Collateral sensitivity and the evolution of drug resistance in *Escherichia coli*.

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

M.Sc. Biology with Specialization in Data Science

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Ottawa, Ontario

Abstract

Recently, collateral sensitivity networks have created excitement for designing new therapies that could reduce rates of evolution of antibiotic resistance in pathogens. To explore this idea, I tested the key assumption that collateral sensitivity should reduce the frequency of mutation to resistance. Thirty strains of *Escherichia coli,* each selected for resistance to a single antibiotic, were screened for minimum inhibitory concentration values against twelve drugs to find relationships of collateral sensitivity. Ceftazidime resistant strains showed susceptibility to chloramphenicol. It was therefore expected that growth in sub-MIC concentrations of chloramphenicol would reduce mutation rate to ceftazidime resistance, however, an increase in mutation rate was observed. The results showed that μ was significantly higher in populations evolved in sub-MIC concentrations of cef, compared to plain LB ($p = 2.93x10^{-5}$) For populations grown in LB μ was 0.011 per 10^9 cells, 95% CI [0.019, 0.005] whereas populations grown in LB and sub-MIC cef, μ was 0.21 per 10^9 cells, 95% CI [0.452,0.158]. There are two likely hypotheses, the first is that the increase in mutation is due to antibiotic related SOS response, and the second is that multi-drug-resistance was selected for in both cases. This highlights the need for evolutionary considerations in designing new drug therapies, as apparent patterns of collateral sensitivity may not be a sufficient criterion.

Acknowledgements

It is my belief that the Scientist is the most noble of all God's creatures. To be clear, I do not mean God in a strict religious sense but to signify, as Immanuel Kant did for his age, that science and religion are not so different. They both require a kind of faith enshrined in their devoted. I further believe that anyone who tells you otherwise is lying, or trying to sell you something. In the latter case, the false idea that the practice of Science can offer us any control over nature. This idea is a lie, yet somehow it is true. We live longer, better, and with more opportunity as a species today than ever before, solely because of Scientists. However, do not let the general scientific institution impress you, for not all scientists are *Scientists*. Therefore, I say that the Scientist is the most noble creature. For a lifetime, the Scientist endures the very human task of drowning in their own mortality as it makes liars of us all, but more than others, they flail in a desperate search for elusive Truths as they bob between the waves. The Scientist bears it all in good faith that their contributions will, one day – through a kind of prayer – bear fruits for those they will never meet. It is a kind of selflessness we cannot expect from evolutionary theory, and thus it is perhaps the least scientific way of life, thus making Scientists the least scientific creatures, and so the most noble. For there is nothing noble in the calculation of humanity, only the arduous Truth behind it. Our greatest achievements as a species come soundly from this self-denying, and self-imposed madness, this eternal paradox. As my proof, any true Scientist would read this here and rightly call it nonsense. I am not yet a Scientist, but wish to acknowledge the Scientists who so earnestly tried to convert me. To my committee, my family, friends, lab mates and especially Ally – the best *Scientists* I know.

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1 Chapter: Framing the Problem

"Everything in excess is opposed to nature" – Hippocrates

1.1 Recent Trends

Understanding the evolution of antimicrobial resistance (AMR) may prove to be an important asset to combat the emerging global health crisis of drug-resistant pathogens. With the globalization of humans and animals, has come the globalization of pathogens. Never has the gene pool for AMR been so accessible to these organisms, nor the selective force against them so strong (Gandon *et al.*, 2016). The last ten years have seen a dramatic increase in both proportion and absolute number of bacterial pathogens resistant to multiple drugs.

When the forefather of antibiotics, Alexander Fleming, accepted his Nobel Prize in 1945, he warned against the ease with which bacteria could and would acquire resistance. Just three years later 38% of *Staphylococcus aureus* strains in a London hospital were resistant to penicillin (Barber and Rozwadowska-Dowzenko, 1948). Currently 90% of strains in the UK (Johnson *et al.*, 2012), and almost all strains in the US are resistant to penicillin. Many other antibiotics are on similar trajectories. For example, methicillin-resistant *S. aureus* (MRSA) is reported at 50% prevalence rates in some communities in the US (Klevens *et al.*, 2007). There are many other worrisome trends, like organisms producing extended spectrum beta-lactamases (EBSL), that can inactivate even 3rd generation cephalosporins by hydrolysis. ESBL producing organisms are often resistant to multiple drugs, and even classes of drugs like co-trimoxazole (64%), gentamicin (9%), and ciprofloxacin (68%)(Pitout and Laupland, 2008). Though many of these studies are performed in hospital settings, there has also been a rise in resistant infections acquired in

the general community. This is even true for low AMR countries like the Netherlands where a survey of 1,713 asymptomatic, urban-dwelling people found that 8% had ESBL producing Enterobacteriaceae in their stool samples, where 10.6% was the prevalence found in symptomatic patients of the same region (Huttner *et al.*, 2013).

From hospitals to communities, AMR is a global problem. In 2014, the World Health Organization (WHO) requested national data from its 194 member states. They required samples of at least 30 isolates, and supplemented missing national data with existing research to present a global picture of surveillance for a broad range of pathogens and drug classes. The report showed that around the world, for *E. coli, K. pneumoniae,* and *S. aureus,* resistance to commonly used antibacterial drugs exceeded 50% in many settings. (World Health Organization, 2014). As governments move forward to address this issue, understanding how selective pressures in various environments can influence the occurrence of AMR will need to be at the center of any effort, lest there be a risk of failing to address this problem and even exacerbating the crisis at an exorbitant cost to public funds, and to humanity.

1.2 Consequences & Cost Modelling

A thorough global modelling study on the continuing rise of AMR, commissioned by the UK government and conducted by the RAND corporation, predicted that by 2050, between 11 million and 444 million lives, and between $2.1 trillion and $124.5 trillion dollars, would be lost compared to a world without AMR (Taylor *et al.*, 2014; O 'Neill, 2016). It can be difficult to estimate the exact cost and so, as the RAND model has done, researchers often model the cost efficiency of addressing antibiotic resistance, rather than

true cost of infection on loss of individual productivity, disruption of economic systems, and additional spending for containment (Smith, 2013). This often results in the gross underestimation of the true cost.

There are significant costs to quality of life, should AMR continue its current trajectory. Since antibiotics were first introduced, many aspects of modern medicine have been built upon the ability to safely mitigate risks associated to complication by infection (Smith, 2013). In the case of general surgery, the CDC estimated that more than half a million patients per year in the United States will develop surgical site infections (SSI). Patients who develop SSIs are 60% more likely to enter intensive care, and have twice the mortality rate of patients without SSIs (Bratzler and Houck, 2005). A dramatic rise of antibiotic resistance would affect all areas of medicine, from cancer treatment, to minor surgery, and would drastically change the standard of living the western world has become accustomed to.

For example, preference for Caesarian section has risen and now accounts for 14.7% to 49% of live births in the western world, varying by region, where the single highest risk factor is postpartum infection (Smaill and Grivell, 2014). Caesarian sections represent an interesting case, as many are deemed medically unnecessary, and the procedure can increase the risk of maternal mortality and morbidity (Shearer, 1993). A rise in AMR, that would make Caesarian sections unfeasible due to risk, represents a loss of two kinds. The first is a loss of a lifesaving procedure in the cases where it is used to mitigate serious birth complications, and the second is a loss of patient autonomy to determine their course of treatment. It would be reasonable to expect losses of the same kind in many areas of medicine.

1.3 Historical and Current Approaches

The written history of germs, disease, and their policies dates as far back as Ancient Athens as described by Thucydides. He spoke of the contagiousness of the plague of Athens after the Peloponnesian War: "there was the awful spectacle of men dying like sheep, through having caught the infection in nursing each other." (Dale, 1849). Germ theory, the idea that disease is caused by interactions of unique infectious microorganisms with a host, wasn't readily accepted by the medical community until the mid-nineteenth century (Benson, 2015). This represents roughly a two hundred year gap between when bacteria were first described by Antony Van Leeuwenhoek in 1676, and their first acceptance as probable cause of diseases (Porter, 1976). If there is a common theme in the shared history of humanity and bacteria, it is that while knowledge has often come quickly, action has always come slow.

In the history of antibiotic stewardship, the threat of resistance was known almost as readily as penicillin was made available. In the U.S., education programs for physicians began in the 1950's as an effort to combat rising rates of AMR, but they were ineffective in curbing the over prescription of antibiotics. AMR saw a steady rise through the 1970s even as externally imposed restrictions were introduced to limit the prescription of antibiotics in hospitals. It wasn't until stewardship programs introduced in the 2000s gave infectious disease experts the authority to deny inappropriately prescribed antibiotics that over-prescription began to change. It is still a major issue in many regions, including and especially areas of the developing world (Kazanjian, 2016).

Since then, AMR has been considered an emergent global threat, and a major public health problem according to the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) (ECDC, 2009; Efsa, 2009; World Health Organization, 2014; The White House Administration, 2015; WHO, 2015, 2016). Leading approaches propose breaking strategies down into three main areas: AMR in animals and the food chain, in the environment and community, and within the health care system (Roca *et al.*, 2015). Globally, there has been some support shown in the form of national plans, national coordination, and progress reports published (WHO, 2015).

In animals, antibiotics have been used to promote the growth of livestock, and to prevent and treat infections. The industrial scale use of antibiotics has increased the selective pressure, both for harmless and pathogenic stains, that have a risk of infecting humans through direct contact, the food chain, or environmental pollutants. One example of this can be traced to the bla_{ESBL} and bla_{AmpC} genes. The bla_{ESBL} gene confers resistance to a variety of β-lactams including penicillins, the first four generations of cephalosporins, as well as monobactams. The bla_{AmpC} β-lactamases confer resistance to penicillins, and first to third generation cephalosporins(Pfeifer, Cullik and Witte, 2010). Both genes have been shown to be transmitted from Enterobacteriaceae of food animals to pathogenic *E. coli* and *Salmonella* strains (Liebana *et al.*, 2013). Similarly, a recent finding of a plasmid-based resistance gene to colistin, a 'last resort' antibiotic often only used in humans for very difficult infections, was also attributed to antibiotic use in pigs (Liu *et al.*, 2016).

A 2009 committee hosted by the European Food Safety Authority (EFSA) called together many major policy stakeholders to put forward recommendations to reduce the rise of resistance from food animals and the food chain. Based on current trends in veterinary medicine and agricultural practice, they recommend a banning of antibiotics as growth promoters and limiting preventative dosing of food animals, reducing transmission by developing alternative treatment strategies and increasing hygiene throughout the food chain, and raising awareness among farmers, veterinarians, and food handlers by developing educational materials. They also call for better collaboration and communication between surveillance systems for humans and animals. The committee specifically called for the reservation of fluoroquinolones and third and fourth generation cephalosporins to only be used in the cases of tough infections, and the reduction of the use of methicillin. In order to accomplish this they have been standardizing the list of agents to be monitored across networks in Europe, increased monitoring efforts, especially where there are gaps for certain strains, and by conducting more regular screens not just when infection occurs, but on healthy animals (Efsa, 2009).

In communities, policy bodies have played a role in tracking antimicrobial consumption data, rates of prevalence outside clinical settings, as well as proposing new metrics to present a single measure of resistance and use of an antibiotic (Laxminarayan and Klugman, 2011). There is also a need for increased education and awareness campaigns at the community level, including better packaging to improve understanding of antibiotic use (Roca *et al.*, 2015). Unfortunately, at the community level many of the challenges require substantive policy solutions, and public funds which depend on strong political commitments (Spellberg *et al.*, 2011).

The health care system is a risky environment that provides two key factors that increase the risk of AMR: there is a constant influx of new pathogens, and high levels of antibiotic consumption. When overlaid with the intricacies and particularities of individual clinical settings, and regional differences in policies, it creates a challenging problem space for which there is no one solution. Some of the complexities that act as determinants of increased antibiotic use include, but are not limited to, relative humidity, healthcare expenditure proportional to gross domestic product, feelings of distrust, proportion of population aged > 65 years, and availability of treatment guidelines. Determinants that contributed towards less antibiotic use include but are not limited to: restrictions on marketing activities towards prescribers, population density, number of antibiotics available, educational attainment and degree of atheism (Blommaert *et al.*, 2014).

It has been argued that at the clinical level, stewardship programs should be mandatory. An ideal example would include embedded microbiology labs that could screen patients at admission for any incoming resistant strains. In general, hospitals require greater reporting mechanisms for susceptibility testing, and greater restrictions that limit and manage the use of antibiotics in hospitals, all with the goal that the allocation of resources, down to the drug dosage, should take into account the pathogen itself, the time of exposure, patterns of drug exposure, and risk of transmission (Fischbach, 2011; Roca *et al.*, 2015).

Since the 2000s, many countries have released national strategic plans outlining similar approaches to combat the rise of AMR. In the Western world this includes the European Union in 2001(Conselho da União Europeia, 2001), the WHO with its

European member states in 2011(WHO Regional Office For Europe, 2011), followed by the U.K. in 2013 (Department of Health and Department for Environment Food and Rural Affairs, 2013), Canada (Public Health Agency of Canada, 2014, 2016) and the U.S. (The White House Administration, 2015) in 2014 and 2015, with the WHO announcing a global strategic action plan in 2015 (WHO, 2016).

As of 2015, regions leading in policy strategies that meet the WHO criteria of being a financed national plan with multi-sectoral input, are South-East Asia, where 45% of reporting member states have such a plan, followed by the European region with 43% of reporting member states. Clearly there is work to be done, but key knowledge about the issue is still missing. Without a diligent understanding of the evolutionary mechanisms behind this threat and a consideration of the root cause of the problem, there is a tremendous risk that current policy programs will not only fail to address the problem, but in fact cause increases in AMR.

1.4 Public Health and Clinical Approaches

Some examples of policy in action include drug cycling, drug restriction programs, and combination therapies. Drug restriction programs are based on the idea that reducing the prescription of an antibiotic will decrease the prevalence of resistance to that drug. It has been a successful tactic for many respiratory infections, but only where the population of the pathogen was dominated by a single kind of resistance mutation. It has seen less success in cases where the resistance gene has been located on a plasmid (Enne, 2009). The factors underlying success of these programs are complex, but can be framed as three variables: the resistance cost of a resistance mutation to an organism,

how clonal a resistant population of bacteria is, and whether there is co-selection for other antibiotics. In cases of low cost of resistance, little clonal structure, and co-selection, restriction programs have been challenged. For example, an 8-year restriction program failed to reduce plasmid-carried sulphonamide resistance among *E. coli* in the population (Enne *et al.*, 2001).

Drug cycling programs are a preventative measure to AMR that can involve cycling several drugs in a clinical setting with similar antimicrobial activity, but they start and end with the same drug. The rationale is that resistance acquisition is a result of drug exposure, and so limiting the clinical use of a drug by including it in a cycling program reduces the risk of AMR. A second premise is that the fitness cost of resistance to the first drug will result in the loss of those mutants over the course of the cycle, leaving a sensitive population when the first drug is reintroduced again at the end (Andersson and Hughes, 2010). The actual efficacy of this strategy has not been concretely proven and is questioned by consideration of practical limitations that include incomplete antibiotic dosage, non-uniform drug action within the patient, variability in the cost of resistance, and the risk of compensatory mutation. (Imamovic and Sommer, 2013).

Drug cycling programs can often fail when resistance mutations carry no *in vivo* cost, which allows the resistance mutation to persist. For example, in the case of acquired fosfomycin resistance in *P.* aeruginosa, AMR was both highly likely to arise and had no observed fitness costs in terms of virulence, colonization, persistence, ability to cause lung damage, or lethality (Rodríguez-Rojas *et al.*, 2010). Even in the case where a resistance mutation does incur a fitness cost, it can persist if the fitness cost can be

recovered by a compensatory mutation that ameliorates the initial fitness cost (MacLean and Vogwill, 2014; Melnyk, Wong and Kassen, 2015).

Combination therapies, or the combining of antibiotics during treatment, are a clinical method to improve treatment efficacy and prevent the rise of resistance (Bell, 2013). The underlying principle is that the probability of AMR arising for drug A and B will be the probability of drug A and B multiplied, and thus less likely. However, the study of how drugs interact is much more complicated than this and can be explained as several molecular archetypes: combinations where the drugs inhibit targets in different pathways, combinations that inhibit distinct parts of the same pathway, and combinations that inhibit the same target in different ways. Generally speaking molecular archetypes help estimate the strength of a drug combination (Fischbach, 2011). For example, the directly observed treatment, short-course (DOTS) regimen for tuberculosis, is a treatment combination of four drugs: isoniazid, rifampicin, ethambutol, and pyrazinamide, where at least three drugs are known to have different cellular targets in different pathways. It is a drug combination that has improved the treatment of resistant tuberculosis globally, and was thought to reduce risk of resistance due to a shortened treatment period, however, the DOTS regimen is constantly challenged by new resistant strains (Cox, Morrow and Deutschmann, 2008).

The remaining two options can still be effective, but face greater challenges due to smaller diversification of targets. A combination therapy that targets two targets in the same pathway is Augmentin. The ß-lactam amoxicillin is combined with the beta-lactamase inhibitor clavulanate to prevent the degradation of amoxicillin (Lee, Yuen and Kumana, 2003).

In addition to molecular archetypes, there are also drug interactions to consider. Synergistic interactions result in a greater than expected killing effect, and antagonistic interactions result in a less than expected killing effect. Synergistic drug interactions are often preferred in clinical use, but how drug interaction affects the rate of mutation, or adaptation hasn't fully been explored. A large-scale screen exploring drug interactions found that synergistic drug pairings can increase the rate of antibiotic resistance in populations as compared to single drug treatments. This was shown for a synergistic pairing of doxycycline and erythromycin in hundreds of replicate *E. coli* populations, evolved in parallel. However, this experiment also showed that increase in rate of adaptation was limited by the availability of resistance mutations for both drugs (Hegreness *et al.*, 2008).

Experiments that model AMR evolution during combination therapies argue that antagonistic and synergistic drug pairs may have distinct tradeoffs in environments with high resource competition, where fitness costs are more pronounced. Synergistic drug pairings increase selective pressure, which fosters an increased risk of antibiotic resistance for the same level of inhibition of a single drug. Antagonistic pairings can increase risk of resistance because of longer treatment times, but also provide single-drug resistant mutants less selective advantage due to the fitness cost of resistance in a low resource environment. One optimization model describes this tension as a trade-off between treatment efficiency and prevention of resistance, where an optimal limit to drug synergy exists at the point where higher synergy no longer decreases treatment time, but would still increase the risk of resistance (Torella, Chait and Kishony, 2010).

An interesting aspect of combination treatments is how they may be informed by nature. There are many examples in *Streptomyces,* and other bacteria demonstrating that mono-production of secondary metabolites or antibiotics, is a rarity in nature. Natural synergies like this are thought to be contingent, and to have co-evolved by chance, as production of synergistic metabolites often occur in separate bio pathways. A strong example of this is the co-production of clavulanic acid and β-lactam antibiotics in *S. clauvuligerus* (Ward and Hodgson, 1993). They act synergistically because clavulanic acid is a natural inhibitor of β-lactamases, but both metabolites are manufactured by completely unrelated biosynthetic pathways. When combined with β-lactams, it has been shown to be more effective against β-lactam-resistant bacteria, than just using β-lactams alone. Clinically this has been reflected in the use of augmentin, or a combination of clavulanic acid and methicillin (Challis and Hopwood, 2003).

1.5 Antibiotic Mechanisms of Action and Collateral Sensitivity

Some bacteria possess an intrinsic resistance to antibiotics, usually due to some inherent structural or functional characteristic. The simplest form of inherent resistance can come from the absence of a viable drug target, as is the case for triclosan in *Pseudomonas.* This is due to an insensitive allele of *fabI* that encodes an additional copy of the target enzyme for triclosan (Zhu *et al.*, 2010). Many Gram-negative bacteria possess resistance due to an inability of drugs to cross their outer membranes. For example, vancomycin acts by preventing crosslinking of peptidoglycans in Gram-positive bacteria. However, due to its inability to cross the outer membrane of Gram-negative bacteria, the drug cannot access its target in the periplasm (Tsuchido and Takano, 1988).

Bacterial populations can acquire or evolve resistance through a host of mechanisms such as reduced membrane permeability, increased expression of efflux pumps, changes to target sites because of mutation, modifying or protection of target sites through methylation or other mechanisms, and direct inactivation of antibiotics through hydrolysis or transfer of a chemical (Blair *et al.*, 2015).

Limited accessibility of a drug to its target is a common mechanism of AMR. Commonly, hydrophilic drugs can diffuse through major porins in the outer membrane, like OmpF and OmpC. However, a downregulation, or a replacement of these porins with more selective porins, would lead to reduced permeability and increased resistance (Tenover, 2006). These mechanisms have been shown to be involved in other groups of Enterobacteriacaea, such as *Pseudomonas*, and *Acinetobacter* where porin-related reduction in membrane permeability confers increased resistance. It has led to previously undocumented resistance to newer drugs like carbapenems, and cephalosporins where it was thought resistance was commonly due to enzymatic degradation (Baroud *et al.*, 2013).

Another means of limiting access is through an increase of efflux pumps that actively transport antibiotics out of the cell. Efflux pumps, when not specific, can confer resistance across multiple classes of drugs also known as multidrug resistance (MDR) pumps. Among some of the more clinically dangerous families of MDR pumps are the tripartite resistance nodulation division (RND) pumps, which confer MDR to a wide range of antibiotics due to their generality of substrate (Piddock, 2006). RND pumps have been found alongside other AMR genes on the IncHI1 plasmid in *Citrobacter*

freundii, pointing to easily transferable, and robust mechanisms of MDR for other pathogenic strains (Dolejska *et al.*, 2013).

Modification can result in protection as well, as is the case in *Staphylococcus aureus* that acquire point mutations in the *mprF* gene. *mprF* encodes a multipepetide resistance factor that tags anionic phospholipid phosphatidylglycerol with L-Lys. These modifications change the phospholipid content of the membrane, which reduces the membrane-binding affinity of daptomycin, preventing it from disrupting the membrane (Mishra *et al.*, 2013).

Most antibiotics contribute to cell death by binding specifically to their target, and preventing its normal action. If the target acquires a mutation that causes a structural change to the target, it can reduce the binding affinity with the drug, resulting in resistance. A classic example of this is with *gyrA*-mediated quinolone resistance. DNA gyrase is a tetramer that consists of two A subunits, and two B subunits, encoded by the *gyrA* and *gyrB* genes that reduce negative supercoiling by ligating and re-ligating DNA during replication. While there are many documented mutations, one specific example can be found the *gyrA* S83L/D87N double mutation. These mutations results in a decrease of quinolone affinity due to a structural change of the binding site (Maruri *et al.*, 2012; Zayed *et al.*, 2015).

Recent discoveries of methyltransferases have also identified target modification without genetic modification as a means of antibiotic resistance. For example, the erythromycin ribosome methylase (*erm*) family of genes alter the active site of 16S rRNA through methylation, effectively protecting the site from antibiotic disruption (Talebi *et al.*, 2016). The *erm* genes are often carried on plasmids, making for a clinically

dangerous mechanism of gene transfer. While it was previously thought that protection by methylation was not clinically prominent, more Enterobacteriaceae throughout North America, Europe, and India have been detected carrying methyltransferase genes (Fritsche *et al.*, 2008; Hidalgo *et al.*, 2013).

There are other methods of target site protection, including the *qnr* family of genes that encode pentapeptide repeat proteins (PRPs) that can rescue topoisomerase IV and DNA gyrase from quinolone activity.

In contrast to target modification, there are resistance mechanisms that destroy or inactivate antibiotics to disrupt their antimicrobial properties. Inactivation by hydrolysis is a major, and well understood pathway to resistance dating back to the first discoveries of β-lactamases (Dowson, Coffey and Spratt, 1994). Thousands of enzymes have been catalogued that also inactivate their target drug by hydrolysis, including not just β-lactams, but aminoglycosides, phenicols, and macrolides as well. β-lactamases were followed by the extended-spectrum β-lactamases (ESBLs), which include a wider range of activities against new generation cephalosporins that were introduced, like ceftazidime (Liebana *et al.*, 2013). The EBSLs have been widely and robustly transferred across species, facilitated by insertion sequences like IS*ecp1*, and transfer on plasmids to many other species (Poirel, Bonnin and Nordmann, 2012). As a result, two β-lactamases, CTX-M14 and CTX-M15 have become the two most widely isolated globally. Where CTX-M15 is often seen in clinical *K. pneumoniae* strains, and CTX-M14 seen in *E. coli* strains in outside communities (Dhanji *et al.*, 2011; Zhao and Hu, 2013).

While there is often one target for an antibiotic, there are often a multitude of mechanisms by which antibiotic resistance for that target can arise. For example, with

fluoroquinolone resistance in *E. coli* resistance can arise from mutations to *gyrA*, preventing binding to the target, mutations in the MarR family repressors, like *marR* can increase expression of AraC family activators like *marA*, and thus RND efflux pumps like *acrAB* , or acquisition of *qnr* genes that protect the target, or from acetyl transferases that can inactivate quinolones (Blair *et al.*, 2015).

Often increased resistance to a single antibiotic can be accompanied by increased resistance to multiple other antibiotics, like in the case of *marR* mediated quinolone resistance in *E. coli* (Pal, Papp and Lazar, 2015). By contrast, in the case of collateral sensitivity, genetic adaptation to one antibiotic results in *sensitivity* to other antibiotics (Imamovic and Sommer, 2013). For example, adaptation to aminoglycosides was shown to confer sensitivity to a broad range of antibiotics. The postulated mechanism involved identifying a broad class of genes expected to influence the membrane electrochemical potential. It is hypothesized that a reduction in the proton motor force (PMF) across the membrane, reduces respiration required for aminoglycoside uptake. This would also decrease the efficacy of PMF-dependent major efflux pumps such as the AcrAB transporter. The result would be resistance to aminoglycosides, but a hypersensitivity to several other antibiotics that would normally be cleared by the AcrAB transporter such as quinolones, tetracyclines, and β-lactams (Lázár *et al.*, 2013).

Collaterally sensitivite mutations have been implicated in slowing AMR evolution in lab experiments simulating proposed alternating-drug combination therapies. In an experiment, strains of *S. aureus* were evolved in single-, alternating, and mixed-drug antibiotic environments, where the drugs were chosen with distinct mechanisms of action. Alternating an antibiotic daily was shown to slow the rate of evolution of

resistance to at least one of the component drugs. The drugs tested were trimethoprim(tmp), a dihydrofolate reductase inhibitor, neomycin (neo), a ribosome-targeting aminoclycoside, and ciprofloxacin (cip), a fluoroquinolone that targets the DNA gyrase/topoisomerase complex (Kim, Lieberman and Kishony, 2014). This demonstrates a potential for drugs that show collateral sensitivity, or cross-resistance, to allow for treatments that would slow the rate of evolution, when used at effective doses.

Similarly, a large collateral sensitivity screen showed that gentamicin and cefuroxime could be deployed cyclically to select against resistance to either drug in *E. coli*. AMR was evolved in *E. coli* to 23 clinically relevant antibiotics and then tested for collateral sensitivity against all 23 drugs. From this experiment, substantial collateral sensitivity for nine antibiotic classes was identified, including prominent general relationships of collateral sensitivity networks between aminoglycosides, polymixins, and tetracyclines. This study then tested if collateral sensitivity could be used to slow the rate of AMR to drugs showing collateral sensitivity. Gentamicin and cefuroxime exhibited a collateral sensitivity relationship, and a fluorescent protein expressing strain of *E. coli* were evolved in gentamycin to 32X the MIC of the wildtype. After exposure to 4μg/mL of cefuroxime the gentamicin resistant strain was eliminated. From this same population, the remaining wildtype was evolved in cefuroxime to 128X the MIC of the wildtype and exposed to 1μg/mL of gentamycin that showed a similar killing effect as before. This experiment demonstrates the possibility of using collateral sensitivity as a basis for alternating drug treatment (Imamovic and Sommer, 2013). To elaborate these claims and fully explore a potential defense against AMR, it will be crucial to understand how general this phenomenon is across drug classes as well as for different pairings of drugs.

1.6 Experimental Aims

Given what is known about collateral sensitivity relationships, it is expected that in a case where resistance to A confers sensitivity to B, growth in the presence of B should select against mutations conferring resistance to A. This expected because the increase in sensitivity to drug B should eliminate mutants resistant to A, thus reducing the number of colonies that appear when plated, lowering the number observed mutants. Similarly, it is expected that the mutation rate of resistance to A, when grown in the presence of B should also be lower.

To test this hypothesis, I identified sensitivity relationships between two drugs, A and B, for multiple resistance mutants per drug. Two conditions were tested in estimating mutation rate, a permissive environment with LB broth, and an antagonistic environment with antibiotic concentrations of drug A, or B far below the MIC value for the wild type. Replicate populations were grown and plated on above MIC concentrations the opposite drug in the pair. In this experiment, the drugs chosen from the collateral sensitivity screen were chloramphenicol and ceftazidime because CEF mutants showed resistance to cef, and all of the CEF strains showed susceptibility to cm. Sensitivity to a drug was not found for most other strains, which is why this pair was chosen.

Contrary to our prediction mutation rate was seen to increase in sub-MIC chloramphenicol and ceftazidime, and a statistically significant amount in the sub-MIC environment of ceftazidime. This was unexpected, and requires further investigation to assess the generalizability of claims about mutation rate, and in a larger scope the degree to which relationships of collateral sensitivity can be relied upon to slow the rate of AMR evolution.

2 Chapter: Methods

2.1 Strains

Thirty strains (Table 1) selected from antibiotic containing environments were tested for collateral sensitivity relationships between twelve drugs (Table 2). These strains were derived from *E. coli* K12 (MG1655), and were selected from Luria Delbruck assays on 1-8X the MIC for the wildtype per drug (Table 1) by another researcher. The previous researcher performed whole genome sequencing with an Illumina MiSeq to analyze mutational profiles. Mutations that have been confirmed by other experiments as well as new putative resistance mutations are listed (Table 1).

2.2 Antibiotic Preparation

Antibiotics were sterilized using 0.22μM filters and prepared at a stock concentration of 10mg/mL. Antibiotics were prepared according to the work of Andrews (Table 2).

2.3 Minimum Inhibitory Concentration Assay

Strains were streaked from -80°C glycerol stocks onto 1.5% agar plates containing Luria Broth (LB) and incubated overnight at 37°C. Single colonies were picked into 10mL liquid LB in a shaking incubator overnight at 120rpm and 37°C. The overnight liquid colonies were diluted by a factor of 10^2 and dispensed at 100ul/well into 96-well plates. 100ul of diluted antibiotic were added to row A, producing the starting concentration as outlined in Table 1, to a total volume of 200uL. Dilutions were performed for rows 1-7 by taking 100uL from the previous row and mixing with the next

row. The last row was left as a no-antibiotic control. After being grown over night the 96 well plates were measured at 600nm in a Biotek Elx808 plate reader. The minimum inhibitory concentration was defined as the first reading where optical density readings were more than 50% less than the previous rows. MICs were determined for all 30 strains against 12 antibiotics with two technical replicates with test ranges as indicated in (Table 2). All dilutions were performed using an Integra Viaflow Assist.

Table 1 List of strains studied in this study, sorted by gene of resistance mutation. Mutation notation is according to (den Dunnen and Antonarakis, 2001) where * is a stop, and fs is frameshift.

Strain	Selected on	Gene	Mutation
Cm2	cm	acrR	E118*
Cm4	cm	acrR	E98fs
Cm1	cm	acrR	indel; possible intergenic
Cm3	cm	acrR	A151fs
Cef3	cef	cpxA	L15_T16 deletion
Gn11	gn	cyoA	rearrangement
Gn12	gn	cyoA	I127fs
Gn13	gn	cyoA	F102fs
Km14	kn	cyoA	W82*
Cef8	cef	envZ	P248S
S83L	cip	gyrA	S83L
Cip1	cip	gyrA	S83A
Cip3	cip	gyrA	D87Y
Cip5	cip	gyrA, valS	D87G, G611G
Cip8	cip	marR	R77H
Cip2	cip	marR	delta 72-82
Cip6	cip	marR	R94C
Amp4	amp	marR	V1fs
Tet8	tet	marR	H120fs
Cef4	cef	marR, yqek	L46fs, S20L
Cef7	cef	rfaG	E289fs
Cef6	cef	rfaH	Trp4*
Rif1	rif	rpoB	I572L
Rif7	rif	rpoB	I572S
Kn6	kn	ubiB	Y176*
Km12	kn	ubiF	Q120*

Kn4	kn	yejO	R134C
Km11	kn	ygfB	rearrangement
Col4	col	yiaW-aldB	intergenic

Table 2 List of antibiotics used in this study, organized by drug class, and mechanism of action. Range for MIC indicates the range of concentrations used to determine the MIC. For experimentally determined MICs see Table 4.

Drug Name	Abbreviation	Class	Target	Range for MIC (ug/mL)	Solvent
Naladixic Acid	nal	Quinolone	DNA Gyrase	3.9 - 250	H_2O
Levofloxacin	levo	Quinolone	DNA Gyrase	0.016 – 250	H_2O
Ciprofloxacin	cip	Quinolone	DNA Gyrase	0.016 – 250	0.1M NaOh*
Rifampacin	rif	Rifamycin	RNA polymerase	3.9 - 250	DMSO
Ampicillin	amp	β-lactam	Cell Wall	3.9 - 250	Sat. $NaCO^3$
Ceftazidime	cef	β-lactam	Cell Wall	0.016 – 250	Sat. $NaCO^3$
Meropenem	mer	β-lactam	Cell Wall	0.016 – 250	H_2O
Kanamycin	kn	Aminoglycoside	Protein synthesis, 30S	3.9 - 250	H_2O
Gentamycin	gn	Aminoglycoside	Protein synthesis, 30S	3.9 - 250	H_2O
Streptomycin	strep	Aminoglycoside	Protein synthesis, 30S	3.9 - 250	H_2O
Colistin	col	Polymyxin	Lippopolysaccharide	0.016 – 250	H_2O
Chloramphenicol	cm	Amphenicol	Protein synthesis, 50S	0.016 – 250	EtOH

2.4 Modified Luria-Delbrück Assay

Luria-Delbruck assays were performed to estimate the apparent rate of mutation under different conditions. Drug pairs were chosen from the MIC screen if two sets of mutants showed resistance to the antibiotic they were exposed to previously, and susceptibility to the reciprocal mutant's drug. For these sets four Luria-Delbruck (L-D) Assays were performed (Luria and Delbrück, 1943). Assays normally involve growing overnight cultures and diluting to a small number of cells, ~100 or 10^{-6} dilution after growth. The dilutions were then grown in at least thirty replicate populations overnight and selected on 1-8X the MIC of a desired antibiotic. The number of mutants on selective plates were then counted. A small fraction of each replicate population was diluted and plated on rich media without antibiotic at 10^{-5}, 10^{-6}, and 10^{-7}, dilutions to calculate the overall population size. In the modified L-D assay, two parallel assays were conducted from the same initial overnight population, where one set of replicate populations were grown in rich media, and another in sub-MIC levels of an antibiotic.

The two drugs tested for this experiment were chloramphenicol and ceftazidime. In the first two experiments, wild type MG1655 was streaked from glycerol stocks frozen at -80°C onto 1.5% agar plates containing LB. They were incubated overnight at 37°C and single colonies were picked and transferred to 10mL of liquid LB. Liquid colonies were placed into a shaking incubator overnight and grown at 37°C, and 120 rpm. The overnight liquid colonies were diluted by a factor of 10^6, to contain ~100cells/100μL. In a 96 well plate, 30 populations were grown in plain LB, and 30 populations were grown in chloramphenicol at 0.25X the MIC concentration for the wild type (1.2μg/mL). The 96 well plate was incubated overnight at 37°C. 10uL from each population was serially

diluted to a concentration of 10^{-6}, 10^{-7}, and 10^{-8} at a volume of 100μL. These dilutions were spot plated onto plain LB in four replicates of 25μL to determine a population count N. The remaining 90μL of each replicate in the 96 well plate was spread onto 1.5% agar plates, containing either LB with no antibiotic, or LB + ceftazidime at ~3.5X MIC (0.8μg/mL). Both sets of plates were incubated overnight at 37°C. The serial dilution plates were counted the next day for population estimates, and the antibiotic containing plates were counted for number of mutants. This experiment was also done in reverse, where MG1655 was grown first in ceftazidime at ~0.25X the MIC concentration of the wild type (0.06μg/mL) , and plated on 1.5% agar plates containing LB and ~3.0X MIC concentration of chloramphenicol (15μg/mL)

2.5 Estimating Mutation Rate with FALCOR

Mutation rate was estimated using the Ma-Sandr-Sakar Maximum Likelihood Estimator (MSS-MLE)(Sarkar, Ma and Sandri, 1992) method through a web tool called the Fluctuation Analysis Calculator (FALCOR)(Hall *et al.*, 2009). As was described, a small number of cells was used to inoculate parallel cultures in non-selective, and low antibiotic environments. These parallel cultures were then plated on selective environments to determine the number of mutants in each colony, *r*. Dilutions of parallel cultures were plated onto rich media to calculate the total number of viable cells, N_t. The number of mutants that appear, *r*, reflects the mean number of mutations that occur during the lifetime of a cell, μ. μ is called the apparent or estimated rate of mutation because it is a measure of mutation followed by selection on an antibiotic plate. Mutants that appeared early will produce more colonies on the selective media. Therefore, the ratio of r/N_t can vary greatly, even if the mean number of mutants that occur in the

lifetime of a culture, *m,* is the same between colonies. The MSS-MLE uses an initial estimate of *m* to calculate the probability of observing *r* mutants, p_r. Where based on *r*, a value of *m* is estimated to satisfy the equation for each population. The mutation rate, μ, is calculated for each colony as well as a ratio of m/N_t. The median μ is then calculated, and confidence intervals determined using the standard curve for values of μ (Hall *et al.*, 2009, Appendix A)

3 Chapter: Results

3.1 Wild Type & Strain MIC values

MIC assays were conducted on 30 strains (Table 1), with 12 drugs (Table 2), where 29 strains were evolved in resistant environments containing a single antibiotic. The wild type strain, MG1655 assayed and compared to known literature values for similar lab strains (Andrews 2001, Table 4).

Table 3 Experimental MIC values for MG1655 compared to similar lab strains as an average of n = 2, for each MIC.

Drug	Literature Value (µg/mL)	Experimental Value (µg/mL)	Std. Error
RIF	16	14.44	±0.84
NAL	4	3.93	±1.37
AMP	4	3.14	±0.47
CM	4	5.46	±3.21
KN	1	2	±0.00
GN	0.5	0.5	±0.00
COL	0.5	0.45	±0.03
CEF	0.25	0.24	±0.09
LEVO	0.03	0.06	±0.00
MER	0.02	0.06	±0.00
CIP	0.02	0.38	±0.13
STREP	N/A	2.63	±0.63

For resistant lab strains, MICs were calculated as a Log_2 transformed ratio of the resistant strain MIC to the wild type. MICs were organized by drug class. Overall error bars were not included for MIC results as two technical replicated were used, and were identical in most cases.

For the quinolones tested, all the quinolone resistant strains showed varying degrees of resistance compared to the wild type, with S83L being the most resistant to all

quinolones, except for the CIPR8 strain, which was more resistant to cip. Only one other strain, KN6, showed increased susceptibility to nal. Two strains, CEF3 and CEF6, showed increased susceptibility to levo, and eight strains, CEF3, CEF6, CM2, KN14, GN11, GN12, GN13, and COL4, showed increased susceptibility to cip (Figure 1).

For the β-lactam antibiotics, almost all the resistant strains showed increased resistance compared to the wild type. The greatest overall increase in resistance was to amp, with more than one third of the strains showing a 2X increase in resistance to amp compared to the wild type. Only one strain, CIPR5, had increased susceptibility to amp compared to the wild type. One strain, GN11 showed increased susceptibility to cef. Two strains, CEF3 and KN12, showed increased susceptibility to mer (Figure 2).

For the aminoglycoside antibiotics, all strains showed increased resistance to streptomycin. All the quinolone strains except S83L and CIPR1 showed increased susceptibility to kn. The same was true for gn, except CIPR8 which showed increased resistance to gn like S83L. Nine other strains, CEF6, CEF7, CM1, KN6, KN12, RIF1, RIF7, COL4, and AM4 showed increased susceptibility to kn. Ten other strains, CEF4, CEF6, CEF7, CEF8, CM3, CM4, KN6, RIF1, RIF7, and COL4 showed increased susceptibility to gn. All the quinolone resistant strains also showed increased resistance to col, and ten other strains, CEF3, CEF6, CEF7, CM2, KN6, KN14, GN11, GN12, GN13, and COL4 showed increased susceptibility to col compared to the wild type (Figure 3).

Only two strains, CM1 and RIF1, showed increased resistance to rif. Of the remaining strains, ten showed no difference from the wild type and the rest had increased susceptibility to rif. All the quinolone resistant strains showed increased resistance to

cm, except for CIPR5. All the CM strains, as well as TET8, and AMP4, showed increased resistance to cm. The remaining strains showed some increase in susceptibility to cm compared to the wild type, notably the KN and GN strains (Figure 4).

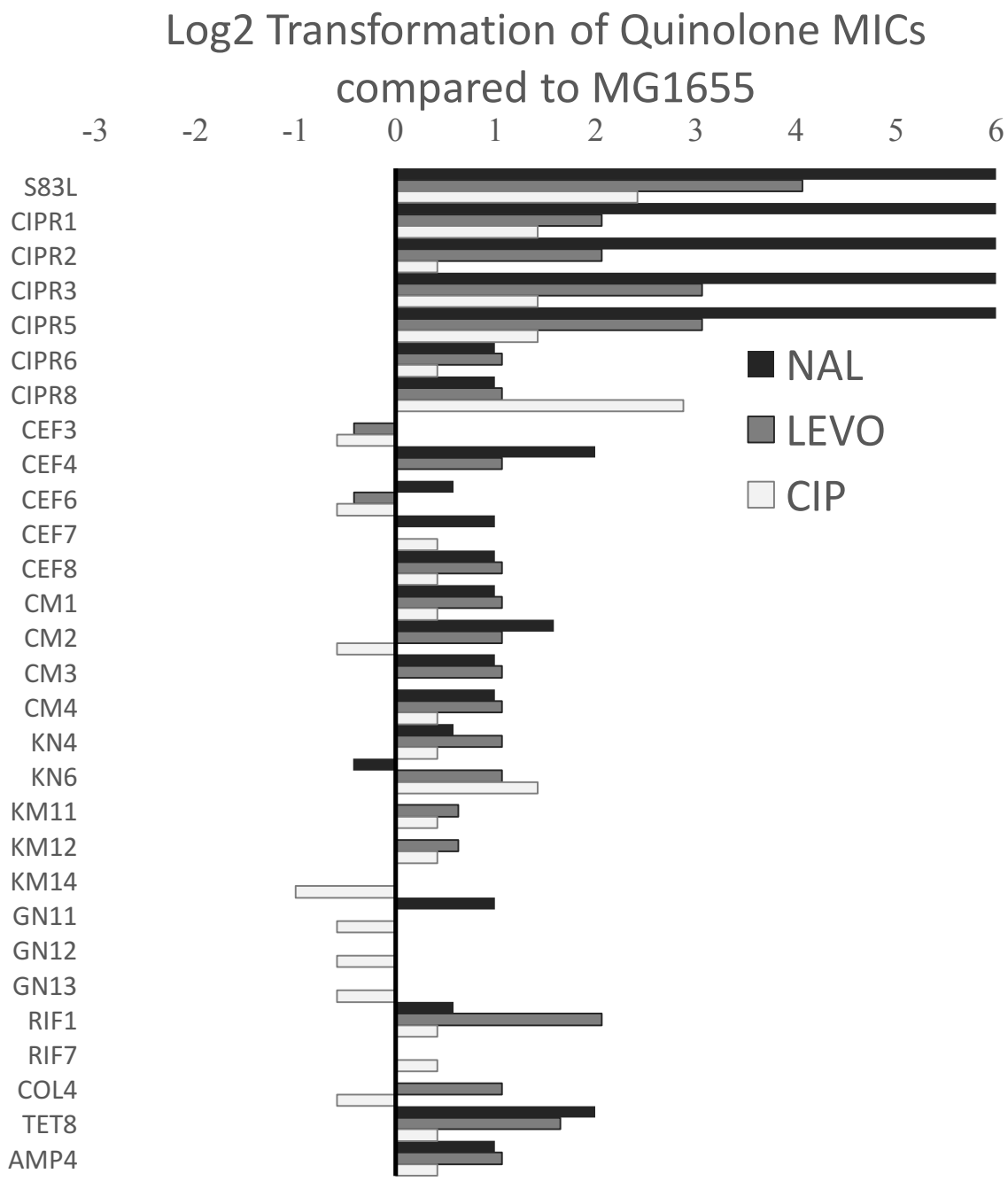

Figure 1 Log$_2$ ratios of MICs of thirty evolved strains, as compared to the wild type to three quinolones, naladixic acid, levofloxacin, and ciprofloxacin. Positive ratios show resistance (to the right of the y-axis, negative ratios show susceptibility (to the left of the y-axis.)

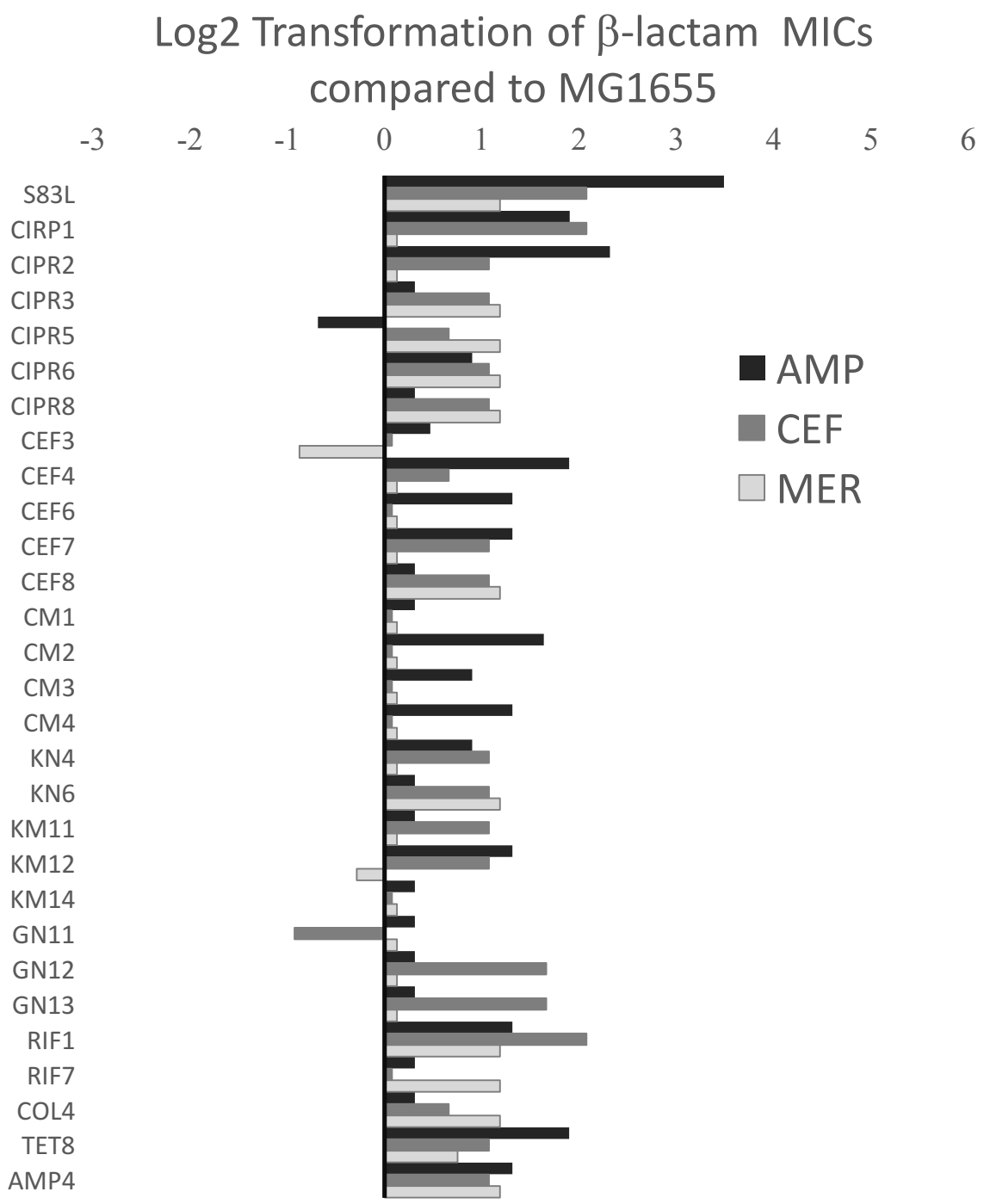

Figure 2 Log$_2$ ratios of MICs of thirty evolved strains, as compared to the wild type to three ß-lactams, ampicillin, ceftazidime, and meropenem. Positive ratios show resistance (to the right of the y-axis, negative ratios show susceptibility (to the left of the y-axis.)

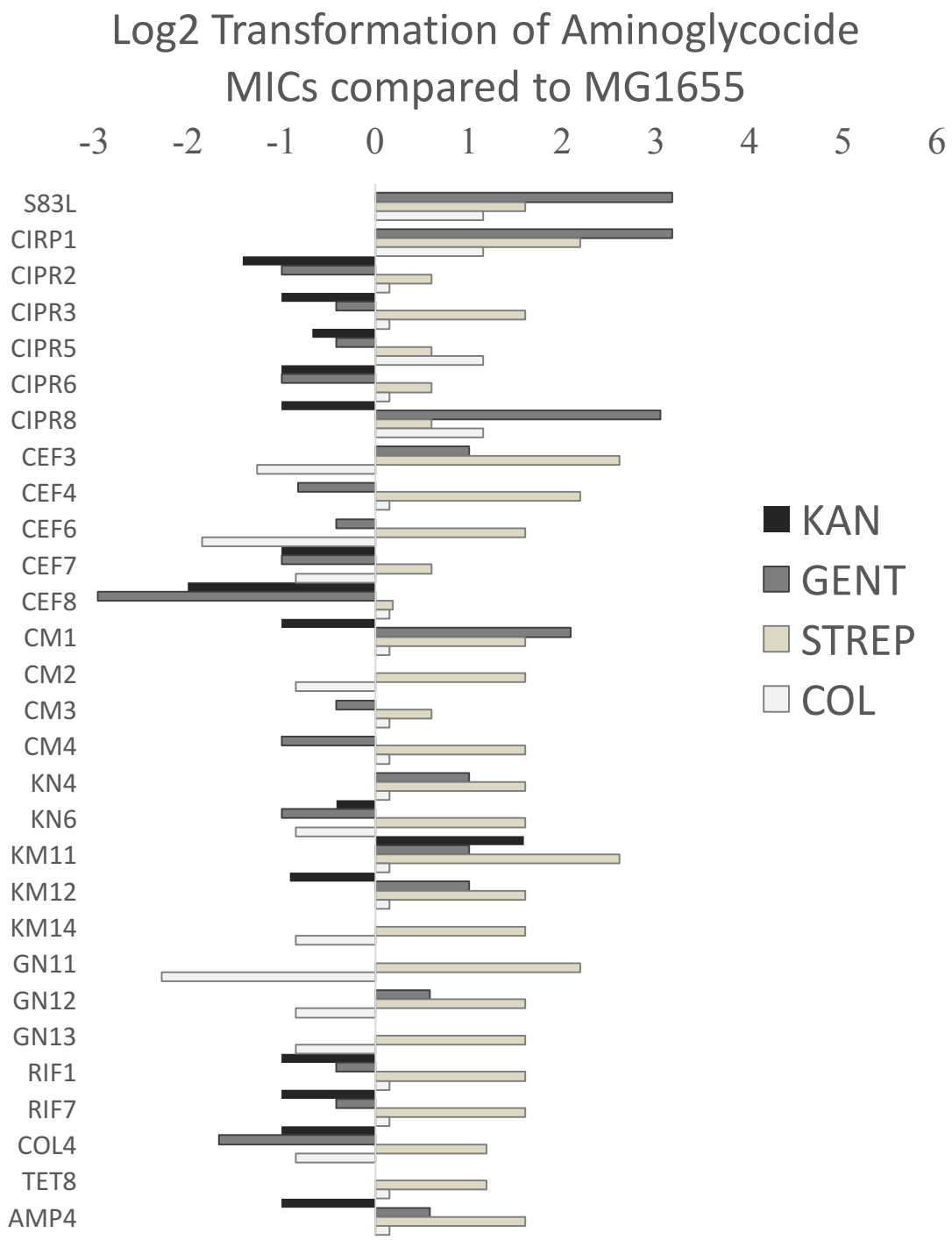

Figure 3 Log_2 ratios of MICs of thirty evolved strains, as compared to the wild type to four aminoglycosides, kanamycin, gentamycin, streptomycin and colistin. Positive ratios show resistance (to the right of the y-axis, negative ratios show susceptibility (to the left of the y-axis.)

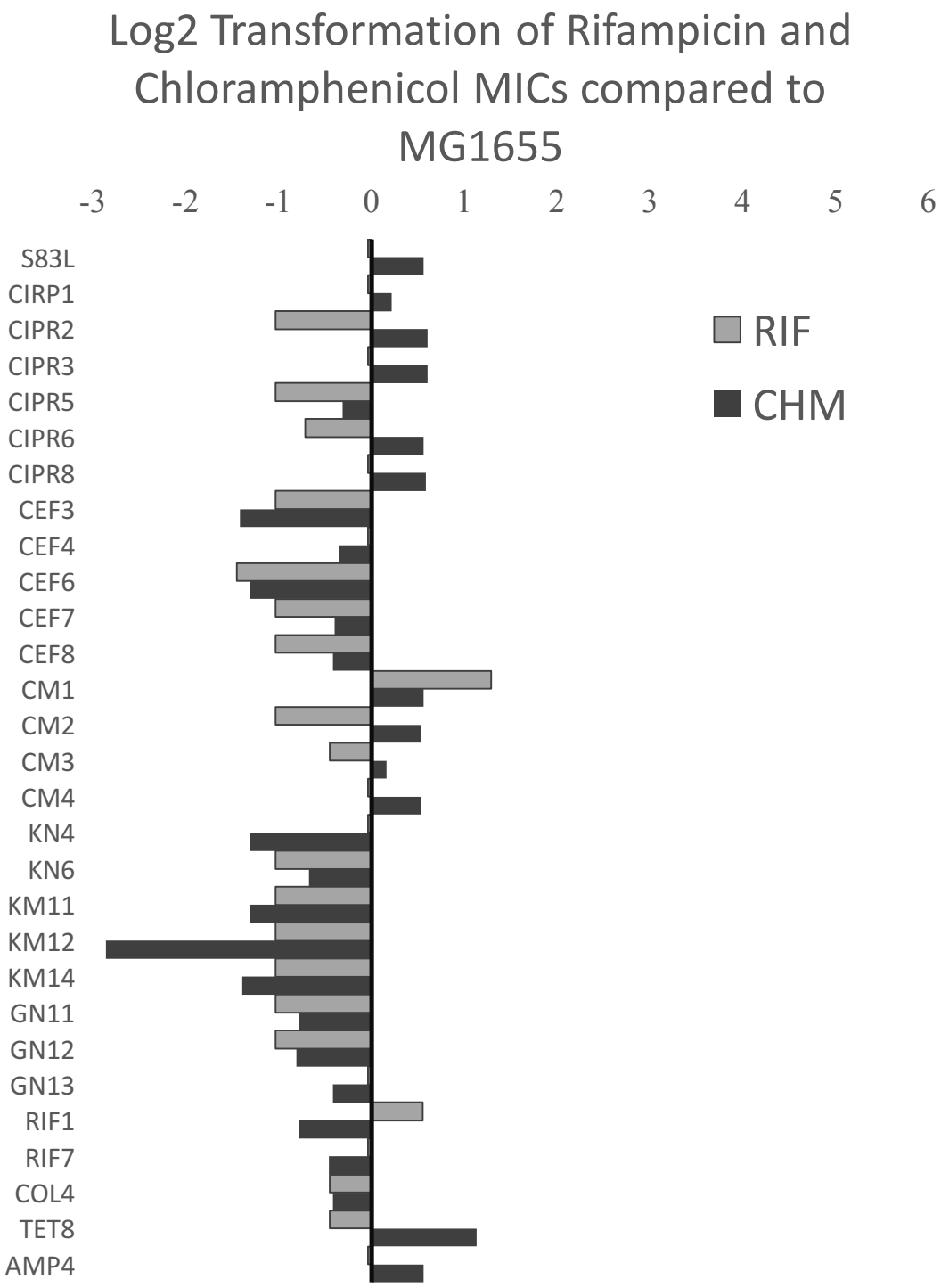

Figure 4 Log_2 ratios of MICs of thirty evolved strains, as compared to the wild type to rifampicin, and chloramphenicol. Positive ratios show resistance (to the right of the y-axis, negative ratios show susceptibility (to the left of the y-axis.)

3.2 Analysis of Collateral Sensitivity

From the MICs, mutually sensitive drug pairings were identified in an array. Collateral sensitivity pairs were identified as ideal candidates if all strains resistant to drug A showed susceptibility to a drug B, and all strains resistant to drug B showed susceptibility to drug A. There were cases of mixed susceptibility across resistant strains evolved in the same antibiotic where only some mutant strains showed increased susceptibility. Ideal cases were hard to identify, but certain potential relationships were identified. In general, quinolone resistant strains showed increased susceptibility to kn and rif, but showed mixed susceptibility to gn. Mutants evolved in cef showed mixed susceptibility to levo, cip, mer, gn, and kn, and all mutants evolved in cef showed increased susceptibility to col, rif, and cm. Mutants evolved in cm showed mixed susceptibility to gn. All mutants evolved in kn showed increased susceptibility to nal, col, rif, and cm. All mutants evolved in gn showed susceptibility to cip, col, rif, and cm. All mutants evolved in rif showed susceptibility to gn, kn, and cm (Figure 5).

There were a few cases of what appeared to be ideal, or near ideal cases of mutual collateral sensitivity. Quinolone resistant mutants showed susceptibility to kn, and KN6 showed susceptibility to one of the quinolones, nal (Figure 6). Quinolone resistant strains showed mixed susceptibility to gn, while gn resistant strains showed increased susceptibility to one of the quinolones, cip (Figure 7). Mutants evolved in cef showed a mixed susceptibility to gn, and one mutant evolved in gn showed increased susceptibility to cef (Figure 8). Mutants evolved in cm showed a mixed susceptibility to gn, and all mutants evolved in gn showed increased susceptibility to cm.

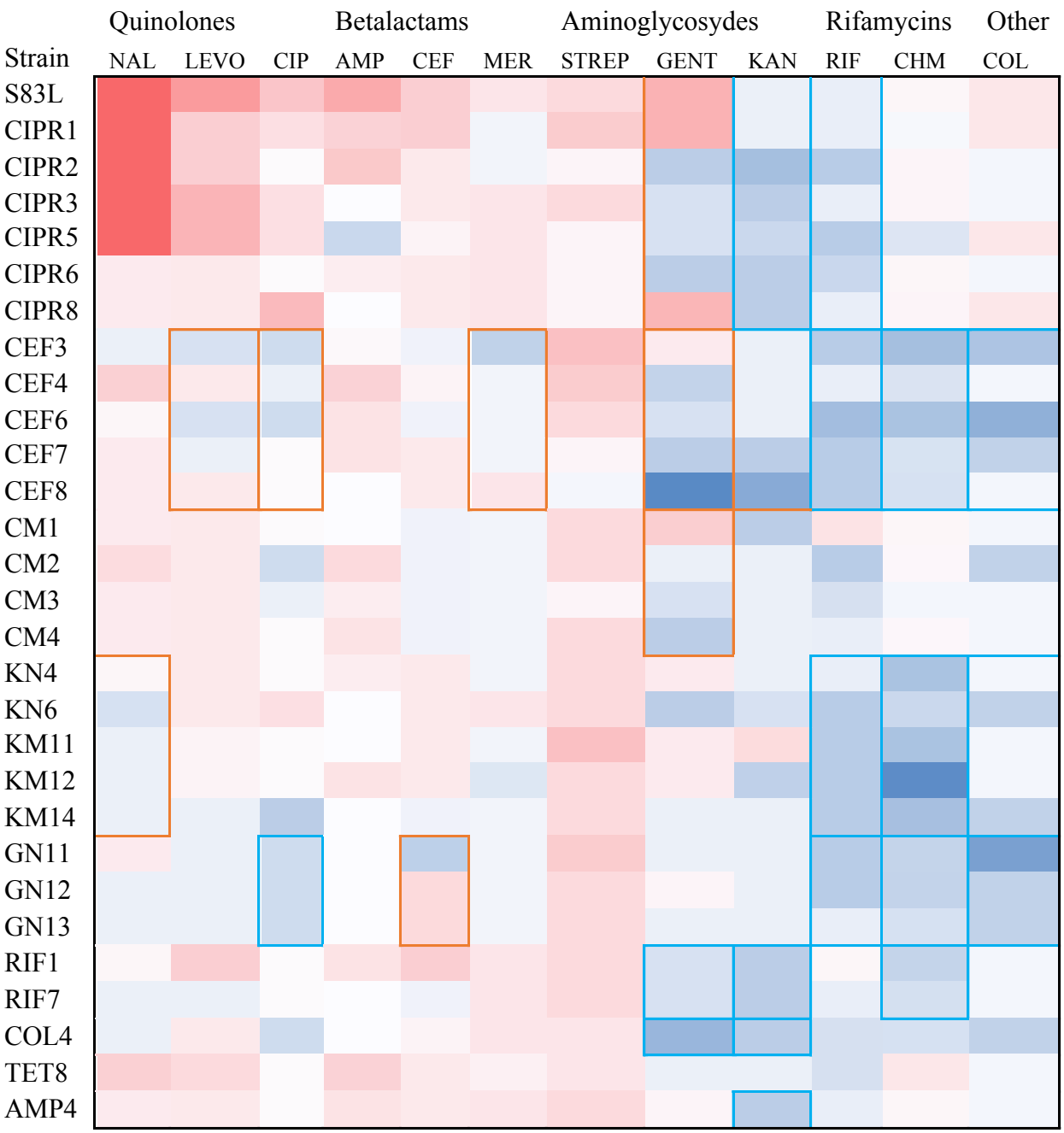

Figure 5 Array showing resistance (red) and susceptibility (blue), for twelve different antibiotics tested for 30 strains previously evolved in antibiotic containing environments. Cases where all evolved strains were susceptible to a drug were boxed in blue, cases with weak susceptibility, or partial susceptibility across strains were boxed in orange.

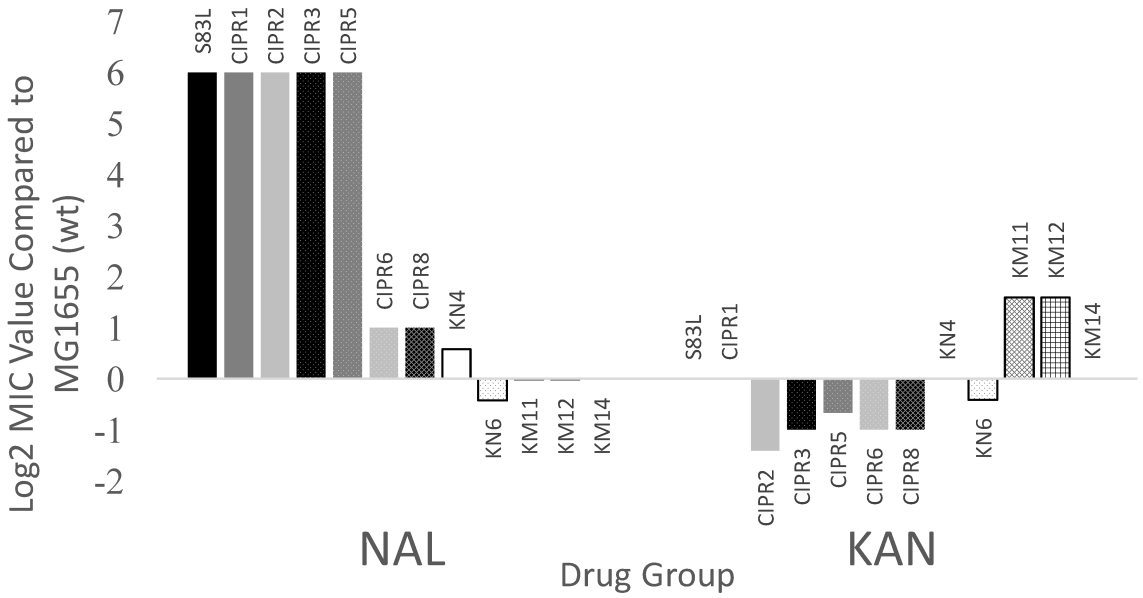

Figure 6 Comparative MICs for nal and kn, across quinolone resistant mutants and KN strains. Quinolone resistant mutants, CIPR2, CIPR3, CIPR5, CIPR6, and CIPR8 show some susceptibilty to kn, and KN6 showed some susceptibility to nal and kn.

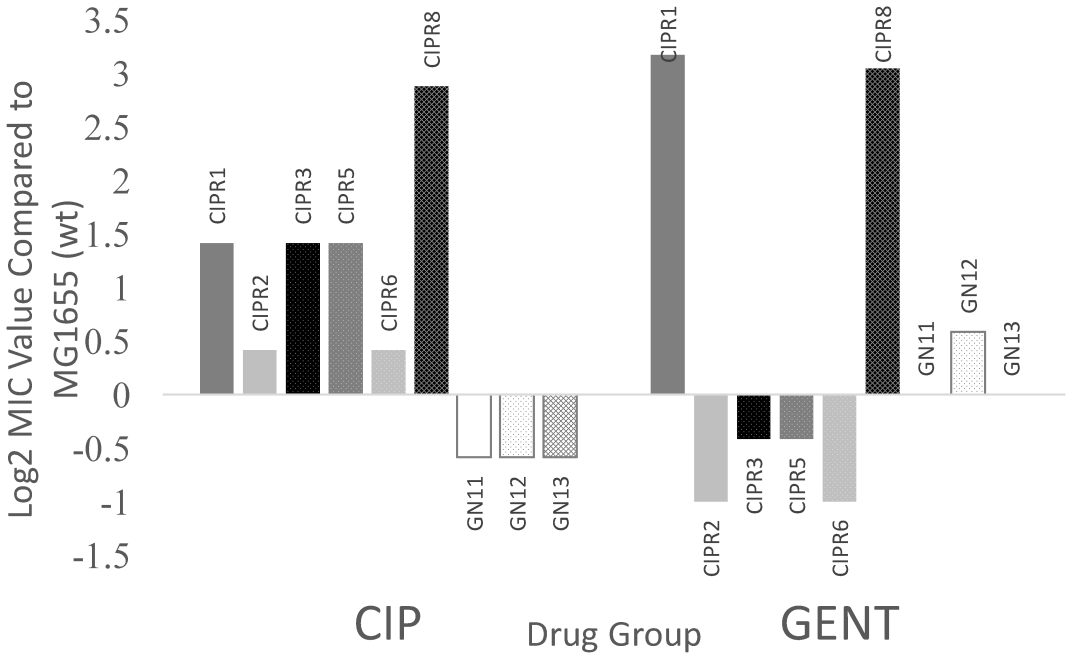

Figure 7 Comparative MICs for cip and gn, across quinolone resistant mutants and GN strains. Quinolone resistant mutants, CIPR2, CIPR3, CIPR5, and CIPR6, show some susceptibility to gn, and GN strains show some susceptibility to cip.

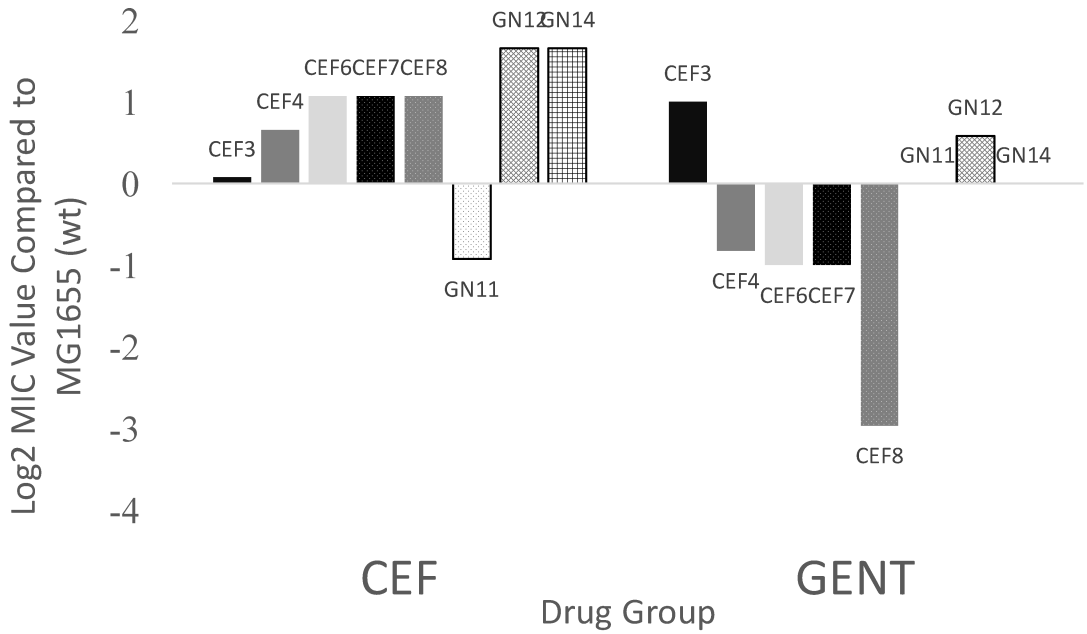

Figure 8 Comparative MICs for cef and gn, across CEF and GN strains. CEF strains, CEF4, CEF6, CEF7, and CEF8, show some susceptibility to gn, and GN11 showed some susceptibility to cef.

3.3 Determination of Drug Pairs for Luria-Delbruck Assays

Given what is known about collateral sensitivity relationships, it is expected that in a case where resistance to A confers sensitivity to B, growth in the presence of B should select against mutations conferring resistance to A. Similarly, it is expected that the mutation rate of resistance to A, when grown in the presence of B should also be lower.

Therefore, when selecting a suitable collateral sensitivity pairing to test this hypothesis, cases where *both* susceptibility and resistance arose in a group of resistant mutants were excluded. This was on the assumption that the selective environment allowed for an availability of resistance mutations that could confer resistance to both drugs in the pair. From this criterion, collateral sensitivity pairs were chosen based on cases where all mutants in each group showed either no difference in sensitivity compared to the wild type, or showed unanimous sensitivity across all mutant strains. Of the remaining relationships, cef and cm were identified to be a good fit as mutants

evolved in cef were unanimously sensitive to cm, and cm showed little difference from the wild type in terms of cef resistance (Figure 9).

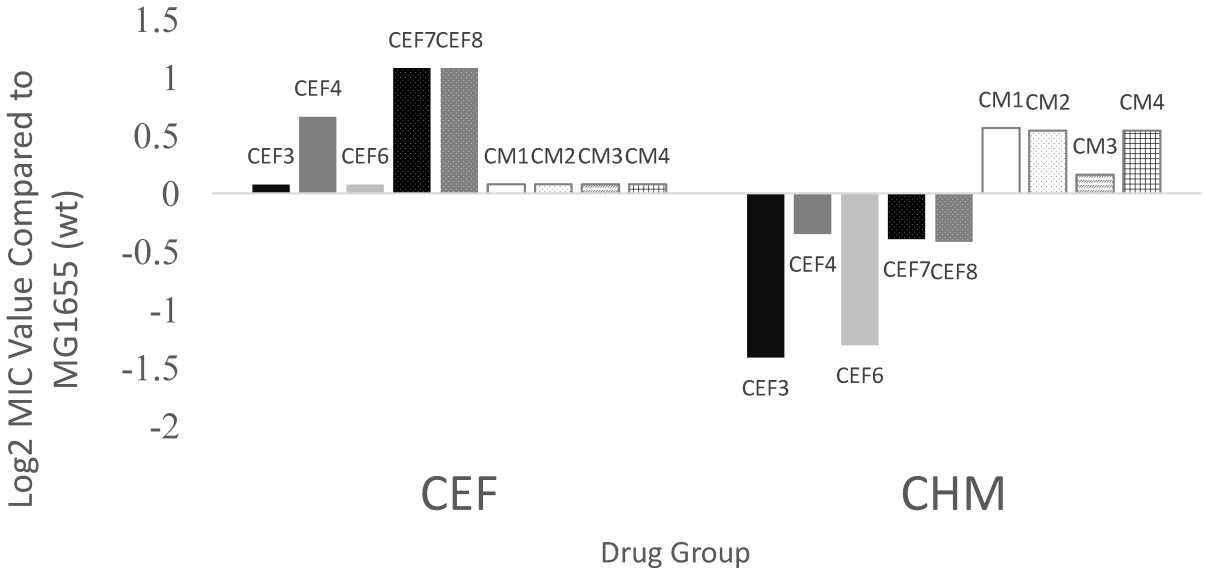

Figure 9 Comparative MICs for cef and cm, across CEF and CM strains. All CEF strains showed some susceptibility to cm, and all cm showed little difference from the wild type for cef resistance.

A control was selected for testing in the Luria-Delbruck assay from a relationship where there was no clear collateral sensitivity. That is, either there was little, or no difference from the wild type in measures of resistance, or there was mixed sensitivity and or resistance acquired. Quinolone resistant mutants and GN resistant mutants strains were a good candidate, as GN strains had equal MIC values for levo to the wild type, and the quinolone mutants were either very resistant to gn, or slightly susceptible (Figure 10).

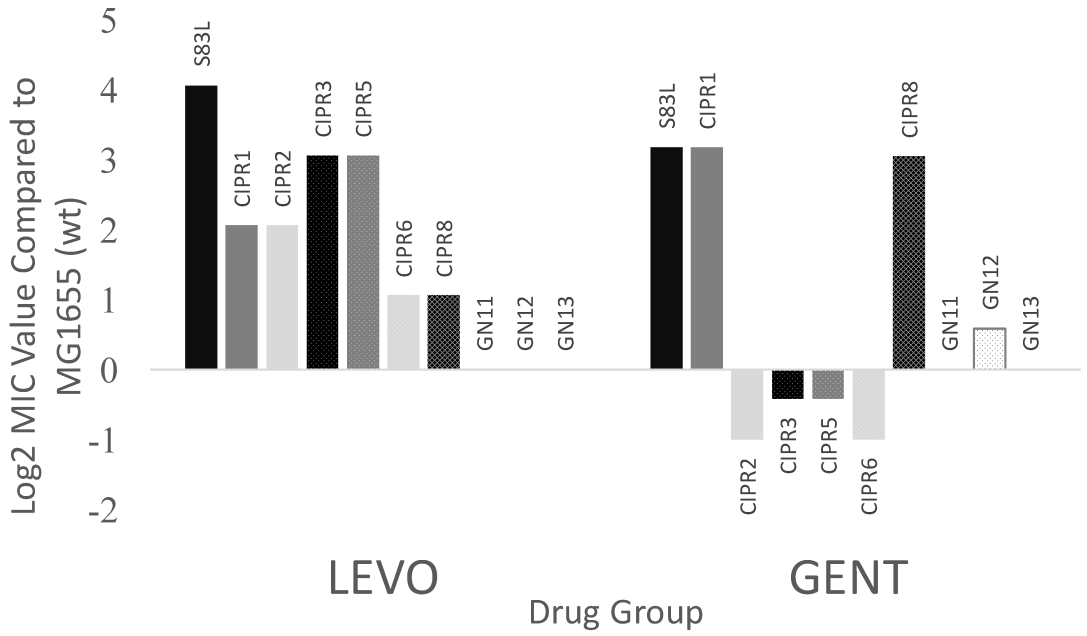

Figure 10 Comparative MICs for levo and gn, across quinolone resistant mutants and GN strains. There were mixed results for resistance and sensitivity for quinolone mutants to gn, and the GN strains showed little difference from the wild type for levo resistance.

3.4 Rate of Mutation in Rich-Media vs. Low-Level Antibiotic Environments

I first tested the prediction that the susceptibility of cef-evolved mutants to cm would select against any cef-resistance mutations arising during growth in LB+cm. This would result in fewer Cef^R mutants, and a lower estimated mutation rate to Cef^R per generation, when compared to populations grown in LB without antibiotic. Multiple independent populations of E. coli MG1655 were grown from small inocula in a sub-MIC concentration of cm, and plated to LB+Cef at 3.5xMIC. Unexpectedly, populations grown in sub-MIC cm yielded a higher estimated mutation rate than populations grown in plain LB, however it was not statistically significant ($p = 0.23$). For populations grown in LB $m = 4.669$ and $\mu = 0.2407$ per 10^9 cells, 95% CI [0.3084, 0.1795]. For populations

grown in LB and sub-MIC cm, m = 6.269 and μ = 0.3777 per 10^9 cells, 95% CI [0.4724,0.2896] (Figure 11).

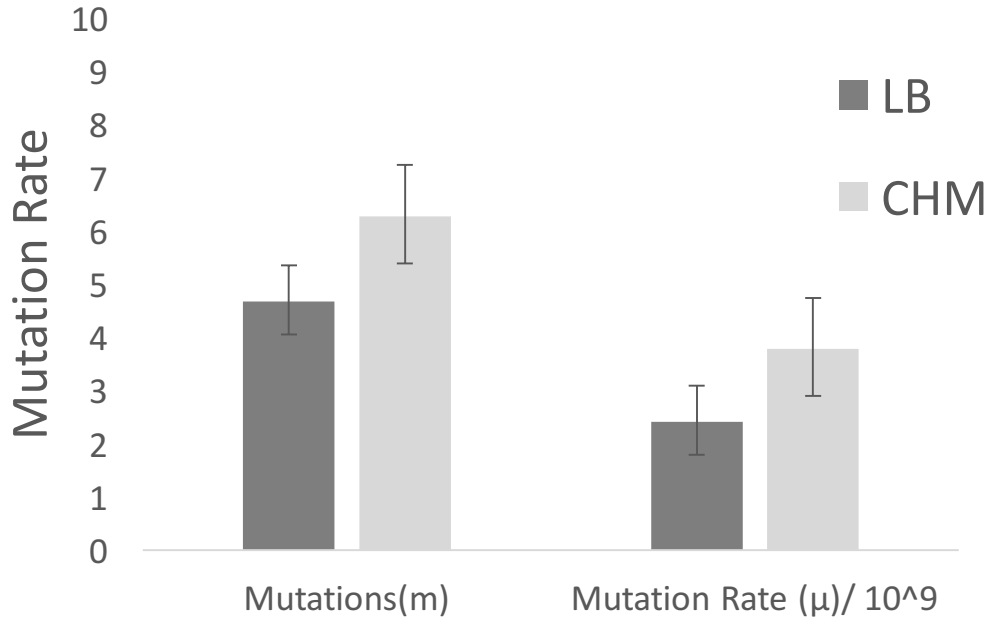

Figure 11 Mutations per culture (m) and estimated mutation rate per 10^9 cells (μ) for groups of 30 MG1655 populations grown in either plain LB (dark grey) or LB with sub MIC cm (light grey), then selected on 3.0x MIC cef plates. μ was estimated using Ma-Sandri- Sarkar Maximum Likelihood Estimator (MSS-MLE) Method.

The experiment was done in reverse to test if the relationship was bi-lateral. That is, if when grown in sub-MIC cef, would the effect on mutation rate persist, despite there being no obvious sensitivity of CM strains to cef. It was hypothesized that due to the lack of cef susceptibility in strains evolved in cm, there would be no difference in μ. The results showed that μ was significantly higher in populations evolved in sub-MIC concentrations of cef, compared to plain LB ($p = 2.93x10^{-5}$) For populations grown in LB m = 0.33 and μ = 0.011 per 10^9 cells, 95% CI [0.019, 0.005]. For populations grown in

LB and sub-MIC cef, m = 0.585 and μ = 0.21 per 10^9 cells, 95% CI [0.452,0.158] (Figure 12).

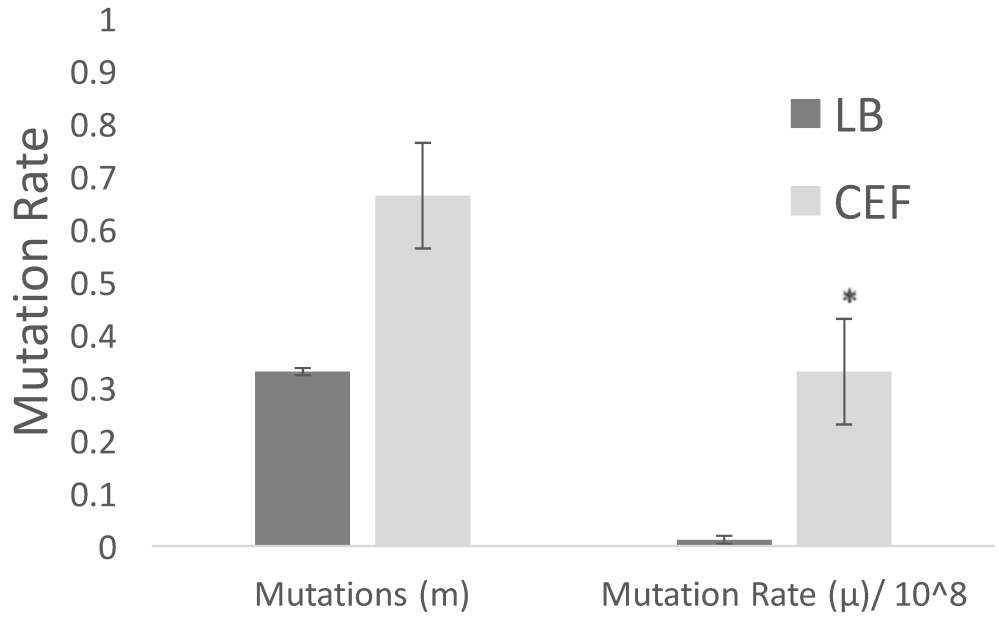

Figure 12 Mutations per culture (m) and estimated mutation rate per 10^9 cells (μ) for groups of 30 MG1655 populations grown in either plain LB (dark grey) or LB with sub MIC cef (light grey), then selected on 3.5x MIC cm plates. μ was estimated using Ma-Sandri- Sarkar Maximum Likelihood Estimator (MSS-MLE) Method. * Indicates statistical significance.

The second control, using the gn and levo, was used to verify if mutation rate increases merely due to the presence of antibiotic. The experiment was conducted by growing a small number of cells in either sub-MIC gn, or plain LB and selecting on levo. There was no difference in μ between cells grown in sub-MIC gn, or cells grown in plain LB (p = 0.9) For populations grown in LB m = 0.623 and μ = 0.109 per 10^8 cells, 95% CI [0.168, 0.059]. For populations grown in LB and sub-MIC gent, m = 0.289 and μ = 0.188 per 10^8 cells, 95% CI [0.201,0.082] (Figure 13).

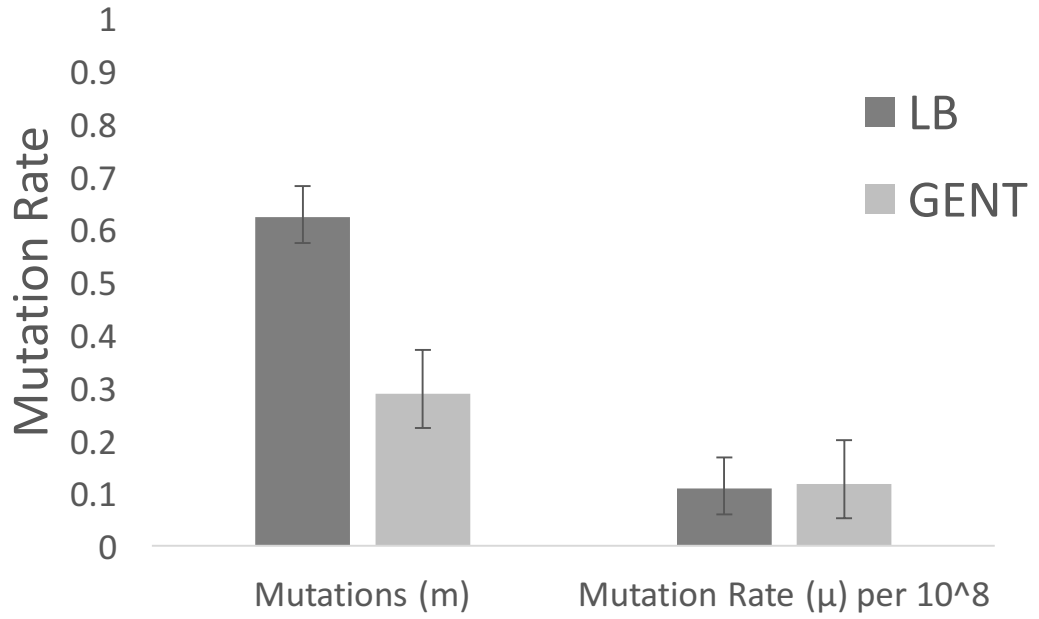

Figure 5 Mutations per culture (m) and estimated mutation rate per 10^8 cells (μ) for groups of 30 MG1655 populations grown in either plain LB (dark grey) or LB with sub MIC gn (light grey), then selected on 3.5x MIC levo plates. μ was estimated using Ma-Sandri- Sarkar Maximum Likelihood Estimator (MSS-MLE) Method.

3.5 Summary of Results

It was hypothesized that growing MG1655 in sub MIC concentrations of cm, with a collateral sensitivity relationship to cef, would reduce the apparent mutation rate when selected for on plates containing cef. The number of mutations per culture (m), and the per-cell mutation rate (μ) did not drop in wild-type strains that were grown in a sub MIC environment containing cm, compared to a rich-media environment, and were in fact elevated slightly, but it was not statistically significant ($p = 0.23$). The reverse relationship was also found when the wild type was first grown in sub-MIC concentrations of cef, and then selected on cm, and it was statistically significant ($p = 2.93 \times 10^{-5}$).

As a control, it was expected that two drugs with weak, or no relationships of collateral sensitivity would have no effect on mutation rate. MG1655 was grown in sub MIC gn and selected on 3.5x MIC levo plates. No differences in mutations per culture or mutation rate (μ) as calculated by the MSS-MLE method were found from a fluctuation assay.

4 Chapter: Discussion

4.1 Collateral Sensitivity

Collateral sensitivity, or the phenomenon whereby the acquisition of resistance to one drug results in sensitivity to a different drug or treatment, may present an opportunity to be leveraged in the design of new treatments. The phenomenon itself has been documented by studies involving *E.* coli (Imamovic and Sommer, 2013; Lázár *et al.*, 2013; Oz *et al.*, 2014), the malaria parasite *Plasmodium falciparum* (Lukens *et al.*, 2014), the fungal pathogen *Candida albicans* (Vincent *et al.*, 2013) with regards to extreme fitness costs, and has long been studied in cancer therapies (Pluchino *et al.*, 2008). Collateral sensitivity could inform proposed alternating drug treatments, where two drugs are used one after the other. Experiments have demonstrated *in vitro* that alternating drug treatments can slow the rate of evolution to AMR (Kim, Lieberman and Kishony, 2014), and in the case of pathogens possessing a collateral sensitivity, effectively kill mutants that acquire resistance to either drugs (Imamovic and Sommer, 2013).

There are several key differences in this study in that, mutants were selected by Luria-Delbruck assay, rather than selection experiments. Selection from L-D results in a higher likelihood of selecting single step mutants, rather than selection experiments where resistant genotypes may carry more than one resistance mutation. In certain cases, only single representative strains were used for testing of collateral sensitivity relationships, such as (Imamovic and Sommer, 2013), whereas this study used multiple single step mutants that showed resistance to one drug, offering a broader picture of the fitness landscape regarding collateral sensitivity. Lastly one of the three studies

conducted on *E. coli* (Imamovic and Sommer, 2013) did not involve whole genome sequencing to identify mutants.

However, based on this previous work it was hypothesized that growing MG1655 in sub MIC concentrations of cm, with a collateral sensitivity relationship to cef, would reduce the apparent mutation rate when selected for on plates containing cef. The opposite was observed for MG1655 grown in sub-MIC cm and cef, which was unexpected. This was especially surprising, given the evidence that a collateral sensitivity based alternating drug experiment was effective at killing virtually all resistant mutants (Imamovic and Sommer, 2013), and that cycling experiments had been shown to reduce the rate of evolution to AMR (Kim, Lieberman and Kishony, 2014), as increased rate of mutation, as a proxy for mutability and probability of favorable mutations, leads to increased AMR (Martinez and Baquero, 2000).

4.2 Comparing and Validating MIC Data

Any examination of hypotheses rests on the premise that there was a relationship of collateral sensitivity between the drug pairs tested in this study. As such, I validated the MIC values and collateral sensitivity relationships that I obtained using external sources. The validation of MIC data was done by a comparison of experimentally obtained values for the wild-type *E. coli* strain, MG1655, to a database of MIC values obtained by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). All the MIC values obtained for MG1655, except for ciprofloxacin were well within the normal distribution of EUCAST values of the epidemiological cut-off values (ECOFF) that consist of more than 160k observations for eleven of the twelve drugs used in this study. (Appendix A, Table 4). (Andrews, 2001) was taken as a

secondary verification for MIC values, including rif, which was not catalogued in the EUCAST database for *E. coli* (Andrews, 2001). Experimental values for cip (0.38µg/mL) were close to six times higher than the EUCAST ECOFF values (0.064µg/mL) (Appendix 1). This led to the exclusion of collateral sensitivity relationships containing cip from being tested by Luria-Delbruck assay.

Another issue was that mutants known to be resistant to a drug showed little or no resistance compared to the wildtype in MIC experiments such as GN11 and GN13, which showed no difference in resistance compared to the wildtype MG1655 (Figure 7, 8, 10). This was hypothesized to be due to the MIC experiments not having a high enough resolution to detect differences. For some drugs the 1:1 dilution began at 128 µg/mL where the next step down would be 64 µg/mL. If the MIC for both the resistant strain and the wild type both fell within this range, the assay would not have detected a difference.

Values were also compared to other research on collateral sensitivity in *E. coli*. A similar screen where strains of *E. coli* were evolved in antibiotic containing environments then tested for collateral sensitivity produced many similar results despite the different methods for selecting mutants. For example, MG1655 evolved in gn showed no susceptibility to amp, 2X susceptibility to nal, and 4X susceptibility to col (Imamovic and Sommer, 2013). In this study MG1655 evolved in gn showed no susceptibility to amp, a very weak susceptibility to nal, and a between 2X and 5X susceptibility for col (Figure 5).

Mutants evolved by Imamovic & Sommer in amp showed no change to gn and col, and 4X resistance to nal. This study showed a similar profile, with mutants evolved

in amp showing a slight resistance to gn, almost no change to amp, and 2X resistance to nal (Figure 5). An interesting example was found in comparing strains evolved in the quinolone, nal, as Imamovic & Sommer reported 2X susceptibility to gn, 2X resistance to amp, and no change to col. This was found in almost exact proportion for CIPR6, a quinolone evolved strain with the R94C mutation in the *marR* gene. The R94C mutation is associated with a motif that when mutated, unlike many other *marR* mutations, still binds DNA (i.e. the MarR repressor) but at a 20% deficiency compared to the wild type. It is also associated with increased proteolysis in the cell which may point to other antimicrobial activities (Alekshun, Kim and Levy, 2000). This would explain why out of the quinolone resistant mutants, CIPR6 showed the weakest resistance profile, in that the mar operon was still partially repressed. Other strains, CIPR2, CIPR3, CIPR5 showed similar susceptibility to gn, and similar indifference to col, except for CIPR5 which was 2X resistant to col. However, the S83L strain was resistant to amp, gn, and col.

More generally, many relationships identified in this experiment were confirmed by (Lázár *et al.*, 2013) who identified collateral sensitivity networks in low, and high antibiotic-containing environments. In both experiments the evolution of resistance to quinolones conferred susceptibility to kanamycin, evolution of resistance to kanamycin conferred susceptibility to chloramphenicol, and evolution of resistance to ampicillin conferred susceptibility to kanamycin (Lázár *et al.*, 2013). The control relationship between gn and levo was also confirmed to have no collateral sensitivity (Imamovic and Sommer, 2013). A minor discrepancy was found in that evolution of resistance to kanamycin conferred susceptibility to nal in only one strain KN6, a strain with a mutation to the *ubiB* gene that produces a protein kinase involved in ubiquinone synthesis (Poon *et*

al., 2000). There is some similarity between this observation and the collateral sensitivity observed in aminoglycoside resistant mutants, while *ubiB* was not one of the genes associated with changes to the PMF, thought to be responsible for collateral sensitivity to quinolones (Lázár *et al.*, 2013), it is still thought to be involved in the PMF as most ubiquinones are important electron carriers in the electron transport chain, which generates the PMF (Alberts, Wilson and Hunt, 2008). There are other kinases with similar activity, however, that have been shown to confer resistance to kanamycin (Zaunbrecher *et al.*, 2009). Given the difference in mutant selection, it is unsurprising that there are discrepancies, as many of the single step mutants observed here may have been selected out in longer term selection experiments.

While general trends of collateral sensitivity were observed, and confirmed by previous research, there were marked deviations from the norm that seem to be strain specific as marked by certain mutations. This could be due to experimental differences, however, there is a case to be made for considering the availability of resistance mutations in each environment, and thus the fitness effects of any given mutation in that environment as distinct from general trends of drug resistance and susceptibility. For example, to assume evolution in a kn-containing environment will confer nal-susceptibility may be reasonable, but only based on the availability of kn-resistance mutations that also confer nal-susceptibility for that organism in that environment.

4.3 Hypothesis: SOS response in *E. coli*

The unexpected increase of mutation rate from sub-MIC exposure to both chloramphenicol and ceftazidime has several possible explanations, one is that there was

an increase in mutation rate when the wild type was grown in sub-MIC environments that resulted in a greater number of resistant mutants. Another explanation is that cross-resistance for cm and cef was being selected for by a combination of both environments. A well-studied phenomenon that would account for the increase in the apparent rate of mutation is antibiotic induction of the SOS response in *E. coli*.

Previous experiments have shown that an increase in ß-lactams can initiate an SOS response in bacteria that leads to an increase in mutability (Baharoglu and Mazel, 2011). In *E. coli,* this is thought to be done through a two-component signal transduction system. Two component systems contain a sensor protein that detects a signal, and an effector protein that effects a transcriptional change. Stimulation by a cell signal leads to the auto-phosphorylation of a sensor protein, which in turn phosphorylates an effector protein. Effector proteins bind to operator and promoter sequences of genes to increase or repress transcription that can result in cellular austerity (Miller *et al.*, 2004; Baharoglu and Mazel, 2014).

The two-component signal transduction system in *E. coli* responsible for ß-lactam-induced SOS response is thought to involve penicillin binding proteins (PBPs) and the *dpiAB* operon. The antibiotic effect of ß-lactams comes from the disruption of transmembrane (PBPs) which result in a failure of the cell wall. In *E. coli* disruption of PBP3 increases the expression of the *dpiAB* operon which induces the SOS response by preventing FtsZ polymerization, and thus cell division (Miller *et al.*, 2004).

More evidence has amassed that demonstrates the ability of many classes of antibiotics to activate SOS responses in bacteria that directly increase mutagenesis. For example, quinolones function by disrupting essential DNA topoisomerases, gyrase and

topoisomerase IV. These proteins form double stranded breaks (DSB) in the DNA to unwind the DNA before re-ligating. In the case of ciprofloxacin, it reversibly binds to an intermediate before ligation can occur resulting in free double stranded ends (DSE). This form of DNA damage is thought to be repaired by nucleotide excision repair (NER) or homologous recombination. RecA-single-stranded DNA complexes (ssDNA) help facilitate the process of homologous recombination, but also bind the SOS gene repressor LexA. As many as 40 genes rely on the LexA repressor, but of importance are the expression of nonessential DNA polymerases, Pol II, Pol IV, and Pol V which are error-prone, and whose use can increase mutation rate during DNA repair (Cirz *et al.*, 2005; Baharoglu and Mazel, 2014). Therefore, the presence of ciprofloxacin not only results in an SOS response, but increased mutagenesis that would increase the risk of resistance to cip.

The SOS effect can be activated at sub-MIC concentrations, where lower drug concentration reduces mortality, while the SOS effect increases rate of mutation, due to nonessential polymerase activation, making for a dangerous situation where resistance alleles can fix in a population if they confer a fitness advantage. The discovery of sub-MIC induced SOS responses led to a wider testing of the ability of different drug classes to induce SOS in pathogens. It was found that in addition to ß-lactams, and quinolones, aminoglycosides, chloramphenicol, and tetracycline could induce SOS responses via non-essential polymerases in *V. cholera*, but not in *E. coli* at high concentrations (Baharoglu, Bikard and Mazel, 2010).

Interestingly, this research was expanded when it was found that exposure to sub-lethal concentration of quinolones, ß-lactams, and aminoglycosides were shown to

increase mutation rate in *E. coli* regardless. The increases were associated with increased levels of reactive oxygen species (ROS) which were hypothesized to work in conjunction with SOS response related mutagenesis (Kohanski, DePristo and Collins, 2010). Further work showed that many other agents like ceftazidime, imipenem, trimethoprim, sulfamethoxazole, colistin, and tetracycline could increase mutation rate with sub-MIC exposure in *E. coli*. More interesting was that the elimination of the gene *recA,* that binds the SOS binding protein LexA, resulted in no increase in mutation rate under the same circumstances (Do Thi *et al.*, 2011). This evidence further links ROS species with activation of *recA* DNA repair pathways, and increased mutagenesis but only at sub-MIC concentrations.

Previous evidence showed that sub-MIC ceftazidime, but not chloramphenicol exposure, resulted in increased rate of mutation when selected on rifampicin and fosfomycin plates. However, when grown in sub-MIC chloramphenicol and selected on a plate containing ceftazidime, I observed a non-significant increase in the apparent rate of mutation (Figure 11, 12). Furthermore, this same relationship was not observed where there was not a relationship of collateral sensitivity between strains evolved in environments containing each respective drug (Figure 13). Currently other aminoglycosides like kanamycin have been shown to increase ROS mediated mutagenesis, though gentamycin has not, and I am unaware of sub-MIC experiments for levofloxacin (Kohanski, DePristo and Collins, 2010; Do Thi *et al.*, 2011; Baharoglu and Mazel, 2014).

Ceftazidime is known for being able to increase ROS mediated mutagenesis in *E. coli*, suggesting that the observed increase in apparent mutation rate was in fact expected

for cef (Figure 11). Based on previous research there was no increase in mutagenesis for the same conditions of chloramphenicol exposure, but when selected on two different antibiotics, rifampicin and fosfomycin (Do Thi *et al.*, 2011). In this experiment, the control populations showed a much lower mutation rate when grown in rich media, as opposed to sub-MIC chloramphenicol, and selected on ceftazidime plates. This is interpreted to mean that selection on ceftazidime plates was not a contributor to the increase in mutation rates seen from sub-MIC exposure to chloramphenicol. Therefore, some combination of growing MG1655 in sub-MIC chloramphenicol and selecting on ceftazidime, as opposed to other drugs led to the increase in mutation rate.

Little is known about any *in vivo* drug interaction between ceftazidime and chloramphenicol except for a single case of meningitis treatment where it was thought that the drugs worked antagonistically (French *et al.*, 1985). More broadly speaking, the mechanism of action for ceftazidime targets PBPs in the cell wall, and chloramphenicol targets the 50S ribosomal subunit to disrupt protein synthesis. While no specific interaction data exists for ceftazidime and chloramphenicol, it has been shown that chloramphenicol shows no antagonistic, or synergistic interactions with other drugs that target the cell wall, like cefoxitin (Yeh, Tschumi and Kishony, 2006). Thus, any association between the two mechanisms in this case is unclear. (Kohanski, Dwyer and Collins, 2010).

A way to further investigate this hypothesis would be to redo the experiment but with an SOS initiation gene, like *recA*, knocked out, to see if the increased rate of mutation is recovered under the same conditions. Alternatively,

this might be accomplished by knocking out only the error-prone polymerases Pol II, Pol IV, and Pol V that are expressed during the SOS response.

A more in depth way to confirm the SOS hypothesis would be to perform a bottlenecking experiment where mutants are selected and grown from single colonies repeatedly as their mutations accumulate. By testing this using a sub-mic environment of cef or cm, in comparison to a plain LB environment, mutants could be counted to test if sub MIC exposure is increasing the rate of mutation in sub mic environments.

4.4 Hypothesis: Selection for Cross-Resistance

Regardless of mechanism, it is often a working assumption that measures of mutation rate have varied based on the concentration and kind of selective environment used in fluctuation experiments. The assumption entails accepting that mutation rate would vary proportionally between antibiotic containing selection plates, but only based on the drug used for selection and nothing else (Luria and Delbrück, 1943; Rosche and Foster, 2010). The results shown here challenge that assumption, in that apparent mutation rate was not just contingent on the inoculation environment, and the selection environment, but also the relationship between the two.

An example of this might be that exposure to sub-MIC chloramphenicol may not have increased mutability to resistance for rifampicin or fosfomycin as was the case in Do Thi et al. (2011). However, sub-MIC exposure to chloramphenicol may have increased mutability to resistance for ceftazidime. The exact relationship might be discovered by investigating the mechanism of collateral sensitivity between the two drugs. Considering the mutability of a pathogen from drug in this way, mutability is

defined by one drug's localized ability to create an availability of resistance mutations for another drug. Therefore, it would be incorrect to say that chloramphenicol produced no increase in mutation rate for *E. coli*, but rather chloramphenicol produced no increase in mutation rate for *E. coli* to resist rifampicin, and fosfomycin.

A simple explanation would be that the specific exposure to cef, or cm, or a combination of the two results in the selection of a MDR mechanism, such as mutations in *marR*. Chloramphenicol use has been associated with *marR* mutations, and MDR due to increased expression of the *acrAB-tolR* efflux system multiple species including *E. coli* (Ghisalberti *et al.*, 2005). Ceftazidime exposure has also been associated with *marR* mediated resistance, in addition to mutations to PBP3 genes, which have been shown previously to induce the SOS response (Miller *et al.*, 2004; Chantratita *et al.*, 2011; Tavío *et al.*, 2013). Indeed, all marR mutants in this study, CIPR2, 6, 8, AMP4, TET8, and CEF4, showed increased resistance to cef and cm, except for CEF4 which had increased resistance to cef, and susceptibility to cm. (Figure 5)

It could be that the increase in mutation rate, especially the large increase by sub-mic exposure to cef could be due to PBP3-induced mutations that then increase the probability of *marR* mutations. For sub-MIC cm exposure, *marR* mutants would be produced but to a lesser extent than in cef, which would reflect the results of this experiment. This hypothesis could be tested by repeating the experiment and analyzing mutations in *marR* by PCR. It could also be tested by assessing mutant colonies in an MIC for multidrug resistance. This also demonstrates a key gap in designing alternating treatments based on collateral sensitivity relationships in that a similar mutation like *marR* can arise from many drugs. Therefore, it may be more beneficial to look at

correlations across all mutations, rather than correlations for strains evolved in a specific drug.

4.5 Application for Cycling, and Combination Therapies

To determine a clinical or community policy on broad trends of collateral sensitivity without considering individual fitness landscapes would be dangerous, as it would be imperative to understand the environmental factors that produce kn-resistant, nal-susceptible strains, as more than just the product of evolving a strain in the presence of kn to produce nal-susceptibility. The fear is that alternative availability of mutations in the presence of kn will just result in new strains resistant to kn and nal, thus eliminating the opportunity to leverage relationships of collateral sensitivity at all.

In a clinical context, this kind of information is useful, as it presents the risk thresholds of any clinical or policy practice. For example, it may be true that alternating drug treatments slows the rate of evolution (Imamovic and Sommer, 2013; Kim, Lieberman and Kishony, 2014), but those results were in cases where dosage *in vitro* were highly controlled at the level of MIC. Cases of sub-MIC dosage consistently show increased rate of mutation (Kohanski, DePristo and Collins, 2010; Gullberg *et al.*, 2011).

In terms of how this relates to current proposals, combination therapies are the gold standard, where drug synergy or a greater than expected killing effect, maximizes efficiency. This, however, has been shown to select for multi-drug-resistance except for cases where mutations conferring cross-resistance are rare. Currently a promising alternative is temporally cycling different antibiotics during a treatment, also called alternating drug therapies. Drug pairs that show patterns of collateral sensitivity have been identified as ideal candidates for testing these kinds of therapies as all existing

evidence shows that cycling drugs with this pattern either kill all resistant mutants to both drugs, or slow the rate of AMR evolution. This was shown in the case of gentamicin and cefuroxime in *E. coli*. In general this pattern and treatment proposal are being seen in a cautious but optimistic light, with the belief that collateral sensitivity will contribute to more sustainable use of drugs in the clinic by reducing the rate of resistance evolution (Pal, Papp and Lazar, 2015; Baym, Stone and Kishony, 2016).

The results of this experiment challenge solutions like these to take an evolutionary focus, rather than an antibiotic focus in considering potential solutions. When looking at collateral sensitivity networks to design alternating drug treatments, it would be easy to overlook common routes to MDR that may not be apparent in resistance mutations evolved from the presence of a single antibiotic. Furthermore, the strength of selection can also play a role in determining the result of collateral sensitivity networks (Oz *et al.*, 2014). Therefore, any solution that moves forward with alternating drug therapies must consider evolutionary forces, like strength of selection, the availability of fitness mutations in any given environment, and the interaction of different antibiotic environments with regards to the availability of resistance mutations should it seek to increase the sustainability of existing antibiotics.

4.6 Future Work

While, the observation of increased rate of mutation in *E coli* after exposure to chloramphenicol and selection on ceftazidime was perhaps novel, it would be pertinent to test the generalizability of this case. For example, are there other relationships of collateral sensitivity where previous screens for mutability produced a null result that could be revisited? In the case of aminoglycoside resistance, and it's resulting

susceptibility to multiple drugs, it would be good to test the apparent mutation rate of these relationships. Given an increase in mutation rate it would serve as a basis to investigate underlying MDR mechanisms. It would be worth investigating the other collateral sensitivities identified in this experiment for comparison, then perhaps designing a high throughput screen that might accomplish the same task.

Additionally, it would be worthwhile to investigate molecular specifics of collateral sensitivity. For example, it is known that adaptation to aminoglycosides results in a reduction of the proton-motor-force (PMF) across the inner membrane of gram-negative bacteria, that reduces the efficacy of PMF dependent efflux pumps and thus causes susceptibility to other drugs (Lázár *et al.*, 2013). Similarly, it would be important to investigate the link between *pbp3* mutation in cef resistance, and *marR* acquired resistance in cm.

It would be valuable to attempt to reproduce the collateral sensitivity results from Imamovic & Sommer, 2013 obtained using cefoxitime and gentamycin with the multiple mutant strains outlined in this experiment. The multiple mutant strains may provide a slightly different availability of mutations that would complicate the relationship of collateral sensitivity between cefoxitime and gentamycin. In this experiment, not all GN stains had the same levels of resistance to other cephalosporins like ceftazidime. Perhaps the same might be true for cefoxitime.

Appendices

Appendix A

A.1 EUCAST MIC data for *E. coli* (EUCAST, 2017)

Antibiotic	ECOFF (µg/mL)	Distributions	Observations
Ampicillin	8	49	39270
Chloramphenicol	16	37	11725
Ciprofloxacin	0.064	55	16702
Colistin	2	15	6090
Gentamicin	2	95	44011
Kanamycin	8	4	3860
Levofloxacin	0.25	5	9144
Meropenem	0.125	69	8011
Nalidixic acid	16	7	5190
Streptomycin	16	26	9917

A.2 Maximum Likelihood Estimator Equations

$$\frac{\tilde{r}}{m} - \ln(m) - 1.24 = 0$$

Eq. 1 Lea-Coulson estimation method for m, where $\tilde{r}$ is the median number of mutants per population.

$$p_0 = e^{-m}; \quad p_r = \frac{m}{r} \sum_{i=0}^{r-1} \frac{p_i}{(r - i + 1)}$$

Eq. 2 Calculation for p_r, which is then used to estimate *m*. It is a recursive algorithm that estimates p_r for a given population with the number of mutants, *r*. Estimates of *m* are taken using (Eq 1.) and tested for all possible ranges of *r* between 0 and it's maximum.

$$f(r \mid m) = \prod_{i=1}^{c} f(r_i \mid m)$$

Eq. 3 Maximum likelihood estimation for *m* where initial estimates taken from (Eq. 1) and (Eq. 2) are samples in small numbers until a maximum value is obtained for their products.

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