THE EFFECTS OF GENETIC BACKGROUND ON THE EVOLUTION OF ANTIBIOTIC RESISTANCE AND ITS FITNESS COSTS

By

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ABSTRACT

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Antibiotic resistance is a growing public-health concern. Efforts to control the emergence and spread of resistance would benefit from an improved ability to forecast when and how it will evolve. To predict the evolution of resistance with accuracy, we must understand and integrate information about many factors, including a bacterium's evolutionary history. This dissertation centers on the effects of genetic background on the evolution of phenotypic resistance, its genetic basis, and its fitness costs. To address these issues, I used *Escherichia coli* strains from the long-term evolution experiment (LTEE) that independently evolved for multiple decades in an environment without antibiotics.

First, I examined how readily these LTEE strains could overcome prior losses of intrinsic resistance through subsequent evolution when challenged with antibiotics. Second, I investigated whether lineages founded from different genotypes take parallel or divergent mutational paths to achieve increased resistance. Third, I tested whether fitness costs of resistance mutations are constant across different genetic backgrounds. In these studies, I focused attention on the interplay between repeatability and contingency in the evolutionary process. My findings demonstrate that genetic background can influence both the phenotypic and genotypic evolution of resistance and its associated fitness costs. I conclude this dissertation with a broader discussion about these and other factors that can influence the evolution of antibiotic resistance, and their clinical and public-health implications.

This dissertation is dedicated to my family,
and to that little boy who dreamed of becoming a scientist one day.

ACKNOWLEDGEMENTS

There are many individuals who have encouraged, guided, and inspired me on this journey. First and foremost, I want to thank my Ph.D. advisor, Dr. Richard Lenski. I had the fortune of meeting Rich while I was participating in undergraduate research with Dr. Frank Dazzo. I had become interested in evolutionary biology during this time, particularly as it related to my research work. I therefore applied to the BioMolecular Science Gateway program with aspirations of studying evolution in Rich's lab; I had read about the LTEE in Richard Dawkins' book *The Greatest Show on Earth* and was intrigued by the multitude of interesting questions one could address with such a simple experiment. Even though my knowledge of evolution was limited to the few books I had read, I decided to take a chance and reach out to Rich about a rotation. The rest is history. I could scarcely understand it at the time, but that decision would prove profound on my life. I had set forth on a path of personal and professional discovery, and Rich facilitated the journey. Rich is both incredibly sincere and kind. I learned this fact early on as a rotation student when he invited me and several other lab members to join him and his family for Thanksgiving dinner. Or on the several occasions he would buy the lab ice cream at the Dairy Store, or a few rounds at Crunchy's.

I thank my committee members Dr. Christoph Adami, Dr. Frances Downes, and Dr. Christopher Waters for their guidance and helpful suggestions over these past several years. I also want to thank the Department of Microbiology and Molecular Genetics (MMG); the Ecology, Evolutionary Biology, and Behavior Program; and the BEACON Center communities for encouraging and inspiring me. I also acknowledge the Howard Hughes Medical Institute Gilliam Fellowship and MMG for funding. In addition, I must thank my two undergraduate mentees, Jasper Gomez and Jalin Jordan. It has been a pleasure to work alongside them and bear witness to their

academic and professional successes. Their future is bright, and I am immeasurably proud of them both. They are terrific scientists and friends.

I made some wonderful friends while in graduate school. First, I want to thank Dr. Zachary Blount. My first interaction with Zack was when I was a rotation student. I had opened one of the lab bench drawers and there was a cockroach staring up at me. I was startled, but quickly realized that it was fake. Zack had put it there to mess with the new recruit. My life is enriched because of our friendship. I am also delighted for our conversations on a great many topics. Zack, if you are reading this sometime in the future, remember that "you got the touch, you got the power." I hope to walk many more trails with you, my friend. Second, I want to thank Dr. Thomas LaBar and Josh Franklin for their friendship. In 2017 we began playing regular bar trivia at Crunchy's and it has been wonderful to get to know them. I will cherish the many movie and board game nights with them and Nkrumah Grant, Devin Lake, Elizabeth Baird, Jasper Gomez, Tanush Jagdish, and John Baltusis. Their friendships mean the world to me and they were always there to uplift me during the difficult times of graduate school. I cannot thank them enough.

Lastly, I must thank my family. I had a wonderful upbringing with a sister, mother, and father who believed in me, unwaveringly. My sister, Kaci, is the most caring person I know, and I am so proud of the strong and confident woman she has become. One of the happiest days of my life was watching her walk down the aisle. Kaci is a wonderful mother to Rylee and my best friend. My mother, Lisa, is my stalwart advocate. She gave me the opportunities to follow my own interests and never gave up on me when I failed. She taught me to always keep fighting. My father, Dennis, died shortly after I began graduate school. The most profound lesson I have learned in graduate school – and life in general – is that death ends a life, not a relationship. I continue to learn from my father every day, and I know I inherited his strength of will. I am his monument.

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KEY TO ABBREVIATIONS

AMP – ampicillin

ANOVA – analysis of variance

bp – base pair

CF – cystic fibrosis

CIP – ciprofloxacin

CRO – ceftriaxone

df – degrees of freedom

DM – Davis Mingioli

DNA – deoxyribonucleic acid

dsDNA – double-stranded deoxyribonucleic acid

ESBL – extended-spectrum beta-lactamase

indel – insertion or deletion

IS – insertion sequence

LB – Luria Bertani

LTEE – long-term evolution experiment

MH – Mueller Hinton

MIC – minimum inhibitory concentration

MSW – mutant selection window

NCBI – National Center for Biotechnology Information

rpm – rotations per minute

TA – tetrazolium arabinose

TET – tetracycline

tmRNA – transfer-messenger ribonucleic acid

tRNA – transfer ribonucleic acid

CHAPTER 1: Historical contingency in the evolution of antibiotic resistance after decades of relaxed selection

Work presented in this chapter has been published as Card, K. J., T. LaBar, J. B. Gomez, and R. E. Lenski. 2019. Historical contingency in the evolution of antibiotic resistance after decades of relaxed selection. PLoS Biol. 17:e3000397.

Abstract

Populations often encounter changed environments that remove selection for the maintenance of particular phenotypic traits. The resulting genetic decay of those traits under relaxed selection reduces an organism's fitness in its prior environment. However, whether and how such decay alters the subsequent evolvability of a population upon restoration of selection for a previously diminished trait is not well understood. We addressed this question using *Escherichia coli* strains from the long-term evolution experiment (LTEE) that independently evolved for multiple decades in the absence of antibiotics. We first confirmed that these derived strains are typically more sensitive to various antibiotics than their common ancestor. We then subjected the ancestral and derived strains to various concentrations of these drugs to examine their potential to evolve increased resistance. We found that evolvability was idiosyncratic with respect to initial genotype; that is, the derived strains did not generally compensate for their greater susceptibility by "catching up" to the resistance level of the ancestor. Instead, the capacity to evolve increased resistance was constrained in some backgrounds, implying that evolvability depended upon prior mutations in a historically contingent fashion. We further subjected a time series of clones from one LTEE population to tetracycline and determined that an evolutionary constraint arose early in that population, corroborating the role of contingency. In summary, relaxed selection not only can drive populations to increased antibiotic susceptibility, but it can also affect the subsequent evolvability of antibiotic resistance in an unpredictable manner. This conclusion has potential implications for public health, and it underscores the need to consider the genetic context of pathogens when designing drug-treatment strategies.

Introduction

A population may encounter an environmental change that removes or reduces a selective pressure that was previously important for the maintenance of a trait (Darwin 1859; Lahti et al. 2009). Adaptation to the new environment can therefore affect an organism's fitness in its prior environment. These correlated responses may lead to the functional decay of unused traits over time or, conversely, their maintenance despite relaxed selection (Lahti et al. 2009). However, the evolutionary processes driving these responses are often hard to disentangle because one must rely on retrospective studies and historical inference.

By contrast, evolution experiments with microorganisms provide a powerful approach to study correlated responses. Microbes often have large population sizes and fast generations, and they are amenable to freezing and revival. One can therefore observe evolution in action, directly compare ancestral and derived forms, and simultaneously assess adaptation to one environment and quantify correlated fitness responses in another. Accordingly, numerous studies with bacteria (Chao et al. 1977; Lenski 1988; Reboud and Bell 1997; Cooper and Lenski 2000; Cooper et al. 2001; Ellis and Cooper 2010; Leiby and Marx 2014), viruses (Turner and Elena 2000; Duffy et al. 2006; Agudelo-Romero et al. 2008; Coffey and Vignuzzi 2011; Wasik et al. 2015; Meyer et al. 2016), and yeast (Wenger et al. 2011; Ratcliff et al. 2012; Koschwanez et al. 2013) have found that fitness trade-offs between environments are common.

Trade-offs are often caused by antagonistic pleiotropy, which occurs when a mutation that is beneficial in one environment is deleterious in another. This process can have important public-health consequences when antibiotic-resistance mutations or acquired resistance genes impose costs on bacterial growth and competitiveness relative to their sensitive counterparts in the absence of drugs (Lenski 1997; Andersson and Hughes 2010). Previous studies have shown that pleiotropic

fitness costs are widespread among resistance determinants to diverse drug classes (Nguyen et al. 1989; Schrag et al. 1997; Rozen et al. 2007; Han et al. 2009), although their magnitudes are variable and may also depend on the genetic background (Lenski et al. 1994; Andersson and Hughes 2010; Melnyk et al. 2015; Palmer et al. 2018).

Given that antibiotic-resistance mutations and genes commonly impose fitness costs, one would expect that resistance should decline over time in the absence of antibiotic exposure. However, compensatory evolution often reduces or eliminates these trade-offs (Bouma and Lenski 1988; Schrag et al. 1997; Reynolds 2000; Rozen et al. 2007). Adaptive trends during compensatory evolution have been studied using a number of *E. coli* mutants resistant to the drug rifampicin (Barrick et al. 2010). That study found that the mutants were generally less fit than their sensitive progenitors in a permissive antibiotic-free environment; moreover, the compensatory effects of subsequent beneficial mutations were greater when the resistance was more costly. Thus, compensation exhibited a pattern of diminishing-returns adaptation in that study.

Even when bacteria have no known history of exposure to antibiotics, they may have low level resistance to some drugs because of intrinsic structural or functional features, including their cell envelope and efflux pumps (Cox and Wright 2013). As a consequence, intrinsic resistance may decline in the absence of drug exposure if relevant genes accumulate mutations either by selection or drift in permissive environments (Cooper and Lenski 2000).

A recent study used the *E. coli* long-term evolution experiment (LTEE), and antibiotic resistance as a model trait, to study changes in an organism's capacity to tolerate environmental stresses when it evolves for a long period in the absence of those stresses (Lamrabet et al. 2019). In the LTEE, 12 replicate populations were founded from a common ancestor and have been propagated daily for over 30 years in a medium without antibiotics (Lenski et al. 1991; Tenaillon

et al. 2016). In particular, Lamrabet and colleagues measured changes in mostly low-level intrinsic resistance between ancestral and derived strains isolated from each population after generations 2,000 and 50,000. They found that derived strains were usually more susceptible to most antibiotics than their ancestor, and from multiple lines of evidence they inferred that these losses of intrinsic resistance resulted primarily from pleiotropic side effects of beneficial mutations that arose during the LTEE.

Although the lineage leading to the LTEE ancestor has no known history of exposure to industrially manufactured antibiotics (except streptomycin), it might nevertheless have a history of exposure to similar compounds produced by competitors and to host bile salts. Adaptations that provide resistance to these other stressors, such as those involving the cell envelope and efflux pumps (Thanassi et al. 1997), often confer intrinsic resistance to antibiotics (Cox and Wright 2013). Thus, even the low-level resistance of the ancestor might reflect this prior natural history, and selection was relaxed on these traits in the LTEE environment.

Taken together, the experimental evolution studies described above have two contrasting implications relevant for medicine and public health. First, resistance to antibiotics (including even low-level intrinsic resistance) may decline in the absence of drug exposure. Second, evolution can often compensate for deleterious side effects of mutations, thereby facilitating the maintenance of evolved resistance. The question then arises how readily bacteria can overcome losses of antibiotic resistance that arose during periods of relaxed selection through subsequent evolution in the presence of drugs. In this study, we address this fundamental question by using the LTEE ancestor and derived strains isolated from four populations after 50,000 generations to examine how evolution in the absence of antibiotics affects the bacteria's potential to evolve increased resistance when drugs are introduced. In so doing, we examine the role that genetic background plays in

resistance evolvability (Figure 1.1). Does resistance evolution tend to follow a general trend of diminishing returns (Travisano et al. 1995; Barrick et al. 2010; Wiser et al. 2013; Kryazhimskiy et al. 2014), such that derived strains that are initially more susceptible to a drug can increase their resistance disproportionately relative to their ancestor (Figure 1.1B)? Or is evolvability idiosyncratic with respect to prior evolutionary history (Blount et al. 2008, 2018), such that the relative gains in resistance are independent of a strain's initial susceptibility (Figure 1.1C)?

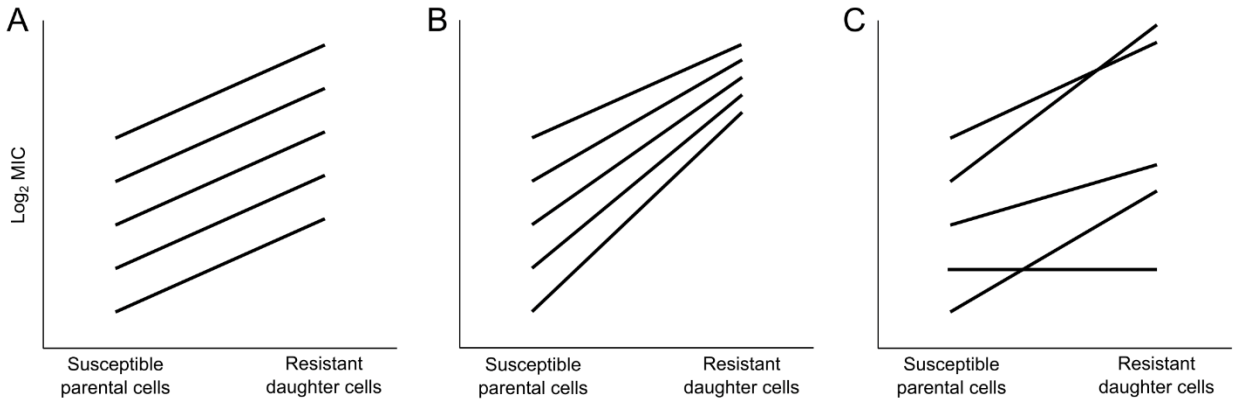

Figure 1.1. Schematic illustration of the evolvability of antibiotic resistance under three scenarios. A strain's evolvability is defined operationally as the maximum increase in resistance from an initially susceptible genotype during one round of drug selection. (A) Null model, with no effect of genetic background on evolvability. (B) Diminishing-returns model, such that backgrounds with low initial resistance are more evolvable than backgrounds that are initially more resistant. (C) Idiosyncratic-effects model, in which evolvability varies among genetic backgrounds but is uncorrelated with their initial level of resistance.

Throughout this chapter, we discuss how differences in genetic background may affect the evolvability of antibiotic resistance. This focus brings to mind the concept of epistasis, whereby the marginal effect of a particular mutation on some phenotype of interest depends on its interaction with another mutation or, more generally, the set of mutations that distinguish genetic backgrounds (Levin et al. 2000; Moore et al. 2000; Trindade et al. 2009; MacLean et al. 2010;

Chou et al. 2011; Khan et al. 2011; Kryazhimskiy et al. 2014; Wong 2017). In the context of our study, epistasis could arise in at least two ways. First, mutations in the same target gene may confer different levels of resistance depending on other mutations that differ between backgrounds. Second, the physiological mechanisms and associated loci underlying resistance may differ across genetic backgrounds. It might seem unexpected that the genetic basis of resistance would differ among closely related backgrounds. However, as we will show, the initial levels of resistance vary among backgrounds, and the physiological mechanisms that allow cells to resist drugs may depend on their concentrations, such that the mechanisms used may also differ across backgrounds. Without a more precise mechanistic understanding at this stage of our work, we cannot distinguish between these forms of epistasis. More generally, we will use the term evolvability (rather than epistasis) because it emphasizes the consequences of these effects for antibiotic resistance.

We confirmed the finding of Lamrabet and colleagues (2019) that the LTEE-derived strains had typically become more susceptible to antibiotics during relaxed selection. However, contrary to our expectation based on a diminishing-returns model, we discovered that these derived strains were usually no more evolvable (and sometimes less evolvable) than their ancestor when exposed to various antibiotics. Instead, idiosyncratic responses dominated over any diminishing-returns tendency, such that the capacity to evolve resistance was hampered on some LTEE-derived genetic backgrounds. These results indicate that evolution and diversification of a single bacterial species in a permissive environment can lead to unpredictable changes in the potential to evolve antibiotic resistance. Our work suggests that methods for predicting, at the strain level, a pathogen's evolutionary potential should be developed in light of the global threat of antibiotic resistance. If successful, such methods could become an integral aspect of resistance surveillance and patient treatment.

Materials and Methods

Bacterial strains

All of the strains used in this study are from the *E. coli* LTEE. In the LTEE, 12 replicate populations were founded from a common ancestral strain called REL606 (Lenski et al. 1991). These populations have been propagated for over 32 years by daily 1:100 transfers in glucose-supplemented Davis minimal (DM) medium without any antibiotics (Lenski et al. 1991), resulting in >73,000 cell generations to date. Samples from each population are frozen periodically at −80˚C. In this study, we quantified the intrinsic antibiotic resistance and evolvability of the ancestor and derived clones isolated from four populations (designated Ara–5, Ara–6, Ara+4, and Ara+5) after 50,000 generations of the LTEE. We chose these strains for two reasons. First, the source populations of these derived clones retained the low ancestral mutation rate, and therefore they accumulated many fewer mutations than their counterparts from several populations that evolved hypermutability (Tenaillon et al. 2016). This characteristic should increase the tractability of identifying candidate alleles affecting resistance evolvability, which we hope to achieve in future work. Second, generation 50,000 is the latest point at which whole-genome sequence data are available for the clonal samples (Tenaillon et al. 2016). We also examined when the Ara+5 population evolved a diminished capacity to increase its tetracycline resistance (as described in the Results) by testing two strains isolated from this population at several earlier time points (generations 500, 1,000, 1,500, 2,000, 5,000, and 10,000). All of the strains used in this study are listed in Appendix A Table 1.

Culture conditions and measurements of resistance and evolvability

All experiments were performed at 37˚C. Bacterial strains were revived from frozen stocks by overnight growth in Luria Bertani (LB) medium. Cells from these cultures were then streaked onto DM agar plates supplemented with 4 mg/mL glucose. We randomly picked single isolated colonies from these plates to start multiple replicate populations in LB. Final population sizes in the LB cultures were approximately $1 - 2 \times 10^9$ cells/mL. When an initially susceptible cell expands into a colony and then a population, new mutations spontaneously occur and increase in number during growth (Luria and Delbrück 1943). The evolution of antibiotic-resistant mutants will therefore originate by independent mutational events in each replicate population (Luria and Delbrück 1943; Kassen and Bataillon 2006).

We define a strain's evolvability as the maximum increase in antibiotic resistance from an initially susceptible genotype during one round of drug selection. Evolvability experiments were performed using Mueller-Hinton (MH) agar (Acumedia, Lansing, MI) supplemented with 1 mg/mL glucose, 0.1 mg/mL magnesium sulfate, 0.01 mg/mL thiamine, and a series of 2-fold dilutions of an antibiotic. We used MH agar because a previous study used this medium to quantify the susceptibilities of LTEE clones to various antibiotics (Lamrabet et al. 2019). Our study, in part, sought to replicate these findings. We chose the four antibiotics in our study because they have diverse cellular targets: ampicillin and ceftriaxone inhibit cell-wall synthesis, ciprofloxacin inhibits DNA replication, and tetracycline inhibits protein synthesis. We prepared stock solutions of each antibiotic following the manufacturers' instructions, which were then stored at −20˚C.

One-milliliter samples of each population were centrifuged at 8,000 rpm for 2 minutes and resuspended in an equal volume of saline. We then plated 100 μL (containing approximately $1 - 2 \times 10^8$ cells) from each suspension onto the antibiotic-amended MH agar plates, and MICs were

evaluated after 48 hours of incubation. For this study, we operationally define a pair of MICs for each series of antibiotic-amended plates as the lowest concentration that prevents either confluent growth or isolated colonies. According to this approach, confluence indicates growth by the susceptible "parental" strain, while isolated colonies are resistant "daughter" mutants. A strain's evolvability was calculated from the difference in MIC between these two genotypes. For each experimental block, putative resistant mutants were confirmed by streaking one randomly chosen colony per strain onto fresh antibiotic-amended MH plates. All clones regrew at the corresponding concentration. This approach indicated that a selected clone was indeed a resistant mutant with a stably inherited increase in its MIC, as opposed to a so-called "persister" that exhibited higher-than-average phenotypic tolerance relative to genetically identical cells (Balaban et al. 2019). Cultures of mutant clones were then frozen at −80˚C in LB medium supplemented with 15% glycerol as a cryoprotectant.

Appendix B Figure 1 provides a schematic representation of our methods for measuring the MICs of sensitive parental strains and their resistant daughter derivatives. In Appendix B Figure 2, we show an image of the resulting plates for one replicate series across a 256-fold (= 28) range of ciprofloxacin concentrations for the LTEE ancestral clone. In this image, one sees confluent growth on the first 3 plates, isolated colonies on the next 2 plates, and no evident growth on the 4 plates with the highest concentrations. Based on these plates, we scored the MIC of the sensitive parental strain as the lowest concentration that inhibited confluent growth, which was 0.0025 μg/mL in this example. We scored the MIC of the resistant daughter derivative as the lowest concentration where even isolated colonies were absent, in this case 0.01 μg/mL. The $\log_2$-transformed difference between these values (i.e., $\log_2$ 0.01/0.0025 = 2 in this example) provides one estimate of the evolvability of the LTEE ancestral strain with respect to ciprofloxacin. We

obtained 32 independent estimates of these MICs and the associated evolvabilities for the ancestral strain against each of the four antibiotics used in our study. We similarly obtained eight independent estimates of the MICs and associated evolvabilities for each of the four 50,000-generation strains used in our study against each of the same antibiotics. Photographs of all of the replicate plate series used to estimate these values have been archived on the Dryad Digital Repository: https://datadryad.org/stash/dataset/doi:10.5061/dryad.g41hg96 (Card 2019).

Experimental design and data analyses

All MIC values were transformed by taking their base-2 logarithm because the antibiotic concentrations were tested across a series of 2-fold dilutions. For each experimental block, an independently isolated LTEE ancestral clone was paired with each derived clone. We had two predictions when we began this study: (i) the derived bacteria would be more susceptible to antibiotics (lower MICs) than their common ancestor as a consequence of the relaxed selection they experienced in the permissive LTEE environment and (ii) the derived bacteria would be more evolvable than their ancestor when challenged with antibiotics, following a general trend of diminishing-returns adaptation.

Statistical tests that rely on normally distributed data were deemed inappropriate for this study owing to the discrete, lumpy nature of the measurements. Instead, we used nonparametric methods. There were also numerous instances in which the derived clones were equal both in MIC and evolvability to the paired assays for the ancestor, and these ties introduced additional complications. Therefore, we used trinomial tests to examine changes in the direction of our expectations relative to the null hypothesis that changes are equally frequent in either direction (Bian et al. 2011). We performed these analyses by individually comparing the four derived clones

with their paired ancestors across each antibiotic. Probabilities were then combined from these independent significance tests using Fisher's method with 8 degrees of freedom (i.e., df = 2*k*; where *k* is the number of comparisons) (Fisher 1934; Sokal and Rohlf 1994). As explained previously (Figure 1.1C), evolvability might be idiosyncratic and therefore not correlated with the initial level of resistance. To assess this possibility, we performed a Kruskal-Wallis one-way ANOVA to test for heterogeneity in evolvability among the LTEE lines. Datasets and the details of our statistical analyses are provided in an R Notebook on the Dryad Digital Repository: https://datadryad.org/stash/ dataset/doi:10.5061/dryad.g41hg96 (Card 2019).

We performed fluctuation tests to estimate effective mutation rates (Luria and Delbrück 1943) for the ancestral strain (REL606) to 4 μg/mL tetracycline, and for a derived clone from the Ara+5 population (REL1162A) to 2 μg/mL tetracycline. The two strains were taken from the freezer and grown overnight in LB broth. Each culture was then serially diluted in saline solution, and fewer than 1,000 cells were transferred into each well of a 96-well plate; each well contained 0.1 mL of LB broth. After 24 hours, we removed the entire volume from each of 84 wells and spread it on an MH agar plate amended with either 2 μg/mL or 4 μg/mL tetracycline for REL1162A or REL606, respectively. The other 12 wells were sampled to enumerate the bacteria using a Coulter counter (Multisizer 4e, Beckman) with a 30-μm aperture; we set a cutoff of 0.2 fL to distinguish cells from background debris and subtracted counts from a sterile LB-only negative control. We incubated the antibiotic-amended plates for 48 hours and we then scored each plate for the absence or presence of one or more colonies, as required for the p_0 method to estimate mutation rates.

Results

Antibiotic susceptibility profiles of the LTEE ancestral and derived clones

Antibiotic susceptibility measurements were generally quite repeatable (Figure 1.2). For each antibiotic, all 32 independent ancestral replicate minimum inhibitory concentration (MIC) measurements were identical. Among the 16 sets of derived-clone replicates (4 clones × 4 antibiotics), the 8 replicate assays gave identical MICs in 2 cases (12.5%), they deviated minimally by a factor of 2 in 12 cases (75%), and in only 2 cases they deviated by a factor of 4 (12.5%). These results provide strong support for the use of our plate-based approach, as described in the Materials and Methods and shown in Appendix B Figure 1, to quantify antibiotic susceptibility profiles.

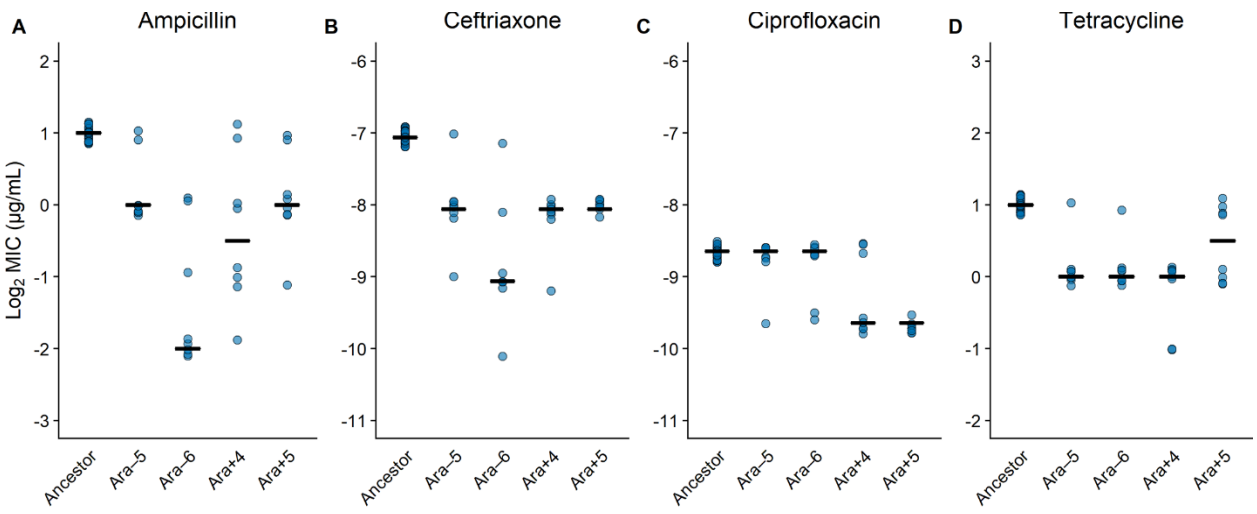

Figure 1.2. Intrinsic resistance usually declined over time in the absence of drug exposure. Comparison of the LTEE ancestor and four independently derived clones sampled after 50,000 generations for their susceptibilities to ampicillin, ceftriaxone, ciprofloxacin, and tetracycline (A–D). MICs are shown on a $\log_2$-transformed scale to reflect the fact that antibiotic concentrations were tested across a series of 2-fold dilutions. In each panel, points show values obtained from 32 and 8 replicate assays for the ancestor and derived strains, respectively. Horizontal bars show the median of the $\log_2$-transformed MIC values for each strain on each antibiotic. The absolute values of the concentrations shown on the y-axis differ among the four antibiotics, but the range is the same in each panel.

Changes in susceptibility under relaxed selection during the LTEE

For each antibiotic, we made 32 comparisons between the MICs of derived clones (4 clones × 8 replicates) against their paired and independently isolated ancestral clones. On balance, we observed increased susceptibility of the strains that evolved under relaxed selection (i.e., in the absence of antibiotics) during the LTEE, consistent with recently published results (Lamrabet et al. 2019) (Figure 1.2). All four derived strains have increased sensitivity to ampicillin (Figure 1.2A), ceftriaxone (Figure 1.2B), and tetracycline (Figure 1.2D) relative to their common ancestor, and two of the derived strains were more susceptible to ciprofloxacin (Figure 1.2C). These trends toward lower resistance are well supported by trinomial tests, as described in the Materials and Methods, and as shown in Table 1.1 and Appendix A Table 2.

Table 1.1. Statistical analyses of declines in intrinsic resistance during relaxed selection of clones sampled at generation 50,000 of the LTEE.

Antibiotic	χ^2	***p***
Ampicillin	40.06	< 0.0001
Ceftriaxone	45.33	< 0.0001
Ciprofloxacin	27.15	0.0007
Tetracycline	41.40	< 0.0001

Analyses were performed using Fisher's combined probability method (df = 8) for multiple independent hypothesis tests of the same hypothesis, with an underlying trinomial distribution for the null.

Evolvability profiles of the ancestor and derived clones

Next, we examined how the prior history of relaxed selection affected the evolvability of antibiotic resistance in the different genetic backgrounds. To address this question, we selected mutants of the ancestral and LTEE-derived strains that survived and grew sufficiently to form colonies at higher concentrations of the four antibiotics than their corresponding parental strains (Appendix B

Figure 1). As described in the Materials and Methods, we operationally define evolvability as the maximum observed increase in antibiotic resistance from an initially susceptible genotype during one round of drug selection (Figure 1.1).

Evolvability measurements tended to be more variable than the MIC measurements. We examined the evolvability of 128 independent ancestral clones across the four antibiotics. There were 73 cases (57%) in which these measurements corresponded to the median for that antibiotic, 49 cases (38.3%) in which they differed by a factor of 2, and 6 cases (4.7%) in which they differed by a factor of 4. Likewise, among the 16 sets of replicates for the LTEE-derived clones, the 8 assays varied by a factor of 2 in 12 cases (75%) and by a factor of 4 in 4 other cases (25%). The greater variation in evolvability measurements in comparison with MIC values among replicate assays is expected given the stochastic appearance of mutations in replicate cultures (Luria and Delbrück 1943). Also, increased resistance can occur through multiple mutational paths (Toprak et al. 2012; Baym et al. 2016), and those mutations affecting one mechanism might confer greater resistance evolvability relative to mutations affecting some other mechanism.

Effects of genetic background on the evolvability of resistance

We examined the possibility of two broad patterns of genetic-background effects with respect to resistance evolvability in our study. First, we asked whether evolvability followed a trend of diminishing returns, such that the more susceptible LTEE-derived genetic backgrounds generally produced mutants with proportionally greater grains in resistance than the ancestor. Both the ancestral and derived strains evolved resistance to varying degrees (Figure 1.3). The evolutionary potential of two of the four derived clones (Ara–5 and Ara–6) was noticeably greater relative to

their ancestor in the ampicillin environment (Figure 1.3A), but there were no clear instances of similar trends in the three other drug environments (Figure 1.3B–D).

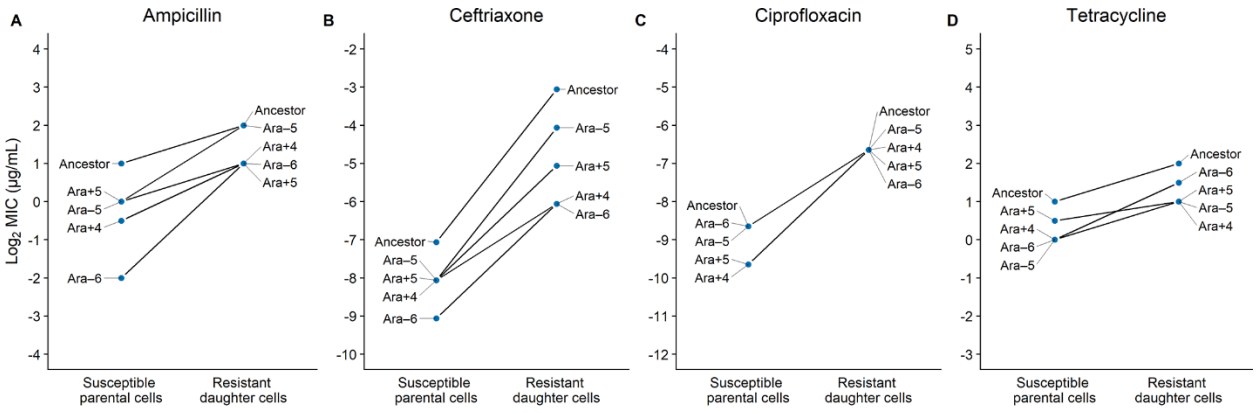

Figure 1.3. Genetic background affects the evolvability of LTEE lines exposed to antibiotics. Lines joining susceptible parental strains with their daughter mutants show the increases in resistance during one round of selection with ampicillin, ceftriaxone, ciprofloxacin, and tetracycline (A–D). If the slope of a derived strain is greater than that of the ancestor, then it has greater evolvability; and vice versa. Median MICs are shown on a $\log_2$-transformed scale to reflect the fact that antibiotic concentrations were tested across a series of 2-fold dilutions. The y-axis ranges for the four drugs have been scaled to the ceftriaxone environment, which had the largest gains in resistance between the susceptible parental cells and the resistant daughter cells.

Overall, there was no statistical support for the diminishing-returns trend, despite the visual impression for the ampicillin treatment. We compared each derived strain's evolutionary potential with its paired ancestor in the four drug environments. We used trinomial tests to quantify the likelihood that each derived strain's evolvability was greater than its ancestral counterpart when tested against the null hypothesis of equally frequent changes in either direction, after taking into account the many numerical ties (Bian et al. 2011). Although the capacity of the derived Ara–5 clone to evolve increased resistance was significantly greater than its ancestor when considered in isolation (Appendix A Table 3), it was marginally nonsignificant when we examined overall trends

for each antibiotic (Table 1.2) using a meta-analysis approach (Fisher 1934; Sokal and Rohlf 1994).

Table 1.2. Statistical analyses of diminishing-returns trends in resistance evolvability of clones sampled at generation 50,000 of the LTEE.

Antibiotic	χ^2	*p*
Ampicillin	14.63	0.0668
Ceftriaxone	0.18	1
Ciprofloxacin	7.88	0.4456
Tetracycline	5.97	0.6511

Analyses were performed using Fisher's combined probability method (df = 8) for multiple independent tests of the same hypothesis, with an underlying trinomial distribution for the null hypothesis.

We then asked whether the proportional resistance gains when exposed to the antibiotics were idiosyncratic among LTEE lines. For example, the capacity to evolve ceftriaxone resistance appeared to be reduced among three LTEE-derived backgrounds (Ara+5, Ara–6, and especially Ara+4) relative to their common ancestor (Figure 1.3B). Similarly, the evolvability of the Ara+5 background with respect to tetracycline appears to be constrained (Figure 1.3D). Indeed, this latter case was the only one in which the mutants of a strain systematically achieved a lower level of resistance than did the mutants of other strains that were initially more susceptible (indicated by the crossing lines in Figure 1.3D). These idiosyncratic tendencies are statistically well supported by Kruskal-Wallis tests. For both ceftriaxone and tetracycline, these tests reject the null hypothesis of homogeneity in proportional resistance increases across the different genetic backgrounds (Table 1.3).

Table 1.3. Statistical analyses of idiosyncratic patterns in resistance evolvability of clones sampled at generation 50,000 of the LTEE.

Antibiotic	χ^2	*p*
Ampicillin	9.19	0.0566
Ceftriaxone	23.45	0.0001
Ciprofloxacin	7.59	0.1077
Tetracycline	10.18	0.0376

Analyses were performed using a Kruskal-Wallis one-way nonparametric ANOVA (df = 4).

Given these idiosyncratic effects of genetic background, we chose to examine one of the cases in greater detail. In particular, we asked when the evolvability of the Ara+5 background declined with respect to tetracycline. To address this question, we examined clones isolated during this population's early history and tested whether they had lost their capacity to evolve tetracycline resistance during a single exposure, to an extent commensurate with the ancestral strain's evolvability. As shown in Figure 1.4, the reduced evolvability was evident in all of the clones isolated from generation 2,000 onward as well as in one of two clones isolated at generation 1,500. With one exception, all of the LTEE-derived parental backgrounds across this time series had the same MIC value as the ancestor (Figure 1.4A). However, the daughter mutants from the later-generation Ara+5 genetic backgrounds had progressively lower levels of tetracycline resistance (Figure 1.4B), which when coupled with the same initial resistance level indicates they had become less evolvable in this respect (Figure 1.4C). A Kruskal-Wallis test decisively rejects the null hypothesis of equal evolvabilities across the entire set of clones ($\chi^2 = 67.89$, df= 12, $p < 0.0001$), and Dunnett's tests comparing the evolvability of each derived clone from LTEE population Ara+5 with that of the ancestor support the temporal break point described above (Table 1.4).

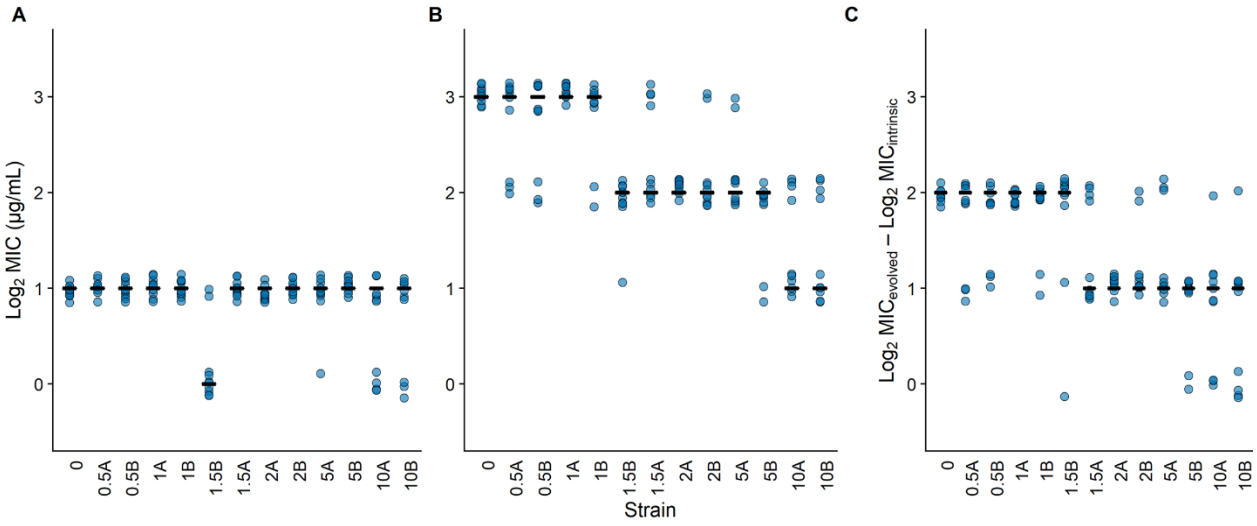

Figure 1.4. Capacity to evolve tetracycline resistance was diminished early in one LTEE lineage. (A) Comparison of the intrinsic tetracycline resistance of the ancestor and a time series of derived strains isolated from the Ara+5 population. Strains are ordered by their time of isolation. The strain identifiers begin with a number corresponding to the generation (in thousands) of their isolation, followed by an arbitrary letter; strains 2A and 2B, for example, are two clones isolated at generation 2,000 of the LTEE. (B) Comparison of the evolved resistance levels after one round of drug selection, based on the MICs of the mutant daughter cells derived from the corresponding parental strains. MICs are shown on a $\log_2$-transformed scale to reflect the fact that the concentrations of antibiotics were tested across a series of 2-fold dilutions. (C) Evolvability is quantified for each strain as the difference in the $\log_2$-transformed MICs of the parental strain and its corresponding daughter mutant. Points show 10 independent replicates per strain. Horizontal bars show the median $\log_2$-transformed MICs (A, B) for 10 replicate assays and the evolvability (C) based on the corresponding 10 paired differences.

Table 1.4. Statistical analyses comparing tetracycline resistance evolvability of clones isolated from the Ara+5 population at different generations to the LTEE ancestor.

Strain	Difference	Lower 95% CI	Upper 95% CI	*p*
0.5A	-0.3	-0.9	0.3	0.6921
0.5B	-0.3	-0.9	0.3	0.6921
1A	0.0	-0.6	0.6	1
1B	-0.2	-0.8	0.4	0.9607
1.5B	-0.3	-0.9	0.3	0.6921
1.5A	-0.6	-1.2	0.0	0.0412
2A	-1.0	-1.6	-0.4	< 0.0001
2B	-0.8	-1.4	-0.2	0.0021
5A	-0.7	-1.3	-0.1	0.0101
5B	-1.2	-1.8	-0.6	< 0.0001
10A	-1.2	-1.8	-0.6	< 0.0001
10B	-1.3	-1.9	-0.7	< 0.0001

Analyses were performed using a Dunnett's test. Strain identifiers begin with a number that corresponds to the generation (in thousands) of their isolation, followed by an arbitrary letter; strains 2A and 2B, for example, are two clones isolated at generation 2,000 of the LTEE.

Multiple factors can contribute to differences in evolvability

The observed changes in evolvability of tetracycline resistance in the Ara+5 population could, in principle, reflect several factors, including differences among genotypes in cell density, mutation rate, and the number of potential mutations that confer sufficient resistance to allow growth at a given drug concentration (i.e., the effective mutational target size). To examine these factors, we performed Luria-Delbrück fluctuation tests (Luria and Delbrück 1943; Pope et al. 2008; Lang 2018) with the LTEE ancestor and 2,000-generation clone 2A at tetracycline concentrations of 4 μg/mL and 2 μg/mL, respectively. We used these different antibiotic concentrations because, in our evolvability assays, the 2,000-generation clone had never produced any mutants that formed colonies at 4 μg/mL (Figure 1.4). We grew 96 replicate 0.1-mL cultures of each strain, starting from small population sizes to ensure mutational independence (Luria and Delbrück 1943; Pope et al. 2008). Because antibiotic-resistant mutants often grow more slowly than their progenitors

(Schrag et al. 1997; Rozen et al. 2007; Han et al. 2009; Andersson and Hughes 2010; Barrick et al. 2010; Melnyk et al. 2015), we used the "p_0" method to estimate effective mutation rates. This method is insensitive to possible differences in growth rate between parent strains and daughter mutants, as it uses only the fraction of the replicate assays that do not yield any mutants (Luria and Delbrück 1943; Pope et al. 2008). For each strain, we used 12 cultures to estimate the population size and 84 cultures to test for resistant mutants at the relevant concentration.

The 2,000-generation clone yielded only about half the cell density as the ancestral strain (8.4×10^7 versus 1.8×10^8 cells per 0.1-mL culture, $p < 0.0001$, based on a two-tailed Welch's *t* test), which indicates one factor that would contribute to its lower evolvability. For the ancestral strain, 11/84 test cultures yielded at least one mutant resistant to 4 μg/mL, while the other 73 cultures yielded none. For the derived strain, 26 test cultures yielded one or more mutants resistant to 2 μg/mL, while 58 cultures produced none. Using the p_0 method, the estimated effective mutation rate for the ancestral strain is 7.7×10^{-10} per cell generation [approximate 95% confidence limits of $4.2 \times 10^{-10} - 1.4 \times 10^{-9}$ based on the uncertainty in p_0 only (Agresti and Coull 1998)], and for clone 2A the estimated rate is 4.4×10^{-9} (approximate 95% confidence limits of $3.0 - 6.4 \times 10^{-9}$).

At first glance, it may seem counterintuitive that the estimated mutation rate was higher for the derived clone than for the ancestor, but recall that we tested this clone at a lower antibiotic concentration. Hence, the two rates are not directly comparable. Moreover, published genome sequences and analyses (Tenaillon et al. 2016) indicate that the underlying point-mutation rate in clone 2A is the same as the ancestral rate. The effective mutation rate that is estimated using a fluctuation test depends on the product of the underlying mutation rate and the effective mutational target size (i.e., the number of mutations that would allow a colony to grow on the antibiotic test

plate). Given the same underlying mutation rate, the difference in the estimated mutation rates implies that the derived clone has a larger mutational target at 2 μg/mL of tetracycline than the ancestral strain has at 4 μg/mL of that antibiotic. These analyses indicate that differences in evolvability between genotypes may reflect differences in several factors—cell density, underlying mutation rate, and effective target size—that depend not only on the genetic background but also reflect complex interactions between the genetic background, potential resistance mutations, and the selective environment.

Discussion

In this study, we addressed a fundamental question about how relaxed selection on a particular set of organismal traits affects their evolvability in situations in which those traits again become advantageous. The traits we studied are resistances to several antibiotics, and the question of how changes in genetic background that occur during relaxed selection affect the subsequent evolvability of resistance has potentially important implications for public health. To address these issues, we examined the capacity of *E. coli* strains to evolve increased resistance to four different antibiotics after they had evolved in a drug-free environment for 50,000 generations as part of the LTEE.

We confirmed that intrinsic resistance tended to decay among the LTEE-derived clones to all four antibiotics we tested (Figure 1.2, Table 1.1). Our results are consistent with a recent study that examined losses of intrinsic resistance in all 12 LTEE lines at generations 2,000 and 50,000 (Lamrabet et al. 2019). Unlike that previous work, however, we then also examined whether and how the LTEE- derived bacteria had changed in their evolvability, specifically their potential to evolve resistance when challenged across a range of concentrations of the same four antibiotics.

We examined two alternative hypotheses that might bear on resistance evolvability. The first is called diminishing returns (Figure 1.1B), and it often characterizes the course of adaptive evolution (Moore et al. 2000; Orr 2005; Barrick et al. 2010; Khan et al. 2011; Wiser et al. 2013; Kryazhimskiy et al. 2014; Passagem-Santos et al. 2018). For example, one study used rifampicin-resistant mutants to examine the relation between their initial fitness costs in the absence of this drug and their ability to reduce or eliminate those costs during subsequent evolution, again in a drug-free environment (Barrick et al. 2010). They first isolated eight *rpoB* mutants after a single round of antibiotic selection and showed that the mutants varied in their fitness defects. The authors then propagated these mutants and detected the first beneficial mutations to sweep to high frequency in those populations. They found that the lower-fitness backgrounds gave rise to mutations that conferred greater advantages than did the backgrounds that initially had higher fitness, in accordance with a diminishing-returns model.

If the evolution of antibiotic resistance after a period of decay under relaxed selection conformed to the diminishing-returns model, then we would expect the more susceptible LTEE-derived backgrounds to be more evolvable than their common ancestor. However, we found little statistical support for diminishing returns in our study (Figure 1.3, Table 1.2). There was one instance in which an individual LTEE-derived clone was significantly more evolvable than the ancestor in the ampicillin environment, and two other clones trended in this direction (Figure 1.3A, Appendix A Table 3). However, the statistical support, even for ampicillin, was marginal at best when the evolvabilities of the four clones were analyzed together to account for multiple tests of the same hypothesis (Table 1.2). In any case, diminishing returns was not typical across the entire set of experiments.

The absence of an overall trend toward diminishing returns might be attributable in part to two methodological issues. First, it might point to a limitation of our plate-based approach, and conventional MIC assays in general, to discern subtle differences in MICs and hence in evolvabilities based on differences in MIC values. That is, slight differences in evolvability may be obscured by the discrete resolution of the assays using 2-fold increasing concentrations of an antibiotic. Consistent with this possibility, the range of initial susceptibilities was greatest in the ampicillin environment, where the trend toward diminishing returns was most evident (Figure 1.3A). An alternative approach that might better capture subtle trends would be to use a continuous culture device that dynamically adjusts drug concentration in the growth medium to match the ongoing adaptive dynamics of the population under study (Toprak et al. 2012). Second, we might have had insufficient statistical power to resolve diminishing-returns trends in evolvability. We tested four LTEE-derived lines and their ancestor, whereas some other studies that show diminishing returns in other contexts have used as many as hundreds of lines (Kryazhimskiy et al. 2014).

There is a third factor—one that is biological, rather than methodological—that could also obscure any tendency toward diminishing returns, and that is idiosyncratic heterogeneity among genetic backgrounds in their evolvability (Figure 1.1C). This pattern occurs when particular mutations that arose during relaxed selection happen to either constrain or potentiate a strain's future evolutionary potential with respect to a given selective pressure. We found that the capacity to evolve ceftriaxone resistance tended to be lower for the derived clones than for the ancestor (Figure 1.3B, Table 1.3). That tendency is in contrast to the ampicillin environment, and it is unexpected given that both drugs are β-lactams that target cell-wall synthesis. In a similar vein, we also demonstrated that the Ara+5 lineage had become significantly constrained in its ability to

evolve tetracycline resistance relative to both its ancestor and the other LTEE-derived lineages (Figure 1.3D).

We conclude, therefore, that historical contingency has played an important role in the capacity of the LTEE-derived populations to respond evolutionarily to changed environments, in particular when challenged with antibiotics. That is, different lineages accumulated genetic differences—even in replicate populations that evolved in the same environment—that influence their ability to evolve and adapt in new directions. Several other microbial evolution studies have also documented cases of historically contingent outcomes. For example, the mutations that accumulated in one LTEE population potentiated the subsequent evolution of a novel metabolic capacity that arose in only that one population, despite comparable time and opportunity in the 11 other replicate populations (Blount et al. 2008; Quandt et al. 2015; Leon et al. 2018). Similarly, an experiment with *Pseudomonas aeruginosa* showed that high-level colistin resistance was potentiated by prior mutations in transcriptional regulators *phoQ* and *pmrB* (Jochumsen et al. 2016). However, the consequences of contingency in these two cases were the opposite of what we saw in our study: namely, evolvability was potentiated in these studies, whereas it became more constrained in ours. Still other studies have found little evidence for historical contingencies affecting evolvability. For example, Travisano and colleagues (1995) isolated a clone from each LTEE population after 2,000 generations in the glucose-limited medium. They then founded three replicate populations from each clone and let them evolve for 1,000 generations in the same environment, except with maltose replacing glucose as the limiting resource. The founding clones had independent histories in the glucose environment, and they varied greatly in their initial fitness in the maltose environment. Despite this initial heterogeneity, however, the populations rapidly

converged on similar fitnesses in the new maltose environment. Thus, adaptation dominated over contingency in that experiment.

Returning to our study, we ask the following questions: When did the evolvability with respect to tetracycline exposure decline in the Ara+5 population? And why, in molecular-genetic terms, did it decline? To answer the first question, we tested clones from throughout this population's history and identified when the bacteria first lost their ability to evolve resistance to the same degree as the ancestral strain. We found that this constraint was already present in one of two clones sampled at 1,500 generations of the LTEE, and it was evident in all of the clones we tested from generation 2,000 and onward (Table 1.4) despite these backgrounds having retained the same initial resistance level as the ancestor (Figure 1.4). These data thus confirm the idiosyncratic effects of genetic background on the evolvability of resistance. By performing fluctuation tests to measure effective mutation rates, we also showed that evolvability can depend in complex ways on several factors, including not only a strain's underlying mutation rate but also the cell density it achieves and the effective mutational target size for a given antibiotic concentration.

With respect to the second question, we do not yet know the answer, but the early timing of the change in evolvability allows us to narrow substantially the genetic possibilities. By combining our phenotypic results with previously obtained genomic data (Tenaillon et al. 2016), we have identified three candidate mutations that alone or in combination could explain this reduced evolvability. These mutations arose in the following genes: *mreB*, which encodes a protein involved in cell-wall structuring; *pykF*, which encodes pyruvate kinase that catalyzes the last step of glycolysis; and *trkH*, which encodes a potassium ion transporter. Interestingly, recent studies have discovered that mutations in *trkH* can cause increased susceptibility to tetracycline through

changes to the proton-motive force, and this relationship may depend upon the genetic background (Lázár et al. 2013; Apjok et al. 2019). In future work, we hope to make genetic constructs that will allow us to investigate the genetic basis for the low evolutionary potential of this background when exposed to tetracycline. We will also sequence some of the antibiotic-resistant mutants that evolved in our experiments to test whether there are any systematic differences among the various strains in the genetic targets of the resistant mutations and whether such differences correlate with the history of relaxed selection and concomitant increased susceptibility.

In summary, we have shown that bacterial evolution in the absence of antibiotic exposure can lead not only to increased susceptibility but also to genetic background-dependent changes in resistance evolvability when cells are exposed to those drugs. The evolution of resistance can thus depend upon previously accumulated mutations in a historically contingent fashion. These findings could have important health implications if evolution in an antibiotic-free environment sometimes erodes not only a pathogen's resistance level but also its potential to evolve greater resistance. We therefore suggest that strategic antibiotic management may benefit not only from surveillance of current resistance levels in pathogens but also from analyses of their potential to evolve increased resistance. This approach could be valuable both on the scale of the individual patient, where effective treatment is paramount, and on a community-wide scale, where judicious efforts to control the spread of drug resistance become critical. We hope that our evolvability-based approach and extensions thereof prove useful in achieving these objectives.

CHAPTER 2: Genomic evolution of antibiotic resistance is contingent on genetic background following a long-term experiment with *Escherichia coli*

Abstract

Antibiotic resistance is a growing health concern. Efforts to control resistance would benefit from an improved ability to forecast when and how it will evolve. Epistatic interactions between mutations can promote divergent evolutionary trajectories, which complicates our ability to predict evolution. We recently showed that differences between genetic backgrounds can lead to idiosyncratic responses in the evolvability of phenotypic resistance, even among closely related *Escherichia coli* strains. In this study, we examined whether a strain's genetic background also influences the genotypic evolution of resistance. Do lineages founded by different genotypes take parallel or divergent mutational paths to achieve their evolved resistance states? We addressed this question by sequencing the complete genomes of antibiotic-resistant clones that evolved from several different genetic starting points during our earlier experiments. We first validated our statistical approach by quantifying the specificity of genomic evolution with respect to antibiotic treatment. As expected, mutations in particular genes were strongly associated with each drug. Then, we determined that replicate lines evolved from the same founding genotypes had more parallel mutations at the gene level than lines evolved from different founding genotypes, although these effects were more subtle than those showing antibiotic specificity. Taken together with our previous work, we conclude that historical contingency can alter both genotypic and phenotypic pathways to antibiotic resistance.

Introduction

Convergent evolution is common in nature. The independent emergence of winged flight in insects and mammals, and of camera-like eyes in vertebrates and cephalopod mollusks, are familiar but striking examples of how evolution can drive distantly related lineages to similar phenotypic outcomes (Conway Morris 2003). For over a century, biologists have sought to understand the processes underlying these patterns and quantify the extent of convergent evolution in the natural world. However, quantifying convergence in nature is difficult for at least two reasons. First, one typically observes only a biased sample of possible outcomes. For example, extinct lineages that evolved different, but ultimately unsuccessful, adaptations usually go undetected, causing one to overestimate the extent of convergence (Woods et al. 2006; Lenski 2017). Second, comparative studies generally cannot account for slight differences in environments or lineages' prior evolutionary histories as causes of divergent adaptation, leading to an underestimation of convergence (Woods et al. 2006; Achaz et al. 2014; Lenski 2017).

Controlled and replicated evolution experiments with microbes allow one to overcome these limitations and more precisely quantify the extents of parallel and convergent evolution (Blount et al. 2018). Populations of bacteria, yeast, and viruses reproduce quickly and grow to large numbers, permitting one to observe evolution over timescales of days to years. One can also propagate many independent lines under identical conditions and characterize evolutionary repeatability at both the phenotypic and genotypic levels.

Evolution experiments with bacteria can also shed new light on the growing crisis of antibiotic resistance. It has been estimated that at least 700,000 people die each year from resistant infections, and the mortality rate has been projected to rise to 10 million by 2050, outpacing deaths from cancer (O’Neill 2016). Alternative strategies are required to combat drug resistance,

especially given the slowing pace at which new drug classes are being developed. Drug discovery through large-scale chemical screens or isolation of natural products is one approach to addressing the problem of resistance (Wohlleben et al. 2016). However, bacteria will likely evolve resistance to these new compounds, as they have to previous antibiotics, diminishing the effectiveness of drug discovery over the long-term.

Alternatively, it might be possible to extend the usefulness of existing therapeutics by channeling evolution toward drug susceptible states with an improved understanding of the factors that shape evolutionary trajectories (Nichol et al. 2015; Baker et al. 2017; Hughes and Andersson 2017; Iram et al. 2019). To that end, the phenotypic and genetic repeatability of resistance evolution has motivated several studies (Toprak et al. 2012; Imamovic and Sommer 2013; Lázár et al. 2013; Baym et al. 2016; Yen and Papin 2017; Card et al. 2019; Maltas and Wood 2019; Nichol et al. 2019). In particular, two landmark studies evaluated the reproducibility of *Escherichia coli* populations evolving in and adapting to increasing antibiotic concentrations in spatially homogeneous (Toprak et al. 2012) and structured environments (Baym et al. 2016). They found strong signatures of genomic parallelism; that is, replicate lines tended to evolve high-level resistance through mutations in a limited set of genes. However, the replicate lines in these experiments were all founded from genetically identical cells, so it is unknown whether selection would target the same genes if the founding genetic background had been different.

Epistatic interactions between mutations (including between resistance mutations and the genetic background in which they occur) can alter adaptive trajectories. Thus, they complicate our ability to predict resistance evolution and design effective treatment strategies. In theory, one can leverage collateral drug responses—where the evolution of resistance to one drug increases a bacterium's susceptibility to other drugs (Imamovic and Sommer 2013; Lázár et al. 2013; Oz et

al. 2014; Chevereau et al. 2015; Yen and Papin 2017; Imamovic et al. 2018)—to forestall or even reverse antibiotic resistance in pathogens. Yet differences in genetic background can make this approach difficult in practice. For example, replicate *E. coli* and *Enterococcus faecalis* populations can take different mutational paths to increased resistance that change their collateral responses to second-line antibiotics (Maltas and Wood 2019; Nichol et al. 2019). Despite stochasticity at the level of individual mutant strains, one can still exploit statistical patterns in resistance profiles across many replicates to optimize drug-cycling protocols (Maltas and Wood 2019). Recent work showed that such an approach could drive populations to eventual long-term susceptibility through intermediate states of high-level resistance (Maltas and Wood 2019).

Replicate populations also accumulate genetic differences as they evolve in permissive environments, and these differences can change their ability to adapt when challenged with antibiotics. We recently used several strains from the *E. coli* long-term evolution experiment (LTEE) to examine the role that genetic background plays in the evolution of drug resistance (Card et al. 2019). In the LTEE, 12 replicate populations were founded from a common ancestral strain and have been propagated daily for over 30 years in an environment without antibiotics (Lenski et al. 1991; Tenaillon et al. 2016). Clones from several populations evolved increased antibiotic sensitivity compared to their ancestor (Card et al. 2019; Lamrabet et al. 2019). We asked whether these strains could compensate for their increased sensitivity through subsequent evolution under drug selection. We found that their evolutionary potential was idiosyncratic with respect to their initial genotype, such that resistance was constrained in some backgrounds but not in others, indicating the role of historical contingency in this process (Blount et al. 2018; Card et al. 2019).

In this study, we sequenced the complete genomes of antibiotic-resistant clones that evolved from several different founding strains during our earlier experiments and used this

information to examine how genetic background affects the genomic evolution of antibiotic resistance. First, we validated our statistical approach by demonstrating that mutations in particular target genes were associated with each of the four antibiotic treatments. We then showed that evolution was also contingent, albeit more subtly, on differences in genetic background, such that resistant lines that evolved from the same genotype had, on average, slightly more mutations in common. These results, taken together with our previous work, indicate that even slight differences in genetic background may complicate one's ability to predict phenotypic and genotypic outcomes of antibiotic resistance evolution.

Materials and Methods

Evolution experiments and bacterial strains

The LTEE has been described in detail elsewhere (Lenski et al. 1991; Lenski 2017). Briefly, 12 replicate populations of *E. coli* were founded from a common ancestral strain called REL606. These populations have been propagated for more than 73,000 bacterial generations by daily 1:100 transfers in a glucose-limited Davis minimal (DM) medium without antibiotics.

In a previous study (Card et al. 2019), we measured the intrinsic resistance of the LTEE ancestor and derived clones isolated from four populations (designated Ara–5, Ara–6, Ara+4, and Ara+5) at generation 50,000 to the antibiotics ampicillin (AMP), ceftriaxone (CRO), ciprofloxacin (CIP), and tetracycline (TET). We also quantified these strains' capacities for evolving resistance by challenging them across a range of concentrations to these same drugs during one round of selection. In this study, we sequenced the complete genomes of a subset of the resistant mutants that evolved during these experiments, and we examined whether the genetic targets of the resistance mutations systematically differed between the four antibiotics and five genetic

backgrounds. Specifically, for each antibiotic treatment we sequenced 4 mutants that independently evolved from the ancestral background, and 3 mutants from each derived background, for a grand total of 64 sequenced mutants (16 mutants × 4 antibiotics) (Appendix A Table 4).

Library preparation and genomic sequencing

Glycerol stocks of frozen samples were grown overnight in 3 mL of Luria Bertani (LB) medium at 37^{o}C with shaking at 250 rpm. Overnight cultures were harvested by centrifugation and genomic DNA was extracted using the E.Z.N.A. Bacterial DNA kit (Omega Bio-tek). DNA was quantified using the QuantiFluor dsDNA system (Promega). Then, 250 ng of purified genomic DNA was used from each sample for library preparation using the Nextera DNA Flex Library Prep Kit (Illumina) per the manufacturer's protocols. The 12 pM final libraries were loaded into a 600-cycle V3 MiSeq reagent cartridge and sequenced on an Illumina MiSeq at North Carolina A&T State University. The resultant FASTQ files of 300-base paired-end reads were deposited to the NCBI Sequence Read Archive.

Mutation identification

We trimmed sequencing reads to remove low-quality bases using Trimmomatic v0.38 (Bolger et al. 2014) on the Galaxy web platform (Afgan et al. 2018). A sliding-window approach was used; reads were clipped when the average quality score was < 20 in a 4-bp window and to a minimum length of 36-bp. Mutations were then identified in the genomes using *breseq* v0.35 (Barrick et al. 2014) with default parameters. We used a version of the REL606 reference genome with updated gene annotations for variant calling (Jeong et al. 2009; Tenaillon et al. 2016).

Each population evolved unique substitutions in *pykF* during the LTEE that distinguish them from one another (Tenaillon et al. 2016). Therefore, we first compared this locus for each resistant clone against its corresponding parental strain to test for possible external and cross-contamination. Strains KJC184 and KJC217 from the CIP and CRO treatments, respectively, were supposed to derive from the Ara+4 and Ara+5 parental backgrounds, respectively, but they had *pykF* alleles corresponding to other backgrounds used in this study. Likewise, strain KJC152 from the CIP treatment was supposed to derive from the Ara+4 background, but its genome was identical to a resistant mutant derived from the ancestral clone. We discarded these three cross-contaminants from our study.

The *breseq* results for each of the other 61 sequenced resistant clones gave information on both its genetic background and the mutations that evolved during our previous antibiotic-selection experiments. We manually curated the results by removing the background-specific mutations (i.e., those that arose during the LTEE), which we did by comparing each resistant clone to its parental strain. We also excluded expansions and contractions of hypermutable short sequence repeats that are unlikely to contribute to stably inherited resistance, and mutations within multi-copy elements (e.g., ribosomal RNA operons and insertion sequences) that may result from gene conversions but cannot be fully resolved using the short-read sequencing data. In addition, we resolved numerous structural variants by manually examining the depth of read coverage across the genome and predictions of new sequence junctions from split-read mapping for each clone (Barrick et al. 2014). To verify the predicted mutations, we applied the genomic changes in each parental background to the REL606 reference genome and reran *breseq*.

In total, we identified mutations in 59 of the 61 antibiotic-resistant clones. Two clones (KJC65 and KJC66) had no clear genetic changes despite exhibiting increased phenotypic

resistance in our earlier study (Card et al. 2019). This discrepancy suggests at least two possible explanations. First, these resistant lines might have mutations that could not be adequately resolved by short-read sequencing, including amplifications of genes or chromosomal regions and inversions bounded by identical sequences (e.g., multi-copy IS elements) (Raeside et al. 2014). Second, these lines might have had unstable genetic changes, including copy number changes in homopolymeric tracts and amplifications, which are often unstable and can lead to hypermutability, phase variation, and other complications (Moxon et al. 1994; Sandegren and Andersson 2009; Blount et al. 2012; Jiang et al. 2019). To look for amplifications that might have been missed by the *breseq* pipeline, we also used another pipeline (Blount et al. 2020) to examine the two genomes without identifiable mutations for evidence of regions with above-average read coverage. However, this analysis did not reveal any amplifications in these two clones. More details can be found in the R Notebook provided on GitHub (https://github.com/KyleCard/LTEE-ABR-mutant-sequencing).

Statistical methods

We quantified the specificity of genomic evolution with respect to antibiotic treatment by comparing the gene-level similarity of mutations between independent resistant lines that evolved under the same treatment versus different treatments. Similarly, for each antibiotic we quantified the specificity of genomic evolution with respect to genetic background by comparing the gene-level similarity of mutations between lines derived from the same parental genotype versus different parental genotypes. Following Deatherage et al. (2017), we included in our comparisons nonsynonymous point mutations, small indels, and IS element insertions in genes or within 150-bp upstream of the start of a gene. However, we modified their approach to also include large

deletions and amplifications if at least one of the affected genes was also found to be mutated in another clone or if there were parallel changes across lines. We excluded from these analyses synonymous mutations, the two clones with no identified genetic changes, and a third clone with only a large amplification that was unique and could not be assigned to any particular gene. A total of 71 mutations qualified based on these criteria.

We then calculated Dice's coefficient of similarity, *S*, for each pair of evolved clones, where $S = 2|X \cap Y|/(|X| + |Y|)$. Here, |X| and |Y| represent the number of genes with qualifying mutations in each clone, and $|X \cap Y|$ is the number of mutated genes in common between them. *S* therefore ranges from 0, when the pair of clones have no mutated genes in common, to 1, when both have mutations in exactly the same set of genes (Sokal and Rohlf 1994; Deatherage et al. 2017; Blount et al. 2020). We then calculated the average of these coefficients for all pairs of clones evolved within the same treatment or from the same parental genotype, S_s, and for all pairs of clones evolved across different treatments or different genotypes, S_d. The difference between these two values serves as a test statistic for the specificity of genomic evolution.

The observed outcome can be seen as one of many possible but equally likely outcomes that could have arisen by chance. One can therefore perform a randomization test to evaluate the significance of the test statistic associated with the observed outcome (Sokal and Rohlf 1994). To do so, we repeatedly rearranged the clones associated with each antibiotic treatment, or the clones within each treatment when testing for background specificity, while maintaining the number and identity of the mutations in any clone (Deatherage et al. 2017). For example, if mutations A and B were found together in the same sequenced clone, we retained their association throughout the procedure but randomly assigned the set to a different clone label. We calculated the specificity test statistic for each of 10,000 permutations of the clone labels. This procedure yields the expected

distribution of the test statistic under the null hypothesis that the similarity among lines is independent of the antibiotic treatment or founding genotype. We then calculated an approximate *p*-value for rejecting this null hypothesis from the proportion of permutations in the expected distribution with a specificity statistic value greater than or equal to the observed value.

To quantify the specificity of genomic evolution with respect to genetic background, we performed an independent randomization test for each of the four antibiotics. Because these tests address the same null hypothesis, we combined the resulting *p*-values using Fisher's method with $2k = 8$ degrees of freedom, where k is the number of comparisons (Fisher 1934; Sokal and Rohlf 1994). We provide the datasets and details of our statistical analyses in an R Notebook on GitHub (https://github.com/KyleCard/LTEE-ABR-mutant-sequencing).

Results

Genomic evolution of strains evolved under four different antibiotic treatments

In our previous study (Card et al. 2019), we isolated antibiotic-resistant mutants that evolved from five different parental genotypes: the LTEE ancestor and four derived clones isolated from the LTEE at generation 50,000. The experiment was performed over one round of drug selection. Here, we sequenced the complete genomes of 64 of the resistant clones, but we discarded 3 that were identified as cross-contaminants (Materials and Methods) (Appendix A Table 4).

The 61 remaining resistant clones had a total of 76 mutations. Forty-five genomes had a single mutation, 11 others had two mutations, and 3 genomes had three mutations (Figure 2.1). Two other clones (both in the tetracycline treatment) had no identifiable mutations; they might have had unstable genetic changes or types of mutations that could not be resolved by our analyses of the short-read sequencing data (see Materials and Methods). In any case, we have excluded

these two clones from the analyses that follow. Twenty-seven of the 76 mutations (35.5%) were single-base substitutions; of these, 22 were either nonsynonymous or nonsense mutations that altered the encoded protein's amino-acid sequence, 1 was synonymous, and 4 occurred in *alaT*, which encodes a tRNA rather than a protein. Five other mutations (6.6%) were in intergenic regions within 150-bp upstream of a gene, which suggests that they affect regulation.

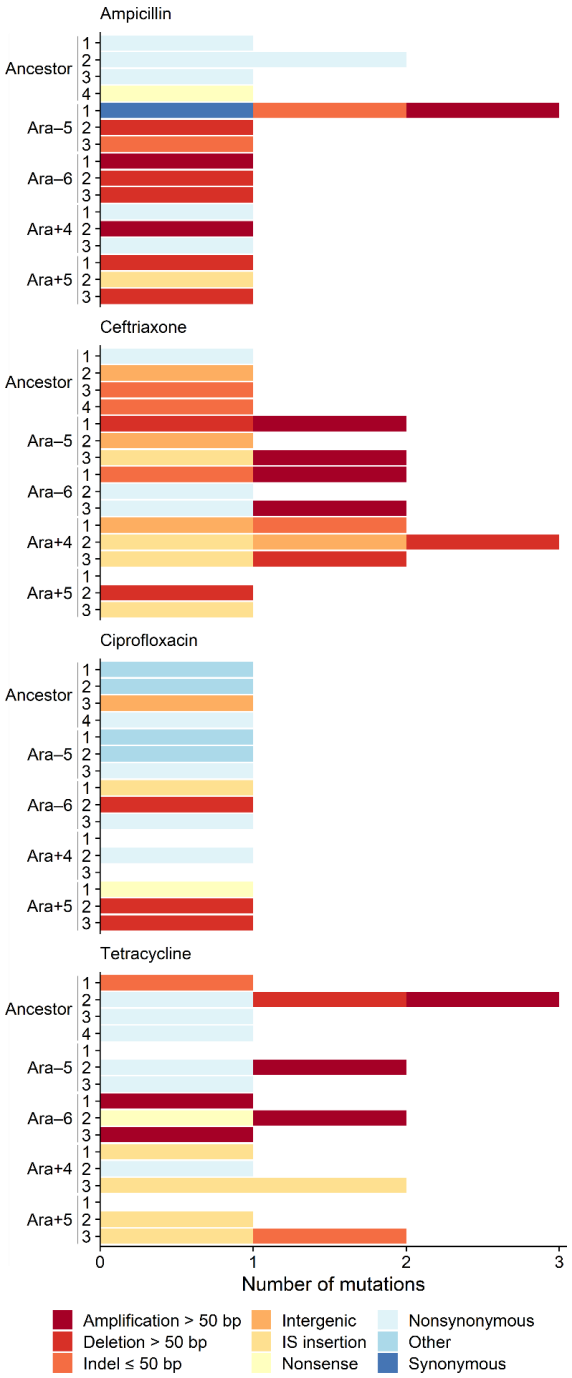

Figure 2.1. Numbers and types of mutations in evolved genomes. Summary of the 76 mutations observed in 61 antibiotic-resistant clones after selection in ampicillin, ceftriaxone, ciprofloxacin, or tetracycline. Mutations are color-coded by the type of genetic change, according to the legend at the bottom. The "Other" category represents mutations in the non-protein-coding gene *alaT*. Evolved genomes are labeled according to their parental genetic background and replicate. Two tetracycline-selected clones (Ara–5-1 and Ara+5-1) had no identifiable mutations (see Materials and Methods).

The largest proportion of mutations in the resistant lines were structural variants. They comprise 44 (57.9%) of the observed changes (Figure 2.1). Eleven of these were IS-element insertions in protein-coding genes; 8 were small insertions and deletions (indels) of less than 50 bp, 13 were large deletions, and 12 were large amplifications. Twelve of the 13 large deletions and 11 of the 12 large amplifications were found in lines derived from the generation-50,000 backgrounds (Figure 2.1). However, 45 of the 61 clones (73.77%) belong to that group, and neither observed distribution deviates significantly from that null expectation (binomial tests, $p = 0.1077$ and $p = 0.1368$ for large deletions and large amplifications, respectively).

Genomic parallelism at the functional level

Antibiotic resistance can arise through mutations that change gene regulation and expression, cell permeability and efflux, and metabolism (Blair et al. 2015). To determine how drug selection acted on these functions in our experiment, we quantified the extent of genomic parallelism in the resistant lines at the functional level – i.e., sets of genes that share broadly defined functions. We used the curated descriptions of cellular processes in EcoCyc (Keseler et al. 2017) to match each mutated gene to an associated function. We excluded large deletions and amplifications when the affected genes do not share a common function.

About 37% of the 57 mutations that fit the criteria for inclusion occurred in regulatory genes, ~26% in metabolic genes, ~21% in genes that encode transporters, and ~11% in genes involved in transcription or translation (Figure 2.2). More mutations in some of these functional categories than in others suggest a pattern of parallel evolution. However, more *E. coli* genes are involved in some functions than in others, and therefore a random mutation is more likely to occur in those categories that constitute a larger proportion of the genome. To examine whether the

observed number of mutations in each category occurred more frequently than expected, we modeled the data using the Poisson cumulative expectation $P(x \geq observed, \lambda)$, where λ is (total number of mutations) × [(combined length of all genes in functional category) / (total genome length)] (Tenaillon et al. 2012). The derived parental backgrounds evolved slightly smaller genomes during the LTEE (Tenaillon et al. 2016), so we used their average genome size (4,602,572 bp) when calculating λ.

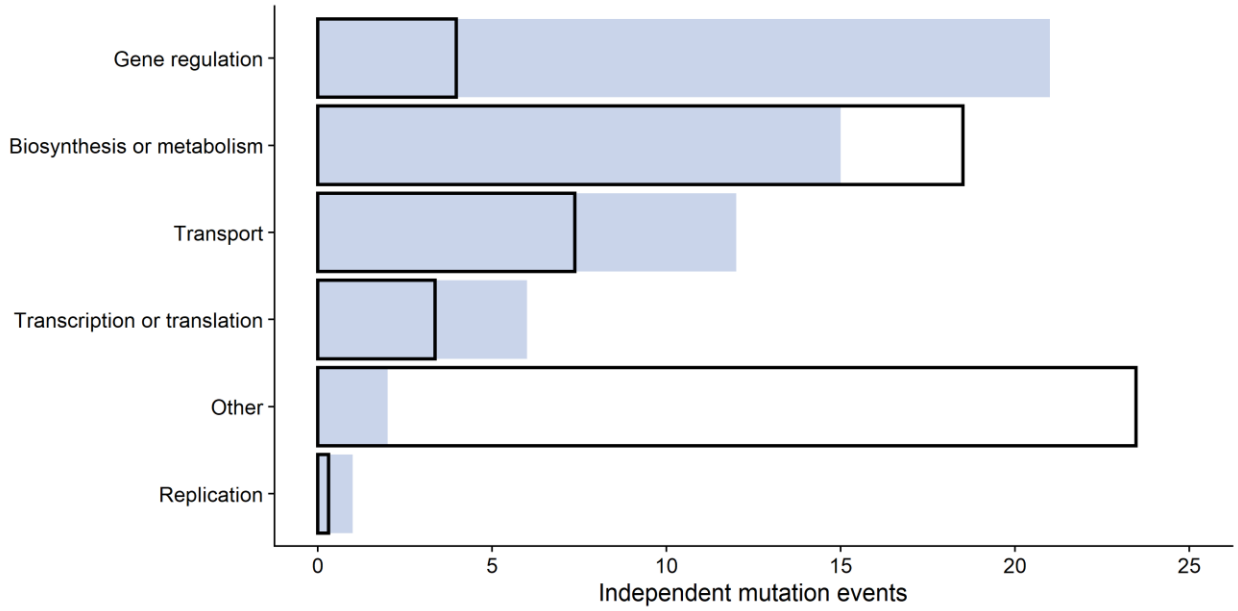

Figure 2.2. Distribution of independent mutations in different functional categories. The observed and expected distributions are shown as shaded regions and outlines, respectively. See text for statistical analysis.

Regulatory genes accrued mutations about 5 times more often than expected from the Poisson distribution (Figure 2.2), and this difference is highly significant ($p < 0.0001$). Genes involved in transport functions had about 1.6 times more mutations than expected by chance, but this difference was marginally non-significant ($p = 0.0723$). Genes involved in transcription or translation had about 1.8 times as many mutations as expected, but this difference was also not statistically significant given the small number of mutations in these targets ($p = 0.1245$). It should be emphasized that this analysis is conservative because it lumps together all of the genes in each functional category. However, mutations in only a subset of these genes are likely to cause resistance. Therefore, the effective mutational target size and the resulting expected number of mutations is presumably much smaller.

Specificity of genomic evolution in the different antibiotic environments

We compared the gene-level similarity of mutations between independent lines that evolved in the same antibiotic treatment and across the four different treatments to evaluate the effect of the selective environment on the genetic paths to increased antibiotic resistance. As described in the Materials and Methods, we computed Dice's coefficient of similarity for each pair of clones using the 71 qualifying mutations that can be assigned to a particular gene. The average within-treatment similarity was 0.089 and the average between-treatment similarity was 0.032 (Figure 2.3). In other words, two clones that independently evolved under the same antibiotic selection had on average 8.9% of their mutated genes in common, whereas those that evolved under different antibiotics shared on average only 3.2% of their mutated genes. A randomization test shows that the 5.7% difference in similarity is highly significant ($p < 0.0001$). Thus, genomic evolution was demonstrably specific with respect to the antibiotic treatment.

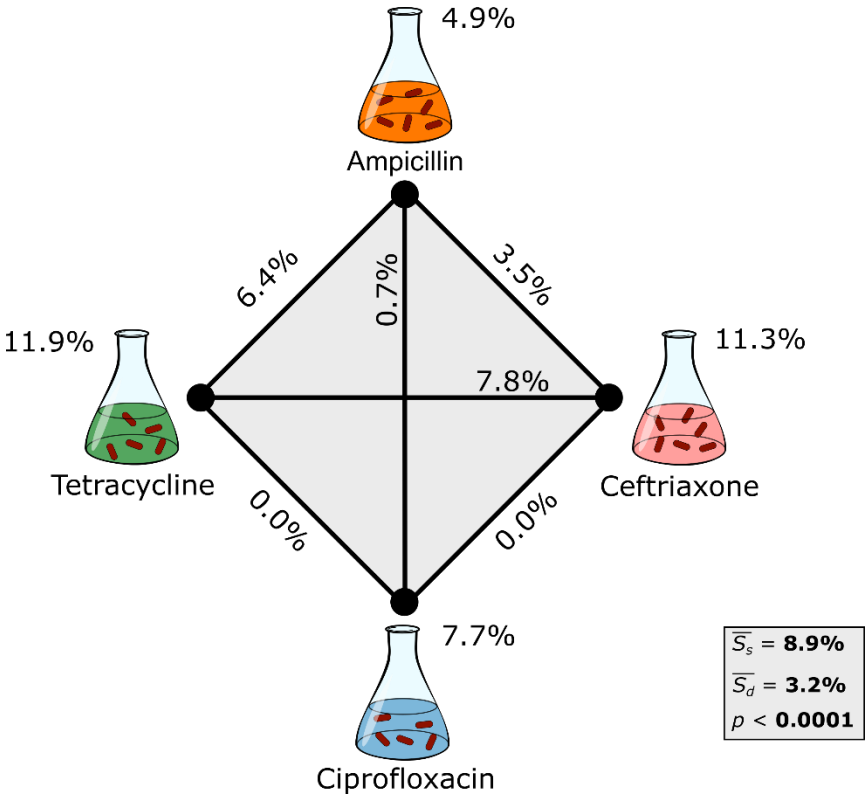

Figure 2.3. Specificity of genomic evolution with respect to antibiotic treatment. Treatments and the edges connecting them are labeled with Dice's coefficient scores that show the average similarity for all clone pairs evolved in the same antibiotic (S_s) and in different antibiotics (S_d), respectively. Only the 71 qualifying mutations (see Materials and Methods) were included in the calculations. The weighted averages of S_s and S_d are shown in the grey box. The difference between these two values indicates the extent to which genome evolution was specific to the antibiotic treatment. The resulting *p*-value was calculated using a randomization test.

The similarity analysis does not reveal the specific genes that contribute to the antibiotic-treatment specificity. To address this issue, we used Fisher's exact tests to identify genes that had an excess of qualifying mutations in the replicate lines evolved under the four treatments (Figure 2.4). We found 5 "signature" genes in which mutations contributed significantly to antibiotic specificity (Figure 2.4, Table 2.1). The *alaT* gene encodes a tRNA; it was mutated in 4 of the 14 CIP-resistant lines, but in none of the other 44 lines with qualifying mutations (Figure 2.4). The *ompR* gene is part of the two-component system that regulates the production of outer-membrane

proteins; it was mutated in 6/14 TET-resistant lines as well as in 4/44 lines that evolved resistance to other drugs. The other gene in this two-component system, *envZ*, was mutated in 2 of the 10 TET-resistant lines that did not have an *ompR* mutation. Two genes, *ompF* and *hns*, were associated with resistance to ceftriaxone (Table 2.1). The former encodes an outer-membrane porin and was mutated in 6/15 CRO-resistant lines along with 3/43 other lines; the latter encodes a histone-like global regulator and acquired mutations in 3/15 CRO-resistant lines and 1 of the 43 lines that became resistant to another antibiotic (Figure 2.4). Finally, a large deletion was found in 3 of the 15 AMP-resistant lines but not in any of the other 43 lines (Table 2.1); this deletion affects multiple genes including *phoE*, which encodes an outer membrane porin (Figure 2.4).

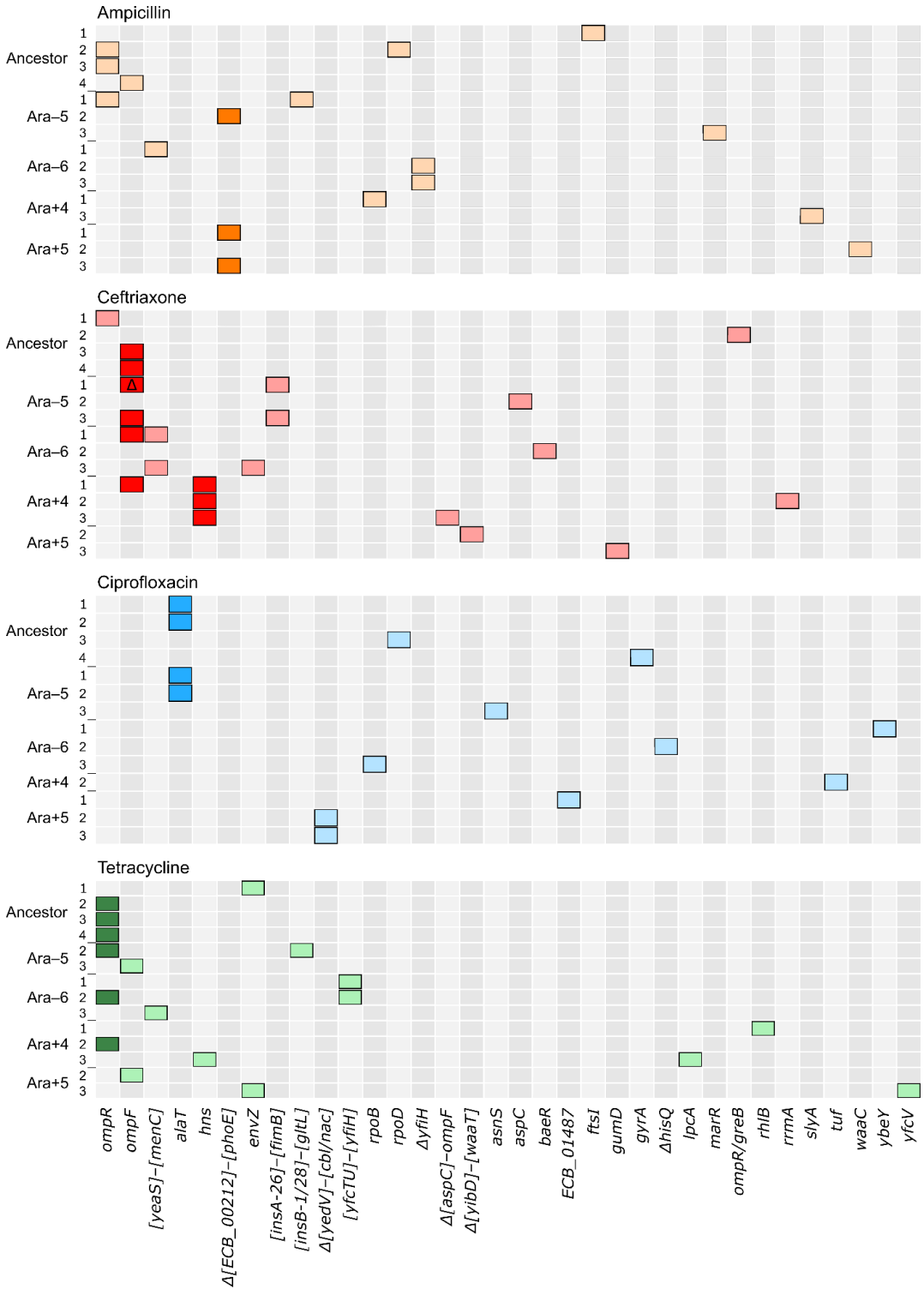

Figure 2.4. Identity of mutated genes in antibiotic-resistant lines. A total of 61 lines (labels at left) evolved from 5 different genetic backgrounds in ampicillin, ceftriaxone, ciprofloxacin, or tetracycline environments. Two (TET Ara–5-1, TET Ara+5-1) had no identifiable mutations; a third (AMP Ara+4-2) had no qualifying mutation that could be assigned to a specific gene (see Materials and Methods). These three lines are not shown. Filled cells identify the 71 qualifying

Figure 2.4. (cont'd)

mutations by the affected genes (shown along the bottom and listed in order of the number of mutations). The darkly shaded cells identify the signature genes, in which mutations are significantly associated with one antibiotic treatment (Table 2.1). A deletion or amplification spanning a given genomic region is indicated when two gene names are shown. If a gene name is shown in brackets, then only part of that gene is affected. If a gene name is preceded by Δ, then those genes are deleted; otherwise, they are amplified. Part of the *ompF* gene is deleted in the CRO Ara–5-1 line.

Table 2.1. Gene targets that contributed to antibiotic-treatment specificity.

Antibiotic	Gene target	*p*
Ampicillin	*[ECB_00212]–[phoE]*	0.0147
Ceftriaxone	*ompF*	0.0064
	hns	0.0493
Ciprofloxacin	*alaT*	0.0024
Tetracycline	*ompR*	0.0087

Statistical significance was calculated using Fisher's Exact Test for the association between antibiotic treatment and genetic targets.

Specificity of genomic evolution with respect to genetic background

We next employed Dice's coefficient of pairwise similarity to quantify the specificity of genomic evolution with respect to the parental strain's genetic background. Using the ampicillin treatment as an example (Figure 2.5A), the clones that evolved independently from the same founding genetic background and from different backgrounds had, on average, 14.8% and 3.1% of their mutated genes in common, respectively, indicating a difference of 11.7%. This trend of greater similarity for clones derived from the same genetic background also occurred in the three other antibiotics (Figure 2.5B–D).

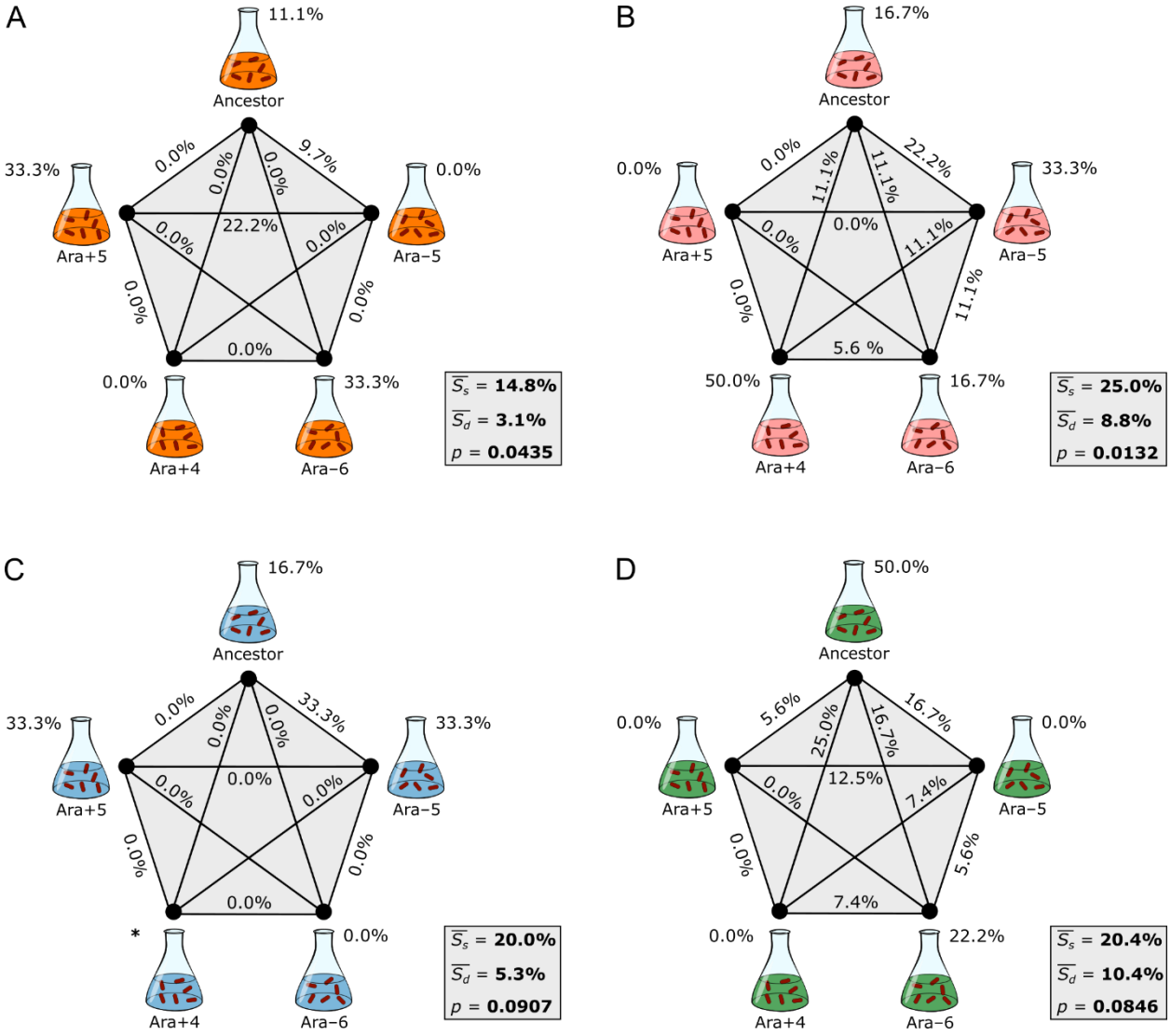

Figure 2.5. Specificity of genomic evolution with respect to genetic background. Five different backgrounds and the edges connecting them are labeled with Dice's coefficient scores that show the average similarity for all clone pairs evolved from the same genetic background (S_s) and from different backgrounds (S_d), respectively, in ampicillin (A), ceftriaxone (B), ciprofloxacin (C), and tetracycline (D). The difference between S_s and S_d indicates the extent to which genome evolution was specific to the genetic background. Two of the three replicates derived from the Ara+4 background in ciprofloxacin were excluded owing to cross-contamination, and S_s cannot be calculated in that case (*). See Figure 2.3 for additional details.

We performed separate randomization tests for the clones in each antibiotic treatment to evaluate whether the effects of genetic background were significant. The associations between genomic evolution and the identity of the parental strain were significant for the lines that evolved

in the ampicillin and ceftriaxone environments (Figure 2.5A and 2.5B), and they were marginally nonsignificant for the lines in the ciprofloxacin and tetracycline environments (Figure 2.5C and 2.5D). When we combined the probabilities from these four independent tests of the hypothesis that differences in genetic background influence the genetic basis of antibiotic resistance using Fisher's method (Fisher 1934; Sokal and Rohlf 1994), the overall trend toward greater similarity (gene-level parallelism) of lines evolved from the same founding genotype was highly significant ($\chi^2 = 24.67$, df = 8, $p = 0.0018$).

In our analysis of genome specificity with respect to antibiotic treatment, we identified several signature genes that contributed significantly to that specificity (Figure 2.4, Table 2.1). We have much less statistical power to identify particular genes that contribute to specificity with respect to genetic background, because only 3 or 4 replicate lines derive from any given background in each antibiotic treatment. Nonetheless, we can identify candidate loci that may contribute to that specificity, which might be further studied in the future. Table 2.2 shows all of those genes that fulfilled both of the following criteria for a given antibiotic treatment: (i) two or more lines derived from the same background had mutations affecting the same gene; and (ii) that background produced at least as many mutations affecting that gene as did the other four backgrounds combined. In the case of each antibiotic treatment, at least two genetic backgrounds have candidate signature genes that fulfill these criteria.

Table 2.2. Candidate genes that may contribute to genetic-background specificity.

Antibiotic	Genetic background	Gene target
Ampicillin	Ancestor	*ompR*
Ampicillin	Ara–6	*yfiH*
Ampicillin	Ara+5	*[ECB_00212]–[phoE]*
Ceftriaxone	Ara–5	*[insA-26]–[fimB]*
Ceftriaxone	Ara–6	*[yeaS]–[menC]*
Ceftriaxone	Ara+4	*hns*
Ciprofloxacin	Ancestor	*alaT*
Ciprofloxacin	Ara–5	*alaT*
Ciprofloxacin	Ara+5	*[yedV]–[cbl/nac]*
Tetracycline	Ancestor	*ompR*
Tetracycline	Ara–6	*[yfcTU]–[yfiH]*

Discussion

How does genetic background affect the evolution of antibiotic resistance? We previously addressed this question by examining the resistance potential of the *E. coli* ancestor of a long-term experiment and derived clones isolated from four populations after generation 50,000. We challenged these strains using a series of drug concentrations, and we found that several strains had a reduced capacity to evolve resistance relative to their ancestor, implicating the role of historical contingency in this process (Blount et al. 2008, 2018; Card et al. 2019). In this study, we asked whether and how genetic background influences the evolution of resistance through chromosomal mutations. We sequenced the complete genomes of 61 resistant lineages that evolved in our earlier experiment to identify the mutations that conferred resistance. We then determined if there were particular signatures of (i) the antibiotic treatment and (ii) the initial background evident from the identities of the mutated genes.

The populations that evolved resistance to the four different drugs in our study exhibited divergent underlying genetic changes (Figure 2.3). This result was expected given that bacteria generally evolve resistance through mutations specific to a drug's mechanism of action (Toprak et

al. 2012; Chevereau et al. 2015; Baym et al. 2016). This specificity was driven by parallel mutations in several genes (Table 2.1). Overall, *ompR* and *ompF* had more mutations than any other genes (Figure 2.4). OmpR is a transcriptional regulator involved in responses to osmotic and acid stress; mutations to OmpR also contribute to antibiotic resistance by altering the expression of the OmpF major porin (Aiba and Mizuno 1990; Chakraborty and Kenney 2018; Choi and Lee 2019). A recent study showed that *ompF* deletions reduce the permeability of β-lactams (e.g., ampicillin and ceftriaxone) across the outer membrane, thus increasing resistance (Choi and Lee 2019). We found that *ompF* mutations were strongly associated with ceftriaxone-resistant lines, consistent with this prior study. However, the evolution of ampicillin resistance occurred through more diverse mutational paths in our experiment. Although there were some mutations in *ompF* and *ompR* in the ampicillin treatment, we saw a significant association of that treatment with deletion of a different outer-membrane porin, PhoE. The down-regulation of this porin also partially modulates the cell's response to osmotic stress (Meyer et al. 1990). In the tetracycline treatment, mutations were more common in *ompR* than in *ompF*, which suggests that altering the expression of other genes in this regulon also contributes to resistance. Mutations in *hns* were associated with ceftriaxone-resistant lines. Nishino and Yamaguchi (2004) showed that deletion of this global transcriptional regulator increases resistance to multiple drugs because it causes overexpression of several efflux pumps. In contrast to the results for the other three antibiotics, the evolution of ciprofloxacin resistance was not associated with mutations in genes related to outer-membrane proteins. Instead, mutations in *alaT*, which encodes an alanine tRNA, were a signature of this treatment. The mechanism behind this resistance is unknown. One possibility is that these mutations modulate interactions that have been reported between this tRNA and tmRNA, which rescues stalled ribosomes after aberrant translation (Gillet and Felden 2001). Enhanced rescue

might directly promote survival or indirectly affect the expression of other vital genes, when cells are treated with this antibiotic.

The signature genes that we observed for each treatment are not the canonical resistance genes for the respective antibiotics (Blair et al. 2015). Ampicillin and ceftriaxone irreversibly bind to transpeptidases and disrupt cell-wall synthesis; ciprofloxacin targets topoisomerase and inhibits DNA replication; and tetracycline targets the ribosome and hinders protein synthesis. Drug resistance often arises through modifications to these targets, yet these changes rarely occur in our study. This discrepancy may reflect two factors: one environmental and the other genetic. First, altering a drug target often confers high-level resistance, but at the expense of bacterial growth rate (Andersson and Hughes 2010; Hughes and Andersson 2017). We used moderate drug concentrations to select for mutants (Card et al. 2019), and the observed resistance rarely reached levels thought to be clinically relevant (EUCAST 2020). This moderate environment should favor mutations that provide sufficient resistance at a low fitness cost, because they will leave more descendants during population growth before treatment, and consequently they will be seen more often after the antibiotic challenge. Second, the *E. coli* used in our experiments are all derived from a B strain that differs in important ways from the K-12 strains that are more widely used in studies of antibiotic resistance (Studier et al. 2009). In particular, *E. coli* K-12 has two major porins, OmpC and OmpF, whereas *E. coli* B expresses only OmpF (Pugsley and Rosenbusch 1983; Schneider et al. 2002). Thus, the use of the *E. coli* B background may well have influenced which genes could mutate to yield resistance in our experiments.

We also found genomic signatures of adaptive divergence associated with differences in genetic background, and these differences are far smaller than those between *E. coli* B and K-12. We sequenced and analyzed resistant lines that evolved from five genetic backgrounds that were

separated in time by only a few decades, and which differed only in the mutations that had accumulated in the antibiotic-free environment of the LTEE (Figure 2.1). Three or four resistant lines independently evolved from each parental background for each of the four antibiotics studied, allowing us to assess the genomic specificity of resistance with respect to the genetic background (Figure 2.5). Although these background effects were more subtle than those showing antibiotic specificity, they are compelling when taken together. Various factors might contribute to the genetic background specificity. Most broadly, epistatic interactions can cause the same mutation to have different effects on resistance, or on its fitness costs, in different genetic backgrounds. The rates at which particular resistance mutations arise may also vary between different genetic backgrounds.

Imagine that the same mutation arises in separate populations founded from two distinct backgrounds. If the mutation confers less resistance in one background than the other, then it may go undetected when those populations are challenged at a high drug concentration. This type of epistasis could therefore generate a signature of genomic specificity of resistance mutations with respect to the genetic background. It is also possible that different genetic backgrounds affect the evolution of resistance by changing the likelihood of certain genomic amplifications or deletions. These types of structural mutations often occur by homologous recombination between IS elements, and they can confer resistance by altering the number of membrane transporters or drug targets (Sandegren and Andersson 2009). Such mutations can also occur spontaneously at very high rates (Cooper et al. 2001; Sandegren and Andersson 2009). In our study, many of the resistance mutations were mediated by insertion sequences, including new copies in the derived backgrounds that previously arose during the LTEE (Tenaillon et al. 2016). The evolution of

resistance in these cases is therefore influenced, at least in part, by changes in the rates at which certain types of mutations arise in the derived backgrounds.

Antibiotic resistance is a growing public-health concern. If the likely routes to resistance can be predicted, at least to some extent, then there exists a potential opportunity to control the emergence of resistance through rational treatment strategies. However, to predict the evolution of resistance with accuracy, we must understand and integrate information about many factors, including a bacterial lineage's genetic history, and how that history may potentiate or constrain its future evolution. The results from this study, together with our previous findings, demonstrate the importance of historical contingency in the evolution of drug resistance at both the phenotypic and genotypic levels. This contingency underscores the importance of accounting for stochasticity, in the past as well as at present, when designing evolutionarily informed treatment strategies.

CHAPTER 3: Idiosyncratic variation in the fitness costs of tetracycline-resistance mutations in *Escherichia coli*

Abstract

A bacterium's fitness relative to its competitors, both in the presence and absence of antibiotics, plays a key role in its ecological success and clinical impact. In this study, we examine whether tetracycline-resistant mutants are less fit in the absence of the drug than their sensitive parents, and whether the fitness cost of resistance is constant or variable across independently derived lines. Tetracycline-resistant lines suffered, on average, a reduction in fitness of almost 8%. There was substantial among-line variation in the fitness cost. This variation was not associated with the level of phenotypic resistance conferred by the mutations, nor did it vary significantly across several different genetic backgrounds. The two resistant lines with the most extreme fitness costs involved functionally unrelated mutations on different genetic backgrounds. However, there was also significant variation in the fitness costs for mutations affecting the same pathway and even different alleles of the same gene. Our findings demonstrate that the fitness costs of antibiotic resistance do not always correlate with the phenotypic level of resistance or the underlying genetic changes. Instead, these costs reflect the idiosyncratic effects of particular resistance mutations and the genetic backgrounds in which they occur.

Introduction

Antibiotics are an essential component of modern medicine. Although they have dramatically reduced the morbidity and mortality caused by severe bacterial infections, their benefits have diminished in recent years because of their overuse in the clinic and in agriculture, which has led to the evolution and proliferation of antibiotic-resistant pathogens. As a result, many infections have become more difficult to treat with mainline drug therapies, and in some severe cases, some pathogenic strains have become resistant to all available drugs. An understanding of the forces underlying and shaping antibiotic resistance is therefore critical to the future health of the human population.

Bacteria can evolve resistance by either spontaneous mutations or horizontal acquisition of resistance genes. Spontaneous mutations commonly confer resistance by altering the cellular target of the antibiotic or increasing its efflux (Blair et al. 2015). Mechanisms associated with horizontal gene transfer include target modification, drug detoxification, and the acquisition of novel efflux pumps (Blair et al. 2015). In either case, resistant variants have a clear advantage over their sensitive counterparts when exposed to the corresponding antibiotic. However, these resistant types often suffer fitness costs because they disrupt the normal functioning of metabolic pathways and physiological processes or increase the energetic burden on the cell (Lenski and Bouma 1987; Nguyen et al. 1989; Andersson and Hughes 2010; Vogwill and MacLean 2015). Resistant types should therefore have lower growth rates than, and be outcompeted by, their sensitive counterparts in the absence of drugs.

A resistant bacterium's competitive fitness, both in the presence and absence of a drug, is an important factor that contributes to its ecological success and thus its clinical impact (Lenski 1997; Vogwill and MacLean 2015; Hughes and Andersson 2017). For example, the fitness of a

resistance mutation determines its likelihood of persisting in a bacterial population prior to drug exposure, its maintenance in a population at a particular drug concentration, and its reversibility when the antibiotic is reduced or removed from the environment (Hughes and Andersson 2017; Santos-Lopez et al. 2019).

The expected time required to reduce the frequency of a resistant mutant in a bacterial population following the cessation of antibiotic use is inversely proportional to the fitness cost of the resistance mutation (Lenski 1997). Although mathematical models can predict the rate of these frequency declines (Levin et al. 1997), these theoretical expectations often are not met under real-world scenarios for at least two reasons. First, some resistance mechanisms are inherently cost free, at least in certain environments. Several mutations in the gene *rpsL* confer resistance to streptomycin, but they have little or no fitness cost in both *Escherichia coli* and *Salmonella typhimurium* (Tubulekas and Hughes 1993), and they even confer a competitive advantage over wild-type strains in some animal infection models (Björkman et al. 1998; Enne et al. 2005). These cost-free *rpsL* mutations are also found in streptomycin-resistant *Mycobacterium tuberculosis* populations, where they may facilitate the long-term maintenance of this resistant type (Böttger et al. 1998; Andersson and Hughes 2010). Similarly, treatment of *Helicobacter pylori* infections with clarithromycin has been found to select for highly resistant commensal *Enterococcus* species that persist for years after drug treatment (Sjölund et al. 2003). This last outcome demonstrates a troubling side-effect of antibiotic use, in which the microbiome can act as both a reservoir for resistance genes and as a conduit for their horizontal transfer to pathogens (Sommer et al. 2010).

Second, pleiotropic costs associated with chromosomal- or plasmid-mediated resistance can often be reduced or even eliminated through subsequent compensatory evolution (Bouma and Lenski 1988; Schrag et al. 1997; Kugelberg et al. 2005; Nilsson et al. 2006; Andersson and Hughes

2010; Barrick et al. 2010). For example, clinically relevant levels of fluoroquinolone resistance occur through the sequential substitution of mutations in several genes (Lindgren et al. 2003). Early genetic changes in the mutational pathway exact a cost on bacterial growth in both laboratory media and mouse models, but the cost can be ameliorated through later resistance mutations (Marcusson et al. 2009). Thus, evolution can restore a bacterial population's ancestral growth rate in the absence of drug selection while simultaneously preserving resistance in the event of future exposure to antibiotics. Moreover, compensatory evolution can sometimes drive multidrug resistance; this outcome has been seen when a genetic change simultaneously provides resistance to a newly imposed drug while reducing the fitness cost associated with resistance to a previous antibiotic (Trindade et al. 2009). Compensatory evolution shows how pleiotropic effects of one mutation can set the stage for epistatic interactions with subsequent mutations.

In general, a bacterium's genetic background can influence the fitness costs of antibiotic resistance. For example, Vogwill and colleagues (2016) examined the costs of rifampicin-resistance mutations in the gene *rpoB* across several *Pseudomonas* species. They found that some mutations vary in their fitness effects across backgrounds, and these costs correlate with transcriptional efficiency. Thus, the same *rpoB* mutation can differentially affect transcriptional efficiency depending on the genetic background, and these idiosyncratic effects in turn lead to heterogeneity in costs. This work evaluated genetic-background effects across a fairly broad phylogenetic scale, while focusing on mutations in a single gene. One can also ask whether genetic background affects the fitness cost of resistance even among recently diverged clones of a single species, and for resistance that has evolved through more diverse mutational pathways.

To address these issues, we evaluated the competitive fitness in the absence of drugs of tetracycline-resistant clones that evolved from several different *E. coli* backgrounds, which

previously diverged during a long-term evolution experiment (LTEE). We ask several questions. First, is there a fitness cost to resistance? Second, is the cost greater for mutants that evolved higher levels of resistance (Figure 3.1A)? Third, do fitness costs vary in an idiosyncratic manner that does not depend on the level of resistance achieved (Figure 3.1B)? Fourth, if there is indeed idiosyncratic variation among lines in the cost of resistance, what factors contribute to that variability? On balance, we found that the resistant lines are indeed less fit than their sensitive counterparts. These fitness costs do not correlate with the level of resistance achieved, nor do they vary among the several genetic backgrounds that we examined (Card et al. 2019). Some variation in cost of resistance occurs even among different mutations in the same gene, on the same genetic background, and conferring the same phenotypic resistance. In any case, further research on the fitness effects of antibiotic resistance should be pursued because of its potential implications for public health and patient treatment.

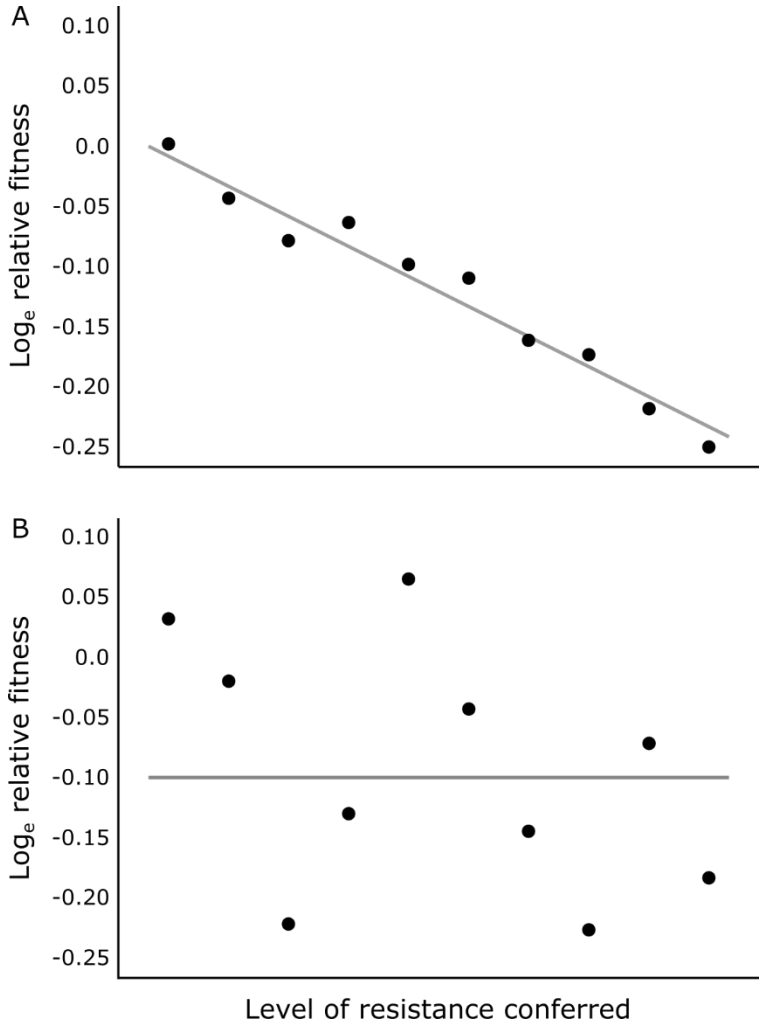

Figure 3.1. Schematic illustration of fitness effects of antibiotic resistance mutations under two scenarios. (A) Tradeoff model, in which the fitness of a resistant line, when measured in the absence of drugs, is negatively correlated with the level of resistance conferred by its mutations. (B) Idiosyncratic model, in which the fitness of resistant lines varies for reasons unrelated to the level of resistance. This idiosyncratic variation might, in principle, reflect differences between genetic backgrounds, mutations in different target genes, different alleles in the same target gene, secondary mutations, and epistatic interactions between the resistance mutations and their genetic backgrounds. The fitness of each resistant line is expressed relative to its sensitive counterpart. A log-transformed relative fitness of 0 indicates no fitness cost associated with resistance, while values below and above 0 represent fitness costs and benefits, respectively.

Materials and Methods

Experimental conditions and bacterial strains

The LTEE has been described in detail elsewhere (Lenski et al. 1991; Lenski 2017). In brief, 12 replicate populations of *E. coli* were founded from a common ancestral strain, called REL606 (Daegelen et al. 2009). These populations have been propagated for over 32 years and 73,000 generations by daily 100-fold dilutions in Davis Mingioli minimal medium supplemented with 25 μg/mL glucose (DM25).

In this study, we examined the competitive fitness of tetracycline-resistant mutants that evolved from the LTEE ancestor and clones sampled from four LTEE populations (denoted Ara–5, Ara–6, Ara+4, and Ara+5) after 50,000 generations. Specifically, we analyzed 4 mutants that independently evolved from the ancestral background, and 3 mutants that evolved from each derived background, for a total of 16 mutants (Appendix A Table 5). We also used three clones as common competitors: REL607, REL10948, and REL11638. REL607 is a spontaneous Ara^+ mutant of REL606, the LTEE ancestor (Lenski et al. 1991). REL10948 is an Ara^- clone isolated from the Ara–5 population at 40,000 generations, and REL11638 is a spontaneous Ara^+ mutant of that clone (Wiser et al. 2013; Lenski et al. 2015). The Ara marker is selectively neutral in the glucose-limited medium; it serves to differentiate competitors during fitness assays because the Ara^- and Ara^+ cells form red and white colonies, respectively, on tetrazolium-arabinose (TA) agar. We used REL607 as the common competitor for REL606 and the four tetracycline-resistant clones derived from it. The 40,000-generation clones served as common competitors for the four 50,000-generation parental clones and twelve resistant mutants that evolved from them; using these common competitors ensured that the differences in fitness were not so large that their densities would fall below the detection limit during the fitness assays.

Fitness assays

Assays were performed in the absence of antibiotics to assess the relative fitness of drug-resistant mutants and their susceptible counterparts. Fitness was measured in an environment identical to that of the LTEE, except the medium contained 250 μg/mL glucose (DM250). Resistant mutants and their sensitive parents each competed, in paired assays, against the same common competitor with the opposite Ara-marker state (Figure 3.2). To set up each competition assay, the competitors were revived from frozen stocks, and they were separately acclimated to the culture medium and other conditions over two days. The competitors were then each diluted 1:200 into fresh medium, and a sample was immediately plated on TA agar to assess their initial densities based on colony counts. The competition cultures were then propagated for 3 days, with 1:100 dilutions each day in fresh medium. At the end of day 3, a sample was plated on TA agar to assess the competitors' final densities. We quantified the realized growth rate of each competitor based on its initial and final density and the net dilutions imposed (Lenski et al. 1991). We then calculated relative fitness as the ratio of the realized growth rate of the clone of interest (either a resistant clone or its sensitive parent) to that of the common competitor. Lastly, the fitness of a resistant mutant in each assay was normalized by dividing it by the relative fitness of the paired assay obtained for its parental strain. We performed a total of 80 pairs of fitness assays (160 competitions in total) to produce 5 replicate estimates of the fitness of each of the 16 tetracycline-resistant mutants relative to its sensitive parent. The relative fitness values were $\log_e$-transformed before the statistical analyses reported in the Results below.

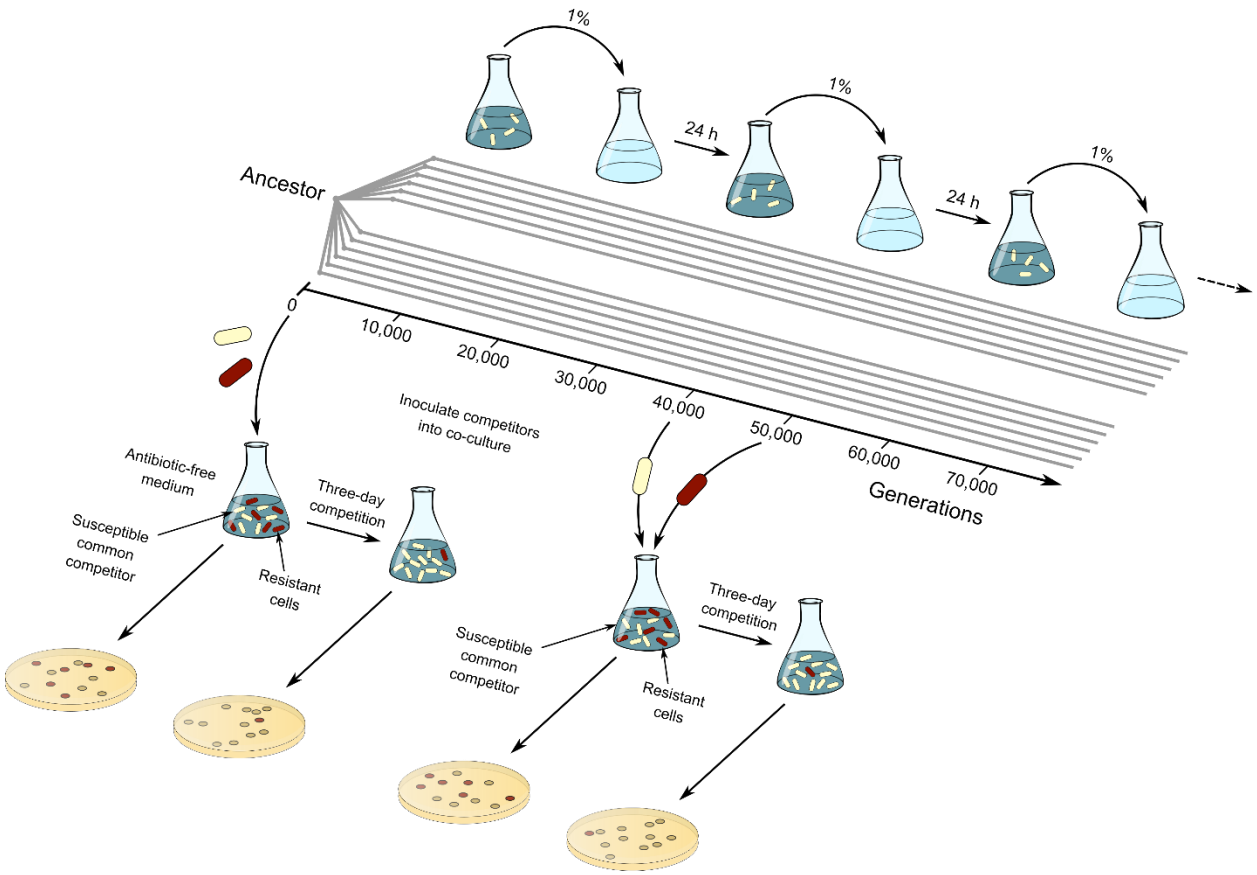

Figure 3.2. Schematic illustration showing the derivation of the strains used in this study and the methods employed to measure the fitness of resistant lines relative to their sensitive parents. Twelve initially identical *E. coli* populations were founded from the same ancestral strain to start the LTEE. A genetic marker distinguishes two sets of six populations each. These populations have evolved for >73,000 generations with daily transfers in a minimal glucose medium. In paired assays, we examined the fitness of tetracycline-resistant mutants (shown in red) that evolved either from the LTEE ancestor or one of four clones sampled at generation 50,000 by competing them against marked susceptible competitors (shown in yellow). We used REL607 as the common competitor for the LTEE ancestor and resistant lines evolved from it, and two 40,000-generation clones (see Materials and Methods) as common competitors for the derived parental strains and their evolved resistant lines. After acclimation to the culture conditions, competitors were mixed at an equal volumetric ratio in a common medium. These cultures were propagated for three days in the absence of tetracycline by serial 1:100 transfers. We quantified each competitor's realized growth rate from the initial and final densities after plating on TA agar, taking into account the net dilution over the three days. These realized growth rates were then used to calculate the fitness of a resistant line relative to its sensitive parent (see Materials and Methods).

Results

Tetracycline-resistant lines have reduced fitness in the absence of the antibiotic

We ask first whether tetracycline resistance is costly, on average, in the absence of the drug. The grand mean of the $\log_e$-transformed fitness of the 16 resistant lines relative to their paired parental strains is -0.0771, indicating that the resistant mutants grow ~7.7% more slowly than their sensitive counterparts during head-to-head competitions with a common competitor. This value differs significantly from the null hypothesis that the resistant lines and their sensitive parents are equally fit (t_s = 2.9973, df = 15, one-tailed p = 0.0045).

Cost of resistance varies among resistant mutants

We measured the relative fitness of each resistant line with 5-fold replication. This replication allows us to test whether the variation in fitness among the 16 tetracycline-resistant lines is simply measurement noise or, alternatively, reflects genetic variation in the cost of resistance. Table 3.1 shows the analysis of variance (ANOVA). The variation among the 16 lines is about 10-fold greater than expected from the variation between replicate assays performed on the same line ($F_{15,64}$ = 10.34, $p << 0.0001$).

Table 3.1. ANOVA on the log-transformed fitness estimates of 16 tetracycline-resistant lines, each measured relative to its sensitive parent.

Source	SS	df	MS	*F*	*p*
Line	0.7948	15	0.0530	10.3384	<< 0.0001
Error	0.3280	64	0.0051		
Total	1.1228	79			

There are many possible reasons why the cost of resistance might vary including mutations in different genes, different alleles even in the same gene, different genetic backgrounds, epistatic interactions between mutations and genetic backgrounds, and so on. In the sections that follow, we examine various possibilities.

Possible reversions of unstable mutations do not explain the variation in fitness cost

We previously sequenced the complete genomes of the 16 resistant lines, and we compared them to their parental strains to identify the mutations responsible for their resistance (Chapter 2). Two lines had no identifiable mutations (Ara–5-1 and Ara+5-1), even though they had increased phenotypic resistance relative to their respective parent strains (Card et al. 2019). This discrepancy suggested that these two resistant lines may have had unstable genetic changes, which might have reverted prior to the genomic analysis and our fitness assays. Potentially unstable mutations include changes in the copy number of oligonucleotide repeats and gene amplifications. We repeated the ANOVA, except excluding the two resistant lines without identifiable mutations. The variation in the cost of resistance remains highly significant in the 14 lines with known, stable mutations ($F_{13,56} = 10.15$, $p << 0.0001$).

Level of phenotypic resistance does not explain the variation in fitness cost

All of the resistant lines evolved during a single round of exposure to tetracycline. However, they vary in the resulting minimum inhibitory concentration (MIC) that they achieved. They also vary in the magnitude of the increase in their MICs relative to their parental strains, which also varied in their MICs. It is possible that mutations that provide greater resistance have higher fitness costs (Fig. 3.1A). To test that possibility, we examined the correlation between the log-transformed

fitness values of the 14 resistant lines and their log-transformed MICs, as previously reported (Card et al. 2019). However, the correlation is not significant; in fact, it is weakly positive (r = 0.1682, two-tailed p = 0.5655). We also computed the correlation between the log-transformed fitnesses and log-transformed fold-increases in resistance, but again the correlation is weakly positive and not significant (r = 0.1002, two-tailed p = 0.7332). Thus, we find no evidence that the variation in the fitness cost of tetracycline resistance is related to the level of resistance conferred by the underlying mutations.

Genetic background does not explain the variation in fitness cost

The 14 tetracycline-resistant mutants with identifiable mutations evolved on five different genetic backgrounds. We asked whether the average cost of resistance differed between the backgrounds. In this case, the ANOVA tests whether the variance in the average cost of resistance for mutants derived from different backgrounds is greater than expected given the variance in the average cost for mutants derived from the same background. This analysis indicates no significant effect of the genetic background on the cost of resistance ($F_{4,9}$ = 0.47, p = 0.7570).

Idiosyncratic differences between mutant lines in the cost of resistance

Neither the level of phenotypic resistance conferred by mutations nor the genetic background in which they arose explains the substantial variation in the fitness effects of tetracycline resistance. Instead, it appears there are idiosyncratic differences in the fitness costs associated with different resistance mutations (Fig. 3.1B). These idiosyncratic effects could, in principle, reflect mutations in different genes, different mutations in the same target gene, secondary mutations that might have hitchhiked with the mutations conferring resistance, or epistatic interactions between any of

these new mutations and the existing mutations that distinguished the different parental strains. Without a much larger number of resistant lines, it is not possible to rigorously disentangle these various sources of idiosyncratic fitness costs. However, by examining and contrasting specific cases, we are able to shed light on some of the sources of these differences.

Two resistant clones, Ara+4-3 and Ara+5-2, have fitness costs that are very similar to one another, but more than double the cost of any of the other 12 resistant mutants (Figure 3.3). Yet these two cases occurred on different genetic backgrounds and have different mutations. Ara+4-3 has mutations in *hns*, which encodes a histone-like global regulator, and *lpcA*, which encodes a phosphoheptose isomerase; Ara+5-2 has a single mutation in *ompF*, which encodes an outer-membrane porin (Chapter 2). We asked whether these two extreme cases are solely responsible for the heterogeneity in fitness costs by performing an ANOVA that excludes them. The variation in fitness costs among the other 12 clones is reduced, but it nonetheless remains highly significant ($F_{11,48} = 4.44$, $p = 0.0001$).

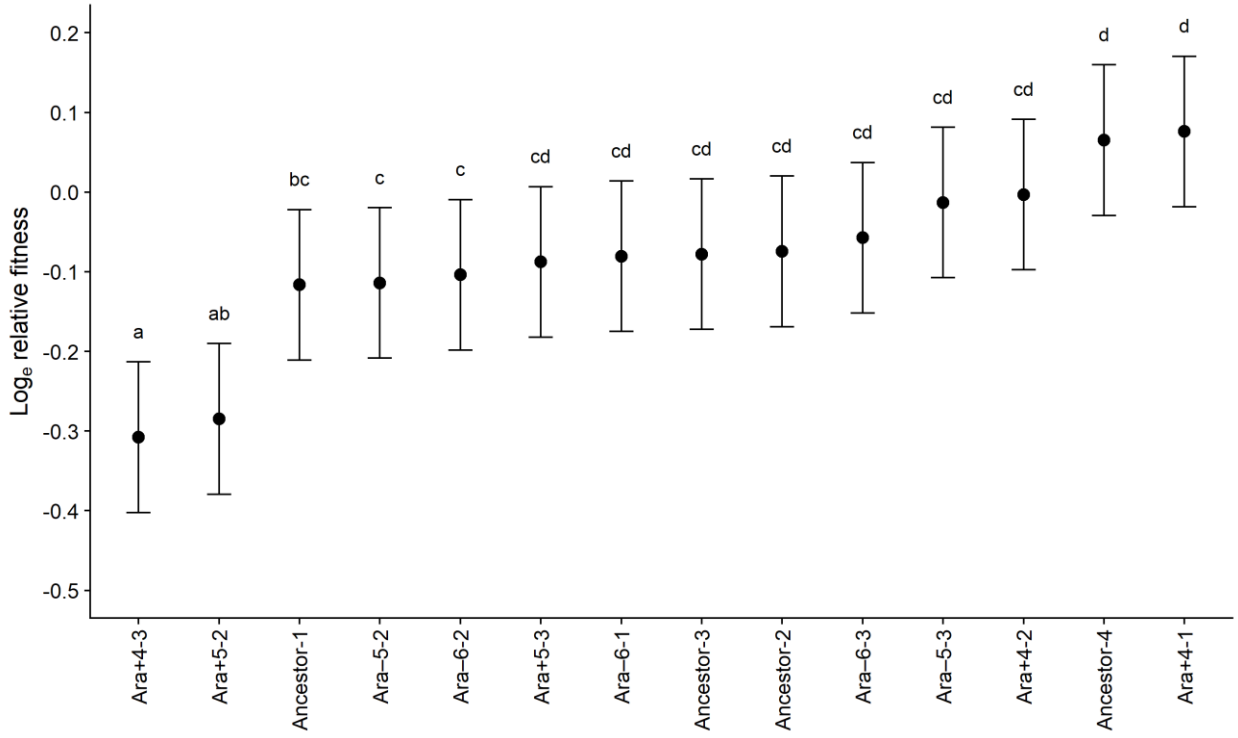

Figure 3.3. Fitnesses of 14 tetracycline-resistant mutants relative to their parental strains. The mutants are arranged from lowest to highest fitness. Each symbol shows the mean $\log_e$-transformed fitness, based on 5-fold replication of paired fitness assays. Error bars show 95% confidence limits calculated using the *t*-distribution with 4 df and the pooled standard deviation estimated from the ANOVA (Table 1). Letters above the error bars identify mutants with relative fitnesses that are not significantly different, based on Tukey's "honestly significant difference" test for multiple comparisons.

Nine of the 14 resistant clones have a single mutation each, while four of them (Ara–5-2, Ara–6-2, Ara+4-3, Ara+5-3) have two mutations, and another (Ancestor-2) has three mutations (Chapter 2). It is reasonable to imagine that in each clone one mutation confers the drug resistance, while the others merely hitchhiked with the resistance mutation. Such hitchhikers might include deleterious mutations that reduce fitness. Therefore, we compared the fitness costs for the resistant clones with and without secondary mutations. The average fitness cost of the clones with multiple mutations is higher (13.8%) than the average of those with single mutations (5.5%), but the

difference is only marginally nonsignificant given the small number of clones in each group and the high variation within each group (Welch's *t*-test, $t_s = 1.4751$, 9.3 df, one-tailed $p = 0.0866$).

It is also interesting to compare the four resistant clones derived from the ancestral LTEE background. All four resistant clones evolved the same level of phenotypic resistance, with MICs that are 4-fold higher than their parental strain (Card et al. 2019). Moreover, all four have mutations affecting the same two-component system that regulates the synthesis of outer-membrane proteins: one clone (Ancestor-1) has a 11-bp deletion in *envZ*, which encodes the sensory histidine kinase; the others (Ancestor-2, Ancestor-3, Ancestor-4) have nonsynonymous mutations in *ompR*, which encodes the DNA-binding response regulator. Even with these striking phenotypic and genetic similarities, an ANOVA shows significant heterogeneity in the fitness of these clones ($F_{3,16} = 4.50$, $p = 0.0180$). We can also compare only Ancestor-3 and Ancestor-4 (each having a single mutation in *ompR* and no other mutation), and the variation in fitness remains significant ($F_{1,8} = 5.71$, $p = 0.0439$). These results show that different mutations in the same target pathway, and even different alleles in the same gene, can lead to different fitness costs of drug resistance.

Discussion

In previous work, we examined the role that genetic background plays in both the phenotypic and genotypic evolution of antibiotic resistance. First, we examined the potential of several different LTEE backgrounds to evolve increased resistance to several antibiotics. We found that evolvability was idiosyncratic with respect to the parental genotype, such that resistance was more constrained in some backgrounds than in others (Card et al., 2019). Genetic differences will accumulate between populations, even if they evolve in the same permissive environment. These differences can unpredictably alter their ability to respond evolutionarily when challenged with antibiotics.

Second, we sequenced the complete genomes of some of these resistant mutants and assessed whether the different initial genotypes took similar or divergent mutational paths to increased resistance (Chapter 2). Again, we found that the initial genetic background is important. On average, the replicate lines that evolved from the same founding genotypes had more gene-level mutations in common than lines derived from different founding genotypes.

The aim of this study was to examine whether and how the genetic background influences the fitness effects of resistance mutations in the absence of antibiotic. In particular, we examined the fitness costs of tetracycline resistance in 16 lines that evolved from five sensitive parental backgrounds. We found that the resistant lines are, on average, less fit than their sensitive counterparts in the absence of the antibiotic. This result is not surprising, given that resistance mutations often disrupt the normal function of metabolic or physiological processes, or impose energetic demands that reduce growth and competitiveness (Andersson and Hughes 2010). We also observed highly significant variation among the resistant lines in their fitness costs (Table 3.1). This variation remained substantial (Figure 3.1) even after we excluded two strains without identified mutations (Chapter 2). These two strains exhibited phenotypic resistance in our earlier work (Card et al. 2019), but that resistance might have been conferred by unstable genomic changes, such as gene amplifications or frameshift mutations in homopolymeric tracts that can cause "phase variation" (Moxon et al. 1994). If so, these unstable changes could have reverted prior to the genomic analysis and the competition assays that we performed.

We then addressed two broad possibilities regarding the variation in fitness cost between the 14 lines with known, stable mutations. First, we asked whether there is a relation between a line's phenotypic resistance and its fitness cost, such that mutations that confer greater resistance are more costly (Figure 3.1A). A meta-analysis of fitness costs across several species and drug

classes by Melnyk and colleagues (2015) supported this association, and the authors suggested it could be understood from evolutionary and mechanistic perspectives. Imagine a population that is well-adapted to one environment and hence near a local fitness optimum. If the environment changes, such as with the addition of an antibiotic, then the population may evolve toward a different optimum through the substitution of new mutations. Mutations of large effect will bring the population closer to this new optimum than mutations of small effect. However, if the environment later reverts to its previous state, then populations that substituted the large-effect mutations will be further from their previous optimum than those populations that acquired small-effect mutations. From a mechanistic standpoint, the increased expression of efflux pumps or drug targets diverts resources from other cellular processes. Also, resistance mutations that change evolutionarily conserved proteins are more likely to disrupt their functions than improve them. In our study, however, there was no significant association between fitness costs and the level of resistance conferred by mutations, whether on an absolute basis or relative to the parent strain.

The second broad possibility is that the fitness costs of resistance can vary for reasons unrelated to the level of resistance conferred (Figure 3.1B). There are several potential reasons for such idiosyncratic variation. One possibility is that the same resistance mutation may have different fitness costs in different genetic backgrounds. In *Campylobacter jejuni*, for example, a C257T mutation in the gene *gyrA* confers fluroquinolone resistance. When fluroquinolone-resistant and -susceptible strains were inoculated separately into chickens, they colonized equally well and each persisted even in the absence of drug exposure (Luo et al. 2005). However, when resistant and sensitive strains were co-inoculated, the resistant variants often prevailed. Further work indicated that this particular *gyrA* mutation was beneficial in some genetic backgrounds, even in the absence of antibiotic, and costly in others (Luo et al. 2005). In our study, by contrast,

the variation in fitness costs among strains was not explained by genetic-background effects, but instead involved several other factors.

One such factor is that resistance mutations can occur in different genes, which can lead to different fitness costs. In this study, the relative fitnesses of clones Ara+4-3 and Ara+5-2 were significantly lower than the other 12 strains. Ara+4-3 is the only line with mutations in either *lpcA* or *hns*. Mutations in the former gene have been shown to confer tigecycline resistance in *E. coli* through modifications to the lipopolysaccharide biosynthesis pathway, and these mutations have moderate fitness costs in vitro (Linkevicius et al. 2013, 2016). The latter gene encodes the global transcriptional regulator H-NS, and mutations in it affect acid resistance (Giangrossi et al. 2005), the modulation of osmotic stress (Lucht et al. 1994), and several other important cellular processes. Changes to this regulator's structure and function might therefore have large fitness costs via widespread pleiotropic effects. The Ara+5-2 clone evolved a 9-bp insertion in *ompF*, which encodes the sole major porin in the LTEE ancestral strain (Crozat et al. 2011); this mutation presumably reduces the cell's antibiotic uptake, but at the expense of acquiring nutrients (Ferenci 2005; Phan and Ferenci 2017). Thus, resistance mutations that affect different cellular pathways and functions can have variable fitness costs, a finding that is consistent with many other studies (Vogwill and MacLean 2015).

Another factor is that mutations in different genes that are part of the same physiological pathway may confer similar resistance levels but have different fitness costs. In our study, four tetracycline-resistant lines derive from the same LTEE ancestor: one had a mutation in *envZ*, while the other three had mutations in *ompR*. These genes encode proteins that comprise a two-component regulatory system that regulates cellular responses to osmotic stress, and which affects antibiotic resistance through altered expression of the major porin OmpF (Chakraborty and

Kenney 2018; Choi and Lee 2019). We observed significant heterogeneity in fitness even among these lines, implying that different changes within this one pathway can impose unique burdens. The evolution of carbapenem resistance in *E. coli* K12 can also occur by mutations in this same two-component system, again with variable fitness costs (Adler et al. 2013). In their study, Adler and colleagues (2013) found that *envZ* mutants had no measurable loss of fitness in the absence of antibiotic, whereas *ompR* mutations suffered a large cost. By contrast, in our study the *envZ* mutation was more costly, which may reflect differences between the *E. coli* K12 and B strain backgrounds or the use of different culture media.

Yet another factor is that different mutations in the same gene can have different costs. The evolution of rifampicin resistance, for example, typically occurs via mutations in several canonical regions of *rpoB*, which encodes the β subunit of the RNA polymerase (Reynolds 2000; Ahmad et al. 2002; Barrick et al. 2010; MacLean et al. 2010; Hall and MacLean 2011). Different alleles have widely varying costs that impact their competitive ability and, moreover, affect the dynamics of subsequent compensatory evolution (Barrick et al. 2010). In our study, two clones derived from the same parent had different nonsynonymous mutations in *ompR*. Both conferred the same level of resistance to tetracycline, but they had different fitness costs in the absence of the drug. Such differences can have important public-health consequences, because a resistant lineage's competitive fitness in the absence of antibiotics is critically important for its long-term persistence in a heterogeneous environment.

More generally, we argue that further studies of the fitness costs of antibiotic resistance are needed, because this phenomenon can inform treatment strategies. Standard clinical practice calls for aggressive treatment to eliminate an infecting pathogen before it has time to evolve resistance (Craig 2001; Drlica 2003; Mehrotra et al. 2004; Abdul-Aziz et al. 2015; Hansen et al. 2020). This

approach is likely beneficial if the population is composed of only drug-susceptible cells. However, if the pathogen population already contains drug-resistant cells, then aggressive treatment may promote the proliferation of the resistant population by eliminating susceptible competitors. To address this problem, an alternative treatment strategy was recently proposed (Day and Read 2016; Hansen et al. 2020). Given that resistance often imposes a cost, resistant variants might be at a competitive disadvantage relative to their sensitive counterparts at low antibiotic concentrations that nonetheless reduce the growth rate of both types. If so, the resulting competition might slow the resistant population's expansion long enough for the immune system to clear the infection.

Both mathematical models (Hansen et al. 2017) and experiments with the LTEE ancestor (Hansen et al. 2020) have shown that competition between susceptible and resistant populations, mediated in part by fitness costs, can indeed slow the time to treatment failure. However, these expectations are complicated by (i) the potential for higher mutation rates, and (ii) idiosyncratic fitness costs that depend on the specific resistance mutation and its interaction with the genetic background in which it occurs. Regarding the first complication, Hansen and colleagues (2020) used a strain with a low mutation rate (Sniegowski et al. 1997). However, six LTEE populations evolved hypermutability by generation 50,000 (Tenaillon et al. 2016), and mutation rates vary in some pathogens by orders of magnitude (Hughes and Andersson 2017). With respect to the second complication, the competitive release of a resistant population should occur faster when fitness costs are lower. Given that the cost may depend on the particular mutation and its genetic background, the time to treatment failure is harder to predict. We think that these issues and their relevance for treatment options are important avenues for future research.

CHAPTER 4: Conclusions and future outlook

Summary

Chapter 1 examined how readily bacteria could overcome prior losses of intrinsic resistance through subsequent evolution when challenged with antibiotics. My co-authors and I focused on the role that genetic background played in this process, with particular attention to the interplay between repeatability and contingency in the evolutionary process (Card et al. 2019). We addressed these questions using *Escherichia coli* strains from the long-term evolution experiment (LTEE) that independently evolved for multiple decades in an environment without antibiotics. We first confirmed that these LTEE-derived strains had typically become more susceptible to various drugs during this period of relaxed selection. We then subjected the strains to a range of concentrations of these same drugs. We found that evolvability was idiosyncratic with respect to initial genotype, such that resistance was more constrained in some backgrounds than in others. These results show that replicate populations accumulate genetic differences, even as they evolve in permissive environments, that affect their ability to adapt when challenged with antibiotics.

In that study we focused on empirical trends in the evolution of phenotypic resistance and the effects of genetic background on those trends. Chapter 2 asked whether a strain's background also influences the genetic basis of resistance. Do lineages founded by different genotypes take parallel or divergent mutational paths to increased resistance? My co-authors and I addressed this question by sequencing the complete genomes of antibiotic-resistant clones that evolved from several parental genotypes during the earlier experiment described above. Using a statistical approach developed to compare mutational targets in populations that evolved under different thermal regimes (Deatherage et al. 2017), we demonstrated that genomic evolution was specific to antibiotic treatment and that particular gene-level mutations were associated with each drug. These results were expected given that antibiotic resistance tends to evolve through ordered mutational

pathways in a limited set of genes (Toprak et al. 2012; Baym et al. 2016), but they served to validate our statistical approach. Next, we examined the specificity of genomic evolution with respect to genetic background. We found that, on average, replicate lines evolved from the same founding genotypes had more mutations in common at the gene level than did lines evolved from different founding genotypes. Taken together, the results from Chapters 2 and 3 demonstrate that a lineage's evolutionary history can alter both its phenotypic and genotypic paths to antibiotic resistance.

Chapter 3 examined whether the fitness cost of tetracycline resistance is conserved across different LTEE backgrounds. Accordingly, my co-authors and I quantified costs by comparing resistant mutants to their sensitive parents in the absence of this drug. On average, the resistant lines had reduced fitness, and there was significant among-line heterogeneity in fitness costs. However, this variation did not correlate with the level of resistance conferred by the mutations, nor did the cost vary significantly across the different genetic backgrounds tested. Instead, these idiosyncratic differences can be explained by mutations in different genetic targets, mutations in different genes that comprise the same physiological pathway, and even different alleles of the same gene. We conclude in this Chapter that fitness costs often depend on idiosyncratic effects of particular resistance mutations and the genetic backgrounds in which they arise.

The multifactorial nature of antibiotic-resistance evolution

Antibiotic resistance is a growing public-health crisis. Efforts to control resistance would benefit from an improved ability to forecast when and how it will evolve. However, to effectively predict evolution, we must integrate information about multiple factors, including a bacterium's evolutionary history. My dissertation centers on the effects of different genetic backgrounds on

the evolution of antibiotic resistance and its fitness costs. Other factors, including the mutation supply rate and population bottlenecks, can also affect evolutionary trajectories and complicate our ability to predict resistance evolution. I discuss these additional factors and their medical implications in the following sections.

Mutational supply rate

The adaptive potential of a population depends upon the number of potential evolutionary paths to improvement and the likelihood of finding each of those paths. Antibiotic resistance can arise through mutations that change gene regulation, cellular permeability and efflux, or the structure of the drug target (Blair et al. 2015). However, not all paths are equal phenotypically because these mechanisms can confer different levels of drug resistance; mutations that reduce drug accumulation in the cell, for example, tend to be less advantageous than those which alter drug targets (Hughes and Andersson 2017). Also, the rate at which these mutations occur depends on both population size and an organism's mutation rate, which together are sometimes called the "mutation supply rate".

The effects of population demography and mutation rates on adaptive potential are not well understood. This situation reflects challenges presented by the great variability of pathogen populations. Clinically relevant population sizes can span many orders of magnitude. Many newborn deaths worldwide have been caused by sepsis (United Nations Children's Fund 2009), and those infections might be driven by bacterial loads as low as 40 cells per milliliter (Stranieri et al. 2018); tuberculosis infections can reach 10^5 cells per milliliter of sputum (Palaci et al. 2007); and titers from meningitis patients can exceed 10^9 cells per milliliter (Bingen et al. 1990; Hughes and Andersson 2017). On balance, large populations should be more evolvable because they are

more likely to sample rare mutations that provide high levels of resistance, although this expectation is complicated by differences in mutation rates between populations.

In vitro mutation rates are typically characterized by the frequency at which detectable mutants arise in a population under selection with a given drug concentration. However, the rate of those mutations with sufficient resistance is likely to vary with the drug concentration (see Chapter 1 Results and Discussion), making this parameter challenging to quantify. For example, the so-called "mutant selection window" (MSW) extends from the minimum drug concentration required to inhibit susceptible variants in the population up to that required to inhibit growth of resistant variants (Drlica 2003; Drlica and Zhao 2007). Moreover, increased antibiotic resistance can arise through mutations in different genes, and these mutations may provide different levels of resistance. At low antibiotic concentrations, mutations in any of these genes can effectively protect the bacteria. However, as the antibiotic concentration rises, the number of potential mutations that confer sufficient resistance (i.e., the effective mutational target size) should decrease. One might therefore overestimate a bacterium's mutation rate at the lower boundary of the MSW and underestimate this parameter at the upper boundary (Martínez and Baquero 2000).

Bacterial populations can evolve elevated mutation rates, typically ranging from 10- to 1,000-fold higher than their wild-type counterparts (Macía et al. 2005; Wielgoss et al. 2013). Hypermutable mutants have a higher probability of discovering rare beneficial alleles compared to nonmutator strains, and therefore these mutants may be favored, at least indirectly (Taddei et al. 1997; Tenaillon et al. 2001; Lenski 2004), in novel or rapidly changing environments (Sniegowski et al. 1997; Jayaraman 2011). For instance, *P. aeruginosa* infections are a major cause of morbidity and mortality in individuals with cystic fibrosis (CF) (Smith et al. 1996). Lung defenses against bacterial colonization primarily occur through ciliary action and neutrophilic

phagocytosis, both of which are limited by the viscous and hyperosmotic environment of the CF lung, resulting in long-term infection (Oliver et al. 2000). This environment, along with recurrent and prolonged drug therapy, can select for *P. aeruginosa* lineages with elevated rates of mutation, in some cases 100-fold higher than the ancestral wild-type (Govan and Deretic 1996; Oliver et al. 2000; Macía et al. 2005; AbdulWahab et al. 2017; Martin et al. 2018). Moreover, these lineages' can have mutation rates that are orders of magnitude higher than the opportunistic, short-term infections of the bloodstream, further suggesting that chronic infections of the lung environment impose repeated, strong selection on *P. aeruginosa* populations (Oliver et al. 2000), which generates second-order selection for higher mutation rates.

Population size is another factor in the relation between mutation supply rate and resistance potential. For example, Adler and colleagues (2013) examined the effect of an extended-spectrum β-lactamase (ESBL)-producing plasmid on the evolution of carbapenem resistance in *E. coli*. They found that the plasmid can increase the mutation rate to ertapenem specifically by 1800- to 6000-fold with an associated rise in resistance levels. They examined whether this observation could be explained by an increased *overall* mutation rate caused by the plasmid's presence. However, mutation rates to a separate antibiotic (i.e., rifampicin) were nearly equivalent between strains with and without the plasmid, indicating that overall mutation rates were not altered. Instead, the authors demonstrated that the ESBL-producing bacteria survived at larger population sizes, and therefore were able to generate more resistance mutations, compared to cells that did not harbor the plasmid.

The studies mentioned above underscore the importance of exploring the roles of population size and mutation rate in the evolution of drug resistance. However, it is not well understood how genetic background interacts with population size and mutation rate to influence evolutionary trajectories to resistance.

Future directions

In my future work, I plan to use *E. coli* strains from the LTEE to examine the contributions of genetic background, population size, and mutation rate to resistance evolvability. The LTEE growth medium contains glucose as the limiting nutrient, along with abundant citrate that is present as an iron-chelating agent. *E. coli* cannot normally use citrate as an energy source under the oxygen-rich conditions of the experiment. However, after ~31,000 generations the population designated Ara–3 evolved the capacity to grow on this substrate (Cit^+) (Blount et al. 2008). The Cit^+ trait caused a several-fold increase in population size. Additionally, the population had the low, ancestral mutation rate until a hypermutator phenotype evolved around 35,000 generations (Blount et al. 2012). As this population's history includes changes in both population size and mutation rate, one could investigate the resistance potential of strains isolated from across this evolutionary time series.

By generation 31,000, the Ara–3 population had evolved nonsynonymous point mutations in genes encoding DNA topoisomerase, efflux pump regulators, and multiple ribosomal proteins (Tenaillon et al. 2016). These mutations could conceivably influence resistance evolution to various drug classes. Nevertheless, rare pathways leading to high-level resistance may become more accessible due to the larger population size after Cit^+ evolution. One objective of this study could be to assess whether an increase in evolutionary potential due to the larger population size outweighs, or is outweighed by, any potentiating or constraining effects of changes in the genetic background (Card et al. 2019). Similarly, resistance potential should also increase when this population evolved hypermutability. However, this response becomes harder to predict for later-generation strains that, owing to hypermutability, have accumulated hundreds of background mutations that may idiosyncratically interact with resistance mutations.

The fitness of a resistant bacterium, including relative to its sensitive counterparts in the absence of antibiotics, can affect its evolutionary success over time (Chapter 3). For example, a mutation that confers less resistance may ultimately prevail over one that confers greater resistance if the latter carries a higher fitness cost than the former (Andersson and Hughes 2010; Melnyk et al. 2015). Discovery of more resistant variants in the short-term may not necessarily equate to their success over longer timescales. To examine this possibility, one could compete mutants against each other and their wild-type progenitors in continuous culture devices (i.e., morbidostats) (Toprak et al. 2012; Gopalakrishnan et al. 2019; Kaznatcheev et al. 2019). Morbidostats steadily increase the drug concentration in a growth medium as the population under study evolves resistance (Toprak et al. 2012; Gopalakrishnan et al. 2019). The evolution of resistance will therefore occur over time with increasing antibiotic concentrations and by the sequential substitution of multiple mutations. One could then sample the evolving populations periodically and perform metagenomic sequencing to examine how the frequency of competitors change over time.

One might also examine the individual effects of population size and mutation rate on evolvability by using a *ΔmutS* variant of the LTEE ancestor. One could compare this hypermutable strain against its wild-type counterpart using the experimental approach as described. Population sizes could be directly controlled by altering the concentration of glucose in the medium. In any case, the ability to predict a pathogen's resistance potential is an important public health goal. Predictions should ideally combine information about a bacterium's genetic context, mutation rate, population size, and the relative fitness of resistance mutations. In this study, one could examine the interaction of these factors on resistance evolution using a well-characterized experimental population of *E. coli.*

Population bottlenecks

As discussed in this Chapter, and throughout this dissertation, evolutionary trajectories to antibiotic resistance can depend upon several factors including the supply of beneficial mutations (dependent upon the mutation rate and population size under selection); a bacterium's genetic context; and the fitness of resistance mutations across varying drug concentrations. In this section, I discuss one additional factor with clinical relevance: the size of the transmitted population that establishes new infections (Hughes and Andersson 2017).

Population bottlenecks play important roles in disease dynamics and the evolution of antibiotic resistance in pathogen populations. The sexual transmission of HIV-1 can occur through stringent bottlenecks as low as one virion particle (da Silva 2012), and in the case of bacterial infections, clonal expansions can drive the spread of tetracycline-resistant Group B *Streptococcus* (Da Cunha et al. 2014). Bottlenecks also reduce genetic diversity, and they may reduce fitness during transmission events if less-fit genotypes survive a bottleneck event and displace their more-fit counterparts (Moxon and Kussell 2017).

Given that genetic variation is the substrate that fuels natural selection, bottlenecks may also impose indirect selection that favors mechanisms that can rapidly regenerate diversity (Moxon and Kussell 2017). Two important mechanisms include hypermutability (discussed above) and phase variation. Phase variation occurs when gene expression is altered, in a reversible manner, by insertions and deletions in short genomic repeats, homologous or site-specific recombination, or epigenetic modifications (Moxon et al. 1994; van der Woude and Bäumler 2004; Moxon and Kussell 2017). Phase variable loci in pathogens can promote virulence by altering cell-surface molecules that modulate host-microbe interactions, and they may also facilitate the maintenance of some antibiotic-resistance determinants as well (Jiang et al. 2019).

Mutation rates, the relative fitness of antibiotic resistance mutations, and population bottlenecks can simultaneously affect evolutionary trajectories of antibiotic resistance. For instance, high-level fluoroquinolone resistance in *E. coli* occurs through the sequential substitution of mutations in several different genes (Lindgren et al. 2003). The first mutation to be selected in this pathway generally occurs in *gyrA*, which encodes the DNA topoisomerase II drug target. Mutations in *gyrA* confer low levels of fluoroquinolone resistance with minimal associated fitness costs (Huseby et al. 2017). However, the second mutation in this sequence is more variable. Mutations in the regulator of the major efflux pump AcrAB–TolC that confer resistance occur at a high rate of 10^{-6} per base pair per generation, whereas mutations in the secondary drug target topoisomerase IV, *parC*, occur at the comparatively low rate of 10^{-9} per base pair per generation. This difference in mutation rate can be understood in terms of different mutational target sizes (Hughes and Andersson 2017; Huseby et al. 2017).

Huseby and colleagues (2017) found that changes in efflux pump regulation predominate when fluoroquinolone resistance is selected for in vitro, in contrast to clinical strains in which second-step mutations often occur in *parC*. The authors hypothesized that this discrepancy might be explained by differences in fitness costs, with *parC* mutations having smaller costs than mutations that upregulate drug efflux. To test their hypothesis, they challenged populations (already containing the first-step mutation in *gyrA*) with increased levels of ciprofloxacin across several population bottleneck sizes. They found that when the population bottleneck was large enough for both variants to survive the bottleneck, the outcome matched that seen in clinical isolates: most replicate lines had mutations in *parC* (Hughes and Andersson 2017). Therefore, this work suggests that the bottleneck size during transmission of this pathogen even between hosts is sufficiently large to maintain the rarer, low-cost alleles compared to the more frequent, but costlier,

alternative solutions. More generally, more stringent bottlenecks might constrain the trajectories of resistance evolution by limiting genetic diversity.

Concluding remarks

"When we try to pick out anything by itself, we find it hitched to everything else in the Universe."

– John Muir, *My First Summer in the Sierra*

In closing, my dissertation research addressed questions about the importance of genetic background on the evolution of antibiotic resistance, including its phenotypic and genotypic patterns, and its associated fitness costs. Nonetheless, several additional factors can alter evolutionary trajectories and complicate our ability to forecast resistance evolution in clinical settings. Thus, an evolutionary perspective might complement and benefit efforts to develop new strategies to limit the emergence and spread of antibiotic-resistant bacteria.

And this work has set me on a trajectory of my own—one that is both fascinating and important for our collective well-being.

APPENDICES

APPENDIX A:

Supplementary Tables

Table A.1. Bacterial strains used in Chapter 1.

Strain	Generation	LTEE Population
REL606	0	-
REL772A	500	Ara+5
REL772B	500	Ara+5
REL962A	1,000	Ara+5
REL962B	1,000	Ara+5
REL1066A	1,500	Ara+5
REL1066B	1,500	Ara+5
REL1162A	2,000	Ara+5
REL1162B	2,000	Ara+5
REL2177A	5,000	Ara+5
REL2177B	5,000	Ara+5
REL4534A	10,000	Ara+5
REL4534B	10,000	Ara+5
REL11339	50,000	Ara–5
REL11389	50,000	Ara–6
REL11348	50,000	Ara+4
REL11367	50,000	Ara+5

All clones were derived from REL606, the ancestral strain of the LTEE.

Table A.2. Statistical significance for changes in intrinsic resistance of the individual clones sampled at generation 50,000 of the LTEE for the four antibiotic treatments.

Antibiotic	Clone	*p*
Ampicillin	Ara–5	0.0080
	Ara–6	0.0039
	Ara+4	0.0080
	Ara+5	0.0080
Ceftriaxone	Ara–5	0.0031
	Ara–6	0.0031
	Ara+4	0.0039
	Ara+5	0.0039
Ciprofloxacin	Ara–5	0.2177
	Ara–6	0.1004
	Ara+4	0.0149
	Ara+5	0.0039
Tetracycline	Ara–5	0.0031
	Ara–6	0.0031
	Ara+4	0.0039
	Ara+5	0.0278

Analyses were performed based on a trinomial distribution, which reflects the many ties in these datasets. The reported *p*-values are one-tailed, which reflects the expectation that resistance should decline under relaxed selection in the antibiotic-free LTEE environment.

Table A.3. Statistical significance for trends of diminishing-returns resistance evolvability of the individual clones sampled at generation 50,000 of the LTEE for the four antibiotic treatments.

Antibiotic	Clone	*p*
Ampicillin	Ara–5	0.0456
	Ara–6	0.1094
	Ara+4	0.1592
	Ara+5	0.8408
Ceftriaxone	Ara–5	0.9481
	Ara–6	0.9969
	Ara+4	0.9969
	Ara+5	0.9722
Ciprofloxacin	Ara–5	0.9250
	Ara–6	0.8778
	Ara+4	0.1964
	Ara+5	0.1222
Tetracycline	Ara–5	0.2895
	Ara–6	0.1946
	Ara+4	0.9250
	Ara+5	0.9722

Analyses were performed based on a trinomial distribution, which reflects the many ties in these datasets. The reported *p*-values are one-tailed, which reflects the directional expectation implied by diminishing returns.

Table A.4. Bacterial strains sequenced in Chapter 2.

Antibiotic*	LTEE parent†	Replicate	Strain
AMP	Ancestor	1	KJC108
AMP	Ancestor	2	KJC109
AMP	Ancestor	3	KJC110
AMP	Ancestor	4	KJC111
AMP	Ara–5	1	KJC114
AMP	Ara–5	2	KJC122
AMP	Ara–5	3	KJC130
AMP	Ara–6	1	KJC115
AMP	Ara–6	2	KJC123
AMP	Ara–6	3	KJC131
AMP	Ara+4	1	KJC112
AMP	Ara+4	2	KJC120
AMP	Ara+4	3	KJC128
AMP	Ara+5	1	KJC113
AMP	Ara+5	2	KJC121
AMP	Ara+5	3	KJC129
CRO	Ancestor	1	KJC212
CRO	Ancestor	2	KJC213
CRO	Ancestor	3	KJC214
CRO	Ancestor	4	KJC215
CRO	Ara–5	1	KJC218
CRO	Ara–5	2	KJC226
CRO	Ara–5	3	KJC234
CRO	Ara–6	1	KJC219
CRO	Ara–6	2	KJC227
CRO	Ara–6	3	KJC235
CRO	Ara+4	1	KJC216
CRO	Ara+4	2	KJC224
CRO	Ara+4	3	KJC232
CRO	Ara+5	1	KJC217‡
CRO	Ara+5	2	KJC225
CRO	Ara+5	3	KJC233
CIP	Ancestor	1	KJC148
CIP	Ancestor	2	KJC149
CIP	Ancestor	3	KJC150
CIP	Ancestor	4	KJC151
CIP	Ara–5	1	KJC154
CIP	Ara–5	2	KJC162
CIP	Ara–5	3	KJC186
CIP	Ara–6	1	KJC155
CIP	Ara–6	2	KJC163
CIP	Ara–6	3	KJC187
CIP	Ara+4	1	KJC152‡

Table A.4 (cont'd)

CIP	Ara+4	2	KJC160
CIP	Ara+4	3	KJC184‡
CIP	Ara+5	1	KJC153
CIP	Ara+5	2	KJC161
CIP	Ara+5	3	KJC185
TET	Ancestor	1	KJC60
TET	Ancestor	2	KJC61
TET	Ancestor	3	KJC62
TET	Ancestor	4	KJC63
TET	Ara–5	1	KJC66§
TET	Ara–5	2	KJC74
TET	Ara–5	3	KJC82
TET	Ara–6	1	KJC67
TET	Ara–6	2	KJC75
TET	Ara–6	3	KJC83
TET	Ara+4	1	KJC64
TET	Ara+4	2	KJC72
TET	Ara+4	3	KJC80
TET	Ara+5	1	KJC65§
TET	Ara+5	2	KJC73
TET	Ara+5	3	KJC81

*AMP, ampicillin; CRO, ceftriaxone; CIP, ciprofloxacin; TET, tetracycline.
†Ancestor, *E. coli* B strain REL606. All other parental strains are clones sampled at generation 50,000 from the indicated LTEE population.
‡Three strains (KJC152, KJC184, KJC217) are cross-contaminants, and they were discarded from all analyses.
§Two strains (KJC65, KJC66) have no identifiable mutations.

Table A.5. Bacterial strains used in Chapter 3.

Evolved tetracycline-resistant clones		
Strain name	**Derived from**	**Freezer ID**
Ancestor-1	REL606	KJC60
Ancestor-2	REL606	KJC61
Ancestor-3	REL606	KJC62
Ancestor-4	REL606	KJC63
Ara–5-1	REL11339	KJC66
Ara–5-2	REL11339	KJC74
Ara–5-3	REL11339	KJC82
Ara–6-1	REL11389	KJC67
Ara–6-2	REL11389	KJC75
Ara–6-3	REL11389	KJC83
Ara+4-1	REL11348	KJC64
Ara+4-2	REL11348	KJC72
Ara+4-3	REL11348	KJC80
Ara+5-1	REL11367	KJC65
Ara+5-2	REL11367	KJC73
Ara+5-3	REL11367	KJC81
Tetracycline-sensitive parental strains		
LTEE population	**LTEE generation**	**Freezer ID**
Ancestor	0	REL606
Ara–5	50,000	REL11339
Ara–6	50,000	REL11389
Ara+4	50,000	REL11348
Ara+5	50,000	REL11367
Strains used as common competitors		
LTEE population	**LTEE generation**	**Freezer ID**
Ancestor	0	REL607
Ara–5	40,000	REL10948
Ara–5	40,000	REL11638

APPENDIX B:

Supplementary Figures

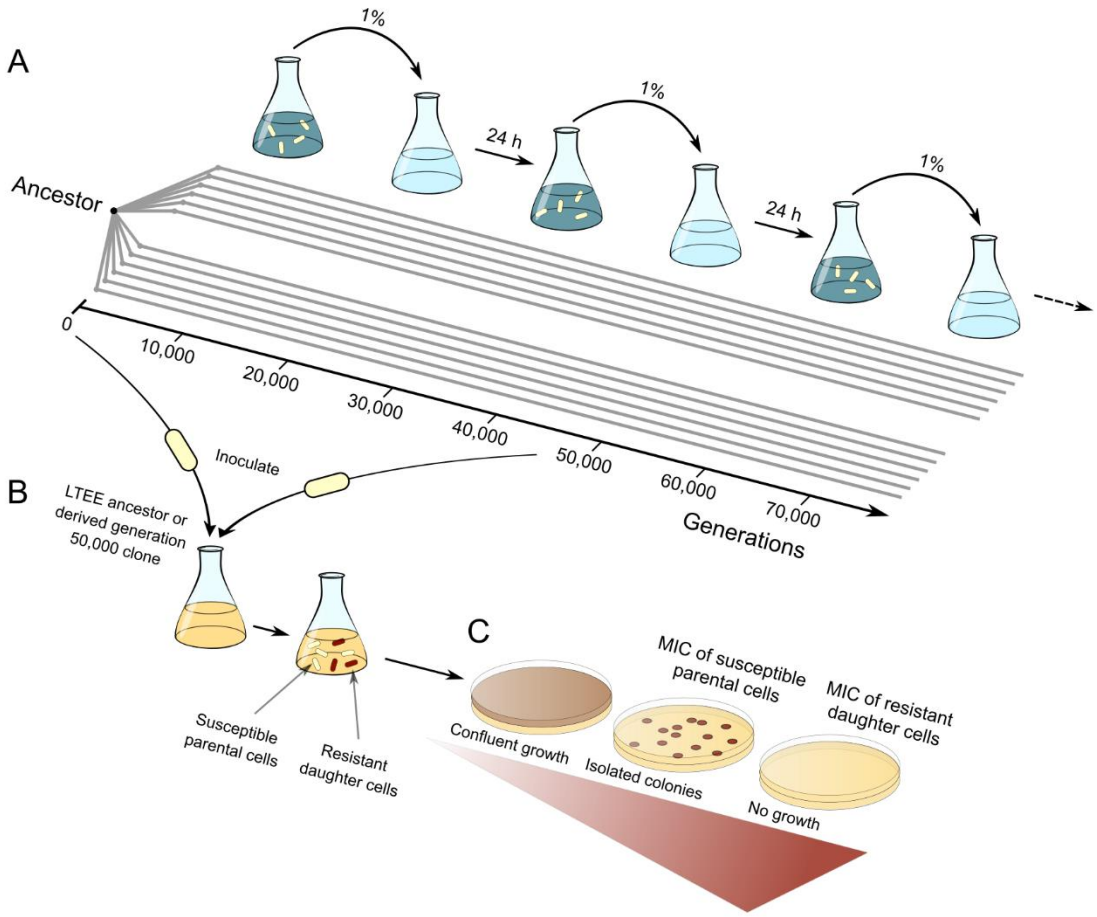

Figure B.1. Schematic illustration of the LTEE and evolvability study design. (A) Twelve initially identical *E. coli* populations were founded from a common ancestor to start the LTEE. These populations have evolved for >73,000 generations with daily serial transfers in a minimal medium without antibiotics. (B) In this study, antibiotic-susceptible ancestral or derived clones from generation 50,000 were inoculated into replicate cultures. A resistance mutation may arise spontaneously and increase in number during a population's expansion, resulting in two genetic variants: the susceptible parental cells and their descendent resistant daughters. (C) These whole populations were then spread onto agar plates supplemented with two-fold increasing concentrations of an antibiotic (shown in red). Minimum inhibitory concentrations (MICs) of these two variants correspond to the lowest antibiotic concentration that inhibits confluent growth and that prevents even isolated colonies, respectively. Resistant clones were confirmed by streaking onto fresh plates with relevant antibiotic concentrations.

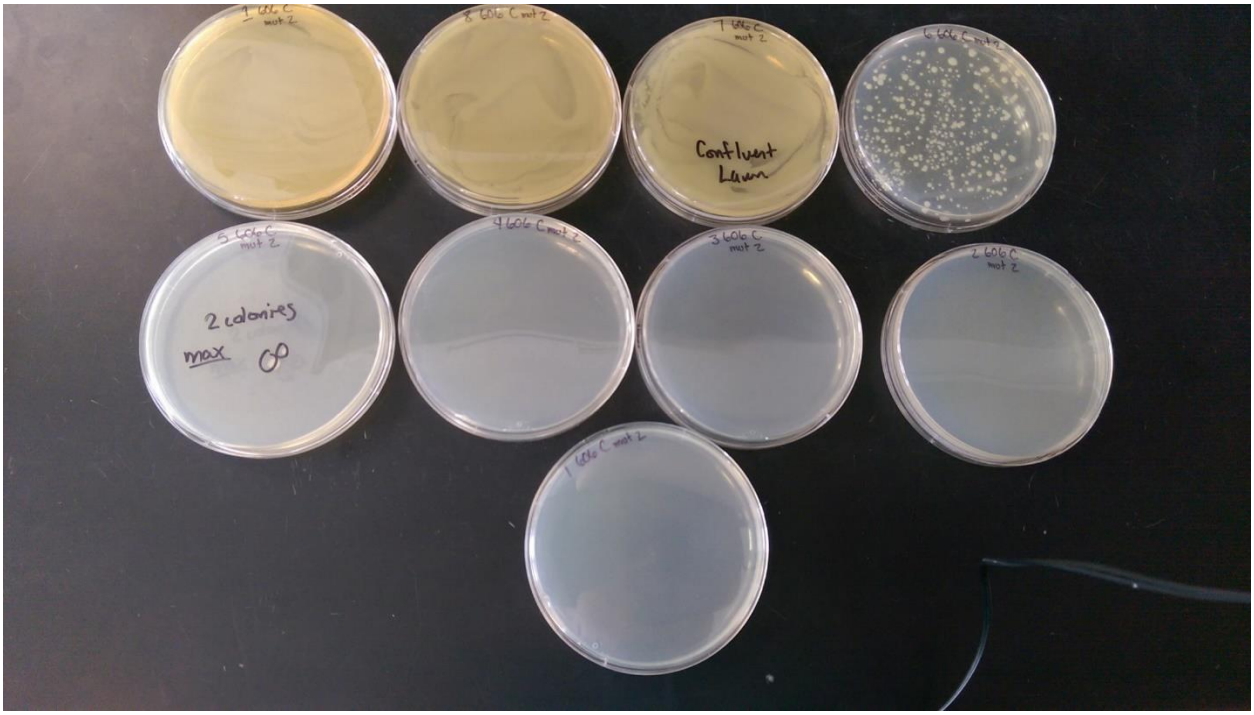

Figure B.2. Experimental plates. Whole populations containing susceptible parental and resistant daughter cells were spread onto MH agar amended with two-fold increasing concentrations of ciprofloxacin (left to right, and down). Confluent lawns of bacterial growth (plates 1–3) consist largely of drug-susceptible cells. Isolated colonies (plates 4–5) are putatively resistant mutants.

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