Compensatory Evolution in Quinolone Resistant *Escherichia coli*

By

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Abstract:

Antibiotic resistance is a major threat to public health, undermining our ability to treat infectious disease. Often, isolates bearing resistance mutations suffer a cost of resistance, that is, lower fitness than their susceptible counterparts. Nonetheless, fitness can be ameliorated by secondary site mutations, known as compensatory mutations. These mutations restore fitness to normal levels without eliminating resistance. Despite the potential importance of compensation for public health strategies to combat antibiotic resistance, relatively little is known about the molecular mechanisms of compensation. Here, we investigated mechanisms of compensation for quinolone resistance mutations in *Escherichia coli.* We found substantial costs of resistance for two genotypes, derived from MG1655 (K-12): a *gyrA* D87G mutant, and a *marR* R94C mutant. Subsequent selection in the absence of antibiotics led to an improvement in fitness, with at least partial retention of quinolone resistance. Whole-genome sequencing was used to identify potential compensatory mutations. Second-site mutations that arose in a *gyrA* D87G mutant encode for proteins involved in cell adhesion, while mutations on the *marR* R94C background occurred in genes with roles in outer membrane function. This work will provide insight into the mechanisms of compensation of the costs of quinolone resistance in *Escherichia coli*.

Key words

Antibiotic resistance, Compensatory Evolution, Resistance, Mutation, Fitness, Epistasis, Second-site mutations

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Statement of Contributions

In this thesis, I carried out all wet-lab experiments, including competition experiments, selection experiments, measurements of minimum inhibitory concentrations, and sequencing. Genome assembly was carried out using a bioinformatics pipeline in the Wong laboratory.

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I. Introduction:

1. Antibiotic resistance:

Since the discovery of penicillin in 1928 and its adoption for broad use during World War II, antibiotics have represented an important part of basic medical treatment (Rice, 2009). Antibiotics are compounds that kill or inhibit the growth of pathogens by interfering with essential bacterial pathways, such as nucleic acid, protein, and cell wall synthesis (Tenover, 2006). Yet in the decades since their introduction, antibiotics have become less effective due to widespread antibiotic resistance worldwide (World Health Organization, 2014). Worryingly, the discovery of new effective antibiotics has slowed remarkably (Projan, 2003), leading to increases in the risk of death or other serious complications due to bacterial infections. Research into the evolution and maintenance of antibiotic resistance is thus central to attempts to control this growing problem.

1.1. Prevalence of Antibiotic Resistance

Antibiotic resistance has become an issue locally and globally for a broad range of microorganisms. The recent World Health Organization antimicrobial resistance global report (World Health Organization, 2014) highlights the most common pathogens and the antibiotics to which they have become resistant (Table 1). From those seven pathogens there are three strains that are of particular international concern, namely *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* (Table 2).

Table1. Common pathogens causing hospital- and community-acquired infections, or transmitted through the food chain, and the classes of antibiotics to which they have evolved resistance (World Health Organization, 2014)

Pathogenic Bacteria	Antimicrobial Agents
Escherichia coli	3rd gen. Cephalosporins
	Fluoroquinolones
Klebsiella pneumoniae	3rd gen. Cephalosporins
	3rd Carbapenems
Staphylococcus aureus	Methicillin "MRSA"
Streptococcus pneumoniae	resistant to penicillin
Nontyphoidal Salmonella	Fluoroquinolones
Shigella species	Fluoroquinolones
Neisseria gonorrhoea	3rd gen. Cephalosporins

*3rd gen. = Third Generation.

Table 2. Three species of pathogenic bacteria causing international concern that are resistant to essential and common major broad-spectrum antimicrobial groups within several World Health Organization (WHO) regions

WHO Regions	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Africa	3rd gen. cephalosporins fluoroquinolones	-	Resistant to Methicillin "MRSA"
The Americas 21 countries	3rd gen. cephalosporins Fluoroquinolones	Cephalosporins	Resistant to Methicillin "MRSA"
Eastern Mediterranean	3rd gen. cephalosporins Fluoroquinolones	Cephalosporins	Methicillin "MRSA"
European	-	Cephalosporins	Resistant to Methicillin "MRSA"
South-East Asia	3rd gen. cephalosporins Fluoroquinolones	Cephalosporins	Methicillin "MRSA"
Western Pacific	3rd gen. cephalosporins Fluoroquinolones	Cephalosporins	Resistant to Methicillin "MRSA"

The evolution of antibiotic resistance threatens to render some clinical infections untreatable, and in general complicates treatment options for many infections. Fluoroquinolone resistance in pathogenic *E.coli* provides a relevant example. Pathogenic *E.coli* causes worldwide infections, such as urinary tract infections, sepsis and food borne infections (World Health Organization, 2014). It has been reported that due to UTIs alone, visits to outpatient clinics reached 7 million, and 100,000 patients required healthcare (Wilson et al. 2004). Such infections were previously treatable with fluoroquinolones, yet in the past 30 years fluoroquinolone-resistant *E.coli* isolates have become widespread (Johnson et al. 2013). In U.S, the prevalence of fluoroquinolone-resistant *E.coli* isolates exceeds 12% in hospitals (Bernard et al. 2008). The same holds true for Canada; between 2007 and 2011, there has been a significant increase in the prevalence of resistance of *E. coli* to broad-spectrum antibiotics, including fluoroquinolones(Karlowsky et al. 2013). Notably, quinolone resistance is linked with increased incidence of mortality (Lautenbach et al. 2005). These numbers are indicative of the greater risks of antimicrobial resistance, and of the necessity of research into the origins and maintenance of resistance, in order to guide health care providers and policy makers in controlling antimicrobial resistance.

1.2. Impact of Antibiotic resistance

Strong antibiotic selective pressure owing to inappropriate antimicrobial consumption to treat human diseases, or to promote the growth of food-producing animals, are major causes of antibiotic resistance (World Health Organization,

2014). This evolution and spread of antibiotic resistance among pathogenic bacteria is increasing as a public health concern since it results in therapeutic failures, leading to hundreds of thousands of deaths annually (Frieden, 2013). For instance, in developing countries, multidrug resistant (MDR) infections threaten health-care measures, because most available drugs have failed to treat community MDR strains, such as *K. pneumoniae*, *S. aureus* and *P. aeruginosa*(Levy, 1998; Levy et al. 2004). Similarly, methicillin-resistant *Staphylococcus aureus* (MRSA), a common cause of hospital infections and the sixth leading of death nationally, accounted for 94,000 infections and over 18,000 deaths in 2005 (Klevens et al. 2007). In Canada, MRSA infections rose sharply from 9.1% to 19.5% between 2005 and 2007 (Zhanel et al., 2008; Nichol et al. 2011) .

Furthermore, antimicrobial resistant infections have become an economic burden, since they result in prolonged illnesses that require increased hospitalization and multiple rounds of drug treatment (Levy et al. 2004).The impacts of infections caused by antibiotic resistant bacteria differ from those with infections with the same susceptible bacteria. Annually, in the United States, the estimated budget to treat antimicrobial resistant infections is between US$ 21 billion and US$ 34 billion (Boucher et al. 2011). Patients with antimicrobial resistant infections have higher costs (~$6,000– $30,000) than do patients with drug-susceptible infections of the same bacteria(Cosgrove, 2006).

2. Evolution of Antibiotic Resistance

Broadly, bacteria can acquire resistance by two major mechanisms: spontaneous mutation of chromosomal genes, or the acquisition of resistance genes located on horizontally transferred mobile elements such as plasmids or transposons (Martinez et al. 2009). Examples of resistant genes transferable by a plasmid include the plasmid-mediated quinolone resistance gene *qnr*. This gene has been found in quinolone resistant *K. pneumoniae* and *E. coli*, and encodes a protein that blocks DNA gyrase inhibition caused by the drug (Martínez-Martínez et al. 1998; Tran et al. 2002). Similarly, β -lactamase resistance in both *K. pneumoniae* and *E. coli* is often caused by plasmid-borne extended-spectrum β-lactamases (ESBL). These enzymes hydrolyze the β-lactam ring of β-lactams, causing resistance (Jacoby et al. 1991).

Antibiotic resistance can also arise via chromosomal mutations, typically via alterations in the nucleotide sequence of the gene(s) that encodes for drug targets or other resistance factors (Maisnier-Patin et al. 2002). For instance, in the case of β -lactam antibiotics, resistance can be attributed to mutations in genes encoding the penicillin-binding proteins (PBPs) that are the targets of β-lactams (Yotsuji et al. 1988;Livermore 1987).

3. Fluoroquinolones

Fluoroquinolones are a class of synthetic broad-spectrum antimicrobial agents, with activity against a wide range of Gram-positive and Gram-negative bacteria (Ruiz, 2003). Fluoroquinolones interfere with topoisomerase II (DNA gyrase) and topoisomerase IV (Drlica et al. 1997), essential enzymes for DNA replication and

transcription (Drlica et al. 1997). Both DNA gyrase and topoisomerases IV are heterodimers consisting of two subunits, namely GyrA and GyrB, or ParC and ParE, respectively (Barnard et al. 2001). These enzymes differ in their functions during replication and transcription: DNA gyrase introduces negative supercoils into DNA in order to relax the packing of DNA due to its positive supercoiling(Barnard et al. 2001). Topoisomerase primarily functions prior to transcription; here, DNA becomes overwound ahead of the transcription bubble, and topoisomerase relaxes this supercoiling. Topoisomerase also has an important role in the decatenation of daughter chromosomes during cell division(Squibb, 2005). As antibiotc targets, DNA gyrase is the primary target in Gram-negative bacteria, while Topisomerase IV is the primary target in Gram-positive bacteria(Hooper, 2001).

Quinolones block replication by preventing the re-ligation of the cleaved DNA that is formed during supercoiling (Cambau et al. 1993; Hooper, 2001; Kampranis et al. 1999). As a result, essential bacterial cellular processes such as DNA replication, transcription, repair, and recombination are compromised, leading to cell death (Cambau et al.1993).

Clinically, fluoroquinolones are used to treat urinary tract infections (UTIs) caused by *E. coli* and *K. pneumoniae* (Kyabaggu et al. 2007), lower respiratory tract infections(Ball et al. 1962), acute uncomplicated cystitis in females caused by *Staphylococcus saprophyticus* (Colgan et al. 2011), and other infections. In the decades following the introduction of fluoroquinolones to clinical use, resistance has arisen in all major pathogens (Ruiz, 2003). According to Canadian Antimicrobial Resistance alliance (CARA) resistance to ciprofloxacin (the most frequently used

fluoroquinolone; CAN-R 2011) has reached 27.6%, 13.4%, and 91.4%% in *E. coli*, *P. aeruginosa*, and *S. aureus,* respectively.

Fluoroquinolone resistance can evolve via several distinct pathways: alterations to the drug targets, efflux, and inhibition of drug uptake (Hooper, 2001; Weigel et al. 2002). In the case of drug target alterations, resistance can be mediated by mutations in the gyrase subunits GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes respectively, as well as the topoisomerase IV subunits ParC and ParE, encoded by the *parC* and *parE* genes respectively (Bagel et al. 1999). Resistance mutations are often found between positions 67 N and 106 N in the GyrA and ParC proteins, such that this region has been termed the " quinolone-resistance-determining-region" (QRDR) (Barnard et al. 2001). In Gram-negative bacteria, where DNA gyrase is the primary target of fluoroquinolones, mutations at Ser-83 and Asp-87 of GyrA are particularly common (Weigel et al. 1998). In Gram-positives, Topoisomerase IV is the drug primary target, and mutations at Ser-79 of ParC and Ser-81 of GyrA (González et al. 1998) are often observed.

Fluoroquinolone resistance can also be gained through mutations in *marR,* a regulatory gene in the marRAB operon, which controls multiple antibiotic resistances (Sulavik et al. 1995) . The *mar* region contains three genes, *marR*, *marA*, and *marB* (Cohen, 1993). *marA* expression, which is normally repressed by MarR, activates the expression of the tripartite efflux pump AcrAB-TolC, as well as the expression of the small regulatory RNAs(sRNA) *micF,* whose expression leads to reduced expression of the *ompF* porin (Alekshun et al. 2001; Chubiz et al. 2011). Consequently, loss-of-function mutations to *marR* result in up-regulation of the

AcrAB-TolC efflux pump and down-regulation of porins, resulting in lower intracellular drug concentrations(Sulavik et al. 1995).

In the case of efflux, regulatory proteins vary between species, but loss-of-function mutations leading to efflux pump up-regulation are common. For example, *nfxB* mutants of *P. aeruginosa* are associated with resistance to fluoroquinolones, owing to up-regulation of the MexCD-OprJ efflux pump (Cohen et al. 1989; Hirai et al. 1987; Join-Lambert et al. 2001). In terms of resistance evolution, mutations that result in up-regulation of drug efflux mechanisms arise at higher rates than mutations in the structural genes for topoisomerases. This is due to the fact that the genetic target in efflux-regulating genes is much larger than the specific amino acid substitutions required for Gyrase or Topoisomerase-mediated resistance (Marcusson et al. 2009).

4. Costs of Resistance:

Pathogenic bacterial strains that have acquired mutations conferring antibiotic resistance often show lower fitness than wild-type strains, a phenomenon known as the "cost of resistance" (Andersson et al. 2010a; Andersson, 2006). Decreased fitness might result from resistance mutations disrupting important physiological processes in the cell, such as cell wall synthesis, DNA replication, or transcription, resulting in impaired growth (Tenover, 2006). Importantly, the biological cost of resistance is a key parameter in determining the stability of resistance in a population, affecting the likelihood that resistant strains will persist in the absence of antibiotic (Andersson et al. 2010b). From a clinical perspective,

addressing the cost of resistance is essential in order to understand the evolution of resistance and to develop efficient strategies to control resistance, since resistance costs influence the rate and trajectory of antibiotic resistance evolution (Andersson, 2006; Hall et al. 2011).

Costs of resistance have been documented for a number of different bacterial species and for a variety of antibiotics (reviewed in Melnyk et al. 2014). The costs of resistance may be expressed as a reduction in virulence, transmission rates, or growth rates (Andersson, 2006). For instance, in *Salmonella typhimurium*, protein synthesis rates are reduced as a result of streptomycin resistance mutations in *rpsL* (encoding the S12 ribosomal protein), as well from fusidic acid resistance mutations in *fusR* (encoding the elongation factor EF-G) (Reynolds, 2000; Ruusala et al. 1984).

Nonetheless, not all resistance mutations carry a cost. In *Campylobacter jejuni,* a quinolone-resistance mutation in *gyrA* was shown to enhance fitness in antibiotic-free medium (Luo et al. 2005). Furthermore, an epidemiological study indicated that rifampicin resistance mutations in the *rpoB* gene of *Mycobacterium tuberculosis* appear to impose a low fitness cost, suggesting that in humans no-cost or low-cost mutations are selected (O'Sullivan et al. 2005). Similarly, in *S. aureus* low-cost rifampicin mutations are favored (Huovinen et al. 2006; O'Sullivan et al. 2005).

In the absence of antibiotic selective pressure, it is expected that susceptible strains are likely to outcompete resistant strains bearing costly resistance mutations. As such it has been assumed that stopping the use of a particular drug will eliminate or reduce resistance in a population (Melnyk et al. 2014). Thus, this

procedure has been applied in different countries with various antibiotics and bacterial species (Enne, 2009). One of the most successful examples of a decline in the drug resistance range occurred in Finland, where about 14% of *Streptococcus pyogenes* became resistant to macrolides in a 5-year period. However, after controls were placed on antibiotic consumption, resistance decreased by 63% (Seppala, 1997). Nevertheless, decreased antibiotic prescriptions are not always effective. For example, in the case of *E.coli* isolates in Sweden, trimethoprim resistance levels did not decrease after intervention, and trimethoprim resistance was frequently associated with resistance to ampicillin and fluoroquinolones (Sundqvist et al. 2009). Such examples suggest that other factors may affect the trajectories of resistance evolution in the face of costs.

5. Compensatory Evolution

Compensatory evolution is one of the factors that may contribute to the persistence of costly resistance mutations. Costs of resistance can be bettered by compensatory mutations: second-site mutations that restore fitness but that do not eliminate resistance (Andersson, 2006). Compensation is a process that can occur in either the absence or presence of antibiotics and has been found in many bacterial species (Andersson, 2010a). Broadly speaking, there are two types of compensatory mutations: extragenic (intergenic) mutations ameliorate the effects of a mutation in one gene by a mutation in elsewhere in the genome, while intragenic mutations occur at the same locus as the resistance mutation (Kondrashov et al. 2002). Numerous examples of both intragenic and extragenic second-site mutations have

been documented (Table 4).

Within different cases, compensatory mutations may provide a fitness benefit only on the drug-resistant background and not in susceptible backgrounds (Maisnier-Patin et al. 2004). These second-site mutations thus represent cases of epistasis. Epistasis occurs when a phenotypic effect of a mutation in one locus depends on the presence of different mutations at another locus (de Visser et al. 2011).

5.1. Reversion

Compensation is not the only mechanism that ameliorates fitness in antibiotic resistant bacteria. Reversion is another potential mechanism whereby resistant strains can recover fitness. Reversion occurs when a mutation back-mutates to the wild type, resulting in a complete loss of resistance (Schrag et al. 1997). However, it has been suggested that second-site mutations are more common than true reversions (Andersson et al. 1999). Since second-site mutations may be found at many loci in the genome, the mutational target size potentially is larger for compensation than it is for reversion (Schrag et al. 1997). For example, Kugelberg et al. (2005) showed that fitness gains in quinolone resistant *P. aeruginosa* were not due to either reversion or intragenic compensation, but rather that extragenic mutations must be involved.

Table 3. Examples of known second-site mutations

Bacterium	Antibiotic	Resistance mutation	Second-site mutation
**E.coli*	Streptomycin	*rpsL*	Intergenic, *rpsD/E*
***S. typhimurium*	Streptomycin	*rpsL*	Intragenic**,** *rpsL*
		rpsL	Intergenic, *rpsD/E*
****M. tuberculosis*	Isoniazid	*katG*	Intergenic, *ahpC*

*(Schrag, et al. 1996; Schrag, et al. 1997)**.**

(Björkman, et al. 1998; Björkman, et al. 1999),** ***(Sherman,et al. 1996)

5.2. The Importance of Compensation:

I am investigating compensatory evolution due to its potential importance in controlling antibiotic resistance. Stopping the use of a particular antibiotic treatment is one of the approaches that has been used to reduce the prevalence resistant strains, based on the assumption that resistance imposes a large fitness cost in an antibiotic-free environment (Melnyk et al. 2014). However, in some cases within clinical settings, resistance is not eliminated in a population after withholding of the drug (Enne, 2009). Compensatory evolution may underlie some failures of this approach: Since compensation involves an increase in fitness without the elimination of resistance, compensated resistant strains may persist (Melnyk et al. 2014) .

Here, I study compensatory evolution in *E. coli* strains resistant to fluoroquinolones. In the current study, I have examined genotypic changes in fluoroquinolone-resistant strains evolved in the absence of antibiotic. Phenotypic data indicate that compensation occurs quickly, and whole-genome sequencing has allowed us to identify putative second-site mutations in different genes in these evolved strains.

The present work aims to better understand the interplay between mutations conferring resistance to fluoroquinolones and second-site mutations, and to gain insight into their effect on bacterial fitness. Such data may allow us to design better strategies that will limit the prevalence of resistance.

II. Materials and Methods:

1. Bacterial strains and growth conditions

Ciprofloxacin (Cip) resistant mutants were available in the lab that were previously derived from a common ancestor, *E. coli* MG1655 (K-12)(Wong et al., 2015). Briefly, these mutants were isolated using a fluctuation assay (Foster, 2000; Luria & Delbrück, 1943). Multiple independent populations of *E. coli* MG1655 were grown in Lysogeny Broth (LB) overnight, and were then plated on LB agar + 25ng/ml Cip and incubated for two days at 30C. Following verification of the Cip^R phenotype, whole genome sequencing was used to identify mutations present in six strains. Five of the six mutants bore a single mutation known to confer resistance to quinolones, while the sixth bears a known Cip^R mutation (*gyrA* D87G)and an additional silent mutation (*valS* G611G) in a gene that has no known impact on Cip resistance (Table 4).

2. Fitness Assays

Competition experiments were performed to estimate the fitness of each mutant in the absence of antibiotic, using a competitive fitness assay against a genotype matched $lacZ^-$ ancestral strain, NCM520. In this experiment, frozen samples of the mutants and ancestral strains were streaked on LB agar and incubated overnight at 30C. Individual colonies were harvested and grown separately in LB for overnight at 30C. Competitions were then conducted by inoculating equal volumes of NCM520 and a given mutant genotype at a 1:100 dilution in LB, followed by a 24-hour incubation at 30C in LB with shaking. Eight

replicate competitions were carried out for each genotype. Relative frequencies of each genotype were estimated from counts of blue and white colonies on LB agar+IPTG+X-gal at the beginning and end of the competition experiment (Figure 1). The white and blue colonies represented the susceptible and resistant strains respectively. While it is possible that *de novo* Lac^- mutants would also appear as white colonies, thus confounding the data, white colonies never appeared on streak plates of MG1655 or any of its derivatives, indicating that mutation rates to Lac^- are low.

The initial ($white_{initial}$ and $blue_{initial}$) and final counts ($white_{final}$ and $blue_{final}$) of each genotype allow us to determine the relative fitness of each mutant. Fitness was calculated for each of the eight replicates using the following equation:

$$S = (\ln [white_{final}/white_{initial}] - [\ln [blue_{final}/blue_{initial}]) / \#\ generations.$$

Relative fitness *w* was then calculated as 1+s, where the units for both *w* and *s* are in per generation. Here, the competition was allowed to proceed for ~6.6 generations.

3. Laboratory Evolution Experiment

I initiated laboratory evolution experiments using two mutants, Cip^R 5(*gyrA* D87G) and Cip^R 6(*marR* R94C), and the wild-type MG1655. Both quinolone resistant mutants *gyrA* D87G and *marR* R94C were chosen because they suffer substantial costs of resistance (see Results below), and MG1655 is used as a control in order to help distinguish true second-site mutations from general lab-adaptive mutations. Individual colonies were used to initiate 12 replicate populations for each genotype, resulting in 36 experimental populations. Populations were grown in 200 μL of

liquid LB medium in a 96-well plate, shaking at 150rpm at 30C. Every 24 hours, 2 µL of culture was transferred to fresh media for a 1:100 dilution. The experiment was run for 15 days, for a total of ~100 generations of selection.

At day 8 and on the final day (15), populations were streaked to LB agar+IPTG+Xgal to test for contamination, which should show up as white colonies. No contaminants were found. Next, single colony isolates from all starting and evolved populations were subjected to competitive fitness assays against NCM520 with 3 replicates per each, as described above.

4. Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration is a laboratory method to quantify resistance, and is defined as the lowest concentration of drug that results in visible growth inhibition (Carson et al. 1995). For MIC assays, a single colony was picked from each of the evolved lineages and ancestral genotypes. Cultures were grown in LB overnight shaking at 150 rpm at 30C. These overnight cultures were diluted 1:100 in a 2-fold dilution series of Cip, ranging from 12.5-800ng/µL, in a 165 µL total volume. After overnight growth at 30C, the lowest concentration of antibiotic at which there was no visible growth was considered the MIC.

5. Whole Genome Sequencing and Analysis

5.1. DNA Purification & Quantification

For whole genome sequencing, a single colony was picked from each evolved line. DNA was extracted using the One-4-All Genomic DNA Mini-prep Kit (Bio Basic,

Markham, Canada) as described in the manufacturer's manual. Following DNA purification, DNA quantification was performed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, UK), following the manufacturer's protocol in order to precisely quantify DNA concentrations.

5.2. Sequencing:

Library construction was carried out using the Nextera XT kit (Illumina; San Diego, US). Libraries were quantified by qPCR (KAPA Biosystems; Wilmington, US). The loaded library concentrations ranged between 10 and 20 pM. Sequencing was carried out on the Illumina Miseq platform used paired-end 300 bp sequencing, loading twelve to twenty-four samples per cartridge.

5.3. Bioinformatics workflow:

Sequence alignment and SNP calling were performed using the pipeline described in Wong and Seguin (2015). Prior to alignment, reads were filtered using Trimmomatic (Bolger et al. 2014). A minimum quality score of 20 was required for leading and trailing bases, and reads with trimmed using a sliding window approach with a window size of 4 and average quality cut-off of 20. Minimum read-length was set to 36. Paired-end reads were then mapped to the reference *E. coli* MG1655 genome (GenBank accession: NC_000913) using *novoalign* (http://novocraft.com/main/index.php). Following local realignment with GATK(McKenna, 2009), SNPs were called using the *bcftools* utility in the *samtools* package(Li et al. 2009). SNPs with a Phred-scaled quality score of ≥20 and with at

least 3 reads containing the derived nucleotide were considered for further analysis. SNP effects were predicted using SnpEff (Cingolani et al. 2012).

Table 4. Ciprofloxacin resistant strains: list of *E.coli* MG1655 derived quinolone resistance mutants and the susceptible strain NCM520

Strain	Description
Cip^R1	*gyrA*, S83A
Cip^R 2	*marR*, deletion of amino acids 72-82
Cip^R 3	*gyrA*, D87Y
Cip^R 5	*gyrA*, D87G
Cip^R 6	*marR*, R94C
Cip^R 8	*marR*, R77H
MG1655	A $lacZ^+$ ancestral strain WT
NCM520	A $lacZ^-$ ancestral sensitive strain

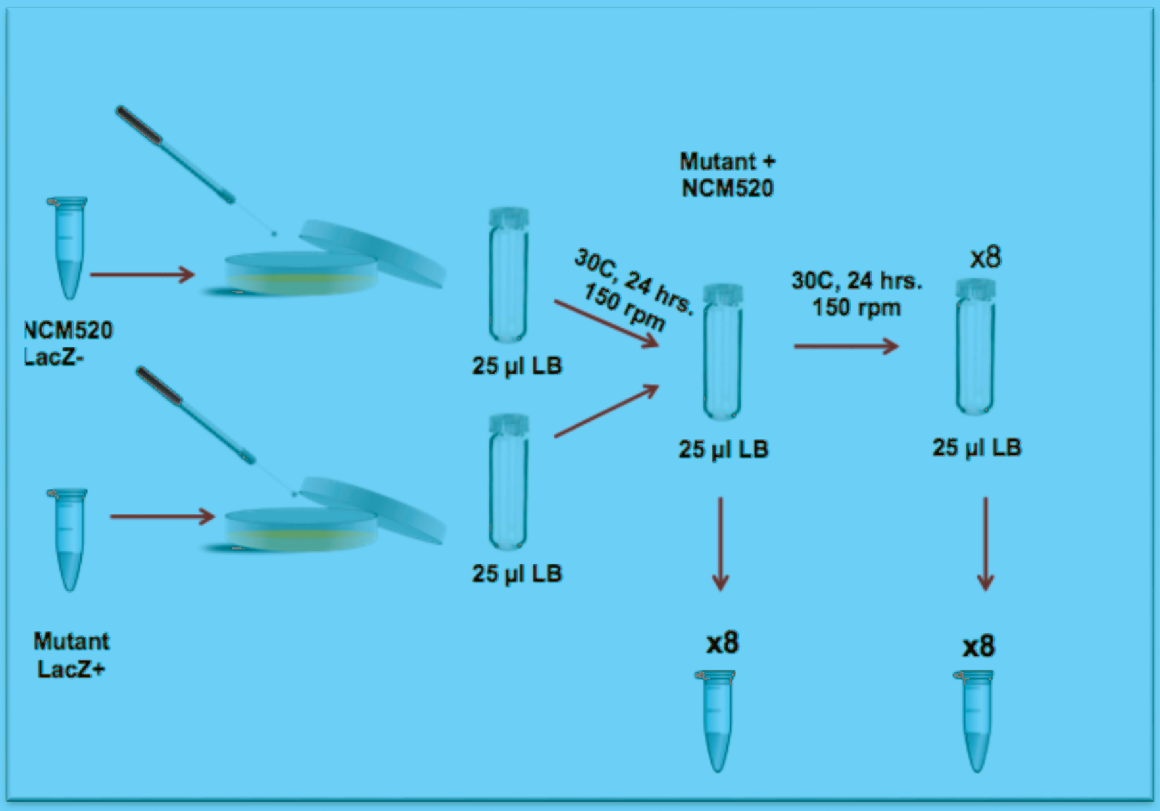

Figure 1: Estimation of fitness via competition experiments. From both LacZ- and LacZ+ strains, cultures were streaked on LB agar. Individual colonies were picked and grown in LB overnight. Finally, equal volumes of the two strains were mixed to compete in the same media; eight replicate competitions were carried out for each genotype. After incubation, dilutions of each culture were plated on LB agar + IPTG +Xgal.

IV. Results

1. Costs of antibiotic resistance

I carried out an initial examination of the costs of quinolone resistance by measuring fitness for six different quinolone resistant genotypes derived from an ancestral Cip^S strain, MG1655. Competitions were carried out in the absence of antibiotic treatment with a genotype matched $LacZ^-$ strain, NCM520. Fitness of the wild-type strain MG1655 was indistinguishable from 1 (Figure 2), indicating that the LacZ marker is neutral.

Results of the competition experiments illustrate that some, but not all, strains carrying resistance mutations suffer lower fitness compared to susceptible strains. Ciprofloxacin resistant genotypes Cip^R1 (*gyrA,* S83A), Cip^R2 (*marR,* deletion of amino acids 72-82), Cip^R3 (*gyrA,* D87Y), and Cip^R8 (*marR,* R77H) have intermediate to high fitness, while both Cip^R5 (*gyrA,* D87G) and Cip^R6 (*marR,* R94C) suffer a substantial cost of resistance (Figure 2, Table 5). There was a significant difference in the fitness costs of resistance mutations between these six genotypes (One Way ANOVA: $P < 0.001$).

2. Laboratory Evolution Experiment

Quinolone resistant *E.coli* mutants Cip^R5 (*gyr*A, D87G) and Cip^R6 (*mar*R, R94C) were chosen to initiate laboratory evolution experiments, because they suffered high costs compared to the wild-type control and other ciprofloxacin mutants (Figure 2, Table 5), and because they bear mutations in different known resistance genes. MG1655 was used as a control in this experiment: mutations

appearing in both MG1655 and Cip^R populations are likely general laboratory adaptations, whereas mutations appearing only in the Cip^R populations are more likely to be true compensatory mutations. Experimental populations were initiated from single colonies in parallel (12 replicate populations per genotype), resulting in 36 evolving lines. Populations were propagated for approximately ~100 generations (15 days) in the absence of antibiotic.

Fitness of a single genotype from each of the evolved populations was measured using a competitive assay against NCM520. Interestingly, in the D87G and R94C evolved replicate populations; fitness was ameliorated, suggesting the presence of second-site mutations. By contrast, the evolved wild-type MG1655 control populations did not substantially gain fitness (Figure 3).

3. Susceptibility Testing

The Cip MIC values for each of the starting and evolved genotypes were measured as the lowest concentration of drug that inhibits the visible growth of bacteria after overnight incubation. Ciprofloxacin was applied in a two-fold dilution series starting with 800 ng/µl. MICs are reported as fold-increase in $\log_2$ MIC over MG1655. Most wild-type evolved lines retained ancestral MIC levels, although increases were noted in some strains. Compare to the wild type, Cip^{R}5 evolved populations showed higher MICs, comparable to or lower than the Cip^{R}5 ancestor. Similar patterns were observed for the Cip^{R}6 evolved populations, with MICs comparable to or lower than the Cip^{R}6 ancestor, but generally greater than the wild type (Figure 4).

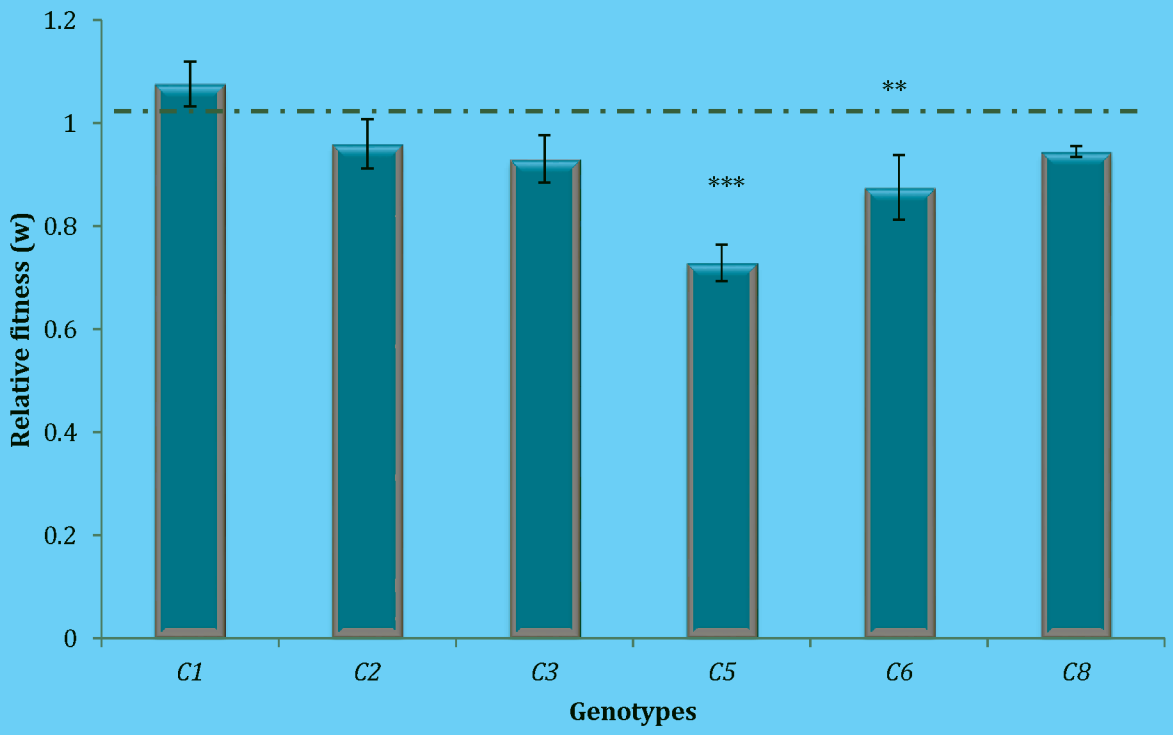

Figure 2: Costs of resistance for six Cip^R mutants. Fitness was measured in the absence of antibiotic via direct competition with a $lacZ^-$, quinolone sensitive strain (NCM520). Four out of six mutants suffer a cost of resistance in the absence of antibiotic. The error bars give +/-1 SE. P-values are indicated on top of the bars (One-sample t-test; ***:P<0.001; **:P<0.01 and *:P <0.05).

Table 5: Costs of resistance for six Cip^R mutants. For each mutant, we determined whether fitness was significantly different from 1 via a one-sided t-test.

Mutant	**Fitness**	***P*-value**	**Standard Error**
Cip^R1 (*gyrA, S83A*)	1.07	0.93	0.04
Cip^R2 (*marR, deletion of amino acids 72-82*)	0.95	0.21	0.04
Cip^R3 (*gyrA, D87Y*)	0.93	0.08	0.04
Cip^R5 (*gyrA, D87G*)	0.72	0. 006	0.03
Cip^R6 (*marR, R94C*)	0.87	0.04	0.06
Cip^R8 (*marR, R77H*)	0.94	0. 005	0.01

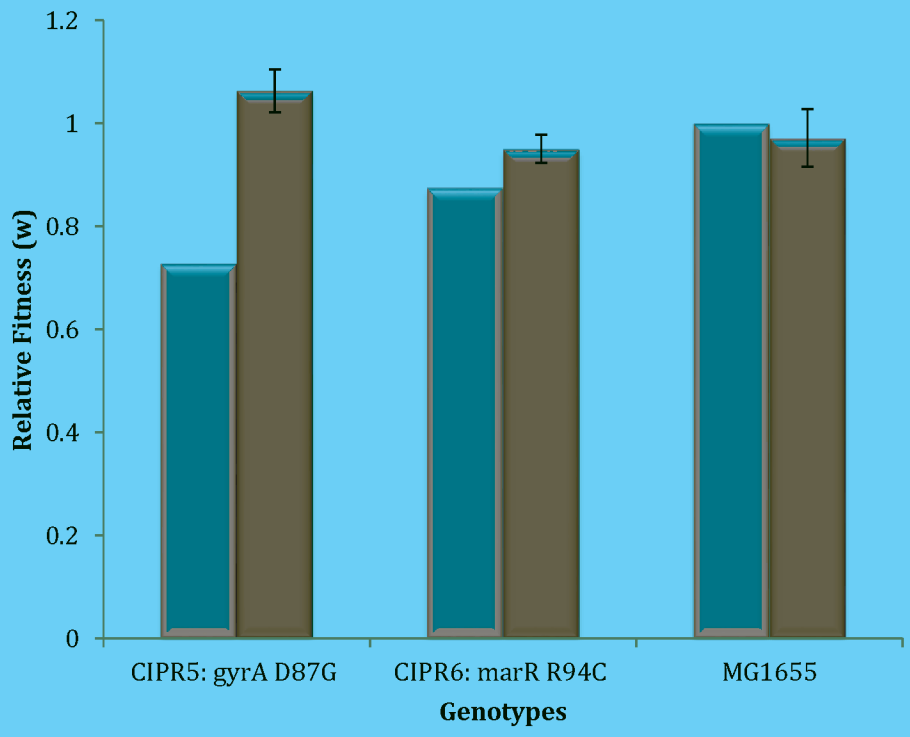

Figure 3: Changes in fitness following selection in the absence of antibiotic. Light gray bars represented fitness of each genotype prior to the laboratory selection experiment. Dark bars give mean fitness (+/-SE) of the 12 evolved populations for each genotype.

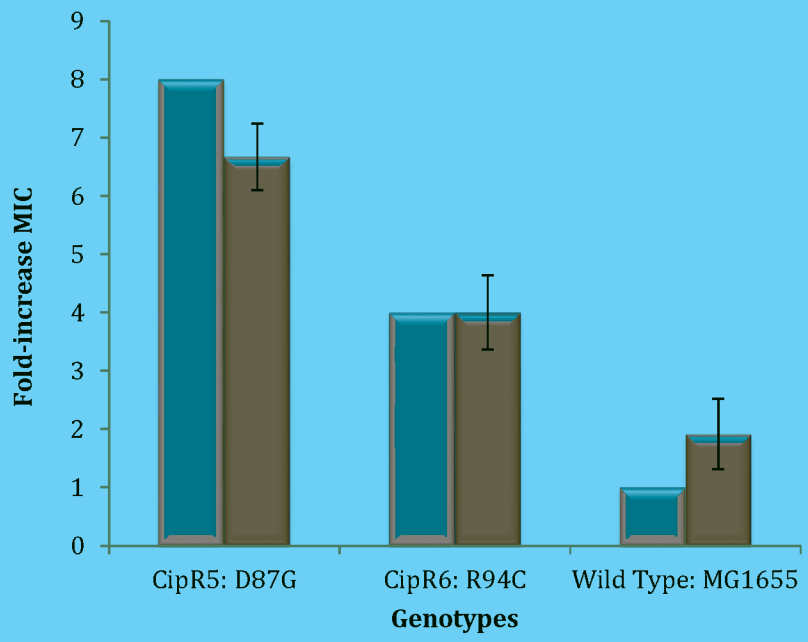

Figure 4: Minimum inhibitory concentration (MIC) of ancestors and experimentally evolved genotypes to ciprofloxacin. Here, the data represent the differences of resistance levels in both ancestor (light bars) and evolved genotypes (dark bars).

4. The relationship between MIC and cost of resistance

It might be expected that high levels of resistance impose higher fitness costs than lower levels of resistance. Thus, for the evolved populations, Cip MIC was regressed against relative fitness. No significant relationship was detected pooling all strains (Figure 5; P= 0.49 , r^2 = 0.01), or when examining only Cip^{R}5-derived strains (Fig.6, *P* =0.61 , r^2 = 0.02) or Cip^{R}6-derived strains (Fig.7, *P* = 0.26 , r^2 = 0.12).

5. Whole Genome Sequencing

To investigate the genotypic changes underlying compensatory evolution, I undertook whole genome sequencing of the complete genomes of single genotypes from all 36 evolved populations, as well as the ancestral strains Cip^{R}5 (*gyr*A, D87G), Cip^{R}6 (*mar*R, R94C) and MG1655. WGS data were obtained using 2x300 bp paired-ends reads on the Illumina MiSeq platform. Following quality filtering, sequencing reads were aligned to the MG1655 reference genome and mutations relative to MG1655 were identified using a standard pipeline (see Materials and Methods for details).

For a given genome, it has been suggested that 10-20 fold coverage is sufficient for accurate mutational identification in laboratory selected microbial strains (Smith et al. 2008). In general, I have achieved sufficient sequencing depth, with a mean of 15.1, 39.2, and 21.6 median coverage for MG1655-, Cip^{R}5, and Cip^{R}6-derived genotypes, respectively. However, analysis showed that some areas of the genome were not sequenced; on average, 81005, 25941and 28613bp achieved zero

coverage for MG1655-, Cip^{R}5, and Cip^{R}6-derived genotypes, respectively. Full and zero coverage data are given in the Appendix (Table 3).

Mutations in the compensated genotypes were identified as those that are present in the derived genotypes, but not in the relevant ancestral strain (MG1655, Cip^{R}5, or Cip^{R}6). Using the current methodology, we are able to detect single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels). Mutations were classified by predicted effect, e.g., missense mutations, synonymous, frame-shift, or stop codons. The sequencing results summarized in Table 6.

Amongst all 36 evolved genotypes, mutations were identified in nine lineages, but not in the remaining 27. We primarily observed mutations with predicted effects on protein sequence (missense, stop, and frameshift), as well as three synonymous mutations. Of particular interest are mutations observed in the Cip^{R}5 and Cip^{R}6 backgrounds, which are candidate second-site mutations (Tables 6 and 7).

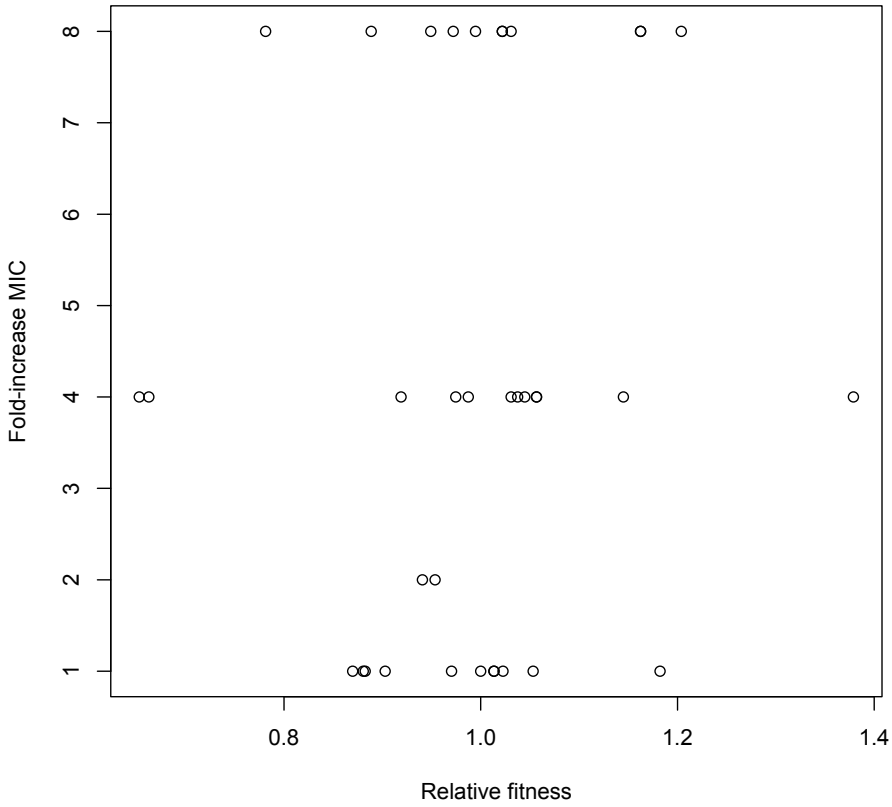

Figure 5: No relationship between MIC and fitness when all evolved strains are pooled.

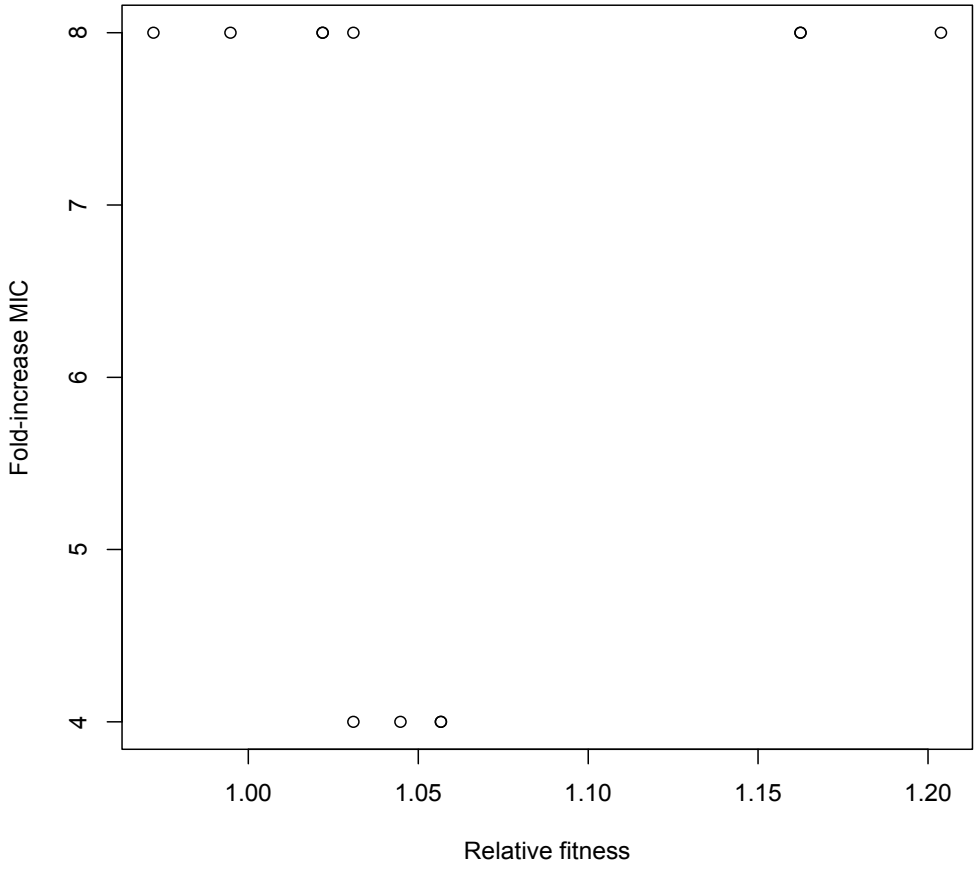

Figure 6. No relationship between MIC and fitness amongst Cip^R5-derived populations.

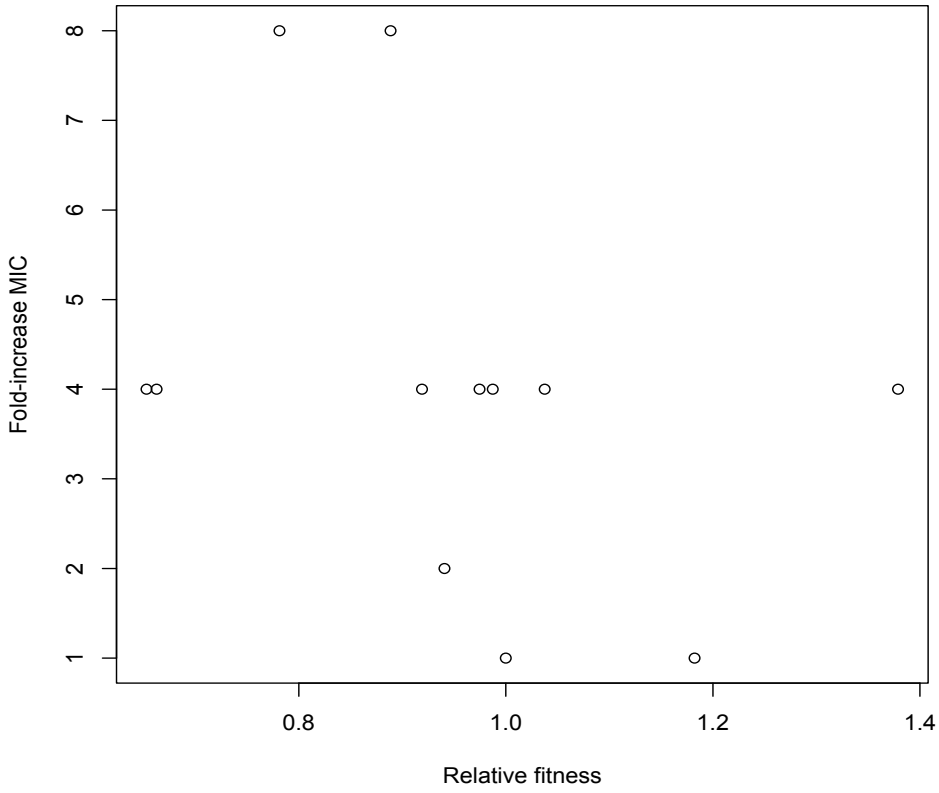

Figure 7. No relationship between MIC and fitness amongst Cip^R6-derived populations.

Table 6. Mutations identified by WGS of genotypes evolved from *gyrA* D87G (Cip^R5), *marR* R94C (Cip^R6), and MG1655 (Wild type).

Genotypes	Gene	Nucleotide alteration	Effect	Mutation type
Cip^R5				
Cip^R5-1				
Cip^R5-2				
Cip^R5-3				
Cip^R5-4	csgE	119 G>C	Gly40Ala	Missense
Cip^R5-5				
Cip^R5-6				
Cip^R5-7				
Cip^R5-8				
Cip^R5-9	ydeR	184 G>A	Gly62Ser	Missense
Cip^R5-10				
Cip^R5-11				
Cip^R5-12				
Cip^R6				
Cip^R6-1				
Cip^R6-2				
Cip^R6-3	yccE	997 A>C	Arg333Arg	Synonymous
	ybhJ	160 C>A	Leu54Ile	Missense

Cip^{R}6-4	oppA	1293_1295delCCA	His431_Gln432del	Deletion
	yebN	74 G>A	Gly25Asp	Missense
Cip^{R}6-5				
Cip^{R}6-6				
Cip^{R}6-7				
Cip^{R}6-8				
Cip^{R}6-9				
Cip^{R}6-10				
Cip^{R}6-11				
Cip^{R}6-12	stfP	289 C>G	Leu97Val	Missense
MG1655				
MG1655-1				
MG1655-2				
MG1655-3				
MG1655-4				
MG1655-5				
MG1655-6	gsiA	1449 del A	Lys483A	Frame shift
MG1655-7	ldhA	345 G>T	Thr115Thr	Synonymous
	yghJ	2148 T>C	Tyr716Tyr	Synonymous
MG1655-8				
MG1655-9	amiC	103 C>T	Gln35*	Stop
MG1655-10				

MG1655-11	lysR	332 A>T	Gln111Leu	Missense
MG1655-12				

*Stop codon

Table 7. Functions of genes in which mutations were detected

Gene	Genotype	Function Description
gsiA	MG1655-6	The glutathione import system ATP-binding protein gsiA
lysR	MG1655-11	Transcriptional activator protein lysR
ldhA	MG1655-7	D-Lactate dehydrogenase
yghJ	MG1655-7	Putative lipoprotein acfD homolog precursor
amiC	MG1655-9	N-acetylmuramoyl-L-alanine amidase amiC precursor
ydeR	Cip^R5-9	Hypothetical fimbrial-like protein precursor
csgE	Cip^R5-4	Curli production assembly/transport component csgE precursor
ybhJ	Cip^R6-4	Hypothetical protein ybhJ
oppA	Cip^R6-4	Periplasmic oligopeptide-binding protein precursor
yebN	Cip^R6-4	Transporter: manganese export protein
yccE	Cip^R6-3	Hypothetical protein yccE
stfP	Cip^R6-12	Hypothetical protein stfP

Discussion

Antibiotic resistance mutations often (although not always) carry a cost, where the fitness of a resistant mutant is lower than that of its susceptible counterpart (reviewed in Melynk et al. 2014). Despite the prevalence of such costs, antibiotic resistance is frequently maintained in pathogen populations when use of a particular antibiotic is stopped (Enne, 2009). It has been hypothesized that compensatory evolution – the accumulation of mutations that alleviate the costs of resistance while not eliminating resistance – may play an important role in maintaining resistance following the cessation of antibiotic use (Andersson et al. 2010a; Melnyk et al. 2014).

In the current study, I examined the evolution of ciprofloxacin resistant genotypes in the absence of antibiotic, in order to gain insights into the prevalence and genetics of compensation. I initiated laboratory evolution experiments from two quinolone resistant *E.coli* mutants, Cip^R5 and Cip^R6, both of which suffer costs of resistance (Figure 1, Table 5). These genotypes represent two major classes of quinolone resistance mutation – Cip^R5 bears a mutation in *gyrA*, which encodes the quinolone target Gyrase A, and Cip^R6 bears a mutation in the efflux and permeability regulator *marR.*

Following 100 generations of selection in the absence of antibiotics, genotypes evolved from both Cip^R5 and Cip^R6 showed evidence for compensatory evolution: Fitness increased over ancestral levels, while resistance was largely maintained (Figures 3 and 4). Notably, the maintenance of resistance strongly

suggests that compensation, rather than reversion, was responsible for the observed increase in fitness. Not all genotypes recovered fitness equally during the selection experiment (Appendix, Table. 1) suggesting that underlying mechanisms of compensation may differ between genotypes.

Mutations arising during compensatory evolution

In order to obtain more detailed knowledge of the molecular changes accompanying compensatory evolution, I used whole-genome sequencing to identify mutations in our evolved lineages. These mutations represent candidate second-site mutations, i.e., those that improve fitness while not eliminating Cip resistance.

Mutations were identified in two genotypes evolved from the *gyrA* mutant Cip^R5. These mutations were found in the *ydeR* and *csgE* genes, both of which have known functions in cell adhesion. *ydeR* encodes a hypothetical fimbrial-like protein in the cell, based on homology to the well-characterized FimA protein (Crépin et al. 2008; Diemen et al. 2005). Fimbriae are important for pathogenic bacteria to establish infection, as they allow the bacteria to adhere to host cells and avoid elimination (Klemm et al. 2000). The *csgE* gene is localized within the *csgDEFG* operon (Hammar et al. 1995), and functions in the production and assembly of curli (Nenninger et al. 2012). Curli are extracellular amyloid fibers produced by enteric bacteria, such as *E. coli* and *Salmonella*, and play important roles in both biofilm formation and adhesion to the cell host surface(Comas et al. 2012; Zogaj et al. 2003). Effects of *csgE* on curli assembly are indicated by altered CsgA fibre morphology in *csgE* mutants, and by an altered ability to bind the diazo dye Congo Red (Chapman

et al. 2010).

It is unclear why mutations in cell adhesion genes should arise during compensatory evolution in a *gyrA* mutant. It is notable, however, that our lab previously observed mutations in the fimbrial gene *fimE* during adaptation of *gyrA* mutants to media containing sub-MIC levels of Cip (Wong and Seguin 2015) Normal adhesion may be disrupted in *gyrA* mutants, especially given the importance of *gyrA* to transcriptional processes. Indeed, studies in *Stapyhlococcus* have shown altered adhesion properties in quinolone-resistant mutants (Bisognano et al. 2000).

Additional mechanisms may also underlie any effects of *csgE* on compensation. In a pervious study, it was found that the overexpression of CsgG causes CsgE to restore erythromycin resistance to cells rendered erythromycin sensitive (Comas et al. 2012). This finding suggested that CsgE participates in gating the outer membrane pore via the GsgG pore in the outer membrane, since CsgE is required for curli fibre biogenesis when CsgG is expressed (Comas et al. 2012). Thus, effects on transport-related processes may also contribute to any role of *csgE* in compensation.

Other potential second-site mutations were found in lines evolved from the *marR* mutant Cip^{R}6 (Tables 6 and 7). Several of the affected genes have known roles in outer membrane function, which is consistent with the effects of *marR* mutations on outer membrane efflux and permeability (Alekshun et al. 1997; Jacoby, 2005). Amongst the Cip^{R}6-derived genotypes, missense SNPs were observed in *ybhJ, yebN* and *stfP*, a deletion was detected in *oppA,* and a synonymous mutation was observed in the *yccE* gene.

The *oppA* gene encodes a periplasmic binding oligopeptide-binding protein and dipeptide transport system (Urbanowski et al. 2000). Previous studies showed that the OppA protein permits and controls the uptake of the aminoglycosides in *E. coli* by increasing the expression of oppA (therefore increasing rise the drug sensitivity) (Kashiwagi et al. 1992). In contrast, Nakamatsu et al. (2007) reported that in *Escherichia coli* K-12 strain, the OppA protein and Opp transport system have no role in sensitivity to aminoglycoside.

yebN (also known as *mntP*) encodes a putative efflux pump, and is regulated by MntR in *E.coli* for manganese homeostasis (Waters et al. 2011). The roles of *yccE*, *ybhJ,* and *stfP* are less clear. *yccE* and *ybhJ* have no known or predicted function, while the *stfP* gene is thought to encode a prophage tail fiber protein.

During the laboratory evolution experiment, the wild type strain MG1655 was evolved as a control. Mutations were detected in five genes, *gsiA*, *lysR*, *ldhA*, *yghJ* and *amiC*. Mutations in these genes are presumably either neutral or are generally lab-adaptive, and were not observed on the Cip^R5 or Cip^R6 background.

The *gsiA* gene encodes for the glutathione import system ATP-binding protein (Wang et al. 2011). Glutathione is the most abundant and important intercellular antioxidant in living cells against diseases and it contains glutamate, cysteine and glycine (Wang et al. 2011). The *lysR* gene encodes for transcriptional activator protein lysR, a member of the LysR family of regulatory proteins (LTTR)(Maddocks et al. 2008). The *ldhA* gene encodes for lactate dehydrogenase in the *E.coli* fermentative pathway (Clark, 1989). The *yghJ* gene is located in the greater *gsp* cluster, and encodes a putative lipoprotein (Yang et al. 2007). Finally, the *amiC*

gene encodes N-acetylmuramoyl-L-alanine amidase amiC, a periplasmic protein with possible roles in cytokinesis (Garcia et al. 2006).

It is somewhat surprising that no mutations were detected in 27 out of 36 populations. While we did not necessarily expect to observe mutations in the MG1655-derived populations, given the general lack of fitness improvement, it is unexpected that no mutations were detected in a number of populations evolved from the fluoroquinolone resistant genotypes Cip^R5 and Cip^R6. Insufficient coverage is unlikely to account for this failure to detect mutations, since sequencing depth exceeded 15-fold in all but one genotype evolved from a Cip^R ancestor, although second site mutations may be present in the small portion of the genome that was not covered (Appendix, Table 3). One possible explanation is that large insertion/deletion events may underlie most compensatory evolution in these lineages. Thus far, our bioinformatic analyses have focused on SNPs and small indels. Analyses are ongoing to identify large indels and rearrangements in our evolved genotypes.

Mutations identified by WGS are candidate second-site mutations, but their appearance in compensated genotypes does not demonstrate that they are in fact compensatory mutations. Site-directed mutagenesis will be required to establish that these mutations are in fact responsible for compensation. In this experiment, potential compensatory mutations will be inserted into the resistant and susceptible backgrounds in order to test their abilities to affect wild type and mutant fitness levels. At a minimum, compensatory mutations must increase fitness on the Cip^R background. Arguably, a truly compensatory mutation should not increase fitness

on the wild-type background. Under this interpretation, compensation is by definition epistatic (Weinreich et al. 2005). Such an event is an example of " sign epistasis", whereby mutations which are neutral or deleterious on one genetic background are beneficial on another (Weinreich et al. 2005). Site-directed mutagenesis experiments would establish whether the mutations observed in this experiment display sign epistasis. Similar experiments have been performed to establish compensatory interactions in the *Adh* gene of *Drosophila melanogaster* (Parsch et al. 1997), and in bacteriophage φX174(Poon et al. 2005).

It is notable that none of the second-site mutations identified in this study are located in either *gyrA* or *marR* – that is, there is no evidence for intragenic compensation. A number of other studies have also suggested that extragenic compensation is likely more common. (Levin et al. 2000; Moore et al. 2000; Reynolds, 2000). For instance, Schrage and Perrot (1996) showed that costs of streptomycin resistance due to mutations in the ribosomal gene *rpsL* were compensated by mutations at other loci in the genome. Similarly, in *M. tuberculosis,* where rifampicin resistance is conveyed by mutations in the *rpoB* gene, whole genome analysis revealed the presence of second-site mutations in ten extragenic regions, such as the *rpoA* and *rpoC* genes (Comas et al. 2011).

In conclusion, our results suggest that compensation happens easily and rapidly, and so is a viable explanation for the persistence of resistance in the absence of antibiotic use. Furthermore, our data shows that extragenic compensation may be more common that intragenic compensation or reversion.

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Appendices

Table 1: Competitive fitness of lines evolved from two quinolone resistant mutants, Cip^{R}5 (*gyr*A, D87G) and Cip^{R}6 (*mar*R, R94C), and the wild-type strain MG1655

Evolved Lines	Mean Fitness Values	Standard Error
Cip^{R}5-1	1.03	0.01
Cip^{R}5-2	1.16	0.01
Cip^{R}5-3	1.05	0.02
Cip^{R}5-4	1.02	0.03
Cip^{R}5-5	0.99	0.05
Cip^{R}5-6	0.97	0.02
Cip^{R}5-7	1.04	0.02
Cip^{R}5-8	1.20	0.06
Cip^{R}5-9	1.03	0.01
Cip^{R}5-10	1.16	0.01
Cip^{R}5-11	1.05	0.02
Cip^{R}5-12	1.02	0.03
Cip^{R}6-1	0.91	0.01
Cip^{R}6-2	0.98	0.02
Cip^{R}6-3	1.03	0.06
Cip^{R}6-4	1.18	0.06
Cip^{R}6-5	0.94	0.02

Cip^R66	1.37	0.11
Cip^R6-7	0.88	0.07
Cip^R6-8	0.65	0.085
Cip^R6-9	0.78	0.09
Cip^R6-10	0.97	0.03
Cip^R6-11	0.66	0.02
Cip^R6-12	1	0.045
MG1655-1	0.90	0.02
MG1655-2	0.94	0.07
MG1655-3	0.88	0.05
MG1655-4	0.88	0.11
MG1655-5	1.05	0.009
MG1655-6	1.14	0.01
MG1655-7	1.01	0.09
MG1655-8	1.02	0.02
MG1655-9	0.95	0.01
MG1655-10	0.86	0.04
MG1655-11	0.97	0.007
MG1655-12	1.01	0.01

Table 2. Ciprofloxacin MICs for ancestral and evolved lines. MICs are expressed as fold-increase over MG1655.

Genotypes	MG1655 MICs	*D87G* MICs	*R94C* MICs
Ancestors	1	8	4
Rep.1	1	4	4
Rep.2	8	8	4
Rep.3	1	4	4
Rep.4	1	8	1
Rep.5	1	8	2
Rep.6	4	8	4
Rep.7	1	4	8
Rep.8	1	8	4
Rep.9	2	8	8
Rep.10	1	8	4
Rep.11	1	4	4
Rep.12	1	8	1

Table 3. Median coverage of reference based alignment for all ancestors and evolved genotypes. Also, data of some proportions in the genome represented low-sequence (Zero) coverage.

Population	Median coverage	Zero Coverage
Cip^R5	38	25941
Cip^R5-1	35	22376
Cip^R5-2	42	23121
Cip^R5-3	25	22440
Cip^R5-4	20	26533
Cip^R5-5	72	21948
Cip^R5-6	71	23301
Cip^R5-7	57	24140
Cip^R5-8	37	20574
Cip^R5-9	20	21445
Cip^R5-10	25	22147
Cip^R5-11	28	22891
Cip^R5-12	40	23613
Cip^R6	16	28613
Cip^R6-1	22	29394
Cip^R6-2	19	47356
Cip^R6-3	33	26588
Cip^R6-4	25	2416567
Cip^R6-5	32	30143
Cip^R6-6	36	27415
Cip^R6-7	44	36442
Cip^R6-8	17	585558
Cip^R6-9	22	28688
Cip^R6-10	19	42135

Cip^{R}6-11	28	35128
Cip^{R}6-12	5	359351
MG1655	7	81005
MG1655-1	13	2458575
MG1655-2	10	2116617
MG1655-3	9	25865
MG1655-4	24	3336085
MG1655-5	25	3584268
MG1655-6	62	289412
MG1655-7	22	27114
MG1655-8	23	38352
MG1655-9	26	28361
MG1655-1	12	29433
MG1655-11	37	28562
MG1655-12	1	36457

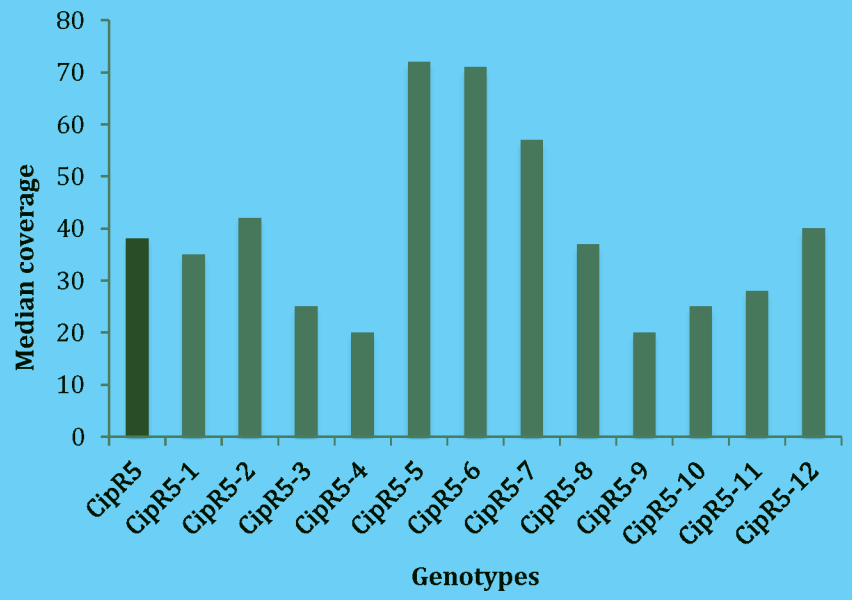

Figure1. Median coverage of Cip^R5 –derived populations

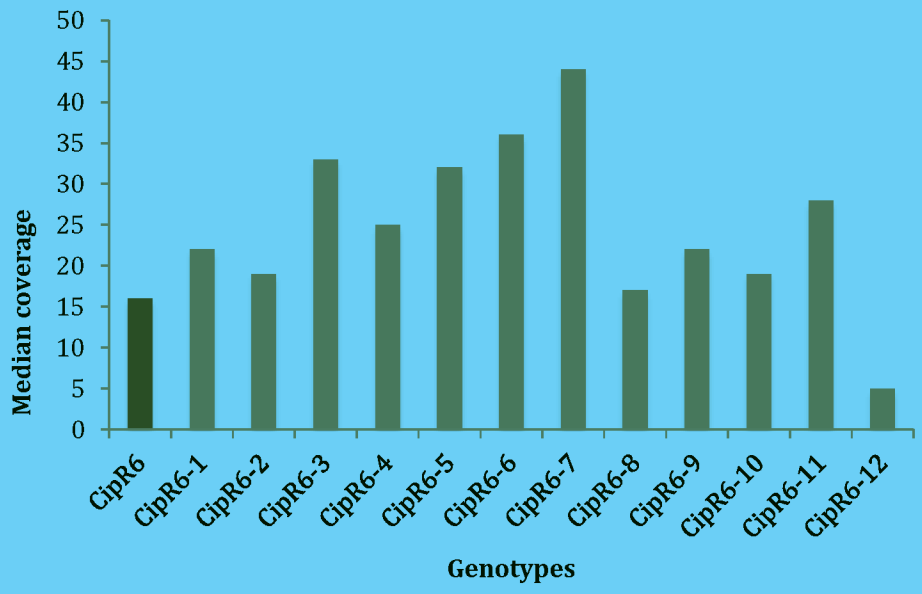

Figure2. Median coverage of Cip^R6 –derived populations

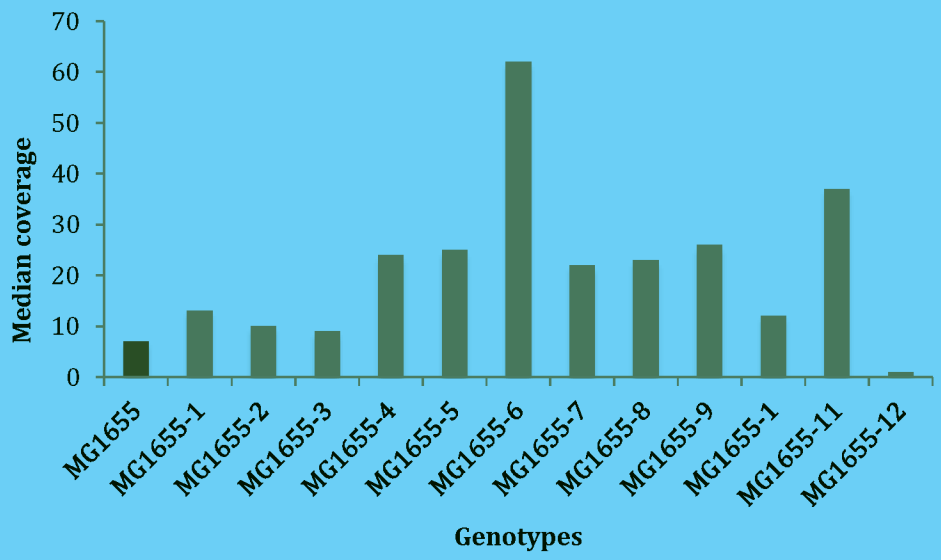

Figure3. Median coverage of MG1655 –derived populations

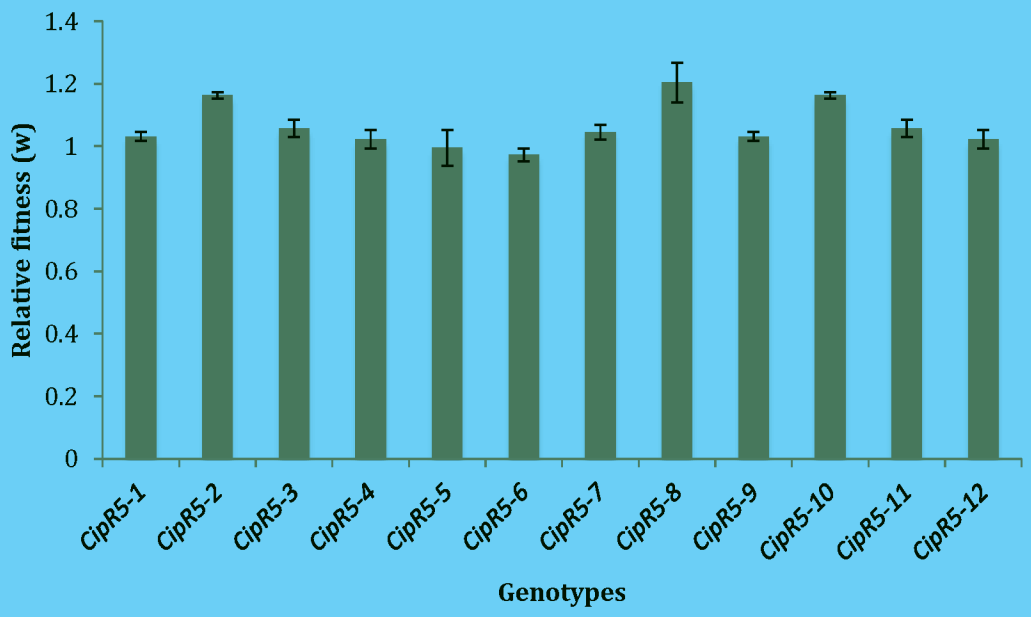

Figure4. Costs of resistance for Cip^{R}5 derived genotypes post laboratory selection experiment

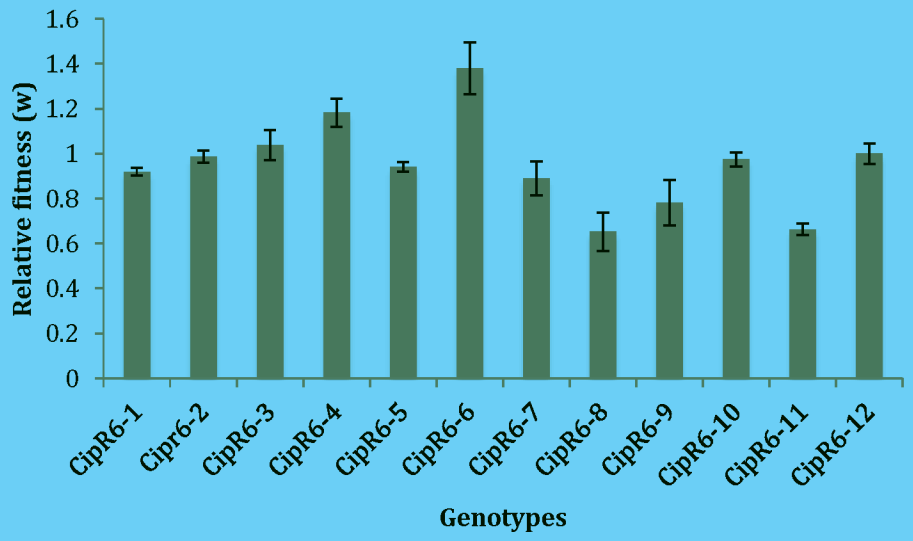

Figure5. Costs of resistance for Cip^{R}6 derived genotypes post laboratory selection experiment

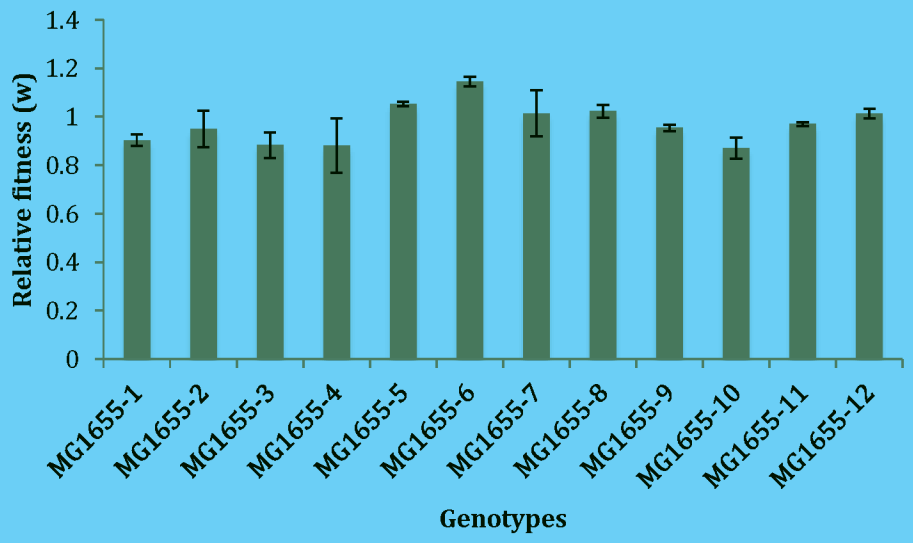

Figure6. Costs of resistance for MG1655 derived genotypes post laboratory selection experiment