s = \pm 0.05$. The second case is “de novo dominated,”
1018 since de novo mutations during the evolution experiment mainly drive the dynamics. In
1019 this case, we use $s = 0.01$ as the mean selection coefficient for pre-existing mutations,
1020 while de novo mutations occur at the rate $\mu = 10^{-5}$ and are strictly beneficial with an
1021 exponential distribution of mean $s = 0.01$. For both of these conditions as well as the
1022 neutral control case, we simulated two replicate populations.

Data and Code Availability

All raw barcode sequencing data used in this study is deposited in the National Center for Biotechnology Information Sequence Read Archive under BioProject accession numbers PRJNA592527 (initial barcode libraries), PRJNA592371 (time points from evolution experiment under increasing drug concentrations), and PRJNA592529 (time points from evolution experiment under constant drug concentrations). All other raw data is included in **Supplementary Tables 1-4**. All custom scripts used to analyze the data are available on request.

Competing Interests Statement

The authors declare no competing interests.

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Author contributions

SB conceived the study. WJ and JL performed the laboratory experiments. MM analyzed sequencing data and developed ecological approach. LG and AWRS performed simulations. MM, LG, AWRS, and SB interpreted the data. MM and SB wrote the manuscript. All authors approved of the final manuscript.

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Figure Captions

Figure 1: Barcoding *E. coli* cells with Tn7 transposon machinery. (A) Map of the helper plasmid expressing the Tn7 transposon machinery (*tnsABCD*). (B) Map of the integration plasmid carrying the barcode cassette and spectinomycin resistance-conferring gene (SpR) nested between the left and right Tn7 arms. (C) Map of the segment undergoing chromosomal integration into the Tn7 attachment site (*attnTn7*). Inset: sequence of the barcode-carrying cassette. (D) Steps for preparation of the barcoded plasmid and chromosomal libraries. See **Methods** for complete details.

Figure 2: Dynamics of barcoded lineage frequencies over evolution experiment. Each row corresponds to a different antibiotic regimen, while each column corresponds to a different replicate. Individual panels show the frequency trajectories for all barcoded lineages in single populations over time of the experiment. Each colored band corresponds to a unique lineage, with its vertical width indicating its frequency at a particular time point. For the top 20 lineages in each population (ranked by mean frequency over time), we assign a unique color to each lineage that is consistent across panels, i.e., the same color represents the same barcode across panels (**Supplementary Table 4**). We use random colors for all lower-frequency lineages. The gray band at the top represents the frequency of reads without identified barcodes. Dots above each plot mark times at which the drug concentration for that population changed (**Extended Data Fig. 2A,B**).

Figure 3: Dynamics of lineage diversity over time. (A) For each population and at each time point, we measured the effective number of barcoded lineages using the diversity index qD (**Eq. 1**; see also **Methods**) for three different values of q , which controls the weight of low- versus high-frequency lineages: 0D (number of unique barcodes, left), 1D (Shannon diversity, center), and ${}^\infty D$ (reciprocal of the maximum lineage frequency, right). Dots above each plot mark times at which drug concentrations changed (**Extended Data Fig. 2A,B**); their colors indicate the drug environment according to the legend. (B) Pearson correlation coefficient between diversity trajectories (1D) from all pairs of populations.

Figure 4: Dynamics of lineage dissimilarity among populations over time. (A) Dissimilarity of lineages for $q = 1$ (Eq. 2; see also **Methods**) between all pairs of populations at generation 120 (left) and the final time point at generation 420 (right). (B) Dissimilarity of lineages among all replicate populations in each drug condition, using $q = 0$ (left), $q = 1$ (center), and $q = \infty$ (right). Dots above each plot mark times at which the drug concentrations changed (**Extended Data Fig. 2A,B**); their colors indicate the drug environment according to the legend.

Figure 5: Reproducibility of individual lineage dynamics. Comparison of final frequencies of all barcoded lineages in low chloramphenicol (CMP) between (A) replicate populations 1 and 2 and (B) replicate populations 2 and 3. The dashed black line marks the line of identity; each panel also shows the Pearson correlation coefficient R and estimated p -value for the frequencies (p -values are numerically indistinguishable from zero). (C) Venn diagram of the top 10 barcodes (ranked by mean frequency over time) in each replicate population from low CMP. (D, E, F) Same as (A, B, C) but for three replicate populations without drug.

Figure 6: Distinct patterns of trajectories from pre-existing and de novo beneficial mutations. (A) Examples of clustered lineage trajectories suggesting the presence of pre-existing beneficial mutations (left) and de novo beneficial mutations (right) from ultra-low chloramphenicol (CMP) replicate 3. Trajectories are partially transparent to show overlap. (B) For each pair of populations, we calculated the relative overlap of lineages in trajectory clusters with apparent pre-existing beneficial mutations (left) and in clusters with apparent de novo beneficial mutations (right) (see **Supplementary Fig. 13** for classification of trajectory clusters). We measured overlap by the Jaccard index, i.e., the ratio of the number of lineages in the intersection to the number in the union. All entries are zero for pre-existing mutations in low trimethoprim (TMP) and for de novo mutations in low CMP because we identify no clusters of these types in those populations (**Supplementary Fig. 13**). (C) Total frequency trajectories of all lineages with apparent pre-existing mutations (left) and with apparent de novo mutations (right) in each population. Dots above each plot mark times at which the drug concentrations changed (**Extended Data Fig. 2A,B**); their colors indicate the drug environment according to the legend.

Replicate 1 **Replicate 2** **Replicate 3** **Low CMP** **Ultra-low CMP** **Low TMP** **Ultra-low TMP** **No drug**

A

Generation 120

Generation 420

B $q=0$ $q=1$ $q=\infty$

A

B

C

D

E

F

1. Extended Data

Complete the Inventory below for all Extended Data figures.

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Figure #	Figure title One sentence only	Filename This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: <i>Smith_ED Fig1.jpg</i>	Figure Legend If you are citing a reference for the first time in these legends, please include all new references in the Online Methods References section, and carry on the numbering from the main References section of the paper.
Extended Data Fig. 1	Initial barcode libraries	Figure_Extended_Data_1.pdf	(A) Distributions of barcode frequencies at different stages of library preparation. For a given frequency on the horizontal axis, the vertical axis shows the number of unique detected barcodes with that frequency. “Raw library” (blue): NextSeq Illumina sequencing of the barcode library as synthesized by IDT (prior to plasmid library creation). “Plasmid library” (red): MiSeq Illumina sequencing of barcodes incorporated into the Tn7 integration plasmid library. “Chromosomal library” (orange): NextSeq Illumina sequencing of the barcode library integrated into <i>E. coli</i> chromosomes and generated

			by PCR performed on chromosomal DNA pooled from four independent extractions. (B) Shannon entropy of nucleotides at each position in the 15 nt barcode for the same libraries in panel (A). The horizontal black line marks the entropy ($\ln 4 \approx 1.386$) of a maximally-random library where all nucleotides are equally abundant at each position. (C) Comparison of individual barcode frequencies in the raw library and in the plasmid library. Points are partially transparent to show their density; the dashed black line marks the line of identity. We also show the Pearson correlation coefficient R and estimated p -value for the frequencies (p -values are numerically indistinguishable from zero). (D) Same as (C) but comparing the plasmid library and the chromosomal library.
Extended Data Fig. 2	Drug concentrations and population growth over evolution experiment	Figure_Extended_Data_2.pdf	(A) Trajectories of low and ultra-low chloramphenicol (CMP) concentrations over time of the evolution experiment. (B) Same as (A) but for trimethoprim (TMP) conditions. (C) Approximate number of cells at the end of each passage for low and ultra-low CMP conditions, along with the populations evolved without drug. Lines are averages over all 14 replicate populations for each condition. (D) Same as (C) but for TMP conditions. (E) Same as (C) but showing the fold-change of population size during each passage on the vertical axis. (F) Same as (E) but for TMP conditions. Periodic oscillations in cell numbers and yields result from the fact that cultures were propagated in two intermittent growth regimes: 9 hours during the day, followed by 12 hours during the night (see Methods). Supplementary Table 2 contains raw OD data for each population at the end of each passage.
Extended Data Fig. 3	Evolution of resistance and	Figure_Extended_Data_3.pdf	(A) At several time points during the evolution experiment, we measured the chloramphenicol (CMP)

	growth traits		IC50 of the barcoded populations evolved in low and ultra-low CMP as well as those evolving without drug. (B) Same as (A) but for trimethoprim (TMP). (C) Growth rate, measured in the absence of drug, of barcoded populations evolved in low and ultra-low CMP as well as without drug. Points represent the mean and error bars represent standard deviation over replicate measurements. (D) Same as (C) but for populations evolved in low and ultra-low TMP. Supplementary Table 3 contains raw growth curve data for the evolved populations at these different drug concentrations.
Extended Data Fig. 4	Trajectories of barcoded lineage frequencies	Figure_Extended_Data_4.pdf	Each row corresponds to a different antibiotic regimen, while each column corresponds to a different replicate. Individual panels show the frequency trajectories for barcoded lineages in single populations over time of the experiment; we only show lineages with mean frequency over time greater than 10^{-4} . For the top 20 lineages in each population (ranked by mean frequency over time), we assign a unique color to each lineage that is consistent across panels, i.e., the same color represents the same barcode across panels (Supplementary Table 4). All lower-frequency lineages are gray and partially transparent to show their density. The dashed black line represents the frequency of reads without identified barcodes. Dots above each plot mark times at which the drug concentration for that population changed (Extended Data 2A,B).
Extended Data Fig. 5	Trajectories of barcoded lineage frequencies under constant conditions	Figure_Extended_Data_5.pdf	Same as Extended Data 4 but for evolution experiments with constant drug concentrations (see Methods).
Extended Data Fig. 6	Dynamics of lineage diversity over time	Figure_Extended_Data_6.pdf	Same as Fig. 3 but for evolution experiments with constant drug concentrations (see Methods).

	under constant conditions		
Extended Data Fig. 7	Dynamics of lineage dissimilarity among populations over time under constant conditions	Figure_Extended_Data_7.pdf	Same as Fig. 4 but for evolution experiments with constant drug concentrations (see Methods).
Extended Data Fig. 8	Trajectories with pre-existing and de novo beneficial mutations under constant conditions	Figure_Extended_Data_8.pdf	Same as Fig. 6B,C but for evolution experiments with constant drug concentrations (see Methods).
Extended Data Fig. 9	Comparison of simulations with different sources of variation	Figure_Extended_Data_9.pdf	We simulated evolutionary dynamics of a barcoded population (see Methods) with different supplies of mutations. We plot trajectories of barcoded lineage frequencies over time for simulated populations with (A) neither pre-existing nor de novo mutations (neutral dynamics), (B) pre-existing mutations only (mean $s = 0.1$), (C) de novo mutations only (mean $s = 0.05$), and (D) both pre-existing mutations (mean $s = 0.01$) and de novo mutations (mixed exponential distribution with 90% deleterious and 10% beneficial). In the top row, each color indicates the relative frequency of a particular lineage at every time point. For simplicity, we show only lineages with a minimum frequency of 10^{-4} (the gray area covers the frequency of all remaining lineages). In the bottom row, we show the frequency trajectories as lines on a log scale; lineages with a minimum frequency of 5×10^{-4} are colored while all other lineages above 10^{-4} are gray. Lineage frequencies represent the estimated frequency from subsampling 10% of the full simulated population.
Extended Data Fig. 10	Simulated dynamics	Figure_Extended_Data_10.pdf	We simulated evolutionary dynamics of the barcoded

	of lineage diversity	a_10.pdf	population for two replicates of each condition: “neutral” (no mutations), “pre-existing dominated,” and “de novo dominated” (see Methods). (A) For each population, we calculated the effective number of barcoded lineages using the diversity index qD (Eq. 1 ; see also Methods) for three different values of q , which controls the weight of low- versus high-frequency lineages: 0D (number of unique barcodes, left), 1D (Shannon diversity, center), and ${}^\infty D$ (reciprocal of the maximum lineage frequency, right). (B) We also calculated the dissimilarity of lineages (Eq. 2 ; see also Methods) among all replicate populations in each condition, using $q = 0$ (left), $q = 1$ (center), and $q = \infty$ (right). All diversity and dissimilarity values are based on perfect sampling of the population.
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Delete rows as needed to accommodate the number of figures (10 is the maximum allowed).

2. Supplementary Information:

A. Flat Files

Complete the Inventory below for all additional textual information and any additional Supplementary Figures, which should be supplied in one combined PDF file.

- **Row 1:** A combined, flat PDF containing any Supplementary Methods, Discussion, Equations, Notes, Additional Supplementary Figures, simple tables, and all associated legends. Only one such file is permitted.

- **Row 2:** Nature Research's Reporting Summary; please provide an updated Summary, fully completed, without any mark-ups or comments. **Please note that this is a required document.**

Item	Present?	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf	A brief, numerical description of file contents. i.e.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
Supplementary Information	Yes	Supplementary_Information.pdf	Supplementary Figures 1-21
Reporting Summary	Yes	nr-reporting-summary.pdf	

B. Additional Supplementary Files

Complete the Inventory below for all additional Supplementary Files that cannot be submitted as part of the Combined PDF.

- Do not list Supplementary Figures in this table (see section 2A)
- Where possible, include the title and description within the file itself
- Spreadsheet-based tables and data should be combined into a workbook with multiple tabs, not submitted as individual files.
- Please note that the *ONLY* allowable types of additional Supplementary Files are:
 - Supplementary Tables
 - Supplementary Audio
 - NMR Data
 - Computational Data

- Supplementary Videos
- Supplementary Data
- Cryo-EM Data
- Suppl. Software

Type	Number If there are multiple files of the same type this should be the numerical indicator. i.e. "1" for Video 1, "2" for Video 2, etc.	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: <i>Smith_Supplementary Video 1.mov</i>	Legend or Descriptive Caption Describe the contents of the file
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 1a: Summary of sequencing data for initial libraries. This table summarizes sequencing of the raw DNA library of barcodes, the library of barcodes on plasmids, and the library of barcodes inserted into the E. coli chromosome, corresponding to Extended Data Fig. 1 . The columns list the total number of reads, the fraction of reads passing the quality condition, the fraction of reads with detected barcodes, the number of unique barcodes before clustering, and the number of unique barcodes after clustering (see Methods).
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 1b: Summary of sequencing data for evolution experiment time points. Same as Supplementary Table 1a but for sequencing each population at various time points during the evolution experiment.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 1c:

		SX	Summary of sequencing data for evolution experiment time points under constant conditions. Same as Supplementary Table 1a but for sequencing each population at various time points during the evolution experiment under constant drug concentrations (Methods).
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 2: Optical densities of evolving populations. Each row corresponds to an evolving barcoded population, and each column corresponds to a time point (passage). Entries are the optical densities at 600 nm of the corresponding population (diluted tenfold) at the end of that passage.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3a: Growth curves of initial population. Growth curve data (optical density at 600 nm) for initial population in a range of chloramphenicol (CMP) and trimethoprim (TMP) concentrations.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3b: Growth curves of evolving populations at passage 8. Same as Supplementary Table 3a but for evolving populations after 8 serial passages.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3c: Growth

		SX	curves of evolving populations at passage 10. Same as Supplementary Table 3a but for evolving populations after 10 serial passages.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3d: Growth curves of evolving populations at passage 12. Same as Supplementary Table 3a but for evolving populations after 12 serial passages.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3e: Growth curves of evolving populations at passage 20. Same as Supplementary Table 3a but for evolving populations after 20 serial passages.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3f: Growth curves of evolving populations at passage 30. Same as Supplementary Table 3a but for evolving populations after 30 serial passages.
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 3g: Growth curves of evolving populations at final time point. Same as Supplementary Table 3a but for evolving populations after 70 serial passages (end of experiment).
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 4a: Top barcodes in evolving populations. For each population, the 20 barcode sequences with the

			maximum average frequency over the evolution experiment. For each barcode we list the average frequency, the final frequency at the end of the experiment, and the hex-encoded color for that barcode used in Fig. 2 , Extended Data Fig. 4 , and Supplementary Fig. 8 .
Supplementary Table	1	Supplementary_Tables_merged.xlsx	Supplementary Table 4b: Top barcodes in evolving populations under constant conditions. Same as Supplementary Table 4a but for the evolution experiment under constant drug concentrations (Methods) with colors used in Extended Data Fig. 5 and Supplementary Fig. 16 .

Add rows as needed to accommodate the number of files.

A **B** **C** **D**

Replicate 1

Replicate 2

Replicate 3

Low CMP

Ultra-low CMP

Low TMP

Ultra-low TMP

No drug

Replicate 1

Replicate 2

Replicate 3

Low CMP

Ultra-low CMP

Low TMP

Ultra-low TMP

No drug

A

Generation 108

Generation 180

Dissimilarity of
lineages ($q=1$) **B** $q=0$ $q=1$ $q=\infty$

AOverlap of lineages with
pre-existing mutations Overlap of lineages with
de novo mutations **B**Total frequency of lineages
with pre-existing mutations Total frequency of lineages
with de novo mutations

A **B**

Supplementary Figures

Supplementary Figure 1: Reproducibility of barcode frequency measurements. We compared measured barcode frequencies between four independent sequencing runs on the initial chromosomal library: **(A)** replicates 1 and 2, **(B)** replicates 2 and 3, and **(C)** replicates 3 and 4. Points are partially transparent to show their density; the dashed black line marks the line of identity. We also show the Pearson correlation coefficient R and estimated p -value for the frequencies (p -values are numerically indistinguishable from zero).

Supplementary Figure 2: Growth curves of the initial population. Growth curves of the initial barcoded population in the presence of **(A)** 0-16 $\mu\text{g/mL}$ of chloramphenicol (CMP) or **(B)** 0-20 $\mu\text{g/mL}$ of trimethoprim (TMP). We defined the MIC for each drug as the lowest concentration of antibiotic at which we observed no growth (see **Methods**). **Supplementary Table 3** contains raw growth curve data for the initial population at these different drug concentrations.

Supplementary Figure 3: Calibration of drug concentration based on population growth. Fold-change in cell density as a function of chloramphenicol (CMP) concentration for barcoded populations at four time points of the evolution experiment.

Supplementary Figure 4: Example of IC₅₀ calculation. (A) Growth curves of cells from a barcoded population evolving in low chloramphenicol (CMP) at generation 48 (passage 8), measured with different concentrations of CMP. (B) For each growth curve in (A), we calculated the area under it and plotted the area as a function of CMP concentration. (C) We similarly calculated growth curve areas for generations 48, 60, 72, 180, and 420 and normalized each by the area of the growth curve with zero CMP at the same time point. The IC₅₀ is then defined as the antibiotic concentration leading to 50% of growth relative to growth at zero CMP.

Supplementary Figure 5: Lineage diversity within and between initial libraries. (A) For each initial barcode library (same as in **Extended Data Fig. 1**, except with the four independent samples of the chromosomal library shown both separate and pooled), we calculate the effective number of barcoded lineages using the diversity index qD (**Eq. 1**; see also **Methods**) for three different values q , which controls the weight of low- versus high-frequency lineages: 0D (number of unique barcodes, left), 1D (Shannon diversity, center), and $^\infty D$ (reciprocal of the maximum lineage frequency, right). (B) Dissimilarity of lineages (**Eq. 2**; see also **Methods**) between all pairs of initial libraries from (A). Columns are the same as in (A): left, $q = 0$; center, $q = 1$; and right, $q = \infty$.

A

B

C

Supplementary Figure 6: Dissimilarity of lineages between populations. Each panel shows the dissimilarity of lineages (Eq. 2; see also **Methods**) between all pairs of populations at a particular time point: (A) $q = 0$, (B) $q = 1$, (C) $q = \infty$.

Supplementary Figure 7: Comparison of lineage frequencies between populations. Each row and each column correspond to a different population (labeled on the vertical and horizontal axes of each panel). Individual panels show comparisons of barcoded lineage frequencies at the end of the experiment between a pair of populations. The dashed black lines mark the line of identity; each panel also shows the Pearson correlation coefficient R and estimated p -value for the frequencies (some p -values are numerically indistinguishable from zero).

Supplementary Figure 8: Comparison of top lineage trajectories between populations. Each row and each column correspond to a different population (labeled on the horizontal and vertical axes of each panel). Individual panels show the traces of the top three (by average frequency) barcoded lineages over time between each pair of populations. The dashed black lines mark the line of identity; each panel also shows the Pearson correlation coefficient R and estimated p -value for the frequencies. Colors of lineage trajectories are consistent across panels and match **Fig. 2** and **Extended Data Fig. 4** (Supplementary Table 4).

Supplementary Figure 9: Comparison of lineage frequencies before and after evolution.

Each row corresponds to a different antibiotic regimen, while each column corresponds to a different replicate. Individual panels compare the initial frequencies of all barcoded lineages with their final frequencies at the end of the evolution experiment in each population. The dashed black line marks the line of identity, while the vertical gray line marks the average initial frequency of a barcoded lineage; this is the null expectation for the initial frequency of the lineage that ultimately fixes.

Supplementary Figure 10: Divergence of lineage dissimilarity over evolution. For each population at each time point, we calculate the dissimilarity of lineages (Eq. 2; see also **Methods**) with the initial population for $q = 0$ (left), $q = 1$ (center), and $q = \infty$ (right). Dots above each plot mark times at which the drug concentrations changed (**Extended Data Fig. 2A,B**).

Supplementary Figure 11: Summary of trajectory clustering. Each row corresponds to a different antibiotic regimen, while each column corresponds to a different replicate. Individual panels show the matrix of Pearson correlation coefficients between all pairs of trajectories used for hierarchical clustering and the resulting dendrogram for each population (see **Methods**). The horizontal dashed lines in the dendrograms mark the thresholds for forming flat clusters. Colors of dendrogram leaves match the corresponding trajectories in **Supplementary Fig. 13**.

Supplementary Figure 12: Distributions of trajectory correlations. Each row corresponds to a different antibiotic regimen, while each column corresponds to a different replicate. Individual panels show histograms of Pearson correlation coefficients between all pairs of lineage trajectories used for hierarchical clustering in each population (see **Methods**). The vertical dashed lines mark the thresholds for forming flat clusters.

Supplementary Figure 13: Clusters of lineage trajectories. Each row corresponds to a single population; the first 10 columns correspond to the top 10 clusters in decreasing order of cluster size (number of trajectories) in each population, while the last column shows trajectories from all other clusters in that population. Individual panels show sets of lineage trajectories that clustered together in our analysis. Trajectories are partially transparent to show their density; dots above each plot mark times at which the drug concentration for that population changed (**Extended Data Fig. 2A,B**). The color of each trajectory cluster matches its corresponding leaves in the dendrogram in **Supplementary Fig. 11**. We label clusters with apparent pre-existing beneficial mutations or with apparent de novo beneficial mutations.

Supplementary Figure 14: Hamming distances among sequences within trajectory clusters.

Each row corresponds to a single population; the first 10 columns correspond to the top 10 clusters in decreasing order of cluster size (number of trajectories) in each population, while the last column shows trajectories from all other clusters in that population. Individual panels show histograms of Hamming distances between all pairs of barcode sequences whose trajectories clustered together in our analysis. The vertical dashed lines marks the expected Hamming distance between two random 15 nt barcode sequences (11.25). Empty histograms correspond to clusters with only one trajectory. The color of each trajectory cluster matches its corresponding leaves in the dendrogram in **Supplementary Fig. 11**. We label clusters with apparent pre-existing beneficial mutations or with apparent de novo beneficial mutations.

Supplementary Figure 15: Comparison of initial frequencies of trajectories in different types of clusters. For each trajectory cluster classified as having pre-existing or de novo beneficial mutations (**Supplementary Fig. 13**), we calculated the variance of initial frequencies of trajectories in that cluster.

Supplementary Figure 16: Dynamics of barcoded lineage frequencies over evolution experiment under constant conditions. Same as Fig. 2 but for evolution experiments with constant drug concentrations (see Methods).

Supplementary Figure 17: Summary of trajectory clustering under constant conditions. Same as **Supplementary Fig. 11** but for evolution experiments with constant drug concentrations (see **Methods**).

Supplementary Figure 18: Clusters of lineage trajectories under constant conditions. Same as **Supplementary Fig. 13** but showing the top 20 clusters for evolution experiments with constant drug concentrations (see **Methods**).

Supplementary Figure 19: Simulated trajectories from pre-existing versus de novo mutations. (A, B) For two simulated populations in the “pre-existing dominated” case (see **Methods**), we show barcoded lineage trajectories with pre-existing beneficial mutations. (C, D) For two simulated populations in the “de novo dominated” case (see **Methods**), we show barcoded lineage trajectories that acquire beneficial mutations de novo. For simplicity, we show the lineages that rise to a minimum frequency of 10^{-4} .

Supplementary Figure 20: Effect of subsampling on estimated diversity. (A) For a simulated population in the “pre-existing dominated” case (see **Methods**), we calculated the effective number of barcoded lineages using the diversity index qD (Eq. 1; see also **Methods**) as in **Extended Data Fig. 10A**, but comparing both the true diversity of the whole population (solid lines) and the estimated diversity based on subsampling 10% of the population (points, with mean and standard error based on three trials). (B) Same as (A) but for a simulated population in the “de novo dominated” case (see **Methods**).

Supplementary Figure 21: Effect of subsampling on estimated dissimilarity of lineages. (A) For two simulated populations in the “pre-existing dominated” case (see **Methods**), we calculated the dissimilarity of lineages (**Eq. 2**; see also **Methods**) between populations as in **Extended Data Fig. 10B**, but comparing both the true dissimilarity of the whole populations (solid lines) and the estimated dissimilarities based on subsampling 10% of the populations (points, with mean and standard error based on three trials). (B) Same as (A) but for simulated populations in the “de novo dominated” case (see **Methods**).