Access to high-impact mutations constrains the evolution of antibiotic resistance in soft agar

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Despite widespread resistance to many important antibiotics, the factors that govern the emergence and prevalence of antibiotic-resistant bacteria are still unclear. When exposed to antibiotic gradients in soft agar plates measuring as little as 1.25 x 11 cm we found that Escherichia coli rapidly became resistant to representatives from every class of antibiotics active against Gram-negative bacteria.

Evolution kinetics were independent of the frequency of spontaneous mutations that confer antibiotic resistance or antibiotic dose-response curves, and were only loosely correlated to maximal antibiotic concentrations. Instead, rapid evolution required unrealized mutations that could markedly decrease antibiotic susceptibility. When bacteria could not evolve through these “high-impact” mutations, populations frequently bottlenecked, reducing the number of cells from which mutants could arise and prolonging evolution times. This effect was independent of the antibiotic’s mechanism of action, and may affect the evolution of antibiotic resistance in clinical settings.

Antibiotic resistance is one of the greatest threats to modern medicine. Antibiotic-resistant organisms are thought to cause over 2,000,000 infections and 23,000 deaths in the United States each year. Historically, preclinical assessment of new antibiotics has focused on measuring the rate with which resistance-conferring mutations occur in vitro. Compounds with low or non-existent spontaneous resistance mutation rates are considered insensitive to the evolution of resistance in vivo, to the extent that some may theoretically be “evolution-proof”. Unfortunately, clinical outcomes are less encouraging. The rate of spontaneous resistance development for clinical antibiotics span from 10⁻⁵ to 10⁻¹¹ resistant mutations per cell per generation, and aren’t linked to the prevalence of resistance. Rapid evolution of resistance during therapy also isn’t correlated to spontaneous resistance mutation rates; the leucyl-tRNA synthetase inhibitor GSK2251052/AN3365 recently failed a phase II clinical trial after rapid evolution of drug resistance in vivo, despite a mutation rate of 1.4 x 10⁻⁷.

The recent development of laboratory evolution chambers has enabled directed evolution of bacteria without labour-intensive sub-culturing, allowing researchers to map the genotypic pathways to antibiotic resistance following growth in etched silicon wafers, moribodostats, and MEGA plates. The latter has also revealed exciting evolution dynamics: bacteria growing in desk-sized MEGA plates encounter wedges of geometrically-increasing antibiotic concentrations up to 20,000x the initial MIC. In this environment, resistant mutants leave behind their susceptible brethren, providing an excellent example of how competition for nutrients can drive the evolution of antibiotic resistance.

Unfortunately, operating these devices requires a combination of specialized expertise and custom-built equipment, limiting their throughput and general utility. To make the evolution of bacteria in vitro more accessible we have designed a compact system based on Soft Agar Gradient Evolution (SAGE). SAGE plates are built from standard petri dishes or similar labware, and can be run in high-throughput without ongoing operator involvement. We validated this system by generating mutants of Escherichia coli (E. coli) individually resistant to twelve antibiotics or antibiotic mixtures, covering every major antibiotic class active against Gram-negative bacteria. We then investigated the kinetics of in vitro evolution, using the fine control over selective pressure provided by the SAGE system to determine the key factors governing the rate of antibiotic resistance. We found that successful strains often exhibited mutation rates distinct from their progenitors, and that mutation supply rates didn’t constrain evolution rates in even small bacterial populations. Instead, evolution rates appeared to be constrained by the limited availability of potential high-impact mutations, unrealized changes to the genome that could markedly increase antibiotic resistance.

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Results

Evolution chamber design. Hard agar gradient plates have been used since at least 1952 for antibiotic susceptibility testing\(^\text{10}\). They are created by sequentially pouring agar wedges of differing composition and allowing diffusion to equalize concentrations across the layers. By controlling the quantity of agar and the slope of the plate, it’s possible to create smooth gradients of arbitrary composition\(^\text{10,11}\). We found that these gradients are similarly stable at soft agar concentrations (0.2–0.75% agar w/v, Fig. 1), that allow bacteria to swim throughout the plate\(^\text{12}\). Bacteria inoculated into plates with antibiotic gradients grow until they encounter limiting concentrations of the antibiotic, at which point resistant mutants are selected for.

Movement through soft agar. To select for efficient chemotaxis, \(E.\ coli\) MG1655, BW25113 \(\Delta\text{mutL}\) and BW25113 \(\Delta\text{mutS}\) were passed three times through 0.25% agar plates, at which point they were able to traverse the plate within 20 hours of incubation at 37 °C. Adding a thin layer of mineral oil both reduced syneresis and decreased the experiment times to approximately 4 hr (Figs S1–3). Bacteria on oil-containing plates had lower densities, with growth restricted to the upper millimetre of the gel (Fig. S3). Increasing the strength of the agar decreased bacterial movement in accordance with previous work on the movement of \(E.\ coli\) in soft agar\(^\text{12}\), while decreasing the agar load led to gels that were too fragile to handle efficiently.

Using the antibiotic ciprofloxacin at 75 \(\mu\text{g/L}\) (~5x the initial bacterial minimum inhibitory concentration (MIC)), we then screened for the evolution of antibiotic resistance in SAGE plates. Circular petri dishes, square petri dishes, machined polycarbonate plates of varying widths, and rectangular 4-well nunclon-treated plates all gave similar results (Fig. S2), with resistant cells emerging after 24 hr of incubation at 37 °C. Due to the similarities in outcomes all further evolution studies were performed with 4-well nunclon-treated plates.

Time-lapse microscopy revealed that \(E.\ coli\) moved smoothly through the agar medium (Fig. S4), propagating in a wave of high density cells\(^\text{9,12}\). Entering agar free of other strains, mutants were able to quickly establish a buffer of discarded cells, blocking competition from faster growing but more antibiotic-susceptible cells. This
is similar to what was observed around the step gradients of MEGA plates, and greatly reduced the impact of loss-of-fitness mutations, provided that these mutations also reduce antibiotic susceptibility.

**Evolution of antibiotic resistance.** When challenged with a variety of antibiotics at a minimum of 5x the MIC, antibiotic resistance emerged in the three _E. coli_ strains under investigation: speed-selected variants of _E. coli_ MG1655 and two strains with elevated mutation rates due to defects in their mismatch repair systems; _E. coli_ BW25113 ΔmutS and ΔmutL13. The magnitude of resistance varied by antibiotic, from 4x to 512x the initial MIC (Fig. 1D, Tables S1–3. Lower levels of resistance could in some cases be further increased by exposing mutants to a second SAGE plate containing 25x the original MIC.

Resistance evolved against every antibiotic tested as well as co-trimoxazole, a 1:19 mixture of trimethoprim and sulfamethoxazole. This included representatives from every major class of antibiotics, as well as antibiotics that don't readily evolve resistance in other _in vitro_ systems. E.g. resistance to doxycycline and chloramphenicol increased 32-fold following passage through their respective SAGE plates, versus a 10-fold increase in doxycycline resistance after twenty-five days of growth in a morbidostat (compared to a 870-fold increase in chloramphenicol resistance over the same time period)7. Similarly, the ease with which we developed resistance to drugs like ampicillin, doripenem, and polymyxin B is at odds with antibiotic resistance in the clinic, which often takes years to gain prevalence in bacterial pathogens14. Clinical resistance is also often linked to the transfer of plasmid-encoded inactivating proteins, which were absent in this study15. For example, resistance to beta-lactams in _E. coli_ is largely due to the production of beta-lactamases, but we were able to readily obtain mutants resistant to ampicillin and doripenem despite the fact that _E. coli_ MG1655 lacks an inducible beta-lactamase (though a copy of _ampC_ is expressed at low levels)15–17.

Both MG1655 and the mutator strains developed resistance at similar levels (Fig. 1D, Tables S1–3). Counter to a theoretical model comparing evolution under uniform and increasing drug concentrations18, the bacterial strains rapidly evolved resistance to both ciprofloxacin and streptomycin, despite the different fitness effects of their resistance-conferring mutations. This may be because the strong founder effect of the system limits competition between naïve and resistant strains, as detailed above.

**Genetic analysis.** Amplification and sequencing of known proto-resistance genes identified several previously reported mutations in strains resistant to streptomycin, ciprofloxacin and trimethoprim (Tables S4 and S5). In particular, all four streptomycin-resistant lineages contained a K42R mutation in RpsL, which has been previously reported to confer near-immunity to streptomycin19,20. To evaluate broader genetic alterations we sequenced the genome of the progenitor strain of _E. coli_ MG1655 and lineages resistant to ampicillin, gentamicin, or polymyxin B (Tables 1 and S6). The ancestral strain was found to have 78 mutations distinct from that of the previously published genome for _E. coli_ MG165521. Some of these were likely acquired prior to this work, but there was an interesting V42A mutation in LrhA, a transcriptional repressor of bacterial motility. This mutation has not been previously reported, but mutants lacking _lrhA_ show enhanced chemotaxis22. It's possible that the chemotaxis screen conducted at the start of this work selected for strains with deficient or altered LrhA function.

| Antibiotic | Resistance-linked mutations | Mutations with unknown effect |
|------------|-----------------------------|------------------------------|
| Ampicillin | AcrB F628L, Aas D649G       |                              |
|            | AcrR G28R                   |                              |
|            | AcrC205G                    |                              |
|            | EmrZ T250M                  |                              |
|            | Pls V545F                   |                              |
|            | MarL A66F, C108F            |                              |
| Gentamicin | CpxA A97T                   | HfrM T133A                   |
|            | DnaF W455R                  | MalF Q115*                   |
|            | FuxA A592V, A608V           | RfaH D106D                   |
|            | SbmA D336s                  |                              |
|            | Yhbs L216P                  |                              |
| Polymyxin B| AcrB G861E                  | Aas D649G                    |
|            | ArrC A192V                  | AsmA R86C, E524fs           |
|            | BamA Q441R, D447G           | HfrM T133A                   |
|            | BasR G53E                   | MalF Q115*                   |
|            | BasL L14P                   | MalF T207A                   |
|            | Gmd F211, T27C              | RfaH D106D                   |
|            | LptD G701R                  | YfrM A106H                   |
|            | LpxC G106S                  |                              |

**Table 1.** Mutations identified through whole genome sequencing. aFor a full list see Table S7. bStrains were collected following passage through the same 5x SAGE plate but different 25x plates. cMutation found in both lineages.
the mutation rates of BW25113 Δ passage through SAGE plates, using the frequency of spontaneous resistance to rifampicin as a proxy measure-
constitutively high mutation rates. We therefore determined the mutation rate of several strains before and after genome sequencing, we were concerned that passage through SAGE plates might select for mutators: strains with
Δ.
Eard of genomic
and the cell counts in each sample were determined via qPCR using universal 16 S primers and a dilution stand-
Representative samples from the growing front and stationary lawn of antibiotic-free SAGE plates were excised,
leading to lineages with rates both above (MG1655 Poly B 1 and 2) and below (MG1655 Poly B 1-1 and 1–2) that
ymyxin B both traverse the outer membrane through self-promoted uptake25 and gentamicin-resistant lineages
the cell envelope, and may be involved in movement of polymyxin B across the membrane. Gentamicin and pol-
not previously linked to polymyxin B resistance, including in MalF, YfiM, and HofM. These proteins are found in
AcrAB and thereby reducing drug uptake24. Similarly, while the polymyxin B-resistant lineages contained a
AcrR and MarR have been previously linked to a 4-fold increase in ampicillin resistance, by increasing expression
to be related to missense mutations in the AcrAB efflux pump and its regulators, AcrR and MarR. Mutations in
 MIC of ampicillin rose 64-fold following passage through SAGE plates. The further increase in MIC appears
5.98
approximately 1.31
×
3.2
×
107). Only cells in the front are under selection, and so the maximal population
×
108)
×
106). This value is likely to be an overestimate, both due to the likely
The time required for bacteria to evolve antibiotic resistance was consistent across replicate experiments
Factors governing the evolution of antibiotic resistance. Maximal antibiotic concentration and slope. The time required for bacteria to evolve antibiotic resistance was consistent across replicate experiments for each antibiotic investigated, but varied between drugs. To determine the factors that governed the evolution of resistance we began to vary the slope and concentration of antibiotic that bacteria were exposed to.
Increasing the maximal concentration of streptomycin in the plates from 5x MIC to 40x MIC and 320x MIC decreased growth across the plate during the first two days of incubation, but had no statistically significant effect on growth at 72 hours. (Fig. 2A). Other antibiotics showed a similar pattern, though with ciprofloxacin increasing the maximal drug concentration from 75x MIC to 125x MIC actually accelerated growth. This may be due to the mutagenic effect of subinhibitory ciprofloxacin concentrations, which could potentially increase the effective

Correcting for mutations in the ancestral strain, we found between 29 and 178 single nucleotide polymorphisms in the evolved strains (Table 1; a full list of mutations can be found in Table S6). In general, resistance appeared to be conferred not by any single genetic change but rather by the additive effects of a small series of
For example, both ampicillin-resistant mutants contained a V5451 mutation in penicillin-binding protein 3, Ftsl. This mutation is linked to a roughly 3-fold increase in beta-lactam resistance in Salmonella spp.23, but the MIC of ampicillin rose 64-fold following passage through SAGE plates. The further increase in MIC appears to be related to missense mutations in the AcrAB efflux pump and its regulators, AcrR and MarR. Mutations in AcrR and MarR have been previously linked to a 4-fold increase in ampicillin resistance, by increasing expression of AcrAB and thereby reducing drug uptake24. Similarly, while the polymyxin B-resistant lineages contained a

### Table 2. Frequency of spontaneous rifampicin resistance before and after antibiotic exposure.

| Lineage               | $f \times 10^6$ | $\mu \times 10^6$ |
|-----------------------|-----------------|-------------------|
| MG1655                | 4.0 ± 2.1       | 1.1               |
| MG1655 Post speed selection* | 64.6 ± 9.8   | 12.8              |
| E. coli BW25113 ΔmutL. | 188 ± 4.3       | 31.1              |
| E. coli BW25113 ΔmutL. Post speed selection* | 39.2 ± 12.8 | 4.3 |
| E. coli BW25113 ΔmutS | 650 ± 134.5     | 82.9              |
| E. coli BW25113 ΔmutS Post speed selection* | 45.3 ± 4.0    | 7.8               |
| Ampicillin 1-1        | 57.3 ± 27.7     | 18.3              |
| Polymyxin B 1         | 312 ± 90        | 66.8              |
| Polymyxin B 1-1       | 17.1 ± 6.2      | 6.4               |
| Polymyxin B 1-2       | 15.6 ± 2.7      | 5.8               |
| Polymyxin B 2         | 71.6 ± 13.4     | 19.9              |

* After selection for efficient swimmers, prior to antibiotic exposure. 

Average of three technical replicates.

Factors governing the evolution of antibiotic resistance. Maximal antibiotic concentration and slope. The time required for bacteria to evolve antibiotic resistance was consistent across replicate experiments for each antibiotic investigated, but varied between drugs. To determine the factors that governed the evolution of resistance we began to vary the slope and concentration of antibiotic that bacteria were exposed to.
mutant supply rate. Of the antibiotics tested, only doxycycline showed a statistically significant difference in growth at 72 hr (10x MIC vs 50x MIC, s = 0.04, two-tailed, unequal variance Student’s t-test. **P value < 0.05, ***P value < 0.01, ****P value < 0.0001).

Figure 2. Passage through SAGE plates as a function of (A) antibiotic concentration and (B) slope. Averages of three biological replicates are shown. Antibiotic concentrations are given as the maximal concentration in the plate, as a function of MIC. The slope in (A) was 8 mm. (B) The likelihood that the observed differences in movement were due to chance was calculated via a two-tailed unequal variance Student’s T test. **P value < 0.05, ***P value < 0.01, ****P value < 0.0001.

Antibiotic dose-response. In SAGE plates selective pressure is restricted to a narrow, high-density band of growing cells (Fig. S4; see above). Varying the maximal antibiotic concentration or slope may reduce the width of this band, constricting the population under selection and causing the prolonged experiment times we observed. As
changing the antibiotic concentration could also alter the mutations required for resistance we investigated the correlation between evolution time and band width through the antibiotic dose-response curve. Compounds with steep dose-response curves will rapidly lower bacterial growth rates as the concentration of drug increases, reducing the width of the growing band. The antibiotic dose-response curve is also closely linked to the mutant selective window in other evolution systems, and is thought to contribute to the slow evolution rate of resistance against antimicrobial peptides like polymyxin B.

However, we found no correlation between the dose-response curves of antibiotics and evolution rates (Table 3). Populations of fast-swimming *E. coli* MG1655 exposed to both doxycycline and polymyxin B required more time to traverse SAGE plates than populations exposed to ciprofloxacin or trimethoprim, though doxycycline had the shallowest measured dose-response curve and polymyxin B the steepest.

The impact of resistance-conferring mutations determines evolution rates. At elevated antibiotic concentrations the dynamics of growth across SAGE plates changed. While bacteria still began by growing in a band of high-density cells, movement at growth-limiting concentrations of antibiotic was slow enough that individual mutation events were visible (Figs 3 and S4). Mutants first appeared as pinpricks of high density cells, growing over time into cones as cells radiated out from their point of origin. In trimethoprim plates these cones quickly coalesced into new bands of high-density cells, which moved across the plate until the concentration of antibiotic was again growth-limiting (Fig. S4).

Mutations were also observed in doxycycline plates, but the change in MIC provided by these mutations was much smaller. As a result, the majority of mutants didn’t form large cones, but instead stopped growing near the point of mutation, leaving behind small nodules of high-density cells (Fig. 3, arrows). Over several days the bacteria in these nodules mutated further, increasing their resistance and allowing further movement across the plate. Increasing the plate’s slope significantly compressed the nodules, reducing the population that could give rise to highly-resistant mutants. This in turn increased the amount of time required for doxycycline resistance to evolve.

Thus, the rate of evolution in SAGE plates is set by the change in antibiotic susceptibility conferred by unrealized mutations. Latent “high-impact” mutations allow strains to re-establish large populations following

| Antibiotic | N value | Median Time to Completion† |
|------------|---------|---------------------------|
|            | 15 × MIC | 25 × MIC                |
| Ciprofloxacin | 3.2   | 2                        |
| Doxycycline | 1.3   | 3                        |
| Polymyxin B | 32.2  | 3                        |
| Trimethoprim | 3.3  | 2                        |

Table 3. Antibiotic dose-response and evolution rates. †Median of three biological replicates. All values in days. N values for *E. coli* MG1655 were calculated after speed selection. Large N values correspond to steep dose response curves.
antibiotic challenge, ensuring that if the concentration of antibiotic once again becomes growth limiting there is a large population from which further mutants can arise. When resistance is conferred by low-impact mutations, the bacteria become trapped in population bottlenecks, reducing the likelihood of further mutations.

With this new framework, we can explain the rapid evolution of bacteria exposed to high levels of ciprofloxacin, trimethoprim, and streptomycin. Resistance to each of these antibiotics is known to occur from a small number of high-impact mutations, several of which were observed in strains following passage through SAGE plates (Table S5). In principle, any antibiotic whose activity can be sharply curtailed by a small number of high-impact mutations will be quickly rendered ineffective by SAGE, regardless of the spontaneous resistance mutation rate or maximal antibiotic concentration.

Population mutation rates appear to vary within SAGE plates. The population bottlenecks that antibiotics create within SAGE plates may be partially alleviated by transiently increasing the mutation supply rate by rapid cell turnover. Antibiotic stress is also known to trigger transiently elevated mutation rates through the SOS response, the stringent response, and the production of reactive oxygen species. However, our work suggests that in SAGE plates these bacteria may also improve their mutation supply rate through the transient evolution of strains with high background mutation rates.

High mutation rates are known to help populations more easily develop antibiotic resistance, and mutator strains are more prevalent in populations of bacterial pathogens. However, we found that mutators were uncommon following exposure of speed selected E. coli MG1655 to gradients of polymyxin B, and that simple passage of E. coli BW25114 ΔmutS through antibiotic-free agar was sufficient to drop the observed mutation rate 14-fold, bringing it close to values recorded for the other strains investigated (Table 2, above). High mutation rates can reduce fitness in the absence of stress and we hypothesize that this is leading to counter-selection later in the plate, when the bulk of the population is antibiotic resistant.

Similar behaviour has been recently observed in E. coli populations exposed to ethanol stress, where population mutation rates frequently rose and fell in response to increasing ethanol concentrations. If this process is occurring in the SAGE system it could explain why we observed relatively small populations of both wildtype progenitor strains and progenitors with mutator phenotypes rapidly developing resistance to antibiotics with low spontaneous resistance mutation rates.

Other environments. Extending these results to bacterial evolution in other systems, access to high-impact mutations may broadly constrain the evolution of antibiotic resistance. Resistant mutants are often less fit, and in liquid environments like that of morbidostats don’t form a significant fraction of the population until mutations broadly constrain the antibiotic significantly inhibits susceptible strains. When bacteria evolve resistance through a series of small number high-impact mutation. Those which lack such convenient resistance pathways will be less susceptible to the evolution of resistance.

Discussion

Using antibiotic gradients in soft agar we evolved mutants of E. coli resistant to representatives of every major class of antibiotic active against Gram-negative bacteria. Our findings show that in soft agar gradients evolution kinetics are constrained by the potential for high-impact mutations. When bacteria develop resistance through high-impact mutations, as with streptomycin resistance, bacteria quickly recover from population bottlenecks following selection, easing passage through future selective barriers. In contrast, factors that have been previously linked to the rate of resistance development, such as the maximal antibiotic concentration, spontaneous resistance mutation frequency, and antibiotic dose-response/mutant selection window had little influence on evolutionary success. Their limited impact in the SAGE plates appears to be in part due to the transient evolution of strains with elevated mutation rates, which increases the mutation supply rate and allows for the generation of resistant mutants from even small bacterial populations. In the absence of antibiotic stress these hypermutators appear to be rapidly replaced by strains with lower mutation rates.

Natural environments are unlikely to contain semi-solid agar gels, but will likely contain antibiotic gradients, potentially allowing for evolutionary dynamics similar to what we have observed. In particular, the human body readily creates drug gradients during both antibiotic therapy and chemotherapy, due to limited penetration into various tissues. If these gradients accelerate the evolution of resistance in vivo, this likely explains why our current methods of evaluating potential new antibiotics are poorly correlated to clinical outcomes. To reduce the emergence of resistance, antibiotic candidates should instead be evaluated by their potential to be nullified by a small number high-impact mutation. Those which lack such convenient resistance pathways will be less susceptible to the evolution of resistance.

Experimental Procedures

Soft agar gradient evolution plates. Sterile 4-well nunclon treated culture dishes were purchased from Thermo Scientific (cat. 167063), and used for the majority of evolution experiments. The hydrophilic surface treatment was not critical for SAGE experiments, but led to more resilient gels due to increased interactions between gel and plate.

Strains. E. coli MG1655 was a generous gift from Éric Déziel, INRS, Canada. E. coli BW25113 ΔmutS and ΔmutL were purchased from the Coli Genetic Stock Center (CGSC) and are part of the Keio Collection.

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Soft agar gradient evolution experiments. Molten 0.25% cation-adjusted Mueller-Hinton agar (MHA) was poured into 4-well plates that were raised on one side 3 mm, 8 mm, or 12.5 mm. Convenien lifters were made from P200 pipette tips, P1000 pipette tips, or a 1/2" role of labeling tape, respectively. Agar was added to half the height of the well on the lower side (0.45 cm), then left to gel at room temperature for 20 minutes. The supports were then removed and a second agar solution was added to an even depth. Plates were incubated overnight at room temperature to allow diffusion between the two layers.

To initiate experiments up to 50 μL of an overnight bacterial culture was inoculated in a line on the side of the well where the concentration of antibiotic was lowest. The wells were then covered with up to 5 mL of mineral oil to prevent desiccation and incubated at 37 °C. After cells had grown throughout the plate mutants were harvested by sampling relevant regions of the plate via pipette. The soft agar extracted was then added to 5 mL of cation-adjusted Mueller-Hinton Broth (MHB) and incubated overnight at 37 °C. Cells were stored at −80 °C in glycerol or used for further experiments.

Minimum inhibitory concentration measurements. Populations extracted from SAGE plates were assessed following standard microdilution procedures. In brief, 50 μL of cation-adjusted MHB containing the antibiotic of interest was mixed 1:1 with fresh media containing approximately 1 × 10⁶ bacterial cells (verified against a freshly prepared McFarland 0.5 standard) to give a final cell density of 5 × 10⁵ CFU. Plates were then incubated at 37 °C for 16–20 hours. Wells lacking bacteria were used as negative controls, and wells with bacteria and without antibiotic were used as positive controls. The MIC was defined as the minimum concentration of antibiotic that results in no visible growth to the naked eye.

Dose response curves and growth rate measurements. To measure the growth rate of E.coli in the presence of different concentrations of each antibiotic, bacteria were added to 96-well plates as described above. The plates were then sealed with parafilm and incubated at 30 °C in a Tecan Sunrise plate reader running Magellan V 7.1. Absorbance was measured at 595 nm every 30 seconds, with 15 seconds of shaking and two seconds of settle time prior to each measurement. Measurements were collected for 23 hours. The data was then exported to Excel. Measurements were zeroed and converted to their natural log. The steepest region of the growth curve was then determined, and a minimum of forty measurements were then fit to a linear curve. These dose response curves were then plotted against antibiotic concentration and fit to a Hill function via linear regression with the Excel Solver tool:

\[
g(c) = \frac{g_0}{1 + \left( \frac{c}{IC_{50}} \right)^n}
\]

where, \(g(c)\) is growth rate as a function of antibiotic concentration, \(c\), \(g_0\) is the growth rate in the absence of antibiotic, and \(IC_{50}\) is the concentration of antibiotic where the growth rate was half \(g_0\). \(n\) is the hill co-efficient, and corresponds to how quickly growth decreases as the antibiotic concentration increases.

Mutation rates. To infer the mutation rate of evolved E. coli lineages we measured the frequency of spontaneous resistance to rifampicin. In brief, each strain was grown in MHB media overnight. Cells were then diluted in fresh MHB and plated in triplicate onto either LB-agar or LB-agar with 100 μg/mL rifampicin. The plates were incubated for 16–20 hours at 37 °C, and the number of colonies on each was counted. The median frequency, \(f\), of each strain was then calculated and used to determine the median mutation rate, \(\mu\), based on the formula:

\[
\mu = \frac{f}{\ln(N_0)}
\]

where \(N\) is the total population, determined by counting cells grown on rifampicin-free agar.

Whole genome sequencing. Strains of interest were grown overnight in MHB, then genomic DNA was extracted using the EZ-10 Bacterial Genomic DNA miniprep kit according to manufacturer’s specifications. Samples were sequenced by Genome Quebec on an Illumina HiSeq. 4000 PE100. Sequence data was then assembled with the A5-miseq pipeline software. Snippy was used to compare the genome assemblies to the reference genome of E. coli MG1655, U00096.3 (https://github.com/tseemann/snippy).

Population size calculations. SAGE plates free of antibiotic were inoculated with 50 μL of E. coli MG1655, then incubated at 37 °C for four hours. Agar blocks containing representative bands and bacterial lawns were then excised with a sterile spatula and heated at 98 °C for five minutes. Cells were mixed 1:10 with distilled, deionized water, heated at 98 °C for three more minutes, and then used as template for qPCR. In brief, 1 μL of template was mixed with 14.6 μL ddH2O, 4 μL 5X EvaGreen qPCR mix, 0.4 μL forward primer (0.1 μM, 16S F27) and 0.4 μL reverse primer (0.1 μM, 16S R1492) [1]. The thermal profile used was 15 min of polymerase activation at 95 °C followed by the PCR cycling stage with 40 cycles (95 °C for 45 sec, 55 °C for 30 sec, 72 °C for 1 min) and ending with a melting curve (95 °C for 15 sec, 55 °C for 15 sec, 95 °C for 15 sec). Results from a dilution series of genomic DNA were used to determine the cell copy numbers.

For cells extracted from the growing front, the reported number of cells was multiplied by 10 to account for the initial dilution, then standardized to the total volume of the excised gel band. This population was then divided by the width of the excised band (~0.75 cm) to give a cell count per cm of the growing front. Values corresponding to cells extracted from the lawn were multiplied by 10, divided 0.05 mL and multiplied by sqrt(1 cm²/0.45 cm) = 1.49 cm² to adjust for the depth of the agar (0.45 cm). The total population of the growing front was estimated by multiplying the width of the lane (2.9 cm), the width of the growing front in the absence of antibiotic (0.3 cm) and the depth of the agar which contained cells (0.1 cm). Results are presented as the average of three independent replicates.
Data Availability

Full MIC testing results and whole-genome sequencing data are included in the supplementary information files.

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Author Contributions
M.H., N.G. and B.F. ran SAGE experiments. N.G. and B.F. performed MIC testing. M.H. completed mutation and growth rate measurements. B.F. conceived of and designed the experiments. All authors contributed to the final manuscript.

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