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Mutation in Antibiotic Resistance: Rate and Fitness

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Abstract of the Dissertation

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Understanding how bacterial populations adapt in antibiotic environments has become important, given rising frequencies of antibiotic resistance. In this series of experiments, *Escherichia coli* are used to understand the mechanisms, timing, and selective forces that dictate how the populations adapt when exposed to the antibiotic streptomycin. We begin with a study of how antibiotic sensitive populations respond to antibiotics in an environment where the concentration of antibiotic grows from zero to above that which would normally kill *E. coli* cells. This was investigated via a novel system using continuous culture techniques. The results indicate that mutations giving resistance to streptomycin are present at low frequencies and immediately sweep to fixation once a threshold concentration of streptomycin is reached. However, resistance mutations of this kind are known to often have fitness costs. The compensatory mutation model holds that mutations that directly mitigate these losses in fitness will come into these populations quickly, raising mean fitness and allowing the resistant strains to successfully compete with their sensitive ancestors. In our experiments, no such direct compensation was found, even when investigated across numerous environments, growth conditions, and timescales. Rather, in all experiments, what was observed was an increase in fitness associated with adaptation to specific environmental conditions, just as would be expected in any experimental evolution experiment using antibiotic sensitive *E. coli* under similar conditions. This implies that after a population gains resistance to an antibiotic, there are little to no lasting negative effects for the population overall.

For my Wife,

Jodi,

whose love and dedication

has made all of the best things

in my life possible.

To her I owe a debt greater than any man can repay in a single lifetime.

Table of Contents

List of Figures, Tables, and Illustrations

Chapter 2

List of Figures, Tables, and Illustrations

Chapter 3

List of Figures, Tables, and Illustrations

Appendix

List of Abbreviations

E. coli	*Escherichia coli*
S. typhimurium	*Salmonella typhimurium*
M. tuberculosis	*Mycobacterium tuberculosis*
MIC	Minimum Inhibitory Concentration
SNP	Single Nucleotide Polymorphism
rpsL	The gene that codes for S12 protein
rpsE	The gene that codes for S5 protein
rpsD	The gene that codes for S4 protein
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
tRNA	Transfer Ribonucleic Acid
DNA	Deoxyribonucleic Acid
mL	Milliliter
µg	Microgram
OD	Optical Density (at wavelength 600 nm)
T5S	Bacteriophage T5 Sensitive
T5R	Bacteriophage T5 Resistant
WGS	Whole Genome Sequencing

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Chapter 1

The History and Significance of Antibiotic Resistance

The modern era of medicine was ushered into existence with the discovery and application of medicines capable of killing bacteria and controlling the infections that they cause. This did not happen in any seminal moment to a single researcher in a solitary lab, as might be depicted in a movie. Rather, this happened over a short but significant period of time due to the work and effort of several groups around the world.

The initial concept of attempting to find chemotherapeutic agents effective against bacteria is credited to Paul Erhlich who reasoned that dyes may be capable of killing bacteria, based upon the results seen in using dye-based stains in order to visualize different species of bacteria under a microscope. Erhlich also later made the claim, quite unaccepted at the time, that by ingesting a medicine, the same effect could be gained as by directly applying the stain to bacteria *in vivo*.

Erhlich's work lead to the development of salvarsan and subsequent sulfa drugs, the first group of compounds known to have medicinal effects against bacterial infections. Sulfa drugs were used with limited success in the early part of the 20th century, particularly during the world-wide wars that plagued the planet during that time. Antibiotics overall, however, did not come into wide use until well after penicillin was

discovered by Alexander Fleming in his lab in September of 1928. Fleming's observation that a fungus was not allowing the growth of staphylococci on a petri plate led him to discover that penicillin was an effective antimicrobial against most gram-positive bacteria.

Contrary to popular belief, Fleming did not manage to change medicine overnight even though he published his findings in 1929. Rather, Fleming found that cultivating the fungus that produced penicillin was difficult and that purification of the drug was even more so. Fleming also suffered the effects of being a famously poor communicator, both in public and private. This hampered his ability to develop collaborative relationships with other scientists, leaving him in a kind of academic purgatory. As such, what would later be called the miracle drug was not well known or understood until the problem of production was taken up by Ernst Boris Chain, Howard Florey, Norman Heatley, and Edward Abraham in at Oxford. The work of these chemists led to the isolation and purification of penicillin along with the discovery of how to prepare aqueous solutions that could be administered to patients.

Even though widespread use of penicillin became a reality in 1940, the world's first true clinical antibiotic was largely ineffective against many bacterial infectious diseases because penicillin killed only gram-positive bacteria. Treatment of gram-negative bacteria would have to wait for the next major advancement in infectious disease control.

Streptomycin was discovered in the lab of Selman Waksman at Rutgers University in 1943. The great benefit of the new antibiotic was that streptomycin was

active against *Mycobacterium tuberculosis* and could be used to treat clinical tuberculosis, which penicillin could not. By 1948, reports of bacterial resistance to streptomycin were being reported in the literature, mainly due to the first randomized controlled clinical trials for a drug conducted by the British Medical Research Council at that time (Crofton and Mitchison 1948). The short timeframe from discovery though development and usage to resistance is not unique to streptomycin. In fact, resistance to just about all known antibiotics followed quickly after clinical or industrial usage of the drug.

By the end of World War II, penicillin had saved countless lives and was known all over the globe. This antibiotic, along with streptomycin and the advances in surgery that took place during the same time (in part thanks to the ability of patients to survive potential surgical would infection via antibiotics), ushered in the modern era of medicine as we understand the practice today. These advances were followed rapidly by equally vital discoveries in genetics with the structure of DNA in 1953 and the avalanche of new antibiotic drug development that occurred from the 1940s through the 1960s (quinolones, 1963; trimethoprim, 1968).

But as these advances accrued, so did setbacks in the efficacy of antibiotics due to antibiotic resistance. Published reports of resistance to penicillin were known in 1943; for streptomycin, 1948. In the second half of the previous century, the rates of resistance of pathogens for all known antibiotics have continually climbed as the number and use of antibiotics has grown. At first, the public health response was to control antibiotic usage with the expectation that rates of resistance would drop as usage diminished. Resistance persisted, even in the face of drug management

practices on both small and large scales. Additionally, the pipeline for new antibiotics has dried up while the costs for the development of new medicines have skyrocketed. Currently, the cost to bring a new drug to market is estimated to be over $500 billon. With costs such as these, drug companies are not interested in developing low profit medicines such as antibiotics. This is particularly true given that the life expectancy of these new and very expensive products can be drastically reduced due to antibiotic resistant strains rising to high frequency.

The Problem of Modernity

Resistance to antibiotic treatments has been, and continues to be, a growing problem (Andersson and Hughes 2010). Typically, in the absence of chemotherapy (Moore et al. 2000) a bacterial population that acquires resistance to an antibiotic, shows lower overall fitness relative to the ancestral population. Research has indicated that resistant strains rapidly overcome much or all of this fitness deficit by means of beneficial mutations called compensatory mutations (Andersson and Hughes 2010).

These compensatory mutations are thought to come into a population soon after resistance and alleviate the associated fitness costs through various mechanisms (Andersson and Hughes 2010). Most of these are specific to the antibiotic being used (Björkman and Andersson 2000). Also, more than one type of compensatory mutation has been observed for a given resistance mutation (Poon et al. 2005).

Researchers have sought to explain the mechanisms of both resistance and compensation by means of Darwinian evolution and population genetics (Levin et al. 1997; Stewart et al. 1998). Empirical data supporting Darwinian and population genetic hypotheses (Schrag et al. 1997) and models (Levin et al. 2000) has come from both *in vitro* and *in vivo* studies of selection before and after mutations for resistance have appeared. The model species in these studies are *Salmonella typhimurium* (Bjorkman et al. 1998), *Mycobacterium tuberculosis* (Böttger et al. 1998), and *Escherichia coli* (Björkman and Andersson 2000).

In 2000, Björkman *et al*. published a study utilizing *S. typhimurium* that indicated distinctly different classes of compensatory mutations in different environments. Specifically, Björkman and colleagues found that the number and type of compensatory mutations differed for resistance in *S. typhimurium* to fusidic acid or streptomycin depending on whether the bacterial population was grown *in vitro* (which for this study means in serial transfer flasks in LB) or *in vivo* (here, injected into mice). Björkman's group found that, for streptomycin, a greater proportion of the compensatory mutations was intragenic when the populations were grown *in vitro* than when grown *in vivo*.

The distinction between intragenic and extragenic compensation is important since the potential mechanisms for these two types of mutations are different (Maisnier-Patin and Andersson 2004). In this review, the authors highlight the potential mechanisms of each of these categories of compensatory mutation. Intragenic mutations would restore or improve of the function of the coded protein product. Extragenic mutations, on the other hand, could act to restore a multi-unit complex, such as ribosomes, which are inhibited by many antibiotics, including streptomycin. While

there are additional mechanisms of compensation, such as efflux, gene dosage effects, and antibiotic modification, the extra/intra-genic mutations are the mechanisms of compensation that are discussed here. In a review of the literature, Poon and colleagues found that 83% of the cases of compensatory mutations were intragenic.

At about the same time as the Björkman publication, Levin et al. (Levin et al. 2000) presented an experiment and accompanying model to explain the population dynamics of compensatory evolution in *E. coli* following selection for resistance to streptomycin in a serial transfer *in vitro* scheme. Specifically, the authors sought to understand why, following cessation of chemotherapy, the resistant mutants of lower fitness are not quickly outcompeted by higher fitness sensitive wild type bacteria and why there are few revertant mutations within the resistant population that return a resistant mutation back to the sensitive state. They proposed what they term the "bottleneck-mutation rate hypothesis" to explain this observation. In this hypothesis, the lack of revertant increase is due to the rate of compensatory mutations coming into the resistant population far exceeding the rate of reversion mutations as well as clonal interference and the repeated bottlenecks in population size associated with serial transfer.

Bottlenecks are an integral part of serial transfer. Would the same results be observed if there were no bottlenecks or is the result common to all methods? While several reviews and studies mention alternative *in vitro* methods, particularly chemostats (Andersson and Levin 1999; Maisnier-Patin and Andersson 2004; Andersson and Hughes 2010), no known studies have been designed to address these questions.

The Björkman study argues that different compensatory mutations are selected *in vitro* and *in vivo*. The Levin group argues that bottlenecks may be restricting the dynamics of adaptation within *in vitro* populations, suggesting that the difference between *in vitro* and *in vivo* compensation may be the presence or absence of bottlenecks. The next logical step is to use an *in vitro* system that eliminates bottlenecks, i.e. chemostats. This is the question addressed with the series of experiments described in chapter 3. The observations were surprising and highlight the need to take more than just fitness deficits and *in vitro/in vivo* conditions into account.

Prior to addressing post-resistance adaptation, however, the evolution of resistance to streptomycin in populations of streptomycin sensitive *E. coli* is investigated. Waksman's streptomycin was so effective against gram-negative bacteria because the mode of action is within the ribosome, necessary to all bacterial cellular processes, as opposed to interfering with cell wall synthesis, as penicillin does.

The ribosome is one of those large complex structures that may be amenable to both intra- as well as extra-genic compensatory evolution when resistance occurs. In the case of streptomycin, resistance centers on the proofreading processes associated with assuring that the correct, or cognate, tRNA with the amino acid called for by the mRNA is incorporated at the proper point into the growing protein peptide chain. In fact, proofreading is integral to how streptomycin acts and how streptomycin resistance evolves in populations.

Prokaryotic Proofreading

The prokaryotic ribosome is made up of two parts, the small ribosomal subunit, 30S, and the large ribosomal subunit, 50S. The 50S subunit is responsible for what we think of when we think about ribosomes, namely, connecting amino acids together to form a polypeptide chain that then folds into a functional protein. 50S is made up of 2 ribosomal RNAs (rRNA) named the 23S and the 5S rRNAs as well as several "L" proteins. These rRNAs and L-proteins come together in a specific way to make the 50S subunit functional. Loss or changes to these proteins affect the functionality of the subunit.

The 30S is the part of the ribosome that decodes mRNA and matches the three nucleotide codon of the mRNA to the 3 nucleotide anticodon of the tRNA. This process is integral to building functional proteins and, thereby, to the proofreading process. As with the 50S, there are several proteins, here called "S" proteins, that are bound up in a specific way with the single long rRNA found in the small subunit, known as the 16S rRNA.

Translation begins on the mRNA during transcription from genes in the cell's DNA. The free end of the transcribed RNA rapidly couples to a free 30S small ribosomal subunit. The first codon triplet, which is always a methionine codon (usually adenine-uracil-guanine, or AUG) binds into the middle site of the 30S recognition complex. This recognition complex is home to three active sites, the A site, the P site, and the E site. The AUG codon binds directly into the P site and is the only codon that does this. Once a start codon is bound in the P site, the second codon binds in the A

site. Following this, the 30S must also bind proteins called initiation factors, noted as IF-1, IF-2, and IF-3.

After the 30S subunit has bound the mRNA and IFs, the formyl –tRNA, carrying the “start” methionine amino acid binds into the P site. After all this, the 30S can marry with the 50S large ribosomal subunit and translation can begin (this “marriage” cannot happen without the three IFs). Only after this marriage can a tRNA (in this case, an aminoacyl-tRNA) bind in the A site. When a cognate aminoacyl-tRNA binds in the A site and when the codon-anticodon match occurs, proofreading proceeds and translation continues. When a noncognate aminoacyl-tRNA binds in the A site, proofreading effectively stresses the mismatched codon-anticodon triplet pair and ejects the incorrect tRNA out of the A site. This frees the A site for binding of the correct, cognate aminoacyl-tRNA.

Proofreading has to two main steps. When an aminoacyl-tRNA comes into the A site, cognate matches between the codon of the mRNA and the anticodon of the tRNA bind tighter than those of noncognate tRNAs. The first step in proofreading is analogous to a time gate in which the probability of a cognate anticodon remaining bound to the codon for the required period of time is much greater than than the probability of a poorly matched noncognate anticodon remaining in the A site. Another way of putting this is that poor codon-anticodon mismatches don’t last, and the tRNA unbinds and leaves the A site.

If, however, the tRNA remains in the A site long enough (here, this is a timespan on the order or milliseconds), then there is a transition to a new chemical conformation

within the ribosome that is partially mediated by the S12 protein in the 30S subunit of the ribosome. S12 helps to stabilize cognate codon-anticodon bonds. However, when the binding is not correct, S12 aids in stressing incorrectly paired nucleotide triplets. This additional stress will then give the noncognate aminoacyl-tRNA a metaphorical push out of the A site. If the tRNA remains bound, the codon-anticodon pairing is strong enough to survive proofreading and the peptide bond between amino acids (AA) is made.

Peptide bond formation happens in the 50S subunit when the tRNA in the P site shifts toward the tRNA bound in the A site tRNA, causing the two amino acids to come close together. The peptide chain is increased in length by one amino acid and then the tRNAs in the A and P sites each move over one space. The P site tRNA moves into the E site, the A site tRNA moves into the P site and the A site is cleared, ready for the next aminoacyl-tRNA to come in and bind, and the entire process starts anew.

Proofreading in *E. coli* cells keeps errors in amino acid incorporation into nascent proteins to about 1 in every 10^4 amino acids. This error rate is much higher than that known for DNA replication (about 1 in 10^{10} nucleotides for *E. coli*). However, protein errors are much more forgiving, as well as shorter-lived, than DNA errors. Additionally, the number of proteins produced is much larger than the copies of DNA in a cell at any time, so a few bad copies can be tolerated fairly well.

The process of proofreading in translation is vital to the story of streptomycin as the antibiotic binds right in the A site, adjacent to the codon-anticodon. This is also where the S12 protein is active, and structural changes to S12 are the phenotype that

often gives rise to streptomycin resistance. As such, many of the streptomycin resistant mutants that will be discussed in the following chapters are mutations in *rpsL*, the gene that codes for the S12 protein. In fact, as will be shown, the role of *rpsL* and the protein that *rpsL* codes for, are vital to the story of the evolution of streptomycin resistant bacteria. The exact mutation in *rpsL* affects the fitness of the bacterium. The mutation in this gene also plays a role in the amount of streptomycin that can be tolerated. A specific change in *rpsL*, and the subsequent selection that occurs, determines how well a mutant survives and reproduces in the environment. How and why this is, in large part, the aim of this thesis.

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Chapter 2

How Bacteria Evolve Antibiotic Resistance

Introduction

Incidents of resistance of bacterial populations to antibiotic treatments are on the rise worldwide (Bush et al. 2011; CMO 2011). In human pathogens, resistant strains represent a growing threat to the public health and foretell of a loss in medical and technological capabilities unprecedented in the history of humanity. The loss of antibiotic treatments would all but cripple not only infectious disease medicine, but all aspects of medicine, including surgery (post-surgical wound management) and oncology (chemotherapeutic immune suppression). Given that antibiotic resistance is an acknowledged and growing problem and given the innumerable negative impacts that the loss of anti-infection treatments would bring, the question remains: how does antibiotic resistance begin?

We know that resistance can arise in susceptible population *de novo* through mutations, horizontal gene transfer from resistant to susceptible strains, and, to a limited extent, efflux (Hall 2004; MacLean et al. 2010). However, very little work has been done investigating how and when these resistance phenotypes enter a population. There has been some excellent work showing that resistance can be selected for in

populations that are in very low (<<MIC) antibiotic environments (Gullberg et al. 2011). However, beyond this, we do not have much experimental evidence for how selection acts upon resistance within susceptible populations exposed to varying levels of antibiotics, although there is a fair amount of theory pertaining to such a question (Levin et al. 1997, 2000; Schrag et al. 1997). These questions, namely when and how does resistance rise in frequency in a population, are basic to our understanding of the nature of antibiotic resistance and in our ability to maintain the foundations of modern medicine. Here, we attempt to look more deeply at how and when a mutation granting resistance to the antibiotic streptomycin comes into and rises in frequency within a population that starts off as being streptomycin sensitive.

Methods

Strain

The ancestral strain used is the DD1953 strain of *Escherichia coli*, a MG1655 derivative of K-12 lab strains used widely. DD1953 is *rpoS*-, having a mutation causing a premature stop codon early in the coding region of the *rpoS* gene, which codes for the σ^S protein. The minimum inhibitory concentration of streptomycin for DD1953 was measured as being 2≥4 μg/mL. DD1953 has been used extensively in our lab, both in serial transfer and especially in chemostat experiments. For the purposes of a molecular marker, resistance to the bacteriophage T5 was used. T5 resistance is a naturally occurring and fairly common mutation (*fhu*) in this strain and has been shown

to be selectively neutral with respect to fitness (Moser 1958) in chemostats under glucose limiting conditions. The ancestral strain stock used for all experiments was grown from a single plated colony of DD1953 in order to insure that the ancestral strain utilized was genetically homogeneous at the start of experimentation. That initial stock was plated with T5 to screen for a T5 resistant mutant. Once a T5 resistant mutant colony was found, a stock of T5 resistant DD1953 was grown from this single colony, insuring a genetically homogeneous stock.

Chemostats

All experimental trials were conducted in chemostats using a protocol modified from that previously described elsewhere (Dykhuizen, Dean, & Hartl, 1987; Dykhuizen, 1993; Hartl & Dykhuizen, 1984). Basically, a chemostat is a continuous culture device that supplies a measured amount of fresh media to an exponentially growing population while removing an equal volume of spent media and dead cells, always keeping the population size (N) constant. For these experiments, the chemostats were all 30 ml experimental volumes with the population size (carrying capacity) to 10^8 cells/ml (N = 3.0×10^9). The experimental temperature was maintained at 37° C at all times.

The media used was Davis Salts minimal media with 0.01% glucose added. Media was metered via a Wizco pump set to deliver 5.625 ml of media per hour to the chemostat chamber (D=0.1875). This rate of dilution results in a generation time of 3.69 hours per generation (Dykhuizen and Hartl 1983). This generation time was held constant throughout all replicate trials.

In addition to the usual single media jar, a second media jar containing the same Davis Salts minimal media and glucose, supplemented with streptomycin at a concentration of 100 µg/ml, was connected to the primary media jar via a siphon line. This siphon line replenished half of the volume of media metered out of the primary jar on a continuous basis. A magnetic stir-bar ensured that the media in the primary jar was well mixed. This means that at T_0, the concentration of streptomycin in the media delivered to the chemostat was 0. As the experiment progressed and more of the media used was replenished with streptomycin media, the concentration of media in the primary jar, and hence, the chemostat, increased. The concentration of streptomycin can be calculated for any time point by using the equation

$$Y_h = \frac{((X_h * Y_{h-1}) + c)}{X_h}$$

Where

h = time point in hours

X = volume of media in primary media jar

Y = concentration of streptomycin

c = constant delivered total mass of streptomycin to primary media jar per hour, here that rate is 281.25 µg/hr

Plating and Population Tracking

A sample was taken from all chemostats at roughly 24 hour intervals. This sample was diluted 100,000-fold and replicate plated on LB agar in Petri plates. The diluted samples were plated 4 times on LB agar and 4 times on LB agar with T5 bacteriophage as a screen for T5-resistant (T5R) clones. The samples were pour plated using soft agar (LB agar with reduced concentration of Difco agar) and then topped with top agar (Difco agar and water). All plates were incubated at 37° C overnight and counted the next day. All plates were counted using a ProtoCol automatic plate reader and ProtoCol software V3.15, both by Synbiosis (Cambridge, UK). The number of T5 sensitive (T5S) colonies was calculated as the mean number of T5R colonies subtracted from the mean number of total colonies. Populations of T5S and T5R can then be tracked over the time course of the experiment.

Sequencing & Bioinformatics

Resistance mutations to streptomycin in *E. coli* strains are most often linked to single nucleotide polymorphisms (SNPs) in the *rpsL* gene. This gene codes for the ribosomal protein S12, which makes up part of the small (30S) subunit of the *E. coli* ribosome. In particular, this protein interacts with ribosomal RNA (rRNA) and messenger RNA (mRNA) at the codon-anticodon recognition site (A site) of the ribosome. This implies that S12 plays a role in codon-anticodon recognition, aminoacyl-tRNA selection, and/or the translational proofreading process (Kurland et al. 1990, 1996; Kurland 1992; Noller and Nomura 1996).

The *rpsL* gene was PCR amplified using forward and reverse primers and then Sanger sequenced at the University of Arizona Genetics Core. These sequence reads were then vetted for quality and aligned to the ancestral *rpsL* gene from the DD1953 strain, as well as the reference K-12 MG1655 genome (Genbank accession NC_000913.3) sequence for *rpsL*. SNPs were identified only when forward and reverse reads of high quality agreed.

Along with *rpsL*, compensatory mutations in the *rpsD* and *rpsE* genes have been reported in the literature (Kurland et al. 1996; Björkman and Andersson 2000a,b). These genes were also sequenced using a similar protocol for comparative reasons. All alignments and assemblies were completed using Geneious bioinformatics software tools (Biomatters Ltd, Auckland, NZ) version 7 or later.

Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MIC) for all strains were found experimentally using optical density (OD) measures in Luria-Bertani (LB) liquid media. Basically, the strain of *E. coli* to be tested was grown overnight in a test tube in LB at 37°C, shaken at 200 rpm. After the population had entered stationary phase, 100 µL of the overnight growth was put into fresh LB media and allowed to enter exponential growth phase. At this point, 100 µL of the growing population was put into an array of fresh test tubes containing 1.9 mL of fresh LB along with a spectrum of streptomycin concentrations, ranging from 0 to 1000 µg/mL. Growth in these tubes was tracked by

measuring optical density at 600 nm at regular intervals with a WPA biowave model CO8000 Cell Density Meter.

Results

Four rounds of experiments were conducted. Each round consisted of two chemostats each inoculated with approximately equal amounts of the two strains, DD1953 T5R and DD1953 T5S *E. coli*. This gives a total of four experimental replicates per round of two chemostats, resulting in 16 replicate experimental runs over those 4 rounds (2 strains per chemostat, 2 chemostats per round, 4 total replicate rounds). Each chemostat was continued for a minimum of 120 hours, although results were usually clear by 96 hours. In 5 of the 16 replicate trials (31.25%), the populations of *E. coli* went extinct within 48 hours, as the concentration of streptomycin in the chemostat approached 7 µg/mL. In 10 replicates, the population was observed to decline to low levels and then rapidly recover within the next 48 hours (Fig. 1: colony counts for experiments). Of the 8 chemostats run (2 chemostats per round), only 1 had no observed streptomycin mutant come into the population at a detectable level within 120 hours. The remaining 7 chemostats had at least 1 streptomycin dependent mutant rise to high frequency within that time frame.

In one replicate, a contaminant was observed at 120 hours that was later identified as belonging to the genus *Bacillus* (Fig. 1H, T5R). This contaminant was at a low frequency (approximately 6.7% of CFU) and was not present at the 96 hour sample,

at which point the *E. coli* strain was extinct. This contamination is not wholly unexpected when we consider that the chemostat was an available niche with all the resources needed for microbial growth. The streptomycin resistant mutant in this chemostat, K42N was at high frequency, but not completely filling the niche space available.

Sequences from the populations that recovered showed that in all cases one of three SNPS had swept to fixation (Table 1). The three SNPs observed all occurred at the same codon position within the *rpsL* gene, codon 42, which codes for the amino acid lysine (K) (codon: AAA), in the ancestor, DD1953 (both strains, T5R and T5S). The observed SNPs were either AAA→ACA (lysine to threonine, K→T), AAA→AAC (lysine to asparagine, K→N), or AAA→ATA (lysine to isoleucine, K→I). The threonine mutant was observed to fix in 7 of the 11 replicates where a streptomycin resistant mutant was observed (63.6%). In three cases (Round 2, Chemostat 1, T5R; Round 4, Chemostat 1, T5R; and Round 4, Chemostat 2, T5S), the K→N was observed. One case (Round 3, Chemostat 2, T5S) was found to be the isoleucine mutant K42I. MICs for all mutants were measured to be greater than 1000 μg/mL for streptomycin.

In order to test whether the discrepancy between the number of trials that resulted in the K42T mutant fixing as opposed to the K42N mutant fixing was an experimental condition or a result of mutant presence/absence in the initial ancestor stocks, a control experiment was conducted. The ancestor strains, DD1953 T5R and DD1953 T5S, were grown overnight in LB. Twenty milliliters of this overnight growth (OD_{600nm} at time of inoculation = 1.96, for density of $1.96x10^9$ and a total population size of $3.92x10^{10}$ cells) was added to 180 mLs of soft agar supplemented with streptomycin

at a concentration of 100 µg/mL. The *E. coli* and soft agar mixtures were then plated in 250 mL glass petri dishes and incubated for 7 days at 37°C. This control was performed in two replicates.

After the 7 day incubation period, a total of 59 streptomycin resistant colonies were observed (total of 59 from 4 plates, see Tables 2 & 3). These colonies were collected and their *rpsL*, *rpsD*, and *rpsE* genes were PCR amplified and then Sanger sequenced. As in the experimental condition, no SNPs were found in *rpsD* or *rpsE* and once again, all SNPs found were in the *rpsL* gene. However, not all of the SNPs found in the control experiment were in the codon 42 position, although most were. The complete list of SNPs from the control experiments is listed in Table 3. Of the 59 streptomycin resistant mutants from the control experiments, 41 (69.5%) were exact matches of the threonine mutant (K42T) observed in the chemostat experiments. This represents the largest portion of the control mutant list and is a close match in terms of percentage to the chemostat experiments (63.6% in experiment versus 69.5% in control). The second most frequent mutant observed from the control experiments was a match to the asparagine mutant (K42N) (7/59 = 11.8% versus 27.3% in experiment). Of course, other streptomycin mutants were also found in the controls (5 others) that were capable of growing in streptomycin concentrations of at least 100 µg/mL.

Discussion

This series of experiments highlights the incredible speed with which a streptomycin resistant mutant can sweep to fixation within a population exposed to low levels of streptomycin. The sensitive clones within the population die off quickly and the resistant mutants, with MICs above 1000 µg/mL, grow rapidly to fill the newly emptied environmental space of the chemostat. We should note here that the MIC of the resistant populations (>1000 µg/mL) is over 143X higher than the streptomycin concentration in the media at the time when the susceptible population has already completely died off (6.99 µg/mL of streptomycin at 48 hrs). The rise was so rapid, in fact, that the overall time to fixation for the streptomycin resistant mutants observed in these experiments was far less than the typical 10-14 day treatment cycle for many antibiotics, including streptomycin. Further, toxic levels of streptomycin are reached at about 430 mg/kg (as per MSDS for streptomycin sulfate: LD50 in rats, oral ingestion) (http://www.pfizer.com/files/products/material_safety_data/284.pdf), far below the 1000 µg/mL level of resistance and still at least 54X higher than the highest concentration of the antibiotic to which the populations were exposed (Fig. 2).

Results from the control experiment indicate that by the time of inoculation, the streptomycin resistant mutants are already present in the population and the shift in environment into one with the antibiotic rapidly selects for the mutants, while also selecting against the sensitive strain. This is evident by the number of mutants that were recovered from the control experiments. If the streptomycin resistant mutants were present in the 20 mLs of inoculum that went into each control petri dish containing agar and streptomycin, then colonies would grow and they would be recoverable. This

is what indeed happened. If the streptomycin resistant mutants had not been present in the inoculum, we would expect a dynamic similar to that observed in the chemostats where no resistant strains were found (see Fig. 1A), namely that the sensitive cells would very rapidly be killed by the antibiotic in the agar, and no mutant colonies would have been expected. Additionally, the distributions of SNPs found in the control experiment were consistent across the two experimental conditions (Tables 2 & 3) and this distribution also correlates well with the distribution of SNPS observed in the chemostat experiments (Table 1).

The total volume of inoculum for the control experiments was 20 mLs of *E. coli* at an OD_{600} of about 1.96. This means that the petri dishes with agar and streptomycin were each seeded with 3.92×10^{10} live, growing cells. The average number of streptomycin resistant colonies recovered from the 4 trial petri dishes of the control experiment was 14.75 (p value of the 4 trials = 0.5238 by G-test of Independence) (Sokal and Rohlf 1995). Put another way, we can calculate a conditional mutation rate for the *rpsL* gene for streptomycin resistance. Taking the four control plates, mutation rate for *rpsL* is calculated to be $1/2.69 \times 10^9$, meaning that we can expect to find a streptomycin resistant mutant with a MIC >1000 μg/mL about once in every 2.7×10^9 *rpsL* gene copies and since *E. coli* are haploid, this becomes a *de facto* cellular mutation rate.

In the chemostat experiments, each chemostat was inoculated with 1 mL of T5S and 1 mL of T5R, both at an OD_{600} of about 0.8. This means that each mL of inoculant contained about 8×10^8 for a total of 1.6×10^9 cells per chemostat inoculation. Given the calculated gene mutation rate, we would expect 59.3% of chemostat replicate trials

to result in an observed streptomycin resistant mutant in the *rpsL* gene (1.6×10^9 total *E. coli* cells/2.7×10^9 expected gene mutation rate). The results in an expectation of 9.5 expected mutants from our 16 experimental chemostat trials. The observation of 11/16 mutants is, therefore, right within the window of our *a priori* expectation.

The chemostat trials support the conclusion of the control experiment that the mutants most probably come into the population during the exponential growth phase prior to exposure to the antibiotic. If the SNPS that are responsible for resistance were to occur after inoculation of the chemostats, we would have to expect that the time of mutation would again be before the level of streptomycin reaches a concentration capable of killing most of the bacterial cells. This must be so because once streptomycin concentrations reach a level that results in bacterial killing, population sizes drop very quickly (Fig. 1), representing a decreasing probability of a SNP occurring, not an increasing one. In fact, keeping pathogen population sizes small is a basic tenet of controlling many infectious diseases, including HIV (Coffin 1995), tuberculosis (Dorman and Chaisson 2007), and malaria (Hartl 2004). This is because host immune systems function well when infection size is controlled, as opposed to the uncontrolled growth of pathogens that warrant antibiotic treatments. By keeping the overall population size of the pathogen in check, the immune system can most probably eliminate the antibiotic resistant mutants that occur through random SNPs before they are selected for and become the dominant strain in an infection.

In fact, extrapolating the results observed here to general infections treated with streptomycin beginning in the 1940s would mean that streptomycin should have been rendered useless as an antimicrobial therapy almost immediately. That is, of course,

not what happened. If many infections can reach pathogen population sizes of 10^{10} (Drlica 2003), similar to our control trial population sizes, we should expect that most cases of streptomycin treatment would have provided for selection of present resistant mutants at rates similar to those seen in the present series of experiments. One reasonable explanation for why this did not occur would be the major component of host/pathogen dynamics not present in the chemostat experiments conducted here, namely, host immune system action. The immune system would have acted on the relatively small population sizes of the resistant mutants along with any persistor cells that escaped chemotherapy, thus ending the line of streptomycin resistant *E. coli* before they had a chance to grow under positive selective conditions and spread beyond the host. While antibiotics were helping the immune system, the implication is that the immune system was also helping to control the spread of antibiotic resistant mutants.

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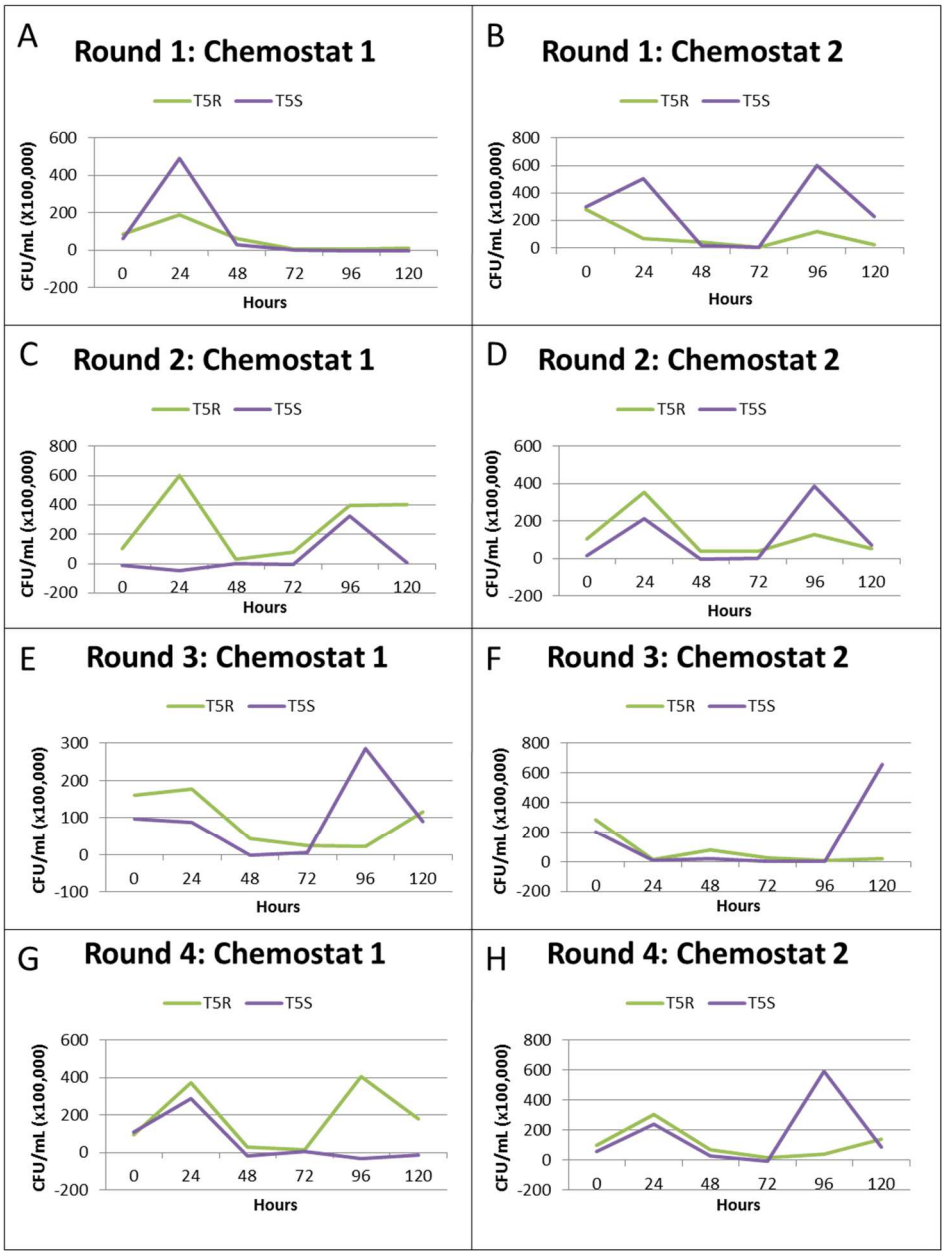

Figure 1A-G: Counts of CFU/mL within experimental chemostats. Within 48 hours, all chemostats indicate that sensitive strains have been significantly depleted to or near 0. In all but one case, the resistant mutant had reached high frequency (when a mutant was present) by 96 hours. The two strains within each chemostat were labelled with or without resistance to T5 bacteriophage. Note that a *Bacillus* contaminant was found in Chemostat 2 of Round 4 at the 120 hour mark that was not present in the 72 hour sample.

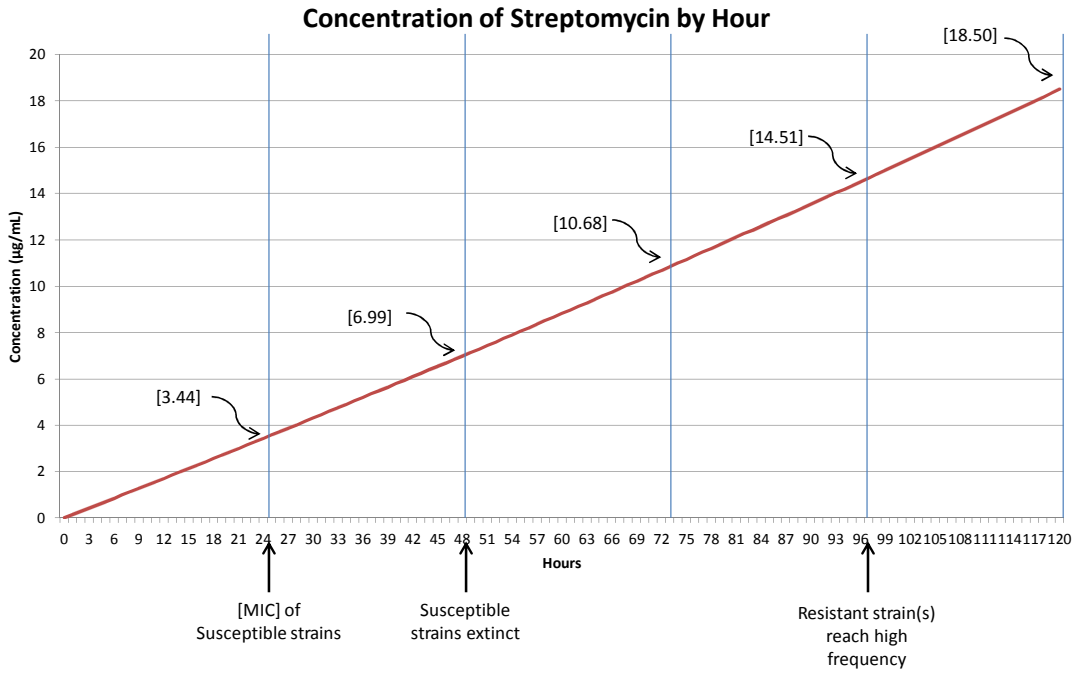

Figure 2: Concentration of Streptomycin over time: As the concentration of antibiotic increased, susceptible strains did not die off at the MIC, but rather were extinct within 24 hours of passing the observed MIC. In almost all cases, resistant strains reached high frequency by the 96th hour.

Round	Chemostat	T5S	T5R
1	1	Extinct	Extinct
	2	K42T	K42T
2	1	K42T	K42N
	2	K42T	K42T
3	1	K42T	K42T
	2	K42I	Extinct
4	1	Extinct	K42N
	2	K42N	Extinct*

Table 1: Rounds and Chemostat Results: The chemostat results each run. In cases where no mutant was observed, the populations both went extinct. Note that in Round 2, Chemostat 1, both the T5R and T5S strains were observed to have a K42T mutation and that in Round 4, Chemostat 2 a *Bacillus* contaminant was found in place of a T5R strain at 120 hours (denoted by *).

	Replicate 1		Replicate 2	
SNP	T5S	T5R	T5S	T5R
K42T	11	11	11	8
K42N	1	2	1	3
K42R	0	0	1	0
K87R	1	2	2	0
P90Q	0	1	0	1
P90L	0	0	0	1
G91D	0	1	0	1
Σ	**13**	**17**	**15**	**14**

Table 2: Mutations found in Control Experiment: No statistically relevant deviation of number or type of mutations was found based upon G-test.

Mutation	Locus	Sequence	Amino Acid	Quantity	Percentage
WT	codon 42	AAA	Lysine	na	
K42T	codon 42	ACA	Threonine	41	69.5%
K42N	codon 42	AAC	Asparagine	7	11.8%
K42R	codon 42	AGA	Arginine	1	1.7%
WT	codon 87	AAA	Lysine	na	
K87R	codon 87	AGA	Arginine	5	8.5%
WT	codon 90	CCG	Proline	na	
P90L	codon 90	CTG	Leucine	1	1.7%
P90Q	codon 90	CAG	Glutamine	2	3.4%
WT	codon 91	GGT	Glycine	na	
G91D	codon 91	GAT	Aspartic Acid	2	3.4%

Table 3: Control Experiment: A complete listing of the 59 streptomycin mutants found in the control experiment. All MICs were measured and found to be greater than 1,000 μg/mL for streptomycin. “WT” indicates wild type ancestral state.

Chapter 3

Fitness, Competition, and Compensatory Adaptation in Antibiotic Resistance

Introduction

The development of the first antibiotics in the early twentieth century revolutionized medicine. The first antibiotics were hailed as “miracle drugs” that would render the infectious diseases that had plagued humanity from the birth of mankind to the pages of history. However, in case after case, soon after an antibiotic was put into wide spread clinical use, antibiotic resistance would become a problem. Antibiotic resistance left these treatments ineffective in a very short period of time.

Streptomycin was discovered in the lab of Selman Waksman at Rutgers University in 1943. The great benefit at the time of the new antibiotic was that streptomycin was active against *Mycobacterium tuberculosis* and could be used to treat clinical tuberculosis, which penicillin could not. By 1948, just five years later, reports of bacterial resistance to streptomycin were being reported in the literature. These reports came mainly out of the very first randomized controlled clinical trials for a drug, which were conducted by the British Medical Research Council (Crofton and Mitchison 1948).

From a population genetics perspective, the rapid increase in resistance is not surprising. The widespread use of antibiotics strongly selects for resistant phenotypes

as these phenotypes are the only bacteria that can grow in an environment where the antibiotic is present. As the number of people undergoing antibiotic treatment increases, so does the overall frequency of resistant strain. This correlation of antibiotic use and the frequency of resistance was quickly understood. Noting the correlation, some argued that reductions in usage of antibiotics would cause lowering rates of resistance among clinically relevant strains. Successes in this approach have been limited.

When Danish authorities banned the use of avoparcin (chemically related to vancomycin) and cephalosporins (β-Lactam) from non-clinical use as growth promoters in livestock, the rates of resistance to these antibiotics did decline (van den Bogaard et al. 2000; Agersø and Aarestrup 2013), although they did not disappear. In Britain, however, following the prohibition of antibiotics as growth promoters in swine farming, the rates of presence/absence for resistant *Escherichia coli* in pig feces showed no decline even years after the ban came into effect (Smith 1975). And when the amount of antibiotics prescribed by physicians for clinical use was reduced in Sweden over a ten year period, the frequency of a penicillin (β-Lactam) resistant clone actually rose, even as the number of hospitalizations for infection decreased (Mölstad et al. 2008). Disappointing results such as these lead Smith in his 1975 paper to label this approach as “the fallacy of assuming that the ecological changes brought about largely by the persistent and widespread use of antibiotics can be reversed simply by resorting to a policy of withdrawal.” If the environmentally mediated change facilitated by the presence of an antibiotic is the force that selects for resistance, why does removal of

that force *not* change the direction of selection and bring the frequencies of resistance to zero[1]?

Further complicating this question was the finding that antibiotic resistant strains often incurred a fitness cost relative to their drug sensitive ancestral strains when grown in antibiotic free environments (Andersson and Levin 1999), making them less fit when the antibiotic was removed. This would suggest that the reduced use approach should be easily successful in reducing and eliminating antibiotic resistance. The reasons that resistant strains were not rapidly outcompeted after cessation of antibiotic use had no reasonable explanation until the role of compensatory mutations was elucidated.

Compensatory mutations are mutations that come into a population that have the effect of reducing the fitness cost associated with the mechanism of resistance to a specific antibiotic drug (for fine reviews, see Andersson and Hughes 2010, 2011). These changes are often suppressor mutations that suppress the negative, fitness robbing phenotypes (molecular or otherwise) that are associated with the antibiotic resistance genotype (Andersson and Levin 1999; Maisnier-Patin and Andersson 2004; Poon et al. 2005). Today, compensatory adaptation is commonly put forward as the mechanism by which antibiotic resistant strains recover fitness costs and compete successfully with initially higher fitness sensitive strains (Levin et al. 1997; Andersson and Levin 1999; Andersson and Hughes 2010, 2011). Compensatory mutations have been observed in several studies that have looked at the loss in fitness that have been

[1] Such statements are not without exception, however. Seppälä and colleagues (Seppala et al. 1997) reported good success in reducing resistance to macrolides in Finland following a reduction in clinical usage over a sustained period of time. Following the implementation of strong reductions in macrolide use in 1991, climbed to a peak of 19% in 1994 and then began declining consistently thereafter. Additionally, they also report similar findings from a reduction in macrolide use in an area of Japan from 1981-1990, with resistance reportedly falling to almost zero.

described as commonly occurring when antibiotic resistance phenotypes are gained (Bouma and Lenski 1988; Schrag and Perrot 1996; Schrag et al. 1997; Björkman and Andersson 2000a,b; Levin et al. 2000; Maisnier-Patin and Andersson 2004; Paulander et al. 2009). Typically, the fitness lost is recovered by means of additional beneficial mutations coming into the population that impart a restored phenotype to the bacterium (Schrag and Perrot 1996). These mutations have been observed both intragenically (in the same gene as the resistance granting mutation) as well as extragenically (in another gene) (Björkman and Andersson 2000a).

Additionally, compensatory adaptation offered an explanation to an associated problem with lower fitness antibiotic resistant strains. The observation was made that there was no evidence of selection for revertants back to wild type sensitive strains among resistant ones even though such reversion would restore the fitness lost. However, compensatory adaptation suggests that revertants were not the only means of restoring fitness. The loss of fitness relative to the sensitive ancestor outside antibiotic laden environments would select for beneficial mutations and bring these compensatory mutations to high frequency. The number of potential mutational targets available would be higher than the single target that would cause a reversion to the previous genotype, making compensatory adaptation much more likely than reversion just as a matter of chance (Levin et al. 1997; Stewart et al. 1998).

Adding further to this narrative was the finding that resistant mutants could not only recover the fitness lost through compensatory adaptation but could have fitness levels in the end that were higher than their sensitive wild type ancestors (Björkman et al. 1998; Hall et al. 2010). This would make compensated mutants unlikely to decrease

in frequency even if clinical use of antibiotics were completely eliminated as these high fitness strains would out-compete their ancestors even in drug free environments.

However, this also lead to a concerning paradox. In samples collected from environments still unexposed to antibiotics (Rwego et al. 2008), as well as from historical collections of pre-antibiotic era bacterial samples (Datta and Hughes 1983; Allen et al. 2010; Haase et al. 2011), phenotyping of samples has revealed that antibiotic resistant clones exist within bacterial populations as part of the natural variation in these populations at low frequencies. If antibiotic resistant genotypes exist with natural populations and these clones have the ability to increase fitness by means of compensatory mutation, why were these clones not at high frequency prior to the widespread use of antibiotics? Put another way: if the resistant mutants have a fitness high enough to survive in competition with the sensitive strains when the antibiotic is removed, why were the resistant strains not there at high frequencies the entire time? How was the antibiotic ever effective at all?

Also troubling is the speed with which resistant strains were observed for classes of antibiotics. Fleming's miracle drug, penicillin, was first used in clinical treatment in 1940, with the first reports of resistance published before 1950. Waksman's streptomycin was introduced in the clinic in 1943. Resistance was already known in the literature by 1948 (Crofton and Mitchison 1948) and the mechanism understood within the following decade (Erdös and Ullmann 1959; Luzatto et al. 1968). In fact, Crofton and Mitchison were the first to raise the questions (albeit not using population biology terminology) of fitness and competition between sensitive and resistant clones as well

as whether there was a baseline frequency of resistant bacteria within the population *prior* to any antibiotic exposure.

Compensatory mutations and suppressor mutations provide very reasonable explanations of the long term viability of antibiotic resistant strains, particularly after the selective pressure of the antibiotic chemotherapy is removed from the environment. Often, these explanations address improvements in fitness in a direct one-to-one ratio with the mechanistic changes that confer the ability of the bacteria to survive exposure to the drug in question. However, these compensatory mechanisms, while often found *in vitro*, are not often found in clinically relevant antibiotic resistant isolates (Andersson and Levin 1999). In fact, evidence of compensatory evolution or suppressor mutations from antibiotic resistant strains isolated from human patients is notably lacking. Yet, laboratory experiments using serial culture techniques have reliably shown just such compensatory adaptation taking place following mutational resistance to antibiotics. One study also found compensatory adaptation in *Salmonella enterica* grown in both flasks and in mice, albeit the types of compensatory mutations differed in each (Björkman and Andersson 2000a).

The present study seeks to investigate the type and mechanism of compensatory adaptation following resistance to streptomycin in *E. coli* grown under continuous culture in glucose limiting conditions, using a gradient of streptomycin concentrations. Continuous culture may provide for closer environmental conditions to the relevant *in vivo* conditions found within a pathogen's host. Thus, chemostats may provide insight into the differences in selection between such environments. Comparisons between continuous culture and serial transfer flasks will be possible by concurrent experimental

evolution of the same strains in the same media in serial transfer environments. Additionally, alternative media or growth conditions will be utilized in order to elucidate the forces at work in adaptation post antibiotic resistance. The experiments employed covered a range of growth conditions as well as time courses in order to shed light on any potential mechanisms.

Methods

Strains

All bacterial strains used are *E. coli*, K-12 MG1655 strains known and utilized in the lab for many years. The ancestral strain for all subsequent experiments is noted as DD1953 and is known to be free of any plasmids, to be antibiotic sensitive and to be *rpoS*- due to a premature stop codon in the coding sequence of the *rpoS* gene. This leaves DD1953 without any ability to utilize σ^S as a stress-inducible global regulator, which has been proposed as a mechanism for the difference in the level of fitness for streptomycin resistant *E. coli* in different media in previous reports (Paulander et al. 2009).

The DD1953 strain served as the streptomycin sensitive ancestral strain for in all experiments. In order to assure genetic homogeneity at the outset, DD1953 was grown from a single *E. coli* colony on a LB agar plate, grown in liquid media and that stock was frozen for long term use.

A screen was used to isolate single colonies of streptomycin resistant mutants of DD1953. Several mutants were observed and grown from single colonies. All streptomycin resistance granting mutations were identified by use of single gene Sanger-type DNA sequencing using defined forward and reverse primers. Pure freezer stocks were prepared and all subsequent experiments were started from the prepared stocks, stored at -80°C. Table 1 contains the strain names along with the mutations found in the *rpsL* gene of all streptomycin resistant mutants, along with additional descriptive data. All mutations were identified in the *rpsL* gene. The *rpsL* gene was PCR amplified using forward and reverse primers and then Sanger sequenced at the University of Arizona Genetics Core. These sequence reads were then vetted for quality and aligned to the ancestral *rpsL* gene from the DD1953 strain, as well as the reference K-12 MG1655 genome (Genbank accession NC_000913.3) sequence for *rpsL*. Mutations were identified only when forward and reverse reads of high quality agreed.

Along with *rpsL*, compensatory mutations in the *rpsD* and *rpsE* genes have been reported in the literature (Kurland et al. 1996; Björkman and Andersson 2000a,b). These genes were also sequenced using a similar protocol for comparative reasons. All alignments and assemblies were completed using Geneious bioinformatics software tools (Biomatters Ltd., Auckland, NZ) version 7 or later. Mutations in *rpsL* have long been known to affect susceptibility to streptomycin by changing the molecular structure of the S12 ribosomal protein. S12 interacts with the A-site of the 30S ribosomal subunit, which plays a role in the proofreading step of codon-anticodon recognition and aminoacyl-tRNA selection in protein synthesis.

Resistance to the bacteriophage T5 was used as a molecular marker in situations where one was desirable, such as in direct competition experiments. Resistance to T5 is due to a single nucleotide polymorphism (SNP) in the *fhu* gene and has been shown to be selectively neutral in chemostat experiments (Moser 1958). T5 resistance (T5R) was generated by streaking FS1 on LB agar plates supplemented with high concentrations (MOI>>2) of T5 bacteriophage virus. A single T5R *E. coli* colony was grown in liquid media for each applicable streptomycin resistant strain as a stock to be used as needed.

Chemostats

All continuous culture experiments were conducted in Kubitschek style chemostats (Dykhuizen 1993). Chemostats were run so as to constantly supply a set amount of fresh media to the 30 mL growth chamber. The amount of media supplied per unit time (the dilution rate, D) was controlled using a Wizco peristaltic pump. By controlling the amount of media available, the population growth rate was maintained in exponential phase throughout the experiment and the generation time can be controlled. Here, the generation time was set to 3.69 hours per generation (6.5 generations per day, D = 0.1875) in order to match the number of generations per day in the 1:100 dilution of flask cultures. The media for all chemostat experiments was Davis salts supplemented with 0.01% glucose (w/v) as the only available carbon source. Spent media and cells left the growth chamber at the same rate fresh media entered. This was done by means of a waste and collection port. All media and growing cells in

the chemostat were constantly mixed using purified and humidified air pumped from the bottom of the chemostat by an aquarium style air pump. This was done so as to maintain proper mixing at all times and to discourage the formation of biofilms on the walls of the growth chamber. The chemostats were maintained at a constant 37°C temperature by means of a water bath.

The chemostats were initially set up and allowed to run for at least 36 hours prior to inoculation with the experimental populations. A sample was taken from the chemostats just before inoculation and plated, without dilution, to verify that the chemostats were free from bacterial contamination at the start. The inoculating sample was grown from frozen stocks overnight in flasks with media identical to that in the chemostat except for a 10X increase in the concentration of glucose. In the morning, 100 µL of the overnight growth was placed into 10 mL of fresh media with 0.1% glucose in a sterile side arm flask and growth in these flasks was tracked by measuring optical density at 600 nm at regular intervals with a WPA biowave model CO8000 Cell Density Meter. When the OD was measured to be 1.7-1.85, the samples were immediately used to inoculate the chemostats. One mL of each strain of this inoculant was used in each chemostat, bringing the total volume of inoculum per chemostat to 2 mL.

Long term chemostats were conducted for a minimum of 10 days and a maximum of 31 days. Samples were collected from the chemostats via the sampling port approximately every 24 hours starting from the time of inoculation. The chemostat samples were diluted 100,000 fold in Davis salts and then replicate plated on LB agar plates with streptomycin. When T5 was being used as a molecular marker, the samples were also replicated on LB agar plates supplemented with streptomycin and T5

bacteriophage (MOI>>2). All plates were incubated overnight at 37°C and CFUs were counted using a ProtoCol automatic plate reader and ProtoCol software V3.15, both by Synbiosis (Cambridge, UK). All plates followed the method previously described (Dykhuizen and Hartl 1981). Basically, plates were made in quadruplicate and the values used in tracking the populations are the average of the 4 replicate plates. T5R and T5S counts were calculated by subtracting the mean number of T5R CFUs from the mean total of all CFUs.

Long term experiment population frequencies were tracked over the course of the experiments (Fig. 1). As adaptive changes were selected for in the chemostat, the frequency of the clone with the positive adaptive mutation increased in frequency until a selective sweep was completed or another positive mutation came into a competing clone and the difference in average fitness between the two competing clones was lowered, in which case, the frequency of the second positive allele would rise in the population. If a second positive mutation occurred in the same clone as the first positive mutation, the rate of change in that clone would increase at the expense of other competing clones in the chemostat. This is so because the chemostat has a constant and limited population size. For *E. coli*, that population size in 30 mL chemostats with the media used here is 10^8 cells/mL (N = 3.0 x 10^9).

All long term chemostat experiments were done in replicate. A total of 4 chemostats were run simultaneously. Chemostats 4-1 & 4-2 utilized the Davis minimal media supplemented with 0.01% glucose and contained a concentration of 100 µg/mL of streptomycin in the media. Chemostat 4-3 had the Davis minimal media, 0.01% glucose, and 16 µg/mL of streptomycin as per Miller (Miller 1992). Chemostat 4-4

served as the negative control and had Davis minimal media with glucose, but contained no streptomycin. An additional condition control was used by growing the same strains using the same media with the 100 µg/mL streptomycin concentration in serial transfer flasks, described in detail below.

Chemostats were changed and the experimental media and population was moved into new sterile chemostats every 10 days or less, so as to avoid the complicating factors associated with any possible build-up of biofilms on the walls of the chemostats. Media and media jars were refreshed every week as well. All streptomycin came from a single batch of prepared stock (10 mg/mL).

Serial Transfer Flasks

Run concurrently with applicable chemostat experiments, serial transfer flasks were an environmental control for the continuous culture experiments. Whereas the chemostats maintained the experimental populations in a constant state of exponential growth, the serial transfer flask populations underwent full cycles of lag phase, exponential growth, and stationary phase each day. This cycle has been suggested as being important to the development and maintenance of antibiotic resistance (Levin et al. 1997), thus was included in the investigation here. The flasks were run in a replicate of two.

Two sterile 250 mL flasks were prepared with 30 mLs of fresh Davis minimal media, 0.01% glucose, and 100 µg/mL of streptomycin. The flasks were initially inoculated with 1 mL of the same inoculant as the chemostats, performed concurrently.

The flasks were placed in an incubator, maintained at 37°C and constantly shaken at 200 rpm for 24 hours.

After the 24 hour growth period, 300 µLs of the experimental sample was transferred into a fresh 250 mL flask with 30 mLs of fresh media. At the time of transfer, a 1 mL sample was also taken for plating (as described above). The 100 fold dilution regime and 24 hour time frame allows for the calculated number of generations to be 6.5 generations in the 24 hour lag-exponential growth-stationary period (Lenski et al. 1991). This insures that the *average* generation time of the *E. coli* in the flasks match the *maintained* generation time in the chemostats, minimizing differences between the experimental replicates.

Growth Curves

Growth curve data for strains were obtained by monitoring optical density (λ = 600 nm) in 96-well plates in a SpectraMax Model 384 Plus microplate reader and SoftMaxPro software (Molecular Devices, Sunnyvale, CA) over a ten hour period. The strains were grown overnight in 3 mL of LB (rich media). The next day, 300 µL of the overnight growth was added to 9 mL of test specific media (3:90 dilution). For this series of experiments, the media was either “rich” (LB broth) or “poor” (Davis salts supplemented with 0.1% glucose). The media-strain mixture was vortexed to insure even distribution. The inoculated media was then put into replicate wells on the 96 well plate, with 180 µLs in each well and at least 1 negative control well per set of technical

replicates. The number of replicates varied from 23-33, depending on how many samples were placed on each plate.

Following inoculation, the 96 well plate was placed into the plate reader and the populations were allowed to grow. The OD was measured every 60 seconds for a period of 10 hours, with mixing of the plate between reads. Typically, the populations reached stationary phase well before the 10 hour mark. Data was analyzed using the grofit package (Kahm et al. 2010) and R statistical software (R Development Core Team 2012). The grofit package assigns a time point for transition into exponential growth as well as into stationary phase, thereby allowing for analysis of length of time spent in lag phase (noted as $\boldsymbol{\lambda}$), as well as the maximum growth rate (maximum slope in exponential phase, denoted as $\boldsymbol{\mu_{max}}$), and the density at the transition into stationary phase (maximum population size, $\boldsymbol{A}$). This allows for quantitative analysis of these measures for each population, using ANOVA. Post-hoc statistical analyses for pairwise comparisons of groups were conducted using Tukey's honest significant differences test (Tukey's HSD), part of the core R statistics program.

Sequencing & Bioinformatics

Resistance mutations to streptomycin in *E. coli* strains are most often linked to SNPs in the *rpsL* gene. This gene codes for the ribosomal protein S12, which makes up part of the small (30S) subunit of the *E. coli* ribosome. In particular, this protein interacts with ribosomal RNA (rRNA) and messenger RNA (mRNA) at the codon-anticodon recognition site of the ribosome. This suggests that S12 has a role in

aminoacyl-tRNA selection and the translational proofreading process (Kurland et al. 1990, 1996; Kurland 1992; Noller and Nomura 1996).

The *rpsL* gene was PCR amplified using forward and reverse primers (Appendix) and then Sanger sequenced at the University of Arizona Genetics Core. These sequence reads were then vetted for quality and aligned to the ancestral *rpsL* gene from the DD1953 strain and the reference K-12 MG1655 genome (Genbank accession NC_000913.3) sequence for *rpsL*. Along with *rpsL*, compensatory mutations in the *rpsD* and *rpsE* genes have been reported in the literature (Kurland et al. 1996; Björkman and Andersson 2000a,b). These genes were also sequenced using the identical protocol. Alignments and assemblies were done using bioinformatics software tools in Geneious (Biomatters Ltd, Auckland, NZ) version 7 or later.

Illumina MiSeq

In order to assess overall changes to the genomes of several of the experimental populations, Illumina MiSeq NGS was employed to sequence the genomes of a sampling of the experimental populations and the ancestral strain DD1953. Miseq sequencing was performed on population samples that were prepared using the Illumina Nextera XT kit for 250 basepair paired reads.

Overall coverage of the genomic sequencing was very low (mean of 9 population samples: 3.822). We believe this was due to a manufacturing problem associated with the lot of reagents sent from Illumina (Illumina personal communication). While the overall coverage depths across the genomic samples were low, the Q30 scores were

acceptably high so as to have a good deal of confidence in the accuracy of the base calls. However, in order to overcome the low coverage without loss of the total data set, the entire bank of MiSeq data was used only to identify potential SNPs across populations when depth of coverage was greater than 5. Associated bioinformatics parameters were also set to conservative values in an effort to utilize this data in a qualitative, rather than quantitative manner.

Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MIC) for all strains were found experimentally using OD measures LB liquid media. The strain of *E. coli* to be tested was grown overnight in a test tube in LB at 37°C, shaken at 200 rpm. After the population had entered stationary phase, 100 µL of the overnight growth was put into fresh LB media and allowed to enter exponential growth phase. After the start of exponential growth, 100 µL of the growing population was placed into a sequence of test tubes with fresh media containing 1.9 mL of fresh LB along with an array of streptomycin concentrations, from 0 to 1000 µg/mL. Growth was recorded by measuring optical density (λ= 600 nm) at regular intervals with a WPA biowave model CO8000 Cell Density Meter.

Chemostat Competitions

When possible, direct competitions between strains were conducted in chemostats in the manner of Dykhuizen (Dykhuizen and Hartl 1980; Hartl and Dykhuizen 1984; Dykhuizen 1993). Using the T5 bacteriophage molecular marker, two strains were inoculated in to a chemostat in approximately equal numbers. Changes in the frequencies of these strains were tracked over 3-5 days via plating as described. All chemostat parameters were as previously described (generation time 3.7 hrs, 37°C) but the concentration of streptomycin was varied to coincide with the context of the competition.

By plotting the natural log of the ratio of CFUs per unit time, the selection coefficient of selection was obtained. From these measures, the relative fitness measure was calculated. As the selection coefficient, s, was calculated by taking the natural log of the ratio of the number of mutant CFUs to the number of ancestral CFUs, the relative fitness, w_R, is calculated by use of the equation:

$$w_R = e^s$$

Additionally, in instances where the proper molecular marker was available, competitions between ancestral and descendent strains is also possible through the use of frozen bacterial stocks.

Long Term Control Experiment

In order to verify that the dynamics observed for the main series of experiments were not unique to the particular mutant utilized, a separate long term control experiment was performed using another streptomycin resistant mutant from the same ancestral stock of DD1953. This long term control experimental series, labeled as experiment 16, was conducted using the same techniques described with identical conditions. Experiment 16 was completed in 4 chemostats, with the concentrations of streptomycin being identical to those in the previous 4 chemostats, namely 2 high streptomycin concentrations (100 μg/mL), a low streptomycin condition (16 μg/mL) and a no streptomycin control condition. This experiment was also simultaneously conducted in flasks with conditions identical to those in the previous flasks (100 μg/mL).

This experiment was conducted over 30 days with the identical generation time of 3.7 hours/generation, for a total of 199 generations. Samples were taken and plated as described; however, there was no molecular marker, just a single strain in each chemostat or flask.

Results

Result 1: Loss of Fitness in Streptomycin Resistant Mutant

The streptomycin resistant mutants listed in Table 1 all lost fitness relative to the streptomycin sensitive ancestor DD1953. The long term chemostat and flask

experiments were inoculated with one or more of these strains. Strains FS1 and FS5 both had identical mutations in the *rpsL* gene that allowed them to survive in streptomycin laden environments. This mutation was at codon 42, which normally codes for lysine (L). In these mutants, the codon is for asparagine (N), making both FS1 and FS5 K42N streptomycin resistant mutants. The MIC for these mutants was measured as being higher than 1,000 µg/mL.

The fitness of FS1 and FS5 was measured to be 0.91 relative to DD1953. This relative fitness was determined by direct competition experiments in chemostats without streptomycin over a period of 5 days. This measured loss in fitness is in line with other published reports of the percentage of lost fitness due to resistance to streptomycin by means of the K42N mutation in *rpsL*, even when measured in species other than *E. coli* (Schrag et al. 1997; Björkman et al. 1999).

Result 2: No Expected Compensatory Mutations

Long term chemostat experiments were sampled and plated daily to understand frequency changes occurring within the populations. Figure 1 illustrates these frequency changes. Chemostats 4-C1 and 4-C2 had high concentrations of streptomycin (100 µg/mL). Chemostat 4-C3 had the low concentration of antibiotic (16 µg/mL), while chemostat 4-C4 served as the negative control and was completely streptomycin free. The results of the long term chemostat experiments did not yield any of the expected compensatory mutations. This was so in all strains from all chemostats in all three of the genes in which compensatory mutations have been

previously reported (*rpsL, rpsE, rpsD*). The sequences of these genes were confirmed using PCR-amplification and Sanger sequencing. The serial transfer flasks, 4-S1 and 4-S2, were also found not to have any compensatory mutations in these candidate genes using the same techniques for the identification of any mutations.

These results are counter to the model in which antibiotic resistant strains of bacteria are sustainable outside of drug-laden environments due to mutations that recover fitness lost relative to the antibiotic sensitive strains. As stated above, this model has been experimentally observed, but such compensatory mutations, identified *in vitro*, are often absent from clinically relevant resistant strains taken from infected hosts. The results here align more closely with the clinically derived strains than from the laboratory derived results.

Result 3: Adaptation and the Recovery of Fitness

Even though none of the expected compensatory mutations were found, these populations all exhibited increases in fitness. This is confirmed through direct competition experiments against FS1 or FS5 (Fig.s 1&2). Overall, the amount of fitness recovered varied, with 4-C1-11S having the highest increase in fitness. These increases in fitness are not surprising, given that population genetics would predict such increases as beneficial mutations come into a population. As a beneficial mutation comes in and increases the fitness of a particular clone, the frequency of that clone would rise at the expense of less fit clones in the population. This leads to selective

sweeps until, eliminating or severely limiting the competing clone. Such dynamics have been seen many times before in both serial transfer and chemostat experiments.

Result 4: Whole Genome Sequencing Sheds No Light on Mechanism

Although none of the expected compensatory mutations were found in any of the candidate genes, fitnesses relative to the streptomycin resistant ancestral strain (FS1 or FS5) did increase in all chemostats as well as in all flasks, without the benefit of the expected compensatory mutations. What is the cause of these increases in fitness?

In order to understand this more fully, population samples were sequenced using Illumina MiSeq. Results of the whole genome testing were hampered due to reagent issues and thus are limited to conservative identification of genomic SNPs. However, even the limited MiSeq data indicates that a fair number of mutations came into these experimental populations over the course of these experiments, with the highest number of mutations, by far, occurring in the 4-S1 flask, where both strains remained at relatively stable frequencies (T5S: 0.487; T5R: 0.513). This implies that the body of mutations across the genomes is responsible for these fitness increases.

Importantly, we must take note of the fact that although there were numerous changes across the genomes of the populations sequenced, and we can attribute fitness increases to these SNPs, there was no clear pattern or repeatability to the numbers or types of SNPs found in the different sample populations sequenced. In fact, SNPs came into just a few genes in common between samples (Table A4), limiting any insights that could possibly be drawn from the array of SNPs identified.

We note here with interest that the flask grown population that maintained stable coexistence between the two competing strains over 286 generations also had the most SNPs within their genomes implies that the population dynamics occurring within this flask differ from all of the other experimental populations, in which one strain would go to high frequency at the expense of the other. In the case of the 4-S1 flask population, population equilibrium was maintained over a long period of time with a high degree of polymorphism. The cause for this coexistence is not currently clear.

Result 5: Growth Curve Analysis and Phenotypic Changes

In order to investigate these fitness changes further, we collected data on the growth dynamics under two media types in 96-well plates. The growth curves were then analyzed to understand some of the underlying phenotypes that contribute to the fitness increases (Fig.s 6 & 7). The growth curve data indicate phenotypic changes of a scale and type very different from accepted hypotheses of compensatory mutation following the gain of resistance to an antibiotic.

Attempting to quantify relative fitnesses of strains based upon the maximum growth rates (μ_{max}) using data from the growth curve experiments yields confounding results. In previously published reports, relative fitnesses have been calculated by comparing generation times (for example, see Björkman and Andersson 2000a, footnote No. 19). These comparisons are predicated on the assumption that the strains involved are competing based upon growth rate alone.

When the same reasoning is applied to the experimental strains here using data from direct competitions in comparison to data on the same strains in the growth curve experiments, the results contradict each other. Of course, the growth curve data collected is for the maximum growth rate, not the average growth rate and the direct competitions conducted in chemostats would have been under conditions of exponential growth at a point nearing saturation, not at the point of maximum exponential growth, as indicated by the growth curves generated (see Figure 4 for example growth curves). Analysis of Variance and post-hoc pairwise comparison tests indicate that changes in μ_{max} were statistically significant between strains at the end of adaptation experiments as compared to the streptomycin resistant mutant at t=0 of long-term experiments (see Appendix for table with p-values for all comparisons).

Result 6: Very Low Fitness Mutants May Behave Differently

In order to investigate whether the relative fitness of the streptomycin resistant mutant at the time of inoculation determines the outcome of compensatory adaptation, an additional long term control was undertaken. The hypothesis tested here was whether or not the ultimate presence or absence of the previously reported compensatory mutations in *rpsD* and *rpsE* could be manipulated by the degree of fitness lost in the mutant relative to the sensitive ancestor. If the results of the long term control differed, that would imply that there is a threshold below which fitness must drop in order to elicit the known compensatory mutations to raise fitness through rapid selective sweeps in the population.

The mutant selected from a screen for streptomycin resistance was the slowest growing mutant found in any screen conducted in the lab. Sanger sequencing of this mutant, 11-49, indicated that the mutation that was responsible for the streptomycin resistance was a P90Q non-synonymous change in *rpsL*. This *rpsL* mutation, P90Q, has only rarely been reported previously in the literature (Holberger and Hayes 2009). Secondly, the P90Q mutation has also been reported as making the cell streptomycin "pseudo-dependent," with a measured growth rate twice as fast in media with 50 μg/mL of streptomycin as when the compound is absent. This pseudo-dependence made direct competition with the DD1953 ancestor an impossibility. However, just by the qualitative observations of 11-49's rate of growth in test tubes, the low fitness of the strain was assured.

In order to inoculate the long term control experiments, 11-49 was grown overnight and the chemostats and flasks were inoculated as per the described protocol. When Sanger sequencing of completed for the inoculant, a secondary mutation in *rpsL* was found. This non-synonymous mutation was identified as K42N, the same mutation as in the FS1 and FS5 strains. This double mutant, labeled as FS8, was found in all 4 chemostats and the 2 replicate flasks for the long term control experiment in t = 0 samples, indicating that the secondary mutation came into the population while growing overnight for inoculation and completed a selective sweep overnight or extremely early in the experimental chambers of the chemostats and flasks.

Subsequent tests indicated that FS8 was able to grow in the absence of streptomycin and had a measured MIC greater than 1,000 μg/mL, just as in the other streptomycin resistant strains. Additional tests also found that the secondary mutation,

K42N, would consistently occur when 11-49 was grown from the initial single colony from the streptomycin resistant screen, suggesting an immediate increase in the ability to grow with the secondary mutation. This may also explain why the secondary mutation comes into the population so readily. If P90Q is dependent upon the concentration of streptomycin for growth, even in moderate concentrations, we can expect a physiological advantage exists for any double mutant that is relieved of this requirement. This may be due to the additional waiting time that would be needed to have streptomycin present in every 30S ribosomal subunit at all times during what should be rapid exponential phase growth, or some other associated aspect thereof, such as a drop in one of the kinetic thresholds associated with cognate aminoacyl-tRNAs binding in the A site and subsequent proofreading steps.

In this sense, the K42N secondary mutation is acting as a compensatory mutation. The K42N mutation would be changing the kinetics associated with tRNA selection, thereby changing the fitness instantaneously in lower or no strep environments. Interestingly, if this is so, then a reversion to *wt* in the P90Q site may confer a fitness increase both in the presence or absence of streptomycin, perhaps explaining the low frequency with which the mutation is found, reflecting the low rate of the mutation happening, multiplied by the low rate of the secondary SNP at the codon 42 site, taken in conjunction with the rate of reversion in the AA 90 locus, all of which would quickly go extinct inside of any sizeable population.

Therefore, the P90Q mutation may act as a bridge mutation, allowing for the survival of a mutant under otherwise killing conditions, regardless of alternative fitness levels, even though this mutant will be very quickly outcompeted by many, if not all,

double *rpsL* mutants. P90Q, then, can be so low in fitness as to be ephemeral, when that short-lived extension even *may allow* for population survival. This would mean that in environments where hard selection exists, there can be transient niches which can impact, or even determine, subsequent genotypes. For instance, there is no way to discern directly whether or not all K42N mutations were the outcome of a wt → P90Q → P90Q -K42N → K42N series of mutational changes to the genotype that, due to selection, occur very quickly. In this line of thinking, whether the K42N change can be considered as compensatory or not can be debated with equal force on both sides of the argument.

Due to the rapid shift from single to double mutant, attempts to quantify relative fitness of the P90Q single mutant to wild type were not feasible. Further, direct competitions with the DD1953 sensitive strain also could not be performed with the double mutant, as the FS8 went extinct in direct competition chemostats within 24 hours (6.5 generations), even when the relative concentration of FS8 to DD1953 at the time of inoculation was greatly increased. However, the FS8 double mutant could be competed with the FS1 single mutant, both of which are streptomycin resistant. When this was done, the fitness of FS8 relative to FS1 was measured in direct competition to be 0.802 (although R^2 was poor and could not be improved in replicates).

Result 7: Use of a Lower Fitness Mutant Does Not Lead to Additional Compensatory Mutations in a Long Term Control

The results of the long term control experiment support the findings of the previous long term experiments in that none of the expected extragenic compensatory mutations were found in the three candidate genes (discounting the secondary K42N mutation as a compensatory mutation). Samples from all chemostats and flasks of this long term control experiment were Sanger sequenced for the three candidate genes every 65 generations over a span of time encompassing 199 total generations. In all samples, across all environments and timescales, no additional genetic changes occurred in these three ribosomal genes.

Interestingly, if we consider the secondary mutation of K42N as an intragenic compensatory mutation, this result would have to be amended to say that the long term control did have results differing from the first set of experiments. However, here, compensation occurs on an extremely short timescale. This would require the secondary (or in this line of thinking, the intragenic compensatory) mutation to always occur as rapidly and reliably as observed here. This would have to be particularly so if any competition, even amongst clones, were to be present in the population. If competition were present, then the P90Q single mutant would be rapidly outcompeted and driven to extinction. Yet, the reliability of the K42N secondary mutation in this series of experiments suggests that the expected waiting time for the next beneficial mutation in *rpsL* might be reduced by the presence of the P90Q, such as the case in

mutator alleles, following which, the original mutation might be counter-selected, as has been suggested previously for antibiotic resistance (Lindgren et al. 2003).

Discussion

In all of the experimental populations measured, fitness increased from the baseline at the time of inoculation. These fitness increases occurred in all populations, regardless of the concentration of streptomycin (high, low, or absent), the number of generations of post-resistant adaptation that was allowed (65-286), or the method of *in vitro* growth utilized (chemostats or flasks). These fitness increases, however, took place without the compensatory mutations that were expected and that have been previously shown to be beneficial in terms of fitness. The fact that populations fitnesses increased is not, however, surprising when we take into account the long list of experimental evolution experiments that have documented that the fitness of a population increases over time as strains adapt to factors such as environment, media, nutrient availability, or any other variable that impacts growth and reproduction (Dykhuizen and Davies 1980; Chao and Cox 1983; Dykhuizen et al. 1987; Lenski and Travisano 1994; Cullum et al. 2001; Poon et al. 2005; Gresham et al. 2008; Barrick et al. 2009).

Previous work has found a number of different SNPs in *rpsL*, the gene in which the streptomycin resistance mutation is usually found, and in genes whose products interact biochemically (which is to say, phenotypically) with *rpsL* products, namely, *rpsE*

and *rpsD* that can provide increases in fitness. The *rpsL* gene codes for the small ribosomal subunit protein S12. S12 is known to interact within the ribosome at the A-site, responsible for codon-anticodon pairing and aminoacyl-tRNA selection during translation. The A-site, along with S12, play critical roles in the proofreading process that safeguards the incorporation of correct amino acids into nascent proteins (Kurland et al. 1990, 1996; Noller and Nomura 1996; Rodnina and Wintermeyer 2001; Noller 2013). The error rate for incorporation of non-cognate tRNAs in the protein synthesis project has been experimentally measured at about 0.1% (Wohlgemuth et al. 2010).

The *rpsE* and *rpsD* genes code for proteins S5 and S4, respectively. These proteins also play a role in the proofreading process and changes to these proteins are known to cause the *ram* phenotype (Ninio 1974; Zaher and Green 2010) which causes faster but more error-prone amino acid incorporation into proteins. This results in the speedier translation of a protein but with the tradeoff of lower accuracy. This faster but more error-prone translation can have a spectrum of fitness effects, depending upon the protein being translated and the amino acid wrongly incorporated. Alternatively, a hyper-accurate phenotype is also known in streptomycin resistance in which the proofreading steps involved in safeguarding proper aminoacyl-tRNA selection, in which S12 is involved, are slowed down such that only exact pairings allow for the cognate codon-anticodon pair to remain chemically intact long enough to allow for peptide bond formation to occur in the P-site of the ribosome. Thus, the prevailing wisdom is that the accuracy of an optimized S12 is counterbalanced by the speed of optimized S4 and S5 proteins in the decoding of mRNAs during translation in wild type ribosomes.

Biochemically, therefore, there was no surprise when compensatory mutations for streptomycin resistance were discovered in *rpsE* and *rpsD*. The narrative had come full circle: the optimized S12 protein had been shifted away from a stable point in order to allow for survival in a streptomycin-laden environment. This shift disrupted the balance between speed and accuracy maintained by the S12-S5-S4 proofreading triumvirate. In order to compensate, S5 and S4 would shift to new kinetic points, thereby reestablishing the optimization that had been disturbed. These three genes then could co-exist without loss of fitness to the cell overall, even after the antibiotic was no longer present in the environment. Speaking in terms of evolutionary biology, a fitness valley was crossed during the selective sweep whereby the streptomycin resistant *rpsL* mutation fixed within the now resistant population. Selection then would be free to increase the frequency of compensatory mutations without consequence in *rpsE* and *rpsD* once those mutations came into the population. Prior to the fitness valley having been crossed, selecting for these compensatory mutations would have been maladaptive, but once the environment shifted to one in which streptomycin was providing for a hard selection for resistance, these SNPs had become advantageous.

The scenario this data provides, however, is one in which a streptomycin resistant strain displays the expected fitness deficit caused by the genetic change that imparts resistance and subsequently increases in fitness but does so *without* usage of any of the compensatory pathways previously described. This contrasts with the hypothesis that the streptomycin resistance granting mutation directs or drives subsequent adaptive changes through to the loss in fitness relative to the antibiotic sensitive ancestor. In fact, the paradigm of fitness lost by virtue of an antibiotic

resistance mutation followed by the whole or partial regaining of fitness by compensating for what is essentially flux through the affected biochemical pathway or mechanism is unsupported in the current series of experiments.

The relative amount of fitness increases varied most widely between the two identical experimental replicates (Fig. 2B) in the high streptomycin chemostats 4-C1 and 4-C2. Also interesting was the fact that relative fitness between one of the high streptomycin chemostats, 4-C2, aligned well with the low and no antibiotic conditions. This suggests once again that after the resistance mutation has increased to high frequency and the minimum inhibitory concentration (MIC) of the strain is above what is necessary in order to survive the chemotherapy, the mechanism of antibiotic resistance need not direct subsequent compensation. In fact, such non-directed post-resistance adaptation cannot be properly called compensatory in that the adaptation would occur along a fitness landscape that is not impacted by the particular phenotypic mechanism of resistance.

Taken together, results 1-4 imply that previously published results that have shown compensatory adaptation as the dominant viable mechanism for the long term survival of antibiotic resistant strains to be incorrect. While compensatory adaptation certainly does achieve the increases in fitness described, the results here, taken in conjunction with previously noted observations of clinically relevant antibiotic resistant strains lacking the identified compensatory mutations, strongly suggest that the main mechanism of sustainability for these strains does not involve compensatory mutations.

If compensatory adaptation is not the dominant mechanism, then how did the six adapted strains from experiment 4 increase in fitness? Whole genome sequencing shed no additional light on the process, as the wide assortment of SNPs across samples failed to yield any discernible pattern. But, we can move one level deeper, below fitness to individual phenotypes beyond just those phenotypes impacted by the resistance mutation. The impact of the K42N mutation on the rate of protein translation has been shown elsewhere. That does not, however, mean that this phenotype is the sole arbitrator of organismal fitness when considering streptomycin resistance. The growth curve experiments allow us to subdivide the growth phenotype into three finer-resolution phenotypes. These phenotypes are: time in lag phase (λ), maximal growth rate during exponential growth (μ_{max}), and the density at the saturation point (A). Looking at these three phenotypes individually, we can break down the fitness of these strains into compartments that may each be under individualized selective pressures within a given environments. By acknowledging the biological truth that all of the phenotypes of an organism will be under selective pressure to improve, particularly following an environmental shift, we can, by taking a finer grained look, allow ourselves to begin to bring the wider picture of fitness into focus. The *de facto* expectation is that these three phenotypes, which are highly intertwined under the larger growth phenotype, would be changing in similar ways at similar points across experimental replicates or strains. If this were found to be true, we could then look for the genetic factors implicated in such changes, just as has been done for the translation rate phenotype with regard to streptomycin resistance in the *rpsL* gene. This would allow for

us to complete what has been called a functional synthesis (Dean and Thornton 2007), allowing for understanding from the level of genotype, through phenotype, to fitness.

However, when we look at the data from the growth curve experiments, the results are not so clear. Firstly, the trends between a nutrient rich environment and the nutrient limited environments do not overlap. Differences in any given strain between λ, μ_{max}, and A vary widely. Much of this can be attributed to the availability of nutrients; however, even this can be counterintuitive: in some cases μ_{max} is higher for poor environments than for rich[2]. Additionally, lower phenotypic ability in one can be, to steal a phrase, compensated by strengths in others (see Figures 5 and 6). Changes in the ability to respond to conditions capable of supporting growth (time in lag phase: λ) do not directly decide upon fitness (*w*) without input from rates of maximal growth (μ_{max}) and saturation density (A) phenotypes. For instance, in the case of 4-C2-12R, λ, μ_{max}, and A are all lower than those of FS1, the streptomycin resistant ancestor. But, because having a shorter time in lag is beneficial, relative fitness of 4-C2-12R is higher than FS1 (w_R of 4-C2-12R: 1.04), even though having lower μ_{max} and A are fitness negatives. Any single phenotype here is not a direct proxy for fitness; all three must be taken together in their given environment.

This last point, that all phenotypes and the environment matter, is illustrated well by one of our results. When flask adapted strains are competed directly

[2] This phenomenon, in which growth can be greater for an antibiotic resistant mutant in limited media when compared to rich media, has been observed previously. In 2009, Paulander and colleagues reported that in *Salmonella enterica* variant Typhimurium LT2, K42N mutants grew faster on limited versus rich liquid media (Paulander et al. 2009). However, their findings suggest that this effect is the result of σ^S induction, whereas that cannot be the case in our σ^S-minus strain. σ^S which is encoded by the *rpoS* gene, is a global stress regulator and, among other things, aids in inducing the start of saturation-mediated dormancy. Our ancestral lab strain, DD1953, induces stationary phase without use of this global stress regulator.

against the low fitness ancestor (FS1 or FS5) in a novel chemostat environmental condition, their relative fitness diverge (Fig. 5). This was even found to be the case even though the 2 flask strains measured (4-S1-44S & 4-S1-44R) were grown and competed together in serial transfer and remained at constant relative frequencies over close to 300 generations (Fig. 3). These two co-adapted strains are just about equal in terms of fitness, else one would have increased in frequency relative to the other over the 286 generations of the long term flask experiment. Yet, when they are independently competed against their lower fitness ancestor under a novel *in vitro* condition, 4-S1-44R has a relative fitness of 0.96 while 4-S1-44S has a relative fitness of 1.02. To use an analogy, this would be like trying to measure the skill level of two well matched baseball pitchers by the outcomes of a series of cricket matches. Some skills transfer nicely, others will not.

These differences in the phenotypic abilities of the two strains competing in flask 4-S1 may, however, be an important illustration of how long term coexistence can be achieved counter to expected population genetic outcomes. Dean and colleagues have argued, and subsequently shown experimentally, that coexistence can be sustained if the competitors are in a fluctuating environment in which environmental variables favor a competitor when that competitor is rare (Dean 2005; Suiter and Dean 2005; Yi and Dean 2013). Their argument rests on the idea that for haploid organisms undergoing asexual reproduction without recombination, the variable most vital to coexistence is the relative number of doublings between the competing strains. If the environment favors competitor A at one point, but a shift in the environment then favors competitor B in

such a way that the overall number of doublings in a set period are equal, then the competitors will coexist for an indefinite period of time.

Here, these conditions may be met without outside manipulation of any environmental variable. In Davis minimal media, the growth curve analysis reveals that the two competitors, 4-S1-44R and 4-S1-44S, which were identical at the time of inoculation, varied in their phenotypic abilities to come out of lag phase (λ), their maximum rate of growth (μ_{max}), and the optical density at which they entered stationary phase (A). The 4-S1-44R strain was better, which is to say faster, at coming out of lag and beginning to grow, giving an initial advantage. But, the 4-S1-44S competitor, once out of lag phase, had a higher maximum rate of growth and entered stationary phase at higher density than 4-S1-44R.

The long term coexistence of these strains, when diluted 1:100 daily into fresh minimal media, results in their fitness being equal (Fig. 3). However, their relative fitnesses are dramatically different when measured against the ancestor in an environment to which they are not adapted, chemostats, even though the media used is precisely the same as that in the flask experiment (Fig. 5). Also interesting to note is that the general outcomes of the chemostat competitions are counter to an unexamined expectation.

In these chemostat competitions, the faster end to lag phase, along with the lower maximum growth rate and earlier entry into stationary phase would be expected, at the face of things, to be negative. Yet, 4-S1-44S actually increases in frequency, indicating higher fitness than the ancestor. On the opposite side of the coin, 4-S1-44R,

with longer lag but higher maximal growth rate and later stationary phase entry seems primed to do well in continuous culture. This is not so. The reasons are interesting and highlight a need on the part of science to look upon situations as the organism in question might, rather than with poorly conceived notions of expected outcomes.

As stated, the media in the chemostat competitions matched that in the flasks. This is a glucose limited minimal media. In the flasks, the strains need to come out of lag phase, enter exponential growth, achieve maximum growth rate as quickly and for as long as possible, and then enter stationary phase. In chemostats, no such repeated shifts between the phenotypes measured occur. The continuous culture conditions within the chemostat mean that a constant supply of a limited resource is available. There is no lag phase, thus an advantage in coming out of lag is no advantage at all. Further, the constant growth rate, at a level determined by the experimenter, not the organism, makes the growth rate an independent variable, incapable of affecting the outcome of the competition. Lastly, there is no stationary phase in chemostats, so the density that a competitor enters stationary phase is moot. Here, 4-S1-44S has higher fitness relative to the ancestor while 4-S1-44R has a lower relative fitness. This outcome is probably determined by how close the rate of doubling set by the researcher is to the maximum rate of growth of the organism. As the doubling time of the chemostats was 3.7 hours, this was much closer to 4-S1-44S's μ_{max} than to that of 4-S1-44R. Meaning that 4-S1-44S grew more efficiently in continuous culture than did 4-S1-44R, yielding the results illustrated in Figure 5.

Along similar lines, the impact of doubling time on the fitness of a streptomycin resistant mutant relative to the sensitive ancestor was investigated by direct competition

in chemostats at two different generation times, 1.5 and 3.7 hours, as well as in flasks. If a slower rate of growth in continuous culture could mitigate the effect on fitness of having a different protein translation rate than the wild type, the overall difference at the level of fitness would be evident in direct competition. No difference in the selection coefficient, *s*, was observed between any of the conditions tested (See Appendix Figure A1 for competition results). The finding that the selection coefficient of the ancestral antibiotic sensitive strain over the resistant strain remains unaffected by a greater than 2-fold shift in doubling time strongly suggests that the factor limiting growth and reproduction, the factors of fitness, is not physiologically time limited. Put another way, the limiting factor in this circumstance is not the limited time available for growth and reproduction, which should be optimized regardless of condition, but is more probably a limiting biochemical constraint. This would be in agreement with Liebig's Law.

Finally, we must consider the results of the long term control experiment. Here, when we used a low fitness streptomycin resistant mutant (P90Q) in *rpsL*, we very rapidly had a compensatory mutation come into the same gene at position 42, namely K42N. This is the same mutation that was found in strains FS1 and FS5 without the P90Q initial mutation. The single mutant was so short-lived that fitness measures were not reliably able to be obtained prior to the secondary mutation rising to high frequency in the population. Lindgren and colleagues have reported an increased mutation rate in flouroquinolone resistance. That is a possible explanation for the repeated K42N mutation here, but we do not have direct evidence in support of such a hypothesis.

Intragenic compensatory mutation has also been reported *in vivo* by Björkman and her coauthors, when they inoculated mice with *S. enterica Typhimurium* that was

streptomycin resistant with a K42N mutation in *rpsL*. However, that intragenic compensatory mutation consisted of at least two mutational steps away from K42N. The asparagine amino acid in this case, as in ours, is encoded by the triplet AAC. The compensated mutant that they recovered after about 50 generations from mice was AGA, which codes for arginine. They reported no intermediate genetic steps. The most parsimonious sequence of mutations that lead from AAC to AGA is AAC → AGC → AGA. In terms of amino acids, this sequence would be asparagine → serine → arginine. Interestingly, Björkman reports a 13X-28X increased mutation rate in the mice grown strains relative to those grown in tubes of LB media.

The question remains as to whether or not the lower starting fitness of the mutant directs subsequent compensatory adaptation. The results here suggest that such fitness deficits may in fact drive evolutionary acquisition of compensatory mutations, with the type and number of these determined by specific genetic background and environmental conditions. Curiously, no additional mutations were noted in our experiment, even when the K42N compensatory mutation came in very early on in the experiment. While an increased mutation rate has been reported elsewhere as a result of antibiotic resistance mutations, no work, to our knowledge, has been done in which the period of time during which these increased mutation rates hold has been attempted.

Also of note is the fact that in the present series of experiments, the low fitness mutant used for this long term control experiment, 11-49, was of such a low fitness that even the double mutant, FS8, was driven to extinction when directly competed with the streptomycin sensitive ancestor in chemostats. This suggests that such a low fitness

mutant would be rapidly eliminated from any population in nature, probably before any compensatory mutation would have the opportunity to increase in frequency beyond just a few singletons within a population. If the magnitude of the fitness lost via an antibiotic resistant mutation dictates where or not compensatory adaptation is required for long term persistence and these compensatory mutations would have to come into a population *and* rise to high frequency before they are outcompeted by other resistant mutants or the full fitness sensitive strain, then this required series is a plausible explanation as to why compensatory mutations are not often found in clinically relevant antibiotic resistant strains. This provides a potential solution to one of the paradoxes related to the persistence of antibiotic resistant strains of bacteria.

Conclusion

The results here, then, are rather mixed. The streptomycin resistant mutant utilized in the long-term experiment (FS1/FS5: K42N in *rpsL*) did show a fitness cost when compared to the streptomycin resistant ancestor (DD1953). Further, after long-term adaptation experiments, in high (100 μg/mL), low (16 μg/mL) and zero streptomycin concentration environments, both in chemostats and serial transfer flasks, all strains increased in relative fitness.

Additionally, none of the previously identified compensatory mutations that were initially expected were found, regardless of environmental variables or the number of generations allowed for growth (range: 65-286). With average population sizes greater

than 3 x 10^9 being achieved everyday both under continuous culture and in serial transfer, more than ample waiting time elapsed during which we would theoretically expect to see compensatory mutations occur and rise to high frequencies. Whole genome sequencing confirmed that numerous mutations came into the experimental populations and were able to rise in frequency over time. The cumulative effect of these apparently selected upon mutations would account for the increases in fitness.

One of the experimental populations, besides increasing in fitness, was also able to establish a stable coexistence between competing strains, contrary to the expectations provided by population genetic theory. The reason this occurred can be explained by Dean's hypothesis for coexistence due to bounded population sizes, which has been shown experimentally, but this would be the first known occurrence when the environment was not manipulated specifically for this purpose. Also of interest is that this particular population is the one with the highest number of SNPs according to the MiSeq data. The possibility that this is in fact an example of stable coexistence due to bounded population space is bolstered by analysis of the growth curve data in which differences in the ability to grow at different stages is clear between the two competing strains.

Compensatory mutation was finally observed in a long term control experiment in which the fitness of the starting mutant was severely lowered by the change in the *rpsL* gene that conferred streptomycin resistance. This mutant was hampered to the extent that a secondary mutation within the same gene raised fitness quickly. Once this second mutation had swept to fixation, further adaptation was as observed in the previous round of long term experiments without further compensatory mutations.

In conclusion, when all the data from these series of experiments are taken together as a whole, the only reasonable conclusion to arrive upon is that, while streptomycin resistance may have been conferred by a single mutation in a known location, all of the subsequent adaptation, in all environments, are the outcome of natural selection acting upon a given strain in a given environment and subjected to the stochastic nature of mutations coming into each population in a manner independent of all the others. This being the case, natural selection acted in the manner normal to all adaptive selection and chose to increase the frequencies of a distinct, random set of SNPs in each trial as they were able to increase fitness overall. No long-term pattern or path is pre-ordained to occur in these populations. Even the repeatability of adaptation within a given strain, seen in elegant work produced elsewhere (Schrag and Perrot 1996; Björkman and Andersson 2000a; Weinreich et al. 2006), falters here for reasons that are unknown and may, in fact, be random.

Such an explanation would be supported by observations of the perseverance of antibiotic resistant strains in a long list of environments (Smith 1975; Mölstad et al. 2008; Andersson and Hughes 2010). This line of reasoning also makes sense when you expand upon one of the explanations offered as to why compensatory mutations occur more often than reversions: the number of compensatory targets simply outweigh the single reversion target (Levin et al. 2000). If this is indeed so, the number of alternative fisherian-type mutations available (Fisher 1930; Orr 2005) in a population with sufficient levels of variation would vastly outweigh the number of possible compensatory mutations. After such adaptation to environmental conditions, compensatory mutations may no longer be adaptive, even in cases such as these,

where such high value phenotypes such as translation speed and accuracy are involved. This may be due to epistatic interactions with new alleles at other locations, the specific parameters imposed by a given environment, or other unforeseen interactions. In any event, the result is clear: evolution continues unabated.

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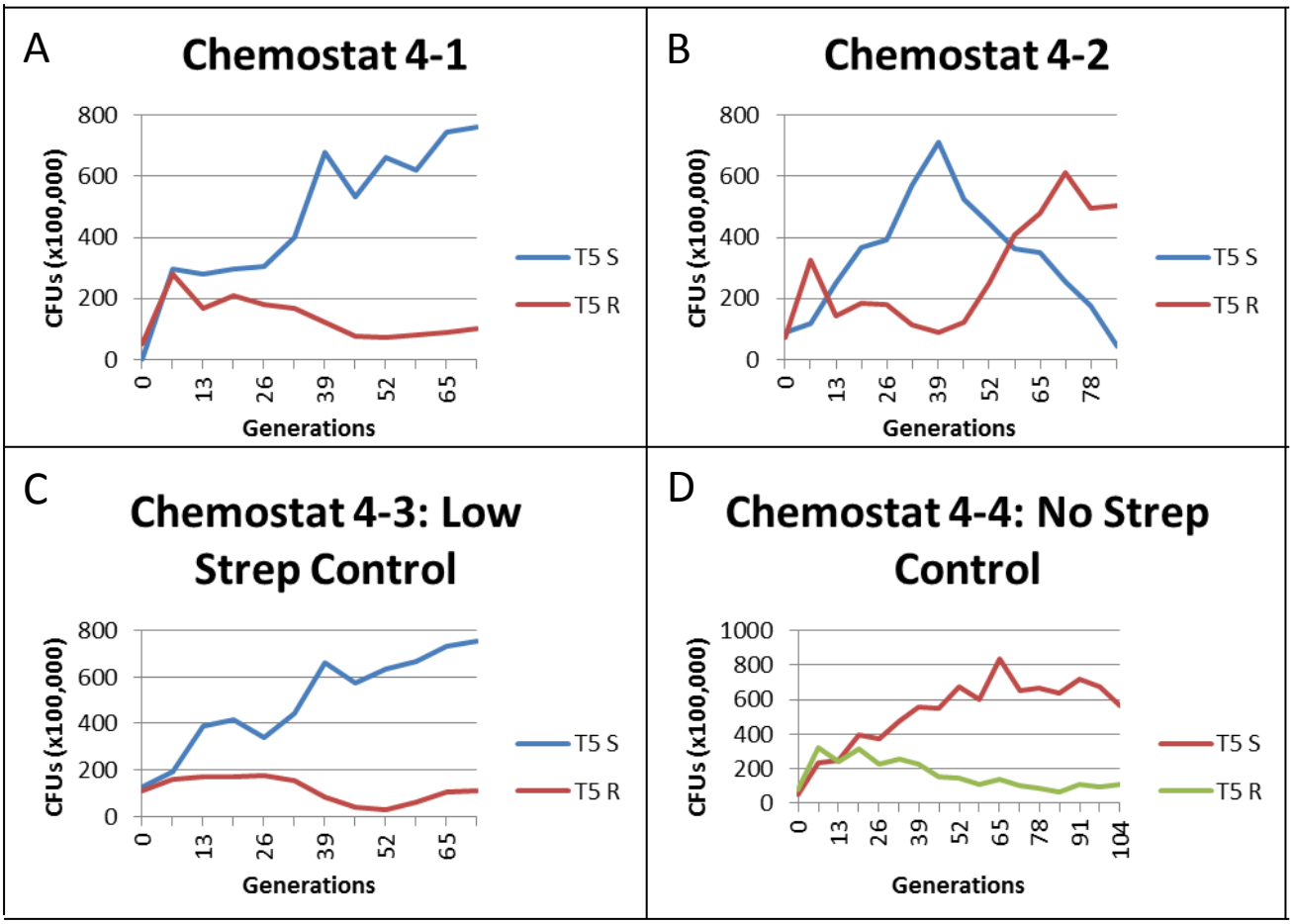

Figure 1: Frequency changes in the long term chemostat experiments. Frequencies were tracked using the system described in Dykhuizen& Hartl (Dykhuizen and Hartl 1981). Changes in relative frequencies over time are easily tracked graphically. In all cases, one strain goes to high frequency at the cost of the other. No differences between the high, low, and no streptomycin environments were observed.

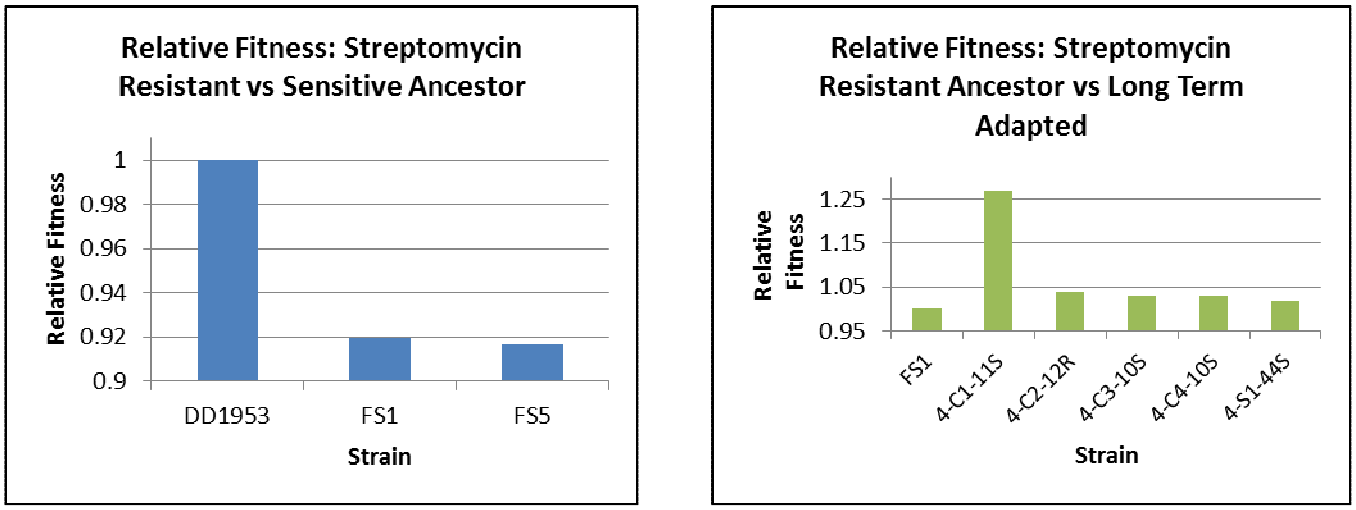

Figure 2: Relative fitnesses of experimental strains. (A) DD1953 is the streptomycin sensitive wild-type ancestor of all subsequent experimental strains. FS1 and FS5 are the streptomycin resistant mutants (*rpsL* K42N). FS1 is T5 bacteriophage sensitive, while FS5 is T5 resistant. The cost of resistance is noted here to be less than 10%. (B) The relative fitnesses of long term adapted strains compared to FS1. All experimental strains increased in fitness as compared to the start of the long term experiment. 4-C1-11S showed the highest increase by far, while 4-C2-12R, the same genetic background in an identical experimental condition showed fitness increases comparable to the strains grown in low streptomycin (4-C3-10S) and the no streptomycin control (4-C4-10S) environments. 4-S1-44S, the flask grown high streptomycin strain also had fitness gains but of the same magnitude, even though 4-S1-44S was allowed to grow *in vitro* for 286 generations. Note the scale of the vertical axis.

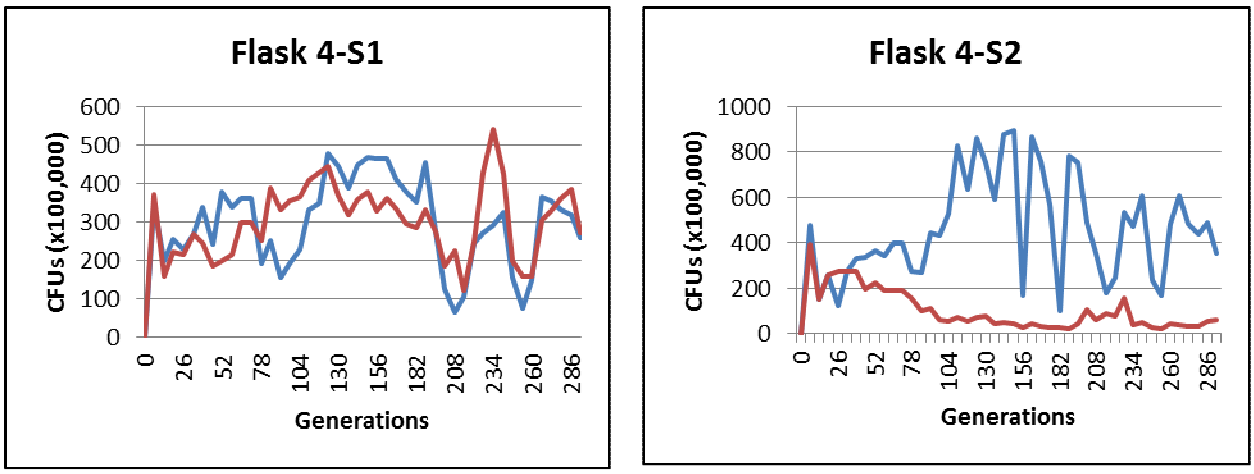

Figure 3: Frequency changes in the serial transfer flask experiments. Flask S1 relative frequencies did not change drastically over 286 generations. This flask also saw the highest number of total mutations come into the genomes (see Appendix for list of mutations). Both flasks allowed for fitness to increase relative to the strain at the starting point.

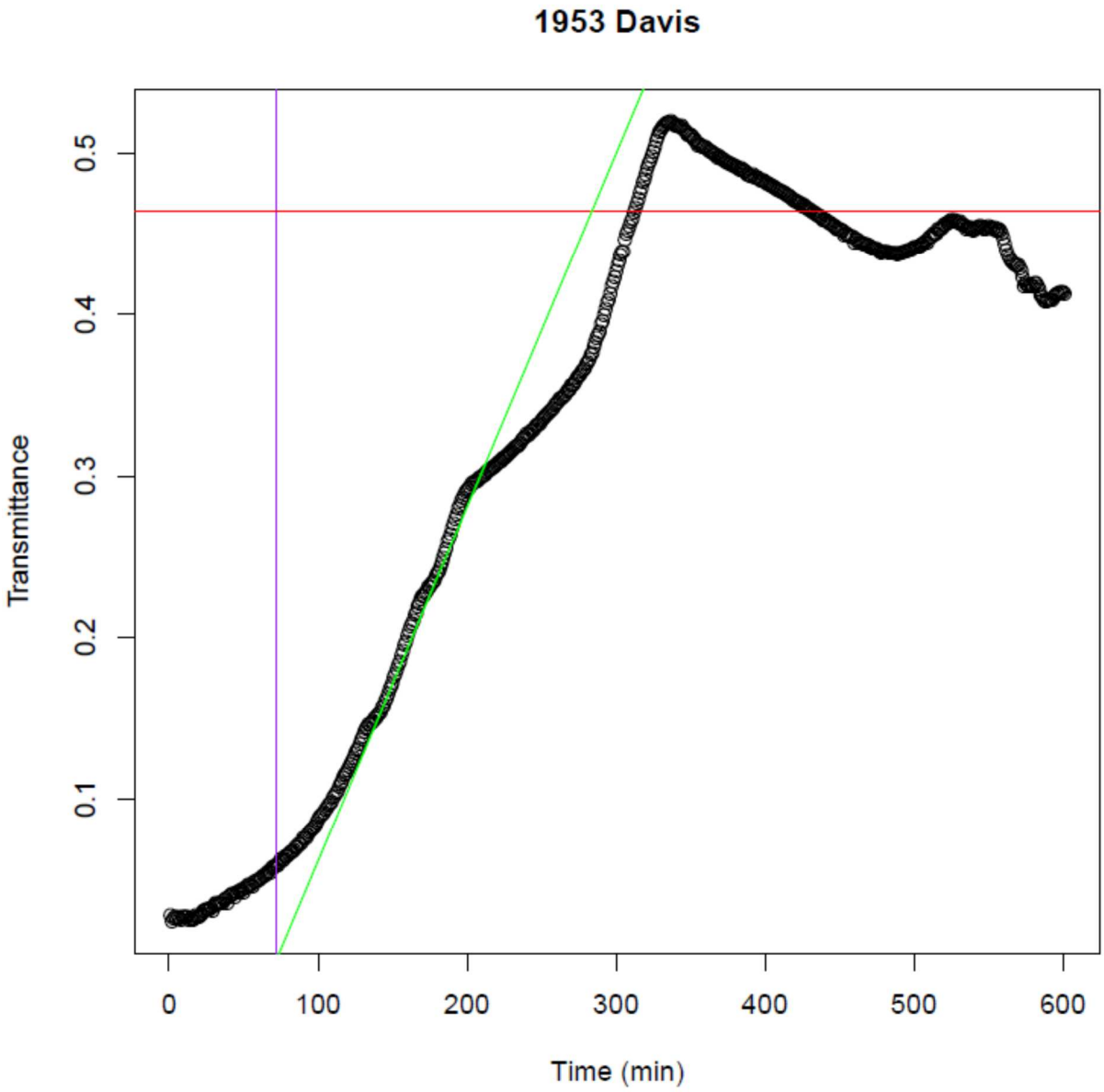

Figure 4: Mean growth curve for DD1953 grown in Davis salts with limiting glucose concentration of 0.1%. Growth was tracked over time using optical density. Analysis of the growth curves for time in lag phase (purple line), maximum slope in exponential growth, μ_{max} (green line), and saturation (red line) were performed using the grofit analysis package in R. Post hoc analyses were then conducted to look for significant differences between strains (Appendix).

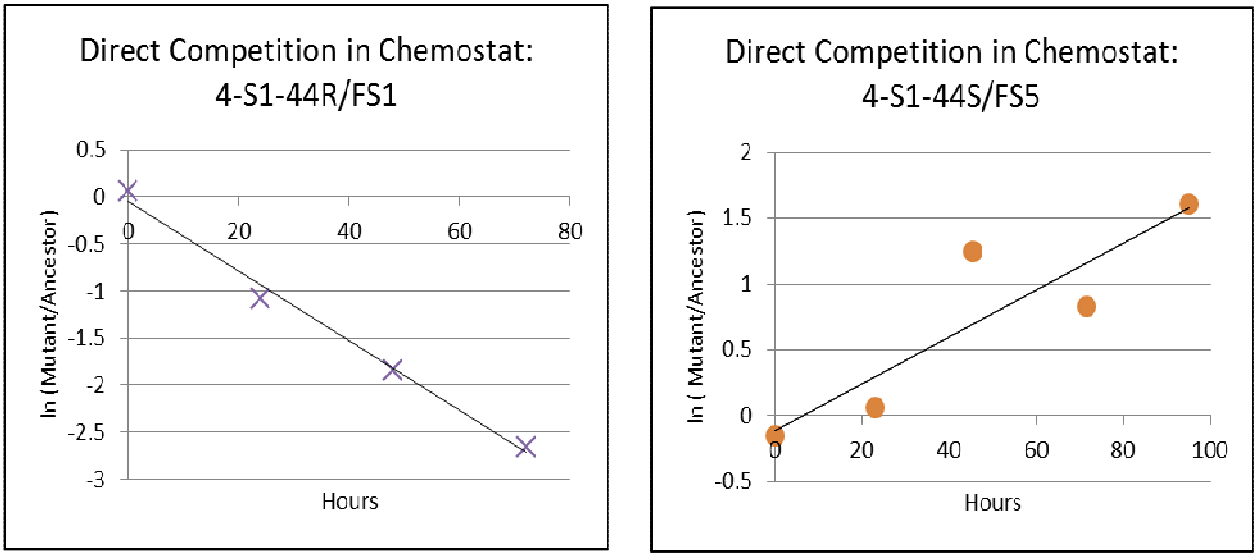

Figure 5: Direct competition in chemostats of the flask adapted strains. (A) Competition of 4-S1-44R versus FS1. The descendent, 4-S1-44R, has a lower fitness than the ancestor in this direct competition (relative w = 0.9635, s = -0.0372, R^2 = 0.9916). The different growth condition of the chemostat relative to the condition to which the mutant was adapted for 286 generations (serial transfer flask) negatively affects this strain. (B) Strain 4-S1-44S shows an increase in fitness (relative w = 1.018, s = 0.01798, R^2 = 0.793) relative to the ancestor. These two strains 4-S1-44R and 4-S1-44S competed in serial flasks for 286 generations at close frequency, arguing that fitnesses were equal (see Fig. 3).

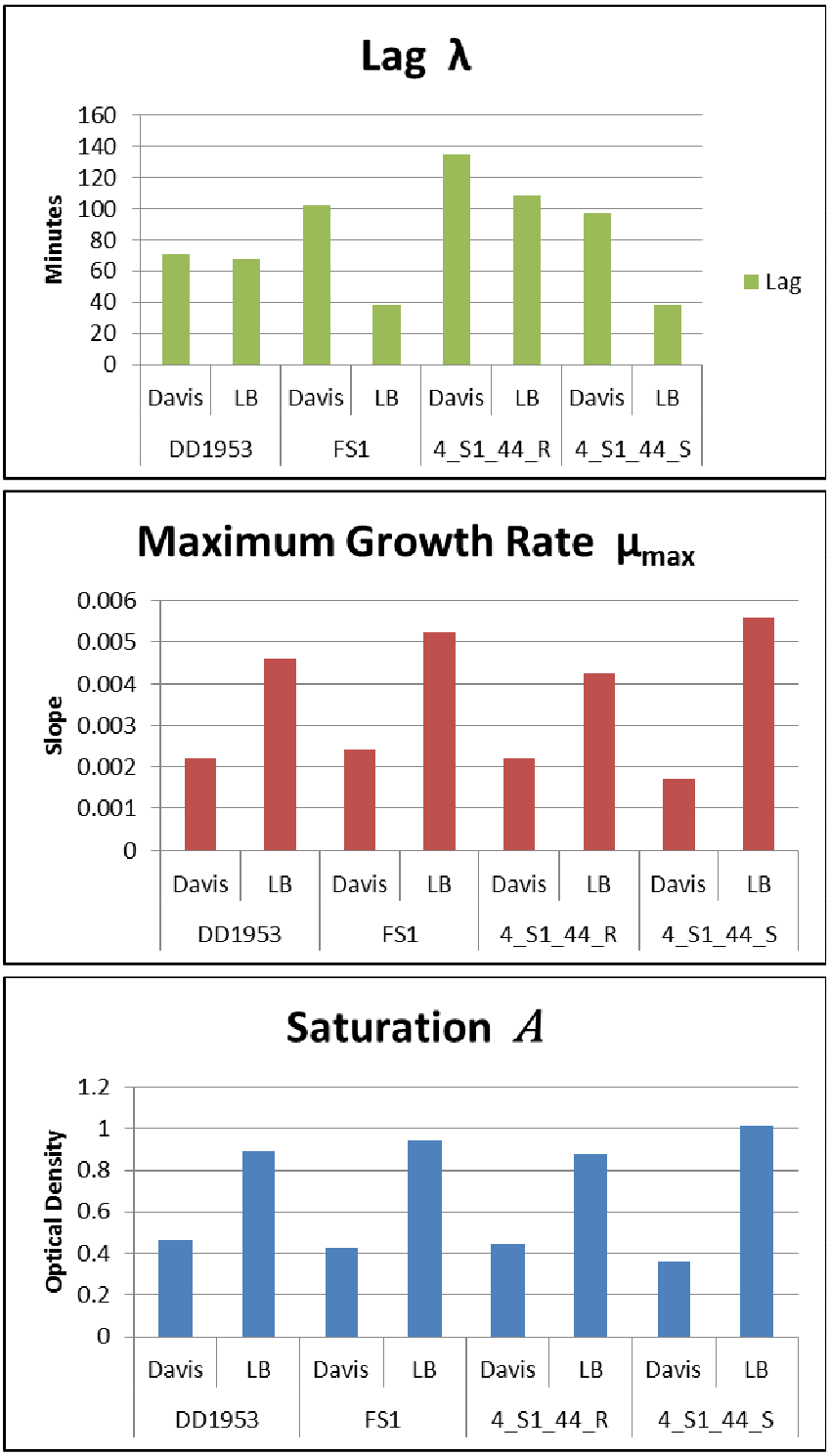

Figure 6: Results of Growth Curve Phenotypes for Flask Adapted Strains: Fitnesses of a strain are the result of all three phenotypes, with deficits in one phenotype being compensated by increases in others.

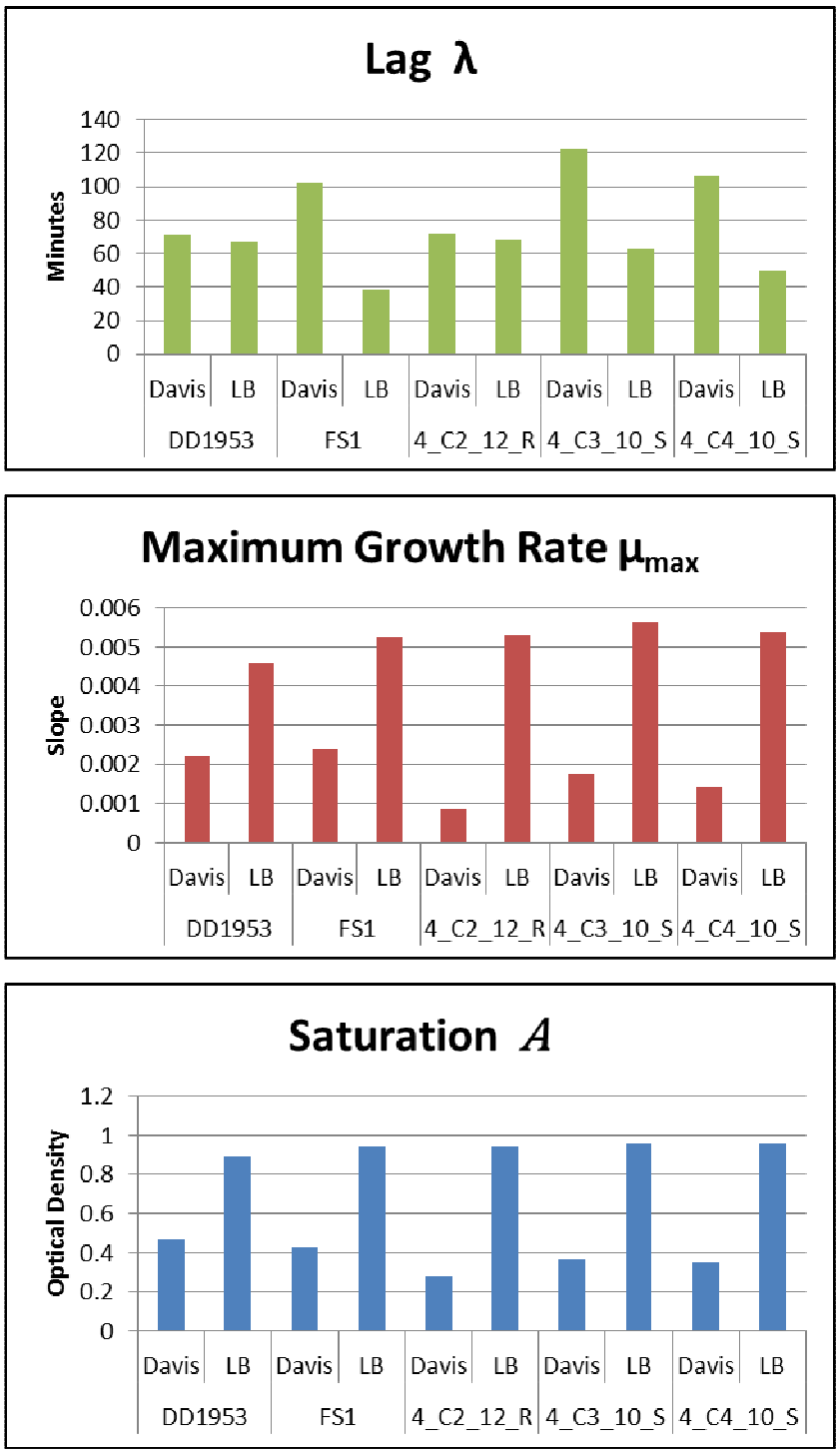

Figure 7: Results of Growth Curve Phenotypes for Chemostat Adapted Strains.

Strain Name	Description	Phenotype	MIC (μg/mL)
DD1953	wild type ancestor	Streptomycin sensitive	2 ≥ MIC ≤ 4
FS1	resistant ancestor strain	Streptomycin resistant, T5 sensitive	≥1000
FS5	resistant ancestor strain	Streptomycin resistant, T5 resistant	≥1000
4-C1-11R	Chemostat evolved, High Streptomycin concentration	Streptomycin resistant, T5 resistant	≥1000
4-C2-12R	Chemostat evolved, High Streptomycin concentration	Streptomycin resistant, T5 resistant	≥1000
4-C3-10S	Chemostat evolved, Low Streptomycin concentration	Streptomycin resistant, T5 sensitive	≥1000
4-C4-10S	Chemostat evolved, No Streptomycin negative control	Streptomycin resistant, T5 sensitive	≥1000
4-S1-44S	Serial transfer evolved, High Streptomycin concentration	Streptomycin resistant, T5 sensitive	≥1000
4-S1-44R	Serial transfer evolved, High Streptomycin concentration	Streptomycin resistant, T5 resistant	≥1000
4-S2-44S	Serial transfer evolved, High Streptomycin concentration	Streptomycin resistant, T5 sensitive	≥1000

Table 1: Strain names, phenotypes, and descriptive information for the strains discussed. The streptomycin sensitive ancestor, DD1953, displays a low tolerance to streptomycin. The single mutation in rpsL, AAA→AAC (K42N) grants high level resistance, with an associated fitness cost (see Fig. 2). Resistance to the bacteriophage T5 (T5R or T5S) was used as a neutral molecular marker throughout all experiments.

Chapter 4

Summary and Conclusions

The results of the experiments described in this thesis seem to be inconclusive. We used a known strain of *E. coli*, and selected a number of streptomycin resistant mutants in chemostats with a gradual increase in the concentration of streptomycin. These mutants all had the ability to grow and survive at high levels of streptomycin, even though they were never exposed to such concentrations. The mutant tested in a long term experimental evolution study, K42N, the most common mutation found in the above selection experiment, did not have any compensatory mutations, contrary to the expectation drawn from the compensatory mutation hypothesis, even though there were a number of replicates. In fact, compensatory mutations were not observed in any of the experiments conducted here, unless the K42N mutation itself is considered a compensatory mutation for the poorest growing mutant observed.

The compensatory mutation hypothesis states that additional mutations will be selected that increase the fitness of a strain with a new antibiotic resistant mutation to wild-type by compensating for the metabolic defects created by the resistance mutation, but leave the strain resistant to the antibiotic. The logic behind the compensatory mutation hypothesis is as follows: 1. The frequency of strains resistant to any particular antibiotic before the use of that antibiotic for therapeutic use is very low (less than 1%). However, if the natural frequency of resistance were high that particular chemical would

not be an antibiotic. 2. It is assumed that the frequency is low because the mutations causing resistance are detrimental. 3. The increase in the frequency of resistance during use of the antibiotic is from selection caused by the use. 4. It was then assumed that discontinuing use of the antibiotic would allow selection against resistance. The frequency of would drop over time. 5. When antibiotic use was discontinued, the frequency of resistance did not drop to the previous levels of before use. 6. Thus, there must be compensatory mutations arising in the resistant strains increasing fitness but not changing the resistant phenotype.

Compensatory mutations as predicted above have been observed by at least two well-known research groups, in two different countries, at two different times in two different species. In *E. coli*, the fitness cost of streptomycin was noted following single nucleotide changes in the *rpsL* gene at codon 42. These changes protected the cell from streptomycin but altered the protein translation rates of the bacterial ribosome, the site of streptomycin action (Schrag and Perrot 1996). When wild type *rpsL* was transduced back into streptomycin resistant strains that had evolved compensatory mutations, the wild type *rpsL* gene had a detrimental effect upon fitnesses in the evolved backgrounds, indicating multiple different compensatory mutation(s) (Schrag et al. 1997).

Following this, Björkman and colleagues, working in *Salmonella*, were able to identify several specific single nucleotide compensatory mutations for streptomycin resistance in the *rpsL* gene or in two functionally interacting genes, *rpsE* and *rpsD* (Björkman et al. 1999; Björkman 2000; Björkman and Andersson 2000a). These three genes produce ribosomal proteins that interact in the proofreading phase of protein

translation in the bacterial ribosome (Bohman et al. 1984; Kurland 1992). Changes in each of these proteins are known to affect protein translation rates (Kurland et al. 1990) in a manner that qualitatively matches the phenotypic data seen in both the Björkman and the Schrag experiments.

Given these results, the question then remains: why did we find no compensatory mutations? Is this due to a fundamental flaw in some aspect of the research conducted here? Is there some environmental cue that was not included here that was included previously? What explains this seemingly negative result and what does this negative result tell us?

There are four possible explanations as to why no compensatory mutations were found in the populations studied here. These possibilities include:

1. Epigenetics
2. Environmental conditions
3. Previous exposure to the antibiotic
4. Strain differences

These four possibilities will be explored in more depth in order to understand whether or not future work should focus on these areas, and if so, how.

Epigenetics

Since bacteria reproduce by fission, half of the parent cytoplasm is distributed to each offspring. If cytoplasmic expression of traits is inherited, then different phenotypes could be found in genetically identical organisms. For the present discussion concerning the evolution of compensatory mutations following antibiotic resistance, there could be selection for epigenetic states that compensate for the effects of the mutation to resistance. In the present study, such a difference would have been manifest by a difference in the relative fitness of the two different epigenetic states of the bacterial cells when compared to the antibiotic sensitive ancestor. No such difference was found. Additionally, the relative fitness of the mutants was comparable to those reported previously in the literature. This is most conspicuous in the published reports where compensatory mutations were seen (Schrag et al. 1997; Björkman and Andersson 2000a,b; Levin et al. 2000). We can not ascribe our observations to epigenetic effects when there is no evidence for them.

Environmental Conditions

The environmental conditions under which compensatory mutations were observed could be relevant only if the conditions under which such adaptation was observed differed in some quantifiable way from ours. Experimental conditions that resulted in compensatory mutations were mostly *in vitro* serial transfer methods in flasks rather than chemostats. One additional study also looked for and found compensatory

mutations *in vivo* in mice (Björkman and Andersson 2000a). However, interestingly, there were different compensatory mutations in the same strains for the same antibiotics between flasks and mice.

Such a result suggests that environment is important for compensatory mutation, as any population biologist would say is so for *any* adaptive path. In order to control for this possibility, we grew our strain in flasks throughout the experiments reported here. In fact, the flask experiments were run for a longer period of time in order to ensure that any lack of observation was not due to a lack of opportunity to fix compensatory mutations. No compensatory mutations were observed in our flask experiments. If there was some aspect of flasks that allowed compensation to occur, then such mutations should have happened here. They did not.

Previous Antibiotic Exposure

We know from previous work that resistance to an antibiotic can be selected even when the amount of antibiotic is very low (Gullberg et al. 2011). In this thesis, the fact that selection for antibiotic resistance occurs much more rapidly than previously expected has also been shown (see Chapter 2). These results, taken together, give a clear expectation that any previous exposure to even low levels of an antibiotic would lead to selection for resistance for that antibiotic. This could happen if media used in these experiments had a contaminating level of antibiotic.

If exposure to the antibiotic had occurred in the ancestral strain used in the present experiments, we can expect to be able to see the signature of past selection in the genotype and phenotype of DD1953. At the most inclusive level, the strain would have been resistant to streptomycin concentrations at least as high as those previously known to the bacteria. The MIC of DD1953 was measured to be below 4 μg/mL. This is far lower than the mean MIC level of 16 μg/mL reported by Miller for *E. coli* lab strains (Miller 1992).

Additionally, if DD1953 had been exposed to streptomycin previously, even to trace levels, there would not have been a fitness difference between the ancestor, which would have been previously exposed, and the streptomycin resistant mutant. The relative fitness between ancestor and mutant was comparable to the fitness costs measured for streptomycin resistant mutants in the studies where compensatory mutations were found. Thus, there is no evidence that our results were caused by previous exposure to streptomycin.

Strain Differences

The final critique of the results reported in this dissertation is that the lack of compensatory mutations here might be the result of using different strains here than in the published work in which compensatory adaptation following antibiotic resistance were found. In one major set of experiments, where compensatory mutations were

found both *in vitro* and *in vivo*, *Salmonella enterica* serovar typhimurium was used (Björkman and Andersson 2000a).

In the second main group of studies in which compensatory adaptation was observed (in flask culture), *E. coli* B, as well as K1/K12 chimera lab strains were used (Schrag et al. 1997). The genetic differences between these strains could be argued as the determining factor in why compensatory mutations were found in these experiments and not in the experiments presented here. The differences between just *E. coli* B and our K-12 MG1655 strain are significant and could play a role in determining adaptive paths after an environmental shift such as the addition of streptomycin.

Perhaps the most pressing future work related to this dissertation might be in repeating the chemostat experiments with the strains used elsewhere to ascertain whether or not the strain difference is a determining factor in the presence or absence of compensatory mutations following antibiotic resistance. However, even with this caveat in mind, we must here acknowledge another fundamentally important aspect of known compensatory mutations. In fact, this concern is highlighted in the published work in which these mutations have actually been observed: compensatory mutations are conspicuously absent from clinically relevant strains of antibiotic resistant bacteria (Andersson and Levin 1999).

The lack of compensatory mutations in clinically relevant antibiotic resistant strains suggests that compensatory adaptation, while one possible mechanism for the long term viability of resistant strains, is not the dominant mechanism for increasing fitness in bacterial populations after antibiotic resistance has evolved. If the post-

resistance adaptive landscape were dominated by compensatory mutations, these genetic changes would be present in many of the antibiotic resistant strains that are currently limiting modern medicine's ability to combat infection through chemotherapy. A modeling study concluded that compensatory mutations would aid in antibiotic resistant clones sweeping to fixation within a population even faster than when they are absent (Handel et al. 2006). In other words, we have every reason to expect that if compensatory mutation were likely to be present then compensatory mutations would be present in these medically relevant drug resistant strains. They are not. This suggests that strains where compensatory mutations are found are strains that are rare.

Conclusion

While the resistance-compensatory mutation narrative has gained recognition as the dominant mechanism of fitness recovery in antibiotic resistance, overwhelming experimental support is lacking. Compensatory evolution through suppressor mutations was not observed in the series of experiments conducted here. However, there is, both here and elsewhere, good evidence that compensatory mutations are not needed in order to provide an explanation of fitness recovery in these circumstances. Adaptive evolution in bacterial populations is known to be fast (Dykhuizen and Hartl 1981), as would be expected in any asexually reproducing microbial species with high effective population sizes. In fact, limiting population size has been an effective strategy for pathogen control in not only bacteria, but viruses as well (Coffin 1995).

Adaptive evolution is dependent upon genetic variation in order to provide the raw materials upon which selection can act, leading to higher fitness variants capable of outcompeting similar genotypes in identical environments. This is the exact understanding of evolution that Darwin himself realized over 150 years ago. The large numbers of individual cells in bacterial populations makes the amount of genetic variation available at any given time larger than that for macroscopic species. Coupled with the short generation time of bacteria, in large part controlled by environmental variables, such as nutrient availability, makes the probability of having a positive mutation upon which selection can act present in a population at a given time higher than is intuitively expected, or, in the case of pathogens, hoped. Ultimately, we may conclude that while compensatory mutations may exist, they are by no means required for survival and may be only one of many ways in which antibiotic resistant populations of bacteria evolve. Such a conclusion would prove, once again, that evolution is a potent tinkerer and that there is, indeed, grandeur in this view of life.

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Appendix

Table A1: Primers utilized in Sanger Sequencing: Throughout all the experiments described, the all DNA samples were PCR amplified using the primers listed below for the three main genes of interest: *rpsL*, *rpsE*, and *rpsD*.

Primer Name	Primer Sequence 5' to 3'	Length (bp)	Melting Temp (°C)	Mol Weight
rpsL-Forward	aaacgtttggccttacttaacggagaacca	30	66	9199.1
rpsL-Reverse	gaagcaaaagctaaaaccaggagctattta	30	63.3	9265.2
rpsE-Forward	cactgcgggtttgagtaattttaatagtct	30	63.3	9242.2
rpsE-Reverse	tggccttcagttctaaggtagaggtgtaag	30	67.4	9317.2
rpsD-Forward	tgcattgtgtcctctctttggtactaagct	30	66	9145.1
rpsD-Reverse	ctcgttttccaggtttgttggagaaagaaa	30	64.6	9276.2

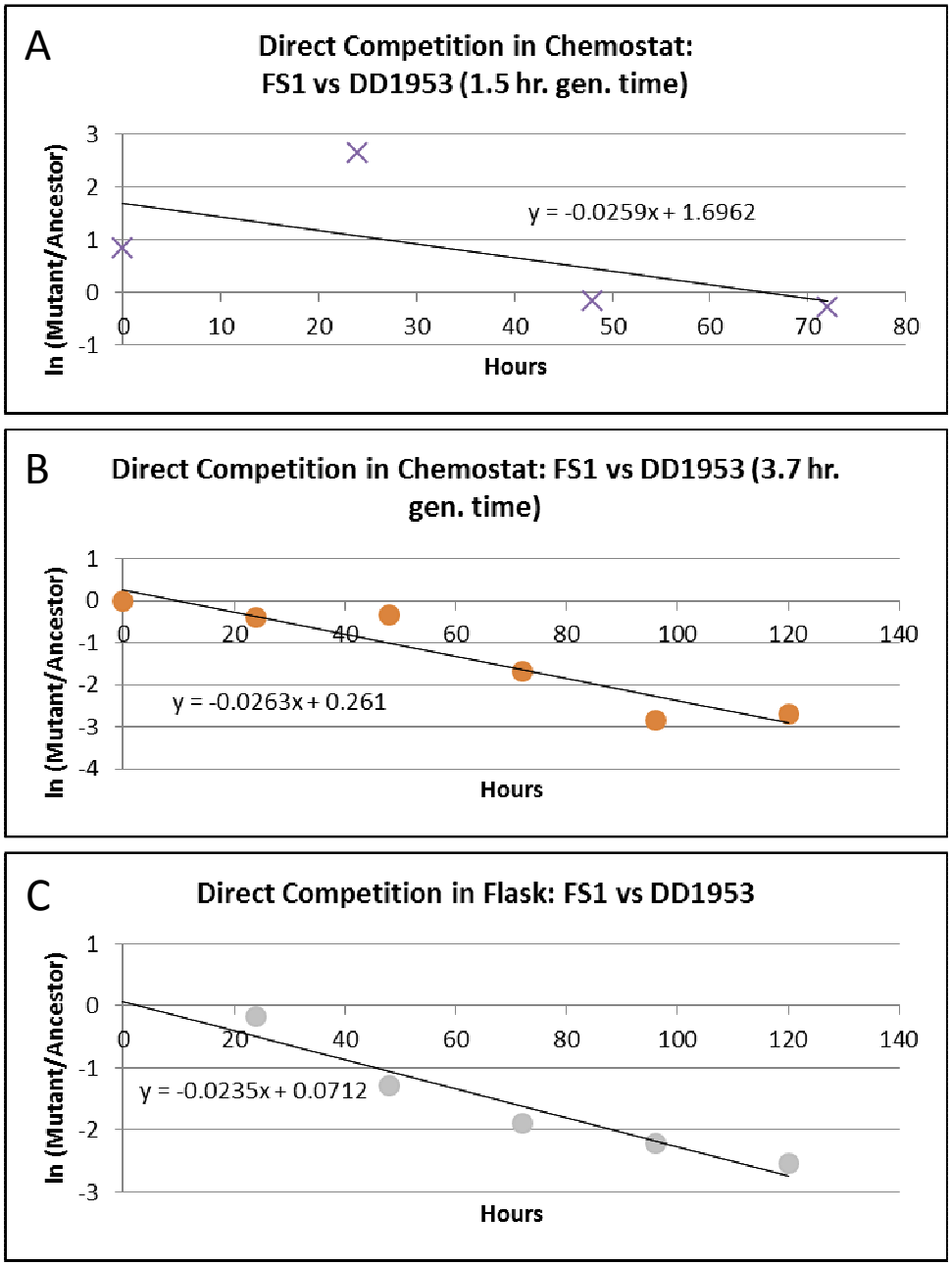
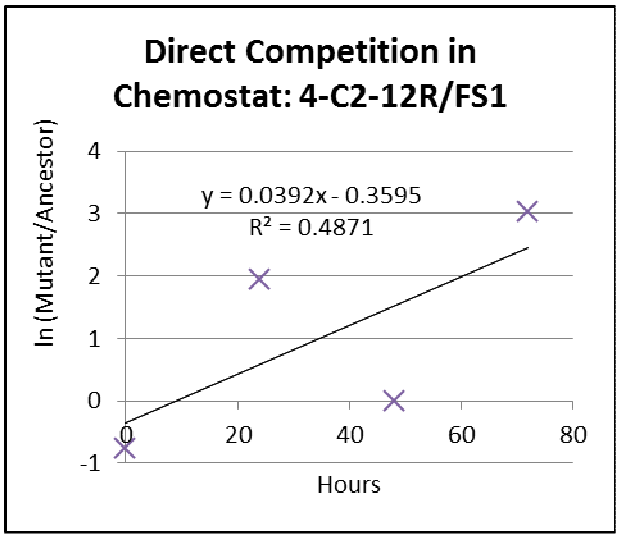
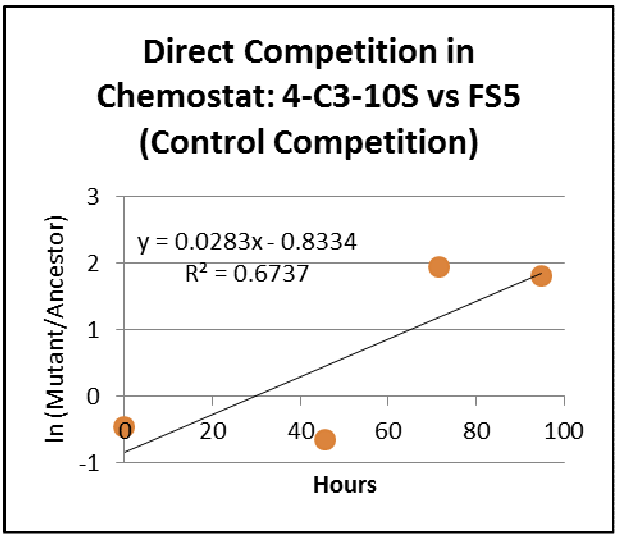


Figure A1: Results of Competitions between the streptomycin resistant mutant FS1 and the sensitive ancestor DD1953 under different generation times. The influence of generation time upon the level of fitness loss imposed was explored in chemostats and flasks. If generation time were important, then the average generation time in a given environment would have a direct impact on how well a strain competed and the specific compensatory mutation that would be most beneficial to a specific strain growing at a specific doubling time. No difference between the selection of coefficients was found by T-test (P=0.182).





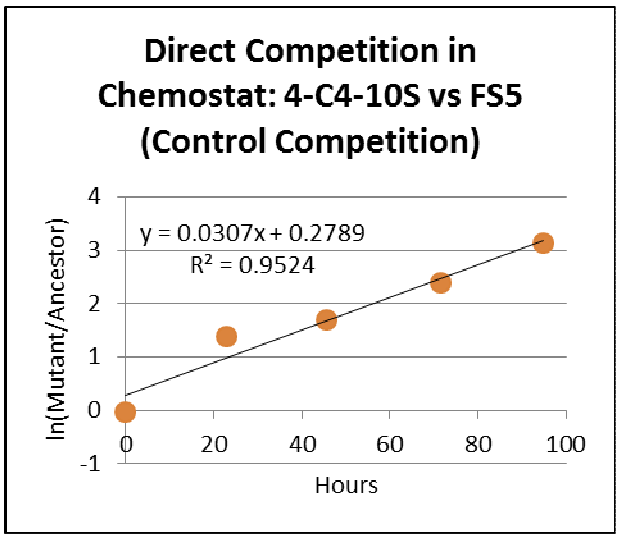


Figure A2: Chemostat Competitions of Additional Long Term Adapted Strains: These are the direct competitions of strains grown in chemostats. (A) One of the high streptomycin concentration replicates relative to the streptomycin resistant ancestor. (B) Results of the competition for the low streptomycin concentration control population. (C) Results for the direct competition of the no streptomycin control population.

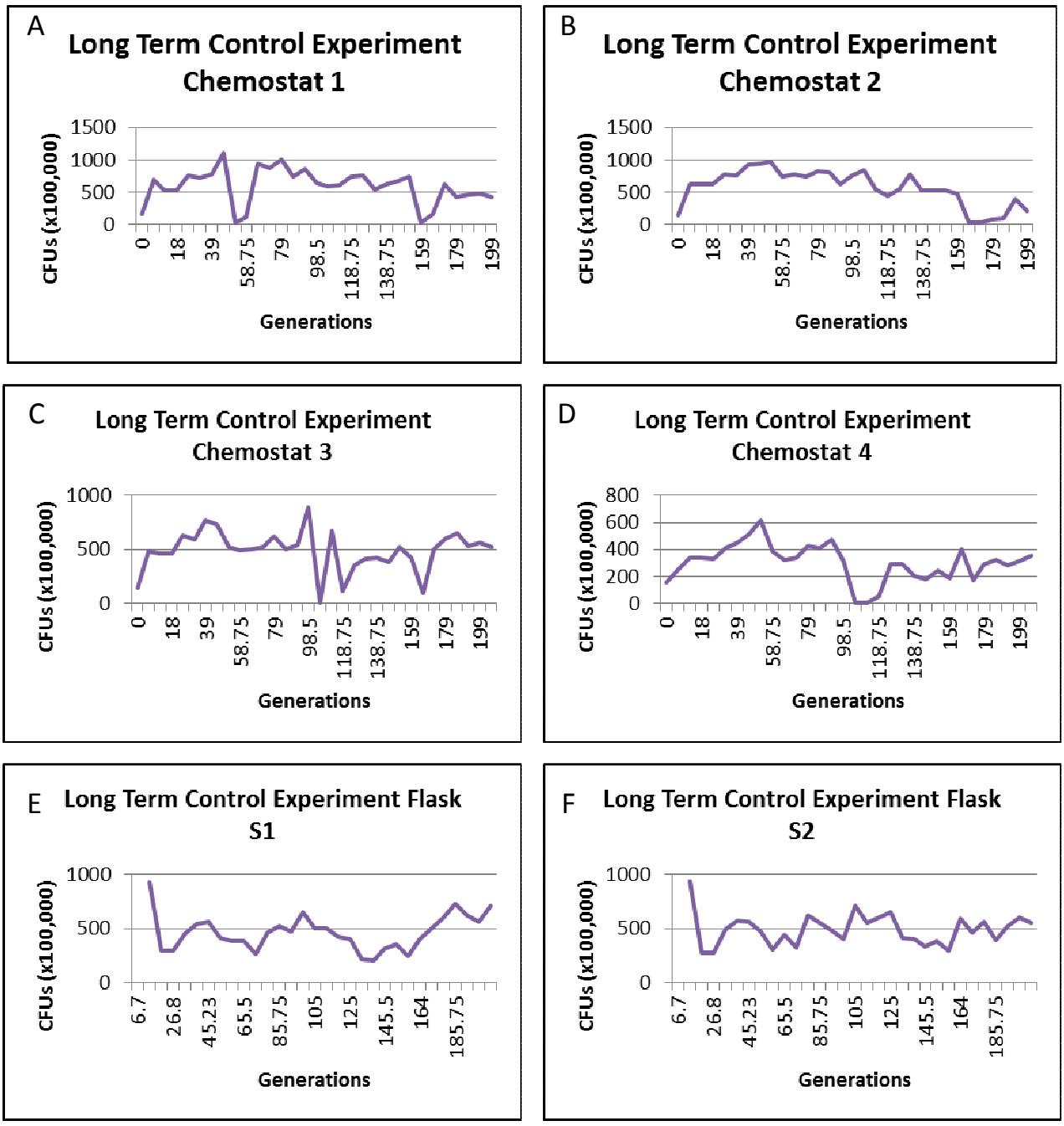


Figure A3: Colony Counts from samples taken daily form the Long Term Control Experiment using the double mutant, FS8. No molecular marker was used in these experiments.

Mean Values for Growth Curve Experiments				
Strain	Media	Lag	Slope	Saturation
DD1953	Davis	70.92409	0.00219	0.46491
	LB	67.22128	0.004591	0.892938
FS1	Davis	102.0857	0.002394	0.425483
	LB	38.56403	0.005229	0.944327
FS5	Davis	124.694	0.001719	0.240981
	LB	145.84	0.003792	0.660924
FS8	Davis	153.6888	0.00162	0.377243
	LB	44.38	0.005904	0.998616
4_C2_12_R	Davis	71.64135	0.000862	0.275926
	LB	68.1678	0.005296	0.944281
4_C3_10_S	Davis	121.9087	0.001749	0.366836
	LB	62.54779	0.005628	0.954079
4_C4_10_S	Davis	106.045	0.00143	0.346928
	LB	49.96787	0.005356	0.955683
4_S1_44_R	Davis	134.6962	0.002183	0.446686
	LB	108.0032	0.004253	0.879103
4_S1_44_S	Davis	97.09289	0.001713	0.361642
	LB	38.19745	0.005585	1.010199
16_1_30	Davis	98.1849	0.001158	0.188261
	LB	86.3092	0.003562	0.440292
16_2_30	Davis	103.8301	0.001241	0.193758
	LB	109.9874	0.004804	0.487767
16_3_30	Davis	124.572	0.001471	0.241836
	LB	67.22162	0.006	0.710663
16_4_30	Davis	121.6779	0.001305	0.226952
	LB	78.54051	0.005754	0.607595
16_S1_30	Davis	127.9253	0.001077	0.185849
	LB	84.68091	0.005456	0.723965
16_S2_30	Davis	123.2319	0.001031	0.172023
	LB	99.57859	0.005587	0.709809

Table A2: Means of Phenotypes for Growth Curve Experiments by Strain and Media Type: Clear differences in parameters measured can be seen by media type, which is to be expected. However, the differences between what phenotypes impart a higher impact upon growth and fitness in which environment(s) is interesting to try to parse and understand. The data suggests that the bacterial perspective on growth and fitness varies drastically from our current level of understanding.

Post-Hoc Tukey's Honest Significant Differences Test Results

Statistically significant values in bold

	Strain		*p value*		
Media	***Ancestral***	***Descendent***	***Lag***	***Slope***	***Saturation***
Davis	DD1953	FS1	***0.00049***	*0.967177*	*0.993356*
Davis	DD1953	FS5	***5.45E-13***	***4.57E-05***	***1.69E-13***
Davis	DD1953	FS8	***1.68E-13***	***6.02E-09***	***0.000546***
Davis	FS1	FS5	*0.097169*	***4.56E-11***	*0.788609*
Davis	FS5	4-C2-12-R	**3.00E-11**	**5.65E-13**	0.999927
Davis	FS1	4-C3-10-S	0.459061	**6.97E-09**	0.577276
Davis	FS1	4-C4-10-S	1	**5.49E-13**	0.079542
Davis	FS1	4-S1-44-S	1	**4.32E-09**	0.471482
Davis	FS5	4-S1-44-R	0.999901	**9.69E-05**	**4.18E-13**
Davis	FS8	16-1-30	**5.55E-13**	**6.73E-07**	**4.75E-13**
Davis	FS8	16-2-30	**5.19E-13**	**0.003145**	**5.45E-13**
Davis	FS8	16-3-30	**0.000368**	0.99951	**6.46E-13**
Davis	FS8	16-4-30	**2.68E-05**	0.075012	**6.87E-13**
Davis	FS8	16-S1-30	0.016059	**1.04E-06**	**5.46E-13**
Davis	FS8	16-S2-30	**7.89E-05**		**1.83E-13**

	Strain		*p value*		
Media	***Ancestral***	***Descendent***	***Lag***	***Slope***	***Saturation***
LB	DD1953	FS1	**0.001961**	**7.83E-10**	0.74836
LB	DD1953	FS5	**1.68E-13**	**5.47E-13**	**1.68E-13**
LB	DD1953	FS8	**0.032992**	**1.68E-13**	**2.21E-06**
LB	FS1	FS5	**1.68E-13**	**1.68E-13**	**1.68E-13**
LB	FS5	4-C2-12-R	**1.68E-13**	**1.68E-13**	**1.68E-13**
LB	FS1	4-C3-10-S	**0.043982**	**0.004352**	1
LB	FS1	4-C4-10-S	0.998642	0.999997	1
LB	FS1	4-S1-44-S	1	**0.030922**	0.177971
LB	FS5	4-S1-44-R	**1.37E-07**	**5.37E-05**	**1.69E-13**
LB	FS8	16-1-30	**1.13E-09**	**1.68E-13**	**1.68E-13**
LB	FS8	16-2-30	**2.96E-13**	**1.68E-13**	**1.68E-13**
LB	FS8	16-3-30	**0.039671**		**1.68E-13**
LB	FS8	16-4-30	**2.38E-05**	0.999849	**1.68E-13**
LB	FS8	16-S1-30	**2.13E-09**	5.17E-05	**1.68E-13**
LB	FS8	16-S2-30	**5.32E-13**	0.069941	**1.68E-13**

(continued on next page)

Davis	DD1953	4-C2-12-R	1		**5.24E-13**	LB	DD1953	4-C2-12-R	1	**3.87E-12**	0.750203
Davis	DD1953	4-C3-10-S	**2.37E-12**	**0.001081**	**0.000461**	LB	DD1953	4-C3-10-S	1	**1.74E-13**	0.32882
Davis	DD1953	4-C4-10-S	**9.83E-05**	**4.99E-12**	**8.66E-06**	LB	DD1953	4-C4-10-S	0.661209	**5.35E-13**	0.271015
Davis	DD1953	4-S1-44-S	**0.0405**	**0.000506**	**0.000393**	LB	DD1953	4-S1-44-S	**0.001488**	**2.78E-13**	**6.18E-07**
Davis	DD1953	4-S1-44-R	**5.49E-13**		1	LB	DD1953	4-S1-44-R	**2.95E-08**	0.060496	1
Davis	DD1953	4-S2-44-S				LB	DD1953	4-S2-44-S			
Davis	DD1953	16-1-30	0.155744		**1.68E-13**	LB	DD1953	16-1-30	0.46725	**2.24E-13**	**1.68E-13**
Davis	DD1953	16-2-30	**6.24E-05**		**1.68E-13**	LB	DD1953	16-2-30	**1.00E-08**	0.942868	**1.68E-13**
Davis	DD1953	16-3-30	**5.22E-13**		**1.81E-13**	LB	DD1953	16-3-30	1	**1.68E-13**	**5.50E-13**
Davis	DD1953	16-4-30	**1.10E-12**		**1.68E-13**	LB	DD1953	16-4-30	0.999698	**1.68E-13**	**1.68E-13**
Davis	DD1953	16-S1-30	**5.21E-13**		**1.68E-13**	LB	DD1953	16-S1-30	0.63301	**5.41E-13**	**5.19E-13**
Davis	DD1953	16-S2-30	**5.63E-13**	**1.68E-13**	**1.68E-13**	LB	DD1953	16-S2-30	**0.000136**	**3.24E-13**	**5.51E-13**
Davis	4-S1-44-S	4-S1-44-R	**1.74E-05**	**0.000896**	**0.023151**	LB	4-S1-44-S	4-S1-44-R	1		**5.70E-09**
Davis	FS1	FS8	**5.26E-13**		1	LB	FS1	FS8	1	**1.27E-12**	0.450875
Davis	FS5	FS8	**0.00016**		**6.20E-12**	LB	FS5	FS8	**1.68E-13**	**1.68E-13**	**1.68E-13**

Table A3: Post-hoc Tukey Honest Significant Differences Results Pairwise Strain Comparisons of Growth Curve Experiments: Statistically significant results of any pairwise comparison is in bold. As with all post-hoc tests, the Tukey is highly conservative.

Strain: DD1953	N= 37		
Variant Frequency (relative to Reference Strain)	gene	Gene Category	Gene Product Description
85.70%	metC	Methionine	Cystathionine beta-lyase; homotetrameric; cysteine desulfhydrase; putative alanine racemase
40.00%	cdd	CytiDine Deaminase	Cytidine deaminase; 2-deoxycytidine deaminase; mutants are 5-fluorodeoxycytidine resistant; binds Zn(II)
40.00%	cheA	Chemotaxis	Histidine protein kinase sensor of chemotactic response; CheY is cognate response regulator; autophosphorylating
40.00%	mlaB	outer Membrane Lipid Asymmetry	Probable phospholipid ABC transporter, quinolone resistance; peripheral membrane protein, cytoplasmic; maintains OM lipid asymmetry; STAS subunit
40.00%	mukB	Mukaku (anucleate)	Chromosome condensin MukBEF, ATPase and DNA-binding subunit; SMC-related protein
40.00%	nuoG	NADH:ubiquinone oxidoreductase	NADH:ubiquinone oxidoreductase subunit G, complex I; NADH dehydrogenase I
40.00%	polA	Polymerase	DNA polymerase I; required for plasmid replication; translesion synthesis; synthetic lethal with ygdG
40.00%	sdaC	Serine deaminase	L-serine:H+ symport permease, threonine-insensitive
33.30%	dapA	DiAminoPimelate	Dihydrodipicolinate synthase
33.30%	icd	Isocitrate dehydrogenase	Isocitrate dehydrogenase, NADP(+)-specific; e14 attachment site; tellurite reductase
33.30%	**pbpC**	**Penicillin-binding protein**	**Penicillin-insensitive murein repair transglycosylase; inactive transpeptidase domain**
33.30%	pgaA	Poly--1,6-N-acetyl-d-glucosamine	Biofilm adhesin polysaccharide PGA secretin; OM porin
33.30%	rpoD	RNA polymerase	RNA polymerase subunit, sigma 70, initiates transcription; housekeeping sigma
33.30%	rutB	PyRimidine UTilization	Ureidoacrylate amidohydrolase; pyrimidine nitrogen catabolism
33.30%	rutB	PyRimidine UTilization	Ureidoacrylate amidohydrolase; pyrimidine nitrogen catabolism
33.30%	sxy	Sexy one	Required for expression of CRP-S-dependent promoters
33.30%	yfaD	Systematic nomenclature	Transposase_31 family protein, function unknown
33.30%	**yfcJ**	**Systematic nomenclature**	**Putative arabinose efflux transporter**
33.30%	yqeC	Systematic nomenclature	Putative selenium-dependent hydroxylase accessory protein
33.30%	zraS	Zn resistance-associated	Two component sensor kinase for ZraP; responsive to Zn(2+) and Pb(2+); autoregulated; regulation of Hyd-3 activity is probably due to crosstalk of overexpressed protein
28.60%	argP	Arginine	Inhibitor of chromosome initiation, transcriptional activator, LysR family; oriC iteron (13-mer) binding protein, competes with DnaA for iteron binding
28.60%	**aspA**	**Aspartate**	**L-aspartate ammonia-lyase; L-aspartase**
28.60%	gnd	Gluconate-P dehyd	6-phosphogluconate dehydrogenase, decarboxylating

28.60%	sfmD	Salmonella fimbriae	Predicted outer membrane fimbrial subunit export usher protein
28.60%	yjbI	Systematic nomenclature	Pseudogene reconstruction, SopA-related, pentapeptide repeats
27.30%	nanC	N-Acetylneuraminate	N-acetylneuraminic acid outer membrane channel protein
25.00%	aceE	Acetate	Pyruvate dehydrogenase, decarboxylase component E1; acetate requirement
25.00%	**hisB**	**Histidine**	**Imidazoleglycerolphosphate dehydratase/histidinol phosphatase; bifunctional enzyme; HAD21**
25.00%	holC	Holoenzyme	DNA polymerase III, chi subunit
25.00%	rplC	Ribosomal protein, large	50S ribosomal subunit protein L3
25.00%	thrS	Threonine	Threonine--tRNA ligase, autogenously regulated; binds Zn(II)
25.00%	yaiT	Systematic nomenclature	Pseudogene reconstruction, autotransporter family; interrupted by IS3B
25.00%	yhiS	Systematic nomenclature	Pseudogene reconstruction, interrupted by IS5T
25.00%	yhiS	Systematic nomenclature	Pseudogene reconstruction, interrupted by IS5T
25.00%	yhiS	Systematic nomenclature	Pseudogene reconstruction, interrupted by IS5T
25.00%	yhiS	Systematic nomenclature	Pseudogene reconstruction, interrupted by IS5T
25.00%	**yhjE**	**Systematic nomenclature**	**Putative MFS transporter, function unknown; membrane protein**

Sample: 4-C2-12R	N = 19		
Variant Frequency	gene	Gene Category	Gene Product Description
40.00%	erfK	L,D-transpeptidase	L,D-transpeptidase linking Lpp to murein, periplasmic
40.00%	lrp	Leucine regulatory protein	Global regulatory protein, Leu responsive; regulator of high-affinity branched-chain amino acid transport system; octameric or hexadecameric functional state
40.00%	pepN	Peptidase	Aminopeptidase N
40.00%	abgA	Aminobenzoyl-glutamate	p-Aminobenzoyl-glutamate hydrolase, A subunit; PGH; manganese-dependent; required for p-aminobenzoyl-glutamate utilization
40.00%	fhuF	Ferric hydroxamate uptake	Siderophore-iron reductase; releases iron from hydroxamate-type siderophores; cytoplasmic
40.00%	opgC	Osmoregulated periplasmic glucans	OPG biosynthetic transmembrane succinyltransferase
40.00%	ydaN	Systematic nomenclature	Zn(II) transporter
40.00%	uhpA	Utilization hexose phosphate	Response regulator of two component system required for uhpT transcription
40.00%	**hisB**	**Histidine**	**Imidazoleglycerolphosphate dehydratase/histidinol phosphatase; bifunctional enzyme; HAD21**
40.00%	ygiN	Systematic nomenclature	Quinol monooxygenase; MdaB and YgiN form an enzymatic quinone reduction-oxidation cycle in vitro; expressed protein
40.00%	**ydeA**	**Systematic nomenclature**	**Arabinose efflux transporter, arabinose-inducible**

40.00%	gpmM	Glycerol P mutase, Mn-dependent	Phosphoglycerate mutase, Mn-dependent, cofactor-independent; iPGM; monomeric
37.50%	**clpB**	**Caseinolytic protease**	**Bichaperone with DnaK for protein disaggregation; disaggregase; protein-dependent ATPase; role in de novo protein folding under mild stress conditions**
33.30%	yhbU	Systematic nomenclature	U32 peptidase family protein, function unknown
33.30%	**pnp**	**Polynucleotide phosphorylase**	**Polynucleotide phosphorylase; exoribonuclease; PNPase component of RNA degradosome; cold shock protein required for growth at low temperatures**
28.60%	ompL	Outer membrane protein	Outer membrane porin L; putative sulpholipid porin
28.60%	ykgA	Systematic nomenclature	Pseudogene, N-terminal fragment, AraC family; interrupted/deleted by IS3A
28.60%	insD1	Insertion Sequence	IS2 transposase B
28.60%	yqhA	Systematic nomenclature	UPF0114 family predicted inner membrane protein; function unknown

Sample: 4-C4-10S	N = 18		
Variant Frequency	gene	Gene Category	Gene Product Description
50.00%	ftsH	Filamentation, temperature sensitive	ATP-dependent membrane protease, complexed with HflCK; regulates lysogeny; mutants are defective in cell growth, septum formation and phage lambda development; mutants rescued by divalent cations; binds Zn(II); hexameric
40.00%	evgS	E. coli homolog of Virulence Gene	Sensor kinase for acid and drug resistance, cognate to EvgA
40.00%	perR	Peroxide	Predicted DNA-binding transcriptional regulator; CP4-6 putative defective prophage
40.00%	nirB	Nitrite reductase	Nitrite reductase [NAD(P)H] subunit
40.00%	ydjH	Systematic nomenclature	Putative sugar kinase, function unknown
40.00%	slyA	Salmonella lysis	Activates crytpic hemolysin gene hlyE; global transcriptional regulator
40.00%	ydbA	Systematic nomenclature	Pseudogene reconstruction, autotransporter homolog; interrupted by IS2D and IS30C; reported to be required for swarming phenotype
40.00%	ydeP	Systematic nomenclature	Acid-resistance protein; required for acid resistance conferred by EvgA overexpression; oxidoreductase homolog
40.00%	dusB	Dihydrouridine synthase B	tRNA-dihydrouridine synthase B
40.00%	gsk	Guanosine kinase	Inosine-guanosine kinase
33.30%	yfaL	Systematic nomenclature	Probable OM autotransporter adhesin; overexpression increases adhesion and biofilm formation
33.30%	**idnK**	**Idonate**	**D-gluconate kinase**
33.30%	yfcJ	Systematic nomenclature	Putative arabinose efflux transporter
33.30%	luxS	LUminescence eXpression	S-ribosylhomocysteine lyase, autoinducer 2 (AI-2) synthesis; functions in quorum sensing; acid-inducible
33.30%	yqiG	Systematic nomenclature	Pseudogene reconstruction, FimD family, interrupted by IS2I; fimbrial export usher protein family

28.60%	lysS	Lysine	Lysine--tRNA ligase, constitutive
25.00%	fucI	Fucose	L-fucose isomerase
25.00%	hslU	Heat shock locus	Heat-inducible ATP-dependent protease HslVU, ATPase subunit; involved in the degradation of misfolded proteins; heat shock protein D48.5

Sample: 4-S1-44	N = 112		
Variant Frequency	gene	Gene Category	Gene Product Description
33.30%	phnD	Phosphonate	Phosphonate ABC transporter periplasmic binding protein; phosphonate catabolism
33.30%	rhaA	Rhamnose	L-rhamnose isomerase
83.30%	pitA	Pi transport	Low-affinity inorganic phosphate transporter; tellurite uptake; arsenate transporter; putative Zn(II) transporter
46.70%	**idnK**	**Idonate**	**D-gluconate kinase**
45.50%	ycbC	Elevated frequency of LYsis	Envelope biogenesis factor; DUF218 superfamily protein
42.90%	bcsE	Bacterial cellulose synthesis	Protein required for cellulose production
42.90%	phnD	Phosphonate	Phosphonate ABC transporter periplasmic binding protein; phosphonate catabolism
41.70%	fabR	Fatty acid biosynthesis regulator	Transcriptional repressor of fabA and fabB
40.00%	tsr	Taxis to serine and repellents	Serine chemoreceptor, methyl-accepting; MCP I; also senses repellents; flagellar regulon
40.00%	tsr	Taxis to serine and repellents	Serine chemoreceptor, methyl-accepting; MCP I; also senses repellents; flagellar regulon
40.00%	thiF	Thiamine (and thiazole)	Adenylyltransferase, modifies ThiS C-terminus; ThiS-thiocarboxylate (ThiS-COSH) synthesis; thiamine-thiazole moiety synthesis; complexes with ThiS
40.00%	fabR	Fatty acid biosynthesis regulator	Transcriptional repressor of fabA and fabB
40.00%	yiaU	Systematic nomenclature	LysR-family transcriptional regulator, function unknown
40.00%	waaL	Systematic nomenclature	LPS core biosynthesis; O-antigen ligase
40.00%	gudP	Glucarate dehydratase	Probable D-glucarate transporter, membrane protein
40.00%	panF	Pantothenate	Pantothenate permease
40.00%	tehA	Tellurite resistance	K+-tellurite, ethidium and proflavin efflux transporter
40.00%	ycdY	Systematic nomenclature	YcdX chaperone; redox enzyme maturation protein (REMP); required for swarming phenotype
40.00%	ygiF	Systematic nomenclature	Inorganic triphosphatase
40.00%	ycdY	Systematic nomenclature	YcdX chaperone; redox enzyme maturation protein (REMP); required for swarming phenotype
40.00%	pepE	Peptidase	Peptidase E, alpha-aspartyl dipeptidase
40.00%	csgD	Curlin sigma S-dependent growth	Transcriptional activator for csgBA and other genes
40.00%	rpsC	Ribosomal protein, small	30S ribosomal subunit protein S3
40.00%	proW	Proline	Glycine betaine/proline ABC transporter permease

40.00%	htrE	High temperature (requirement)	Putative outer membrane fimbrial subunit export usher protein; required for growth at high temperature; induced by heat shock
40.00%	sbcC	Suppression of recBC	DNA hairpin dsDNA 3'-exonuclease SbcCD, Mn(2+), ATP-dependent; ATP-independent 5' ssDNA endonuclease; required for recombinational repair of dsDNA breaks; cosuppressor with sbcB of recB recC mutations; heterodimeric
40.00%	caiT	Carnitine inducible	L-carnitine/gamma-butyrobetaine antiporter; trimeric; BCCT family
40.00%	ybcV	Systematic nomenclature	DUF1398 family protein, DLP12 prophage
40.00%	yjhF	Systematic nomenclature	Predicted GntP family transporter, function unknown
40.00%	tsr	Taxis to serine and repellents	Serine chemoreceptor, methyl-accepting; MCP I; also senses repellents; flagellar regulon
40.00%	ravA	Regulatory ATPase Variant A	Hexameric AAA+ MoxR family ATPase, function unknown; possible molecular chaperone; ATPase stimulated by binding to CadA and ViaA
40.00%	ychF	Systematic nomenclature	Catalase inhibitor protein; ATPase, K+-dependent, ribosome-associated; monomeric
40.00%	ycdY	Systematic nomenclature	YcdX chaperone; redox enzyme maturation protein (REMP); required for swarming phenotype
40.00%	mdtL	Multidrug transporter	Multidrug resistance efflux protein; overexpression confers low-level chloramphenicol resistance
40.00%	ydeM	Systematic nomenclature	Predicted YdeN-specific anaerobic sulfatase-maturating enzyme; Radical SAM enzyme
33.30%	phnJ	Phosphonate	Phosphoribosyl phosphonate carbon-phosphorus lyase; SAM-dependent; phosphonate catabolism
33.30%	yncG	Systematic nomenclature	Glutathione S-transferase homolog, function unknown
33.30%	nuoH	NADH:ubiquinone oxidoreductase	NADH:ubiquinone oxidoreductase subunit H, complex I; NADH dehydrogenase I
33.30%	**insH1**	**Insertion Sequence**	**IS5 transposase**
33.30%	yebT	Systematic nomenclature	MCE domain protein, function unknown
33.30%	menF	Menaquinone (vitamin K2)	Isochorismate synthase, menaquinone biosynthesis
33.30%	yecE	Systematic nomenclature	DUF72 family protein
33.30%	ybdK	Systematic nomenclature	Weak gamma-glutamyl:cysteine ligase activity
33.30%	agp	Acid glucose-1-phosphatase	Periplasmic glucose-1-phosphatase, acidic; has inositol phosphatase activity
33.30%	yfgF	Systematic nomenclature	Cyclic-di-GMP phosphodiesterase, anaerobic; dual domain protein; defective cyclase domain; predicted membrane sensor protein
33.30%	yfiM	Systematic nomenclature	Required for salt suppression of motility and lambda growth; probable lipoprotein, Cys conserved
33.30%	fadR	Fatty acid degradation	Repressor/activator for fatty acid metabolism regulon; fatty acid-responsive transcription factor; fabAB, iclR activator (regulates aceBAK, glyoxylate shunt); fad repressor; homodimeric
33.30%	rlmI	rRNA Large-subunit Methylation	23S rRNA m(5)C1962 methyltransferase, SAM-dependent

33.30%	yidQ	Systematic nomenclature	Probable lipoprotein, function unknown; Cys conserved
33.30%	citG	Citrate	2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase
33.30%	nrdR	Nucleotide reductase	Nrd regulon repressor
33.30%	ribC	Riboflavin	Riboflavin synthase; homotrimer; associated with RibE 60-mer
33.30%	phnE	Phosphonate	Pseudogene reconstruction, phosphonate ABC transporter permease; phosphonate catabolism
33.30%	rtn	Resistance to N4	Predicted membrane-anchored cyclic-di-GMP phosphodiesterase; overexpression confers resistance to phages lambda and N4
30.00%	torD	Trimethylamine oxide reductase	Redox enzyme maturation protein (REMP) for TorA; cytoplasmic chaperone for TorA activation; GTPase; protects TorA TAT signal peptide from degradation
28.60%	tusD	tRNA 2-ThioUridine Synthesizing protein	mnm(5)-s(2)U34-tRNA 2-thiolation step sulfurtransferase; sulfur relay system; C78 accepts persulfide sulfur from C19 of TusA, then probably donates to C108 of TusE; required for swarming phenotype
28.60%	nlpA	New lipoprotein	Lipoprotein-28, inner membrane protein, function unknown
28.60%	yifB	Systematic nomenclature	Competence Mg chelatase ComM homolog, function unknown
28.60%	hemC	Hemin	Porphobilinogen deaminase; neomycin sensitivity
28.60%	tyrB	Tyrosine	Tyrosine aminotransferase; aromatic amino acid aminotransferase; phenylalanine aminotransferase; dicarboxylic amino acid aminotransferase; homodimeric
28.60%	prkB	Phosphoribulokinase	Predicted phosphoribulokinase
28.60%	yejO	Systematic nomenclature	Pseudogene reconstruction, autotransporter homolog; interrupted by IS5K
28.60%	hemC	Hemin	Porphobilinogen deaminase; neomycin sensitivity
28.60%	wbbI	Systematic nomenclature	d-Galf:alpha-d-Glc beta-1,6-galactofuranosyltransferase; involved in lipopolysaccharide biosynthesis
28.60%	wecH	Systematic nomenclature	O-acetyltransferase for enterobacterial common antigen (ECA)
28.60%	ydjO	Systematic nomenclature	Function unknown
28.60%	**insH1**	**Insertion Sequence**	**IS5 transposase**
28.60%	glsB	GLutanimaSe	Glutaminase 2; GlsA2
28.60%	lyxK	Lyxose	L-xylulose/3-keto-L-gulonate kinase
28.60%	barA	Bacterial adaptive response	Sensor histidine protein kinase, pleiotropic; controls the expresion of csrB/C sRNAs; works in concert with UvrY response regulator
28.60%	narY	Nitrate reductase, nitrate regulation	Nitrate reductase II (NRZ), beta subunit
28.60%	pbpC	Penicillin-binding protein	Penicillin-insensitive murein repair transglycosylase; inactive transpeptidase domain
28.60%	ybbW	Systematic nomenclature	Putative allantoin permease, glyoxylate-inducible or allantoin-inducible (anaerobic)
28.60%	proB	Proline	Glutamate 5-kinase, proline biosynthesis; dimeric; feedback inhibition by proline

28.60%	ydjO	Systematic nomenclature	Function unknown
28.60%	ynaJ	Systematic nomenclature	Predicted inner membrane protein, DUF2534 family, function unknown
28.60%	hemX	Hemin	Uroporphyrinogen III methyltransferase
28.60%	ytfE	Systematic nomenclature	Iron-sulfur cluster repair protein; RIC; confers resistance to nitric oxide and hydrogen peroxide; di-iron center
28.60%	mdlB	Multidrug resistance-like	ABC exporter permease-ATPase, function unknown
27.30%	yoaG	Systematic nomenclature	Stress-induced protein, function unknown; involved in cadmium and peroxide resistance
25.00%	cyoE	Cytochrome o oxidase	Cytochrome o oxidase protoheme IX farnesyltransferase subunit
25.00%	**idnK**	**Idonate**	**D-gluconate kinase**
25.00%	hyaE	Hydrogenase 1	Probable HyaA chaperone
25.00%	yigI	Systematic nomenclature	4HBT family thioesterase, function unknown
25.00%	yedV	Systematic nomenclature	Predicted histidine protein kinase sensor, function unknown
25.00%	yjjP	Systematic nomenclature	Inner membrane protein, DUF1212 family, function unknown; H-NS-repressed
25.00%	lyxK	Lyxose	L-xylulose/3-keto-L-gulonate kinase
25.00%	rpsD	Ribosomal protein, small	30S ribosomal subunit protein S4; NusA-like antitermination factor
25.00%	arnT	ara4N (4-amino-4-deoxy-L-arabinose)	4-amino-4-deoxy-L-arabinose(Ara4N):Lipid A transferase; modifies lipid A phosphates with aminoarabinose and confers resistance to polymyxin B and cationic antimicrobial peptides; glycolipid donor is undecaprenyl phosphate-alpha-L-Ara4N; inner membrane pro
25.00%	leuD	Leucine (biosynthesis)	3-isopropylmalate dehydratase small subunit; also called alpha-Isopropylmalate isomerase
25.00%	narZ	Nitrate reductase, nitrate regulation	Nitrate reductase II (NRZ), alpha subunit
25.00%	kefC	K+ efflux	NEM-activatable K+/H+ antiporter
25.00%	yehI	Systematic nomenclature	DUF4132 family protein, YehH paralog
25.00%	yhjE	Systematic nomenclature	Putative MFS transporter, function unknown; membrane protein
25.00%	waaG	Systematic nomenclature	UDP-glucose:(heptosyl)LPS alpha-1,3-glucosyltransferase; LPS core biosynthesis protein; glucosyltransferase I
25.00%	dosC	Direct oxygen sensor cyclase	Diguanylate cyclase, binds oxygen, positive biofilm regulator; cold- and stationary phase-induced
25.00%	nrdD	Nucleotide reductase	Ribonucleoside-triphosphate reductase; class III anaerobic ribonucleotide reductase
25.00%	hemC	Hemin	Porphobilinogen deaminase; neomycin sensitivity
25.00%	waaC	Systematic nomenclature	LPS heptosyltransferase I; LPS core biosynthesis; transfers innermost heptose to KDO
25.00%	ymgI	Systematic nomenclature	Uncharacterized protein
25.00%	thyA	Thymine	Thymidylate synthase; aminopterin, trimethoprim resistance; homodimer

25.00%	pepA	Peptidase	Multifunctional Aminopeptidase A; transcriptional regulator of carAB; DNA-binding role in site-specific recombination mediating ColE1 plasmid multimer resolution; homohexameric
25.00%	insI1	Insertion Sequence	IS30 transposase
25.00%	mdtB	Multidrug transporter	MdtABC-TolC efflux pump, multidrug resistance; heterodimeric RND-type transporter
25.00%	recC	Recombination	RecBCD Exonuclease V subunit, recombination and repair; chi-activated RecBCD recombinase subunit; binds RecB and RecD; may confer processivity on holoenzyme
25.00%	**pnp**	**Polynucleotide phosphorylase**	**Polynucleotide phosphorylase; exoribonuclease; PNPase component of RNA degradosome; cold shock protein required for growth at low temperatures**
25.00%	yfjW	Systematic nomenclature	Predicted inner membrane protein, function unknown, CP4-57 putative prophage remnant
25.00%	hcaR	Hydrocinnamic acid	Transcriptional activator for the hca operon; induced by 3-phenylpropionate and cinnamic acid; autoregulatory
25.00%	iroK	Unknown	3-hydroxypropionic acid resistance peptide
25.00%	yceA	Systematic nomenclature	Putative rhodanese
25.00%	eutB	Ethanolamine utilization	Ethanolamine ammonia lyase, large subunit, adenosylcobalamine-dependent; concerted induction requires both B12 and ethanolamine; heterodimeric

Sample: 4-S2-44S	N = 9		
Variant Frequency	gene	Gene Category	Gene Product Description
40.00%	**aspA**	**Aspartate**	**L-aspartate ammonia-lyase; L-aspartase**
28.60%	gloA	Glyoxalase	Glyoxalase I, lactoylglutathione lyase, Ni(2+) cofactor; inactivated by zinc; dimeric
33.30%	**insH1**	**Insertion Sequence**	**IS5 transposase**
28.60%	mlaD	outer Membrane Lipid Asymmetry	Putative phospholipid-binding protein, signal-anchored; periplasmic; MlaFEDB phospholipid ABC transporter; maintains OM lipid asymmetry
28.60%	ompR	Outer membrane protein	Response regulator for osmoregulation; regulates production of outer membrane proteins
40.00%	thrB	Threonine	Homoserine kinase
40.00%	xapA	XAnthosine Phosphorylase	Xanthosine phosphorylase; purine nucleoside phosphorylase 2, PNP-II; inosine-guanosine phosphorylase; nicotinamide salvage enzyme nicotinamide 1-beta-D-riboside synthase; hexameric
40.00%	ydjN	Systematic nomenclature	Predicted symporter, function unknown
33.30%	yggU	Systematic nomenclature	UPF0235 family protein, function unknown

Table A4: Genomic changes data for several experimental strains are listed. SNP calls were set to conservative levels. Only SNPs at frequencies above 25% of the population sequencing done on the Illumina MiSeq platform are listed. SNPs in genes that were found in more than a single sample are in bold. Few repeated targets of selection were identified and of the gene targets that did have changes in more than one sample, there was no discernible pattern in more than 2 samples (out of the eight population samples sequenced). Analysis of the MiSeq data was limited due to problems with low QC scores caused by a sub-standard lot of reagents produced by Illumina. Gene names and Gene Product Descriptions are from EcoGene (www.ecogene.org). Frequencies reported are in comparison to the E. coli K-12 MG1655 Reference genome, Genbank accession NC_000913.3.

Figure A4: Alignment of rpsL gene for all strains against DD1953 Streptomycin Sensitive ancestor and the E. coli reference sequence (NC_000913.3). Mutations are in the 42nd codon position.

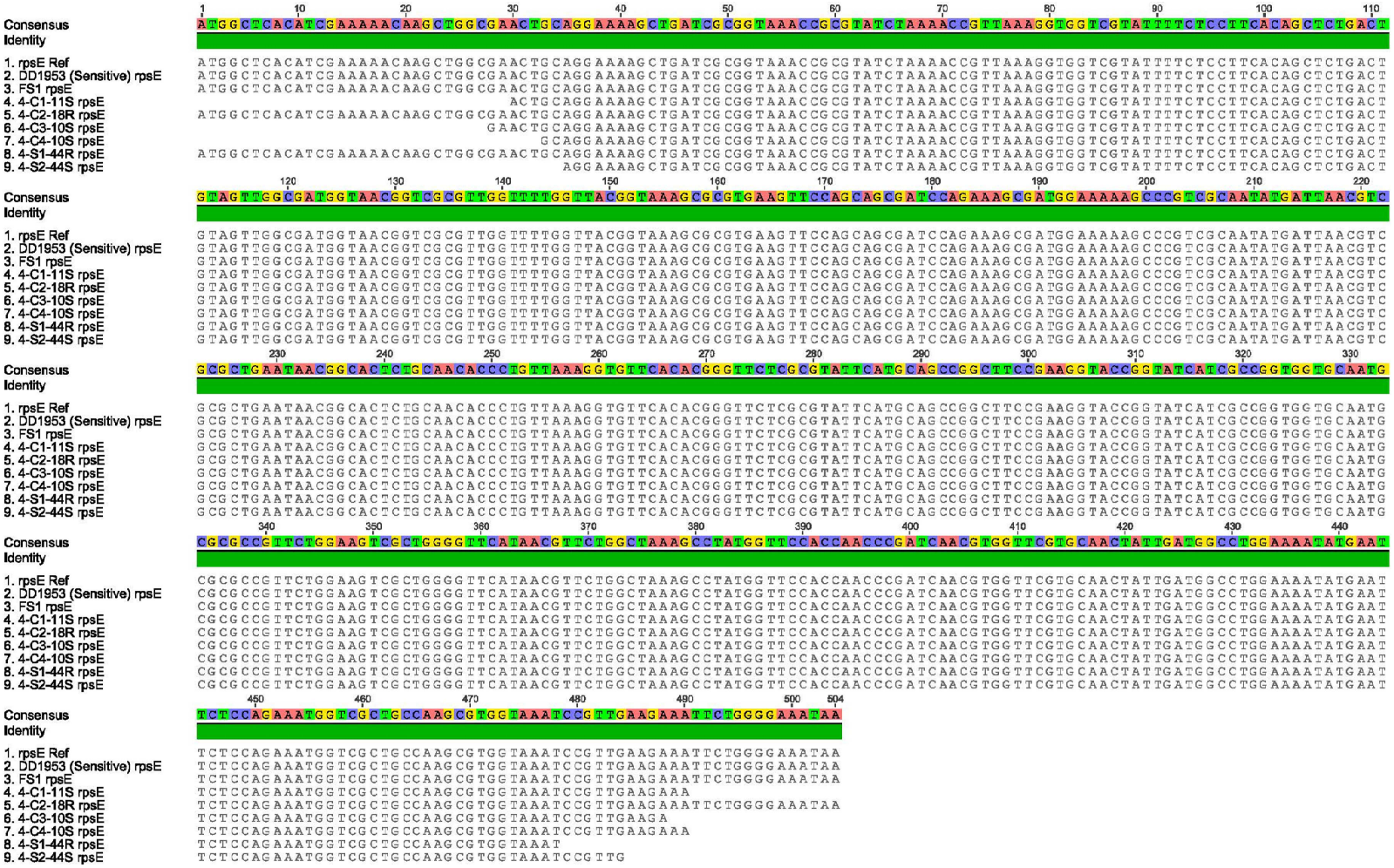


Figure A5: Alignment of rpsE gene for all strains. Note that no compensatory mutations are observed.

Figure A6: Alignment of rpsD gene for all strains. Note that no compensatory mutations are observed