

The effect of environmental heterogeneity on the fitness of
antibiotic resistant *Escherichia coli*

by

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Abstract

The cost of antimicrobial resistance (AMR) is the reduction of fitness of a resistant mutant relative to a susceptible strain in the absence of drug. Costs of resistance are usually estimated in a single environment and on one genetic background; these fitness estimates may not be representative of what happens in nature. I measured the fitness of AMR *E. coli* strains in different environments, including medically and ecologically relevant ones. To do this, a collection of AMR strains of *Escherichia coli* bearing a single resistance mutation were competed against their ancestral strain in 10 different media. The results of this study indicate that laboratory media does not predict fitness in natural environments. We found environments in which resistance alleles suffered no cost, suggesting that these mutants may persist for long periods of time. Data on the fitness of AMR pathogens across environments will help manage their spread.

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“A [persons]’s friendships are one of the best measures of his worth.”

Charles Darwin

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1 Chapter: Introduction

1.1 Antibiotic resistance: History and Background

“There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring ‘fastness’ (resistance).”

Alexander Fleming

Antibiotic resistance (AMR) is one of the greatest public health threats facing humanity. If AMR keeps evolving unchecked, it is predicted that deaths caused by AMR infections will increase from 700,000 per year currently to 10 million per year in 2050 (O’Neill J., 2014). By 2050, AMR will approximately be responsible for more deaths than cancer, cholera, diabetes, measles, and diarrhoeal disease combined (O’Neill, 2016). AMR infections already cost too many lives today: in India alone, 60 000 newborns die due to AMR infections each year (Laxminarayan et al., 2013) and 200 people die from multi-drug-resistant (MDR) tuberculosis each year (O’Neill J., 2014). This means that since the India statistic came out, over 1 million people have died from AMR infections. The economic impact of AMR is also substantial: the cost of AMR to global economic output is estimated at US\$100 trillion (O’Neill J., 2014). In the US alone, more than 2 million infections per year are caused by AMR bacteria, costing the US health care system an extra US\$20 billion per year (Smith & Coast, 2013).

The modern era of antibiotics started with the discovery of penicillin by Sir Alexander Fleming in 1928 (Ventola, 2015). Antibiotics were first prescribed in the 1940s to treat serious infections. Nonetheless, resistance quickly arose in the 1950s and became a clinical problem in the US (Gaze et al., 2013). In response new beta-lactam antibiotics were created – and shortly thereafter methicillin-resistant *Staphylococcus aureus* (MRSA) was discovered (Gaze et al., 2013). Resistance has eventually evolved against every antibiotic that has been developed since then (Gaze et al., 2013).

Since discovery, antibiotics have transformed modern medicine and saved countless lives. They have played a fundamental role in achieving major advances in modern medicine (Gaze et al., 2013). They have successfully prevented or treated infections that can occur in people who have chronic diseases such as diabetes and renal disease, or are immunocompromised like chemotherapy and cystic fibrosis patients, or who have had complex surgeries such as organ transplants (Gaze et al., 2013). Worldwide, antibiotics have also contributed to increasing longevity.

1.2 Mechanisms of Resistance

Antibiotic resistance evolves when a microorganism changes in response to the use of antibiotics. Bacteria can either be intrinsically resistant to antibiotics or acquire this resistance through other means. Intrinsically resistant bacteria have the ability to resist antibiotics as a result of their genetic make-up. An example of this would be if the species in question did not have the target of the antibiotic in their genome. For example,

triclosan does not inhibit the growth of *Pseudomonas* species because this genus has an insensitive allele of *fabI* that encodes an additional enoyl-ACP reductase enzyme, which is the target for triclosan (Zhu, Lin, Ma, Cronan, & Wang, 2010). Bacteria can also acquire resistance through *de novo* mutation or by acquiring a mobile genetic element (MGE). This can happen by several mechanisms that can be classified in three main groups: those that minimize intracellular concentration of the antibiotic, those that modify the antibiotic target, and those that inactivate the antibiotic.

A way bacteria may be or become resistant to antibiotics is reduced permeability, which prevents the antibiotic from entering the cell and accessing its target. Gram-negative bacteria are intrinsically less permeable to antibiotics than Gram-positives, because their outer membrane acts as a permeability barrier (Kojima & Nikaido, 2013). Hydrophilic antibiotics cross the outer membrane by diffusing through the outer membrane porin proteins (Kojima & Nikaido, 2013). Recent studies have now shown that in Enterobacteriaceae, specifically *Pseudomonas* spp. and *Acinetobacter* spp., reduction in porin gene expression contributes to resistance to many drugs such as carbapenems and cephalosporins (Tamber & Hancock, 2003). Selective pressure exerted by carbapenems favours the emergence of mutations in porin genes and genes that regulate porin expression – as was shown in *Escherichia coli* and *Enterobacter* spp. (Lavigne et al., 2013).

Another way bacteria prevent antibiotics from accessing their target sites is by increasing efflux. Bacterial efflux pumps transport many antibiotics out of the cell and

when they are overexpressed, they can confer high levels of resistance (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015). They are a major contributor to the intrinsic resistance of Gram-negative bacteria to many drugs that can be used to treat Gram-positive infections (Blair et al., 2015). Some efflux pumps are specific (Tet pumps) but some transport a wide range of substances – therefore they are called multidrug resistance (MDR) efflux pumps. Although all bacteria have genes that encode MDR efflux pumps, these genes have also been mobilised on plasmids (a mobile genetic element, or MGE) that can be transferred between bacteria. For example, RND (Resistance-Nodulation-Division) efflux pump genes were found to be on an *IncH1* plasmid in *Citrobacter freundii* (Dolejska, Villa, Poirel, Nordmann, & Carattoli, 2013). RND pumps confer clinically relevant levels of MDR and export an extremely wide range of substrates (Piddock, 2006). Well-studied examples of this include the multidrug efflux pump AcrAB in *E. coli* and MexAB in *P. aeruginosa*. The up-regulation of efflux genes seen in multidrug-resistant bacteria is often due to mutation in the regulatory network controlling efflux-pump expression. These mutations can be within a local repressor, a global transcription factor, or intergenic sites that alter the expression of pump genes or their regulators (Kaatz, Thyagarajan, & Seo, 2005)

Many antibiotics specifically bind to their target sites with high affinity, thus preventing the normal activity of the target. Changes to the target site that prevent efficient binding, but that still enable it to carry out its normal function, can confer resistance. If a single point mutation in the gene encoding an antibiotic target can confer resistance to the antibiotic, natural selection in the presence of the antibiotic will select

for this variant and strains with this mutation will flourish and outcompete others. An example of this would be point mutations in *rpoB* that confer resistance to rifampicin in *E. coli*. Rifampicin is a broad-spectrum antibiotic that inhibits bacterial RNA synthesis by targeting a small but highly conserved pocket in the β -subunit of RNA polymerase which is encoded by *rpoB* (Villain-Guillot, Bastide, Gualtieri, & Leonetti, 2007). When rifampicin binds to the rifampicin-binding pocket within the DNA/RNA channel of wild-type RNA polymerase, transcription is blocked such that elongation cannot proceed beyond the first three nucleotides (Hartmann, Honikel, Knusel, & Nuesch, 1967; Kessler & Hartmann, 1977). Mutations in *rpoB* can result in alterations to the structure of the rifampicin-binding pocket and confer rifampicin resistance by decreasing the binding affinity between rifampicin and RNA polymerase (Severinov, Soushko, Goldfarb, & Nikiforov, 1993).

Similarly, point mutations in *gyrA* can confer resistance to fluoroquinolones. The *gyrA* gene encodes DNA gyrase, which can introduce negative supercoils into DNA and remove both positive and negative supercoils. DNA gyrase and topoisomerase IV work together in the replication, transcription, recombination, and repair of DNA. The enzymes transiently break both strands of double-stranded DNA and pass a second DNA double helix through the break, which is then resealed (Kampranis, Bates, & Maxwell, 1999). Quinolones block the reaction and trap gyrase or topoisomerase IV as a drug-enzyme-DNA complex, with subsequent release of lethal, double-stranded DNA breaks (Hiasa & Shea, 2000). Fluoroquinolone resistance by target-enzyme mechanism involves amino acid substitutions in a region of the *gyrA* subunit termed the “quinolone-resistance–

determining region” (QRDR) (Alekhshun & Levy, 2007; Jacoby, 2005). This region occurs on the DNA-binding surface of the enzyme (Morais Cabral et al., 1997) and, for *E. coli*, it includes amino acids between positions 51 and 106 (Friedman, Lu, & Drlica, 2001), with “hot spots” for mutation at amino acid positions 83 and 87.

Modification of the target site can also be an effective means of AMR that does not require a mutational change in the genes encoding the target molecules. In recent years, protection of targets has been found to be a clinically relevant mechanism of resistance for several important antibiotics (Blair et al., 2015). An example of this is chloramphenicol–florfenicol resistance (*cfr*) methyltransferase, which specifically methylates A2503 in the 23S rRNA. Methylation has been shown to confer resistance to a wide range of drugs that have targets near this site, including phenicols, pleuromutilins, streptogramins, lincosamides and oxazolidinones (Long, Poehlsgaard, Kehrenberg, Schwarz, & Vester, 2006).

Bacteria can also inactivate or modify antibiotics, rendering them ineffective. Enzyme-catalysed modification of antibiotics is a major mechanism of antibiotic resistance that has been relevant since the first use of antibiotics, with the discovery of penicillinase (a β -lactamase), in 1940 (Abraham & Chain, 1940). These early β -lactamases, which were active against first-generation β -lactams, were followed by extended-spectrum β -lactamases (ESBLs) that have activity against more drugs, such as oxyimino-cephalosporins (Johnson & Woodford, 2013). As more bacteria carried ESBL genes, the clinical use of carbapenem antibiotics increased. As a result, this has been

associated with an influx of clinical isolates carrying β -lactamases with carbapenem hydrolysing activity (known as carbapenemases) (Queenan & Bush, 2007). In addition, many ESBLs and carbapenemase genes have mobilised and have been transferred onto plasmids.

An antibiotic may also be inactivated by the transfer of a chemical group by bacteria. Chemical groups added to sites on the antibiotic by a bacterial enzyme can cause AMR by preventing the antibiotic from binding to its target site as a result of steric hindrance (Blair et al., 2015). For example, aminoglycosides are particularly susceptible to modification as they tend to be large molecules with many exposed hydroxyl and amide groups (Blair et al., 2015). Aminoglycoside-modifying enzymes confer high levels of resistance to the antibiotics that they modify (Blair et al., 2015).

1.3 Antibiotic resistance in the environment

Antibiotic use in humans and animals carries a risk of selecting for AMR bacteria and antibiotic resistance genes (ARGs) in the environment. Antibiotic resistant bacteria can be found ubiquitously in the environment, for example in soil, fresh water, ocean water, and sewage. This means that humans may get AMR infections from bacteria residing in any of these places. Antibiotic resistance genes in the environment are from both bacteria residing in the environment and from bacteria in human and animal waste, and it can also be transferred between resistant and environmental bacteria by mobile genetic elements (MBEs). Less thought has been given to how human activity may be

causing the evolution of antibiotic resistance in the environment (Wellington et al., 2013).

Environmental reservoirs of antibiotic resistance

Mixing environmental bacteria with bacteria from agricultural systems, clinical, settings, and waste-water treatment plants (WWTP) provides the ideal selective environment for AMR strains to arise – AMR genes can be horizontally transferred across phylogenetically distant microorganisms. These bacteria may have the chance to interact when they are moved into waste water treatment systems, manure composting, and sewage overflow sites on rivers and streams. These environments can act as a “hotspot” for horizontal gene transfer between environmental and clinical bacteria and provide reservoirs for AMR.

Humans can be exposed to antibiotics, ARGs, or AMR bacteria by several pathways, such as: crops that have been exposed to contaminated manure, livestock that have veterinary drugs and resistant flora, fish exposed to antibiotics, groundwater containing residues that is used for drinking water, and salt water that is used for recreation and fishing. Antibiotics have been found in fish in effluent influenced water bodies (Ramirez et al., 2009) and in food crops (Boxall et al., 2006; Farkas, Berry, & Aga, 2007; Kumar, Gupta, Baidoo, Chander, & Rosen, 2005). Exposure can also happen through inhalation of dust from livestock farms.

In contrast to clinics, there are few data available on the epidemiology of antibiotic resistances in the environment (Lupo, Coyne, & Berendonk, 2012). This in turn makes it extremely difficult to make any predictions on the risk of spread and emergence of new antibiotic resistances (Lupo et al., 2012). It is also of concern that sub-MIC levels of antibiotics in the environment may be able to select for AMR (Andersson & Hughes, 2014; Gullberg et al., 2011). For this reason, better knowledge on the environmental reservoir of resistances is fundamental to predict the emergence of new resistances of clinical concern.

The main goal of wastewater treatment is to eliminate organic substances to prevent eutrophication in receiving waters. Antibiotics may end up in sludge, effluent, or in rivers depending on their solubility, polarity, and stability. Wastewater can contain a mixture of pharmaceuticals, biocides, and bacteria. There has been substantial evidence in the past few years that wastewater treatment systems are a reservoir for AMR. For example, a Brazilian study of a hospital sewage treatment system showed that ESBL (extended spectrum beta-lactamase) producing *Klebsiella pneumoniae* were present at all stages of sewage treatment (Costa et al., 2006).

Wildlife and animals as a reservoir for AMR and ARGs may be important in their global spread, with detrimental effects for public health and ecosystems. Notably, bird populations tend to have higher levels of AMR and ARGs because of migratory patterns and high populations densities, even in remote areas (Reed, Meece, Henkel, & Shukla, 2003). The transmission of AMR on the farm site has been confirmed for a wide range of

animals such as pigs (Crombe et al., 2013), cows (Wichmann, Udikovic-kolic, & Andrew, 2014), and insects (Hammer et al., 2016); but their transmission routes have been difficult to disentangle (Vittecoq et al., 2016).

A diverse mixture of antibiotics and other pollutants, their metabolites and resistant bacteria, reaches the aquatic environment through treated and untreated sewage, hospital waste, aquaculture discharges, industrial waste, and agricultural runoff. WTPP are recognized as a major contributor to AMR dissemination into aquatic ecosystems such as rivers, lakes, and oceans (Marti, Variatza, & Balcazar, 2014). A shotgun metagenomic study described the diversity of antibiotic resistance genes in an Indian lake subjected to industrial pollution with fluoroquinolone antibiotics (Bengtsson-Palme et al., 2016). The authors found that the lake harbored a wide range of AMR genes. The levels AMR genes in the lake were estimated to be 7000 times more abundant than in a Swedish lake (Bengtsson-Palme et al., 2016).

To summarize, AMR bacteria can be found ubiquitously throughout the environment. They can be found in fresh water, oceans, waste water, soil, farms, wild animals, and in sediment. Anywhere where nutrient, temperature, and competitive conditions are optimal may serve as a reservoir for AMR. Although antibiotic resistance has become a major threat to human health worldwide, this phenomenon has been largely overlooked in environmental settings.

1.4 Genotype-by-Environment Interactions

Mutations that are beneficial in one environment (such as an environment containing antibiotics) may have different fitness effects in another environment. The fitness cost of antibiotic resistance in bacteria in the environment is a case of genotype-by-environment interaction (G*E) such that different AMR bacteria respond differently to environmental variation. The resulting (G*E) interactions potentially make selection of AMR unpredictable in heterogeneous environments (Hall, 2013). Once antibiotics are used and consumed, bacteria may become resistant to the drugs and are excreted into the environment (O'Neill J., 2014) - which may be a sewage system that leads to a treatment plant and a river, or farm crop fields and overflow ditches. These places may then become environmental reservoirs for AMR if conditions are suitable for bacterial persistence (Levy, 2002). This is concerning because if they are reintroduced into humans or animals and cause an infection, this infection will be resistant to antibiotics.

The fate of an AMR bacterium in a given environment depends on its fitness – how well it can pass on its genes – in that environment (Hall, 2013). Mutations or plasmids that confer AMR to bacteria may cause them to grow better, the same, or worse than they did in the environment that selected for AMR. For example, an AMR bacterial strain that was selected for in ciprofloxacin may have a fitness cost in a natural environment like soil. In the absence of antibiotic, resistant genotypes may have lower growth rates than their sensitive ancestor. Mutations that confer resistance may do so by disrupting some normal physiological process in the cell, which can cause side-effects that result in a change in fitness (Melnyk, Wong, & Kassen, 2015). In the case of plasmid

encoded resistance functions, bacteria must synthesize additional nucleic acids and proteins; this synthesis imposes an energetic burden (da Silva & Bailey, 1986) that causes the bacterium to allocate more energy to this instead of replication. Much still needs to be known about how environment effects the growth of AMR bacteria.

The fitness cost of a mutation can be expressed as the reduction of competitive ability (or fitness) of a resistant mutant relative to the wildtype. According to a meta-analysis by Melynk et al. (2015), the literature on the cost of antibiotic resistance mutations and organisms is sparse, and even in well-known organisms such as *E. coli* costs have only been measured for a handful of resistance mutations in very few media (Melynk et al., 2015). The authors found that resistance mutations in bacteria confer a fitness cost overall – but inferences made from these data are limited because costs will also depend on the environment in which the genotype is growing, due to nutrient type and abundance (Melynk et al., 2015). This is of concern because environmental variation could act as an important factor on adaptive trajectories if the fitness effects of resistance mutations are dependent on environment (Gifford, Moss, & Maclean, 2016). In 2013 Hall found that it was nearly impossible to predict the evolutionary fate of resistant bacteria based on fitness measurements in a single environment, because when these strains undergo subsequent evolution in the absence of antibiotics, they are moved into uncharted areas of phenotype space (Hall, 2013).

Evolutionary microbiologists can use microbial fitness assays to describe evolutionary trajectories and make general predictions about evolution (Orr, 2009).

Fitness assays help researchers detect adaptation to different habitats or locations. In the literature most fitness estimates are obtained by growing the bacterium in a single environment, usually a rich medium such as LB broth, and on a single genetic background (Björkman & Andersson, 2000). However, these fitness estimates in rich media may not be representative of what actually happens in nature, as most environments in the natural world are not rich in nutrients. For example, a study by Hubbard recently showed that the fitness of AMR *E. coli* was dependent on the media in which the experiment was performed (Hubbard, 2018). Remold and Lenski have also confirmed finding G*E interactions in an experiment in which the fitness for 26 genotypes of *E. coli*, differing in a single insertion mutation, was measured in 4 environments (Remold & Lenski, 2001). These data highlight the importance of media consideration and G*E interactions when interpreting the results of evolutionary studies.

1.5 Purpose of the Experiment

To summarize, AMR bacteria can be found ubiquitously in the environment. They can be found in fresh water, oceans, waste water, soil, farms, wild animals, and in health care environments. From a public health perspective, this is important: this means that people might get AMR infections out in the natural world and not just in hospitals. It also means that AMR pathogens might persist in certain environmental reservoirs. The likelihood of persistence in environmental reservoirs depends on the fitness of AMR organisms in those environments. Most laboratory research measures the fitness costs of AMR bacteria in a single laboratory medium, and there is limited data on whether this is

predictive of fitness in the natural world (Melnyk et al., 2015). Fitness measurements allow us to predict evolutionary trajectories – such that we may be able to predict an organism’s persistence in an environment. What is needed is a systematic study of environmental effects on fitness for AMR mutations.

The lack of knowledge about the costs of resistance mutations in various environments limits our understanding of how AMR organisms may persist in the environment. To address this knowledge gap, I investigated the effects of environment on the fitness of AMR mutants of *E. coli*. To do this, each of 6 AMR strains of *E. coli* will be competed against an isogenic, drug sensitive strain in 10 different medically and ecologically relevant environments.

Using these data, I set out to answer these questions:

1. What is the effect of environmental heterogeneity on the fitness of AMR *E. coli*?
2. Can fitness in one environment predict fitness in another?
3. Is there any relationship between productivity and fitness?

Given the results of previous studies (Hall, 2013; Hubbard, 2018; Remold & Lenski, 2001), I predict that fitness will be difficult to predict in heterogeneous environments, and that fitness in one environment will not predict fitness in another.

2 Chapter: Materials and Methods

2.1 Bacterial Isolates

Strains used in this experiment were isolated by Luria-Delbrück fluctuation assays on the corresponding antibiotic in Table 1 by a previous student in the Wong lab and single mutations were confirmed by whole-genome sequencing. The laboratory strain, *E. coli* K-12 (MG1655), was used as the ancestor for the resistant strains. MG1655 has a wild-type *lac* operon and thus forms blue colonies on media containing X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG (Isopropyl β -D-1-thiogalactopyranoside). *E. coli* NCM520 is isogenic to MG1655 but carries a deletion in the *lac* operon and therefore remains white on X-gal and IPTG. Mutant strains will be referred to by their mutation in this document, for clarity.

Table 1. *E. coli* strains used in this study.

Strain Name	Antibiotic	Mutation	Mechanism of Resistance
Cip5	Ciprofloxacin	<i>gyrA</i> D87G	Reduced binding to drug target site
Cip3	Ciprofloxacin	<i>gyrA</i> D87Y	Reduced binding to drug target site
Cip1	Ciprofloxacin	<i>gyrA</i> S83A	Reduced binding to drug target site
S83L	Ciprofloxacin	<i>gyrA</i> S83L	Reduced binding to drug target site
Rif1	Rifampicin	<i>rpoB</i> I572L	Reduced binding to drug target site
Rif7	Rifampicin	<i>rpoB</i> I572S	Reduced binding to drug target site
MG1655	none	none	none
NCM520	none	none	none

2.2 Environments

In this study, environmental media were chosen to examine the effect that they may have on the fitness of AMR *E. coli*. Table 2 below lists the ingredients, temperature, and reference (if applicable) for the medium used. Common laboratory media such as LB broth and M9 + Glucose were used in comparison because they are standard in microbiology experiments. Other media were chosen because they represent environments where AMR pathogens may be found, including media mimicking host environments (lower intestine, synthetic urine, synthetic colon), as well as media representing extra-host environments (river water, soil, sewage overflow). Primary and Anaerobic Sludge were collected by a student in Dr. Banu Ormeci's Laboratory, at Carleton University, Ottawa, Ontario. In-depth description of these media preparation methods can be found in Appendix B.1.

Table 2. Environment ingredient list and preparation instructions

Media	Ingredients	Temperature	Reference
LB Broth	LB Broth Miller – from Bioshop Canada, Burlington, ON Tryptone 10g/L Yeast extract 5g/L Sodium chloride 10g/L Preparation: 25g/L in water	37°C	
Minimal Media (M9) + Glucose	<i>1x Min Salts</i> 6.78g/L Na ₂ HPO ₄ Anhydrous Dibasic 3g/L KH ₂ PO ₄ 0.5g/L NaCl	37°C	

	<p>1g/L NH₄Cl</p> <p><i>Then add (to 1X Salts):</i> 2ml 1M MgSO₄ 100μL 1M CaCl₂ 0.8 % (w/v) glucose</p>		
Minimal Media (M9) + Gluconate	<p>Preparation modified from (Bleibtreu et al., 2013):</p> <p><i>1x Min Salts</i> 66.78g/L Na₂HPO₄ Anhydrous Dibasic 3g/L KH₂PO₄ 0.5g/L NaCl 1g/L NH₄Cl</p> <p><i>Then add (to 1X Salts):</i> 2ml 1M MgSO₄ (100μL 1M CaCl₂ 20mM gluconate final concentration (monosodium glutamate)</p>	37°C	(Bleibtreu et al., 2013) According to this reference, this environment is similar to lower intestine environment.
Synthetic Urine media	<p>Preparation was modified from (Laube, Mohr, & Hesse, 2001):</p> <p>400μL/250mL 1M CaCl₂ 0.73g/250mL NaCl 0.56g/250mL NaSO₄ 0.35g/250mL KH₂PO₄ 0.4g/250mL KCl 0.25g/250mL NH₄Cl 6.25g/250mL Urea 0.28g/250mL Creatinine</p> <p>- pH = 6.0</p>	37°C	(Laube et al., 2001)
Soil Media	<p>Collected on the shore of the Ottawa River on Carleton University on 25/9/17. It had not rained the week previously. Samples were dried out in aerated container until use.</p> <p>Preparation:</p> <p>3g soil in tea bag in 50mL H₂O. Steep for 48 hours.</p> <p>Supplemented with 10g/mL glycerol</p>	25°C	Adapted from (Kraemer & Kassen, 2015)

River water	<p>Collected on the shore of the Ottawa River on Carleton University on 25/9/17. It had not rained the week previously. Samples were frozen at -80°C until time of use.</p> <p>Preparation:</p> <p>The water was passed through a 0.22µM filter to remove particles, then autoclaved for 10 minutes (liq10) to kill bacteriophage present in the water. Glucose was added to a final concentration of 0.2% (w/v).</p>	25°C	Adapted from (Chai, 1983)
Synthetic Colon media	<p>Preparation was modified from (Polzin et al., 2013):</p> <p>6.25g/L Biotryptone 0.88g/L NaCl 2.7g/L KHCO₃ 0.43g/L KHPO₄ 1.7g/L NaHCO₃</p> <p><i>Then add:</i></p> <p>4.0g/L bile salts #3* 2.6g/L D-glucose (0.26% w/v)</p>	37°C	Adapted from (Polzin et al., 2013)
Combined Sewage Overflow	<p>Collected at the Main Street Combined Sewage overflow site (Ottawa, ON) on 1/10/17. It had rained the previous day. Samples were frozen at -80°C until time of use.</p> <p>Preparation:</p> <p>The water was passed through a 0.22µM filter to remove particles, then autoclaved for 10 minutes (liq10) to kill bacteriophage present in the water. Glucose was added to a final concentration of 0.2% (w/v).</p>	25°C	Adapted from (Chai, 1983)
Primary Sludge	<p>Primary Sewage Sludge was collected from Robert O. Pickard Environmental Centre (ROPEC) by a student in Banu Ormeci's Laboratory, Carleton University, Ottawa, ON. The primary sludge was collected from an outlet in a pipe. It has not been treated yet. Samples were frozen at -80°C until time of use.</p> <p>Preparation:</p> <p>The sludge was first passed through circular</p>	25°C	Adapted from (Chai, 1983)

	<p>filter paper with large porosity, then medium porosity, then fine porosity in order to remove solids (GE Whatman circular filter papers).</p> <p>The sludge was passed through a 0.22μM filter to remove particles, then autoclaved for 20 minutes (liq20) to kill microorganisms.</p>		
Anaerobic Sludge	<p>Anaerobic Sewage Sludge was collected from Robert O. Pickard Environmental Centre (ROPEC) by a student in Banu Ormeci's Laboratory, Carleton University, Ottawa, ON. It was collected from an anaerobic digester and has been treated. Anaerobic digestion reduces pathogens, reduces biomass quantity and produces a usable gas as a byproduct (methane). Samples were frozen at -80°C until time of use.</p> <p>Preparation: The sludge was first passed through circular filter paper with large porosity, then medium porosity, then fine porosity in order to remove solids (GE Whatman circular filter papers).</p> <p>The sludge was passed through a 0.22μM filter to remove particles, then autoclaved for 20 minutes (liq20) to kill microorganisms.</p>	25°C	Adapted from (Chai, 1983)

2.3 Yield Assays

Yield assays were completed by inoculating 20 μ L of overnight culture (that was grown in each respective media in Table 2) into 180 μ L of the respective fresh media in a 96-well plate and letting them grow for 18 hours in an orbital shaker at the temperatures corresponding to Table 2. Serial dilutions of the samples were performed so that 50-150 colonies could be counted on a plate per dilution. The plates were incubated at 37 °C in a stationary incubator for 24 hours and then colonies were counted with a ProtoCOL 3

colony counter (from Synbiosis, MD, USA). Each strain had 4 replicates. Colony forming units (CFU) per mL were calculated using this equation:

$$\frac{CFU}{mL} = \frac{(number\ of\ CFU)}{(Volume\ plated(mL))(total\ dilution\ used)}$$

2.4 Competitive Fitness Assays

The fitness of each mutant genotype in each environment was estimated using a competitive fitness assay in which the mutant genotype competed against the ancestral strain MG1655. To begin, an agar plate was streaked with inoculum from a frozen stock (- 80°C in 50% (v/v) glycerol) of each strain and was grown overnight in a 37°C stationary incubator. All strains listed in Table 1 were inoculated by picking a colony off an agar plate and inserting into 200 µL of the medium of interest (Table 2) in 96-well plates with lids (96-well microtest plate with lid, sterile, from Sarstedt, Germany) and parafilm to seal. The experiment consisted of 4 replicates of each strain. The populations were acclimatized to each respective medium for 18 hours at the corresponding temperature (Table 2) in an orbital shaker (150 rpm). Then this culture was diluted 1:10 into the same medium in a 96-well plate for another 18 hours in an orbital shaker (150 rpm) at the corresponding temperatures to ensure that the effect of the glycerol was gone.

After the 18 hours, these cultures of the mutant genotypes and the ancestral MG1655 strain were then diluted 1:100 in the media of interest and mixed at a 1:1 ratio in a 96-well plate, and the plates were allowed to mix in an orbital shaker (150 rpm) for

30 minutes. This initial culture is designated as “time 0 hours.” From the “time 0 hours” mixed culture, 50 μL was taken from each well and placed into a new 96-well plate and frozen at -80°C with 80 μL of 20% (v/v) glycerol in 96-well plates (these samples are “T0”).

The remaining 150 μL of the sample was grown for 24 hours in a 96-well plate in an orbital shaker (150 rpm) at the temperature corresponding to the medium so that both competitors were grown in the competitive medium for 24 hours, and this was designated as “time 24 hours”. After 24 hours, the cultures were frozen at 80°C with 80 μL of 20% (v/v) glycerol (these samples are “T24”). The figure (Figure 1) shown below is a visual schematic of the competitive fitness assay.

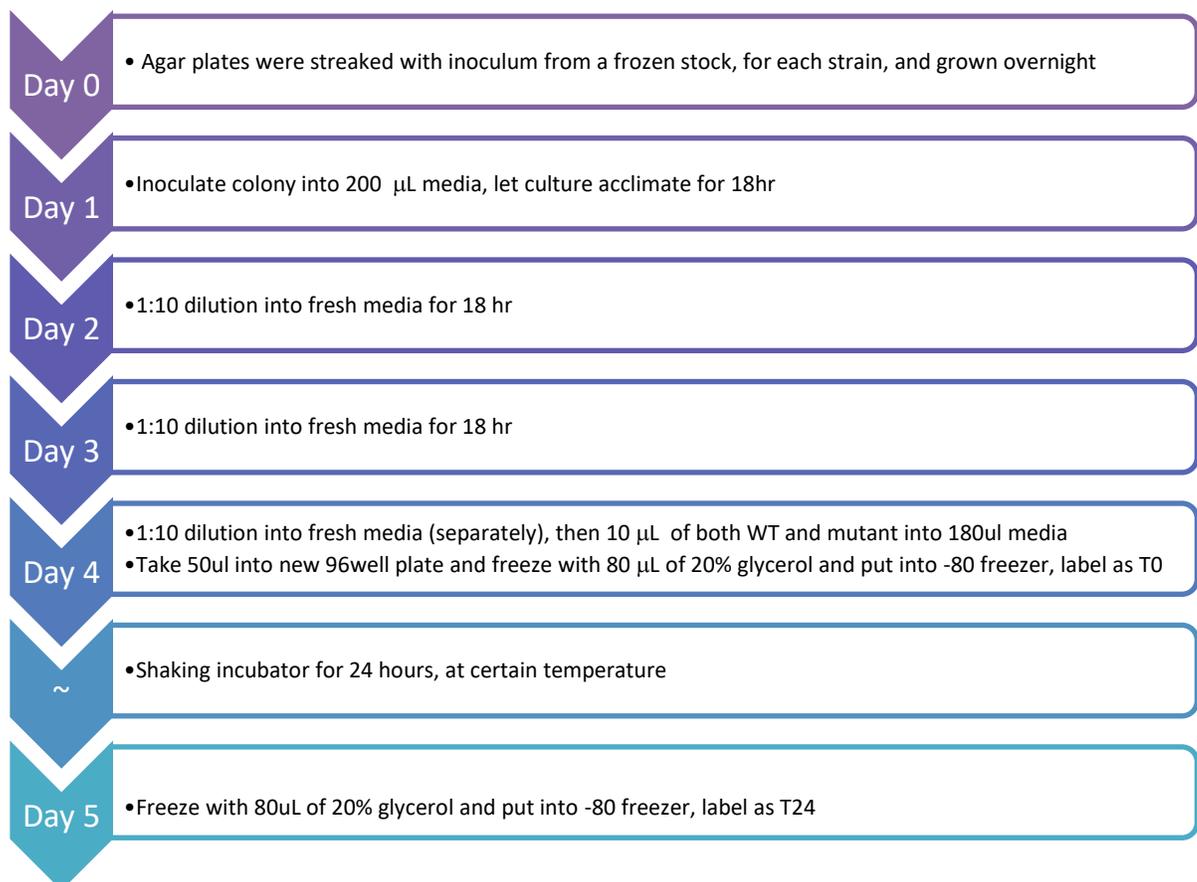


Figure 1. Schematic of competitive fitness assay

The frequency of the AMR mutant allele was determined by sequencing (see below). Competitive fitness assays were completed all at the same time, such that all 6 AMR strains were competed against MG1655 in all 10 environments.

A second set of competitive fitness assays were carried out in which the frequencies of the mutant and WT genotypes were determined by plating on LB+Xgal+IPTG agar plates. Here, NCM520 was used as the competitor instead of MG1655 so that blue/white screening could be used to distinguish between genotypes. These competitions were completed all at the same time for all strains in 5 environments (could not be completed in Anaerobic Sludge, Primary Sludge, Soil, Colon, or Combined Sewage Overflow due to limited quantities of these media).

2.5 Sequencing

Mutant allele frequencies were estimated by next-generation sequencing (NGS). The protocol from Illumina's 16S Metagenomic Library Prep Guide (Illumina, 2013) was adapted for this experiment. Instead of amplifying the 16S region, the region where the mutation is in the AMR strain was amplified with region-specific primers. By sequencing the culture from the competitive fitness assays using primers that amplify the mutation regions, the number of reads of the wild type and AMR strains can be counted and used

to estimate relative fitness measurements. Detailed instructions on the library preparation and steps leading up to sequencing can be found in the Appendix B.2

Firstly, primers were made to amplify a 150 bp region, with the AMR mutation at the center (Table 3). Reverse and forward primers were made for each AMR strain using NCBI's PrimerBLAST program (Ye et al., 2012). To these sequences, Illumina adapter overhang nucleotide sequences were added (Illumina, 2013). Out of the PrimerBLAST queries returned, the top one was selected and then ordered from IDT DNA (Integrated DNA Technologies, Inc., Illinois, USA, www.idtdna.com) as a dried oligo. Optimum annealing temperature was determined by performing a gradient PCR and running it on a 0.8% agarose gel and selecting the temperature at which the brightest band was formed. These primers were tested by PCR and proved to be specific and robust, providing a single solid band on an 0.8% agarose gel. A gradient PCR was performed to determine optimum annealing temperature of all primers.

Table 3. Primer sequences.

Strains	Primer name	Annealing Temperature (°C)	Forward Primer	Reverse Primer	Product Length
<i>gyrA</i> D87G, <i>gyrA</i> D87Y, <i>gyrA</i> S83A, <i>gyrA</i> S83L	<i>gyrA</i> 82-87	60-70	AAAATCTGCCCG TGTCGTTG	GCCGTCGATAGA ACCGAAGT	151
<i>rpoB</i> I572L, <i>rpoB</i> I572S	<i>rpoB</i> I572L	55-70	TACACCCGACTC ACTACGGT	CACCGTCGGTCAC TTTACGA	144

2.6 Bioinformatics and Fitness Calculations

The time “0 hours” and “24 hour” cultures were sequenced using an Illumina MiSeq, using paired-end 300 bp reads. Only the region where the AMR mutation resides was sequenced so that the AMR mutant and wildtype could be counted using the sequencing reads. A custom bash script was then used to clean the reads. In this script, quality was assessed with FastQC (Andrews, 2010), then reads were trimmed with Trimmomatic (parameters: leading=20, trailing=20, window_length=4, window_qual=20, min_length=36)(Bolger, Lohse, & Usadel, 2014). Quality was assessed again using FastQC then reads were merged using Flash (parameters: min_overlap=20, max_overlap=250)(Magoč & Salzberg, 2011). MultiQC was then used to assemble FastQC data for all files before and after trimming (Ewels, Magnusson, Lundin, & Käller, 2016). The “read process” script can be found in Appendix C.1.

Next, another custom bash script was used in order to get the frequency of mutant and wild type reads printed to a .tsv file. In this script, the mutation and wild type allele sequence were searched for in the reads using ‘grep.’ The “Fastq to Freq” script can be found in Appendix C.2.

For each mutant in each environment, the selection coefficient, s , was calculated as per (Dykhuizen & Hartl, 1983):

$$s = \left(\ln \left(\frac{AMR \text{ at } T24}{AMR \text{ at } T0} \right) - \ln \left(\frac{WT \text{ at } T24}{WT \text{ at } T0} \right) \right) / \text{Number of generations}$$

Relative fitness (w) was then calculated as $1+s$, where the units for both w and s are in per generation (Dykhuisen & Hartl, 1983; Wong, Rodrigue, & Kassen, 2012).

2.7 Statistical Analysis and Visualization

All statistical analyses were performed in R version 3.5.1 (“R: A language and environment for statistical computing. R Foundation for Statistical Computing,” 2018) and all graphing of fitness data and yield data was done using GraphPad Prism (GraphPad, 2016). A script including all commands used in R is included in Appendix. Below is a table to summarize R packages and software used.

Table 4. Summary of statistical analyses packages and graphing programs used in this study.

Test/Figure type	Program used	Reference
Two-way and three-way ANOVAs	R packages: R base package readxl	(“R: A language and environment for statistical computing. R Foundation for Statistical Computing,” 2018) (Wickham & Bryan, 2018)
Graphs on fitness data and yield data	GraphPad Prism version 6	(GraphPad, 2016)
Heatmap of correlation values	R packages: ggplot2 reshape2 Hmisc readxl	(Wickham, 2016) (Wickham, 2007) (Harrell, 2018) (Wickham & Bryan, 2018)
Principal Component Analysis	R packages: missMDA FactoMineR factoextra readxl	(Josse & Husson, 2016) (Le, Josse, & Husson, 2008) (Kassambara & Mundt, 2017) (Wickham & Bryan, 2018)

3 Chapter: Results

3.1 Fitness of 10 AMR strains of *E. coli* in 10 environments

“The little beggars are doing just what I don’t want them to.”

Charles Darwin

Relative fitness of six AMR strains (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) was estimated via competitive fitness assays in 10 different media (LB broth, Glucose, Gluconate, Urine, River, Anaerobic Sludge, Primary Sludge, Colon, Combined Sewage Overflow and Soil). We find evidence of variability in fitness between genotypes across environments (Figure 2). For example, while some strains in certain environments have increased fitness in comparison to the WT (such as the *rpoB* mutants in Combined Sewage Overflow) others have decreased fitness (*rpoB* I572L in Soil). Overall, it seems that fitness costs are not common, with most strains staying at similar fitness to the wild type (around 1) or having increased fitness (higher than 1). Unfortunately, the fitness of *gyrA* S83L in LB broth could not be calculated because of sequencing error, so these 4 replicates were not included in analysis.

A two-factor ANOVA (Fitness ~ Environment* Genotype, Table 5) on these fitness data found significant effects of environment ($P = < 2e-16$, below in Table 5), genotype ($P = 1.21e-06$), and their interaction ($P = 2e-16$). Differences between environments are illustrated in Figure 3, which shows the average relative fitness of all

strains in each environment, such that overall fitness in each environment can be visualized. It can be seen, for example, that fitness is generally lower in Anaerobic sludge, and higher in Combined Sewage Overflow and in Gluconate. Similarly, differences between genotypes be seen in Figure 4, which illustrates the average relative fitness of each strain in all the environments, such that overall fitness of each genotype can be visualized.

The significant interaction between genotype and environment (Table 5) means that the genotypes responded differently to different environments. This interaction can also be seen visually in Figure 5 as different genotypes responds to some environments differently – for example *rpoB* I572L has high fitness in Combined Sewage Overflow and low fitness in Soil, while *rpoB* I572S has high fitness in both. This can also be seen in Figure 6, where average relative fitness of genotypes plotted against environment and each genotype has a separate plot.

Table 5. A two-way ANOVA on fitness estimates for 6 AMR genotypes in 10 environments.

Independent Variable	Df	Sum Sq	Mean Sq	F	P (*<0.05)
Environment	9	2.244	0.24928	25.236	< 2e-16
Strain	5	0.385	0.07691	7.786	1.21e-06
Environment*Strain	44	4.522	0.10278	10.405	< 2e-16

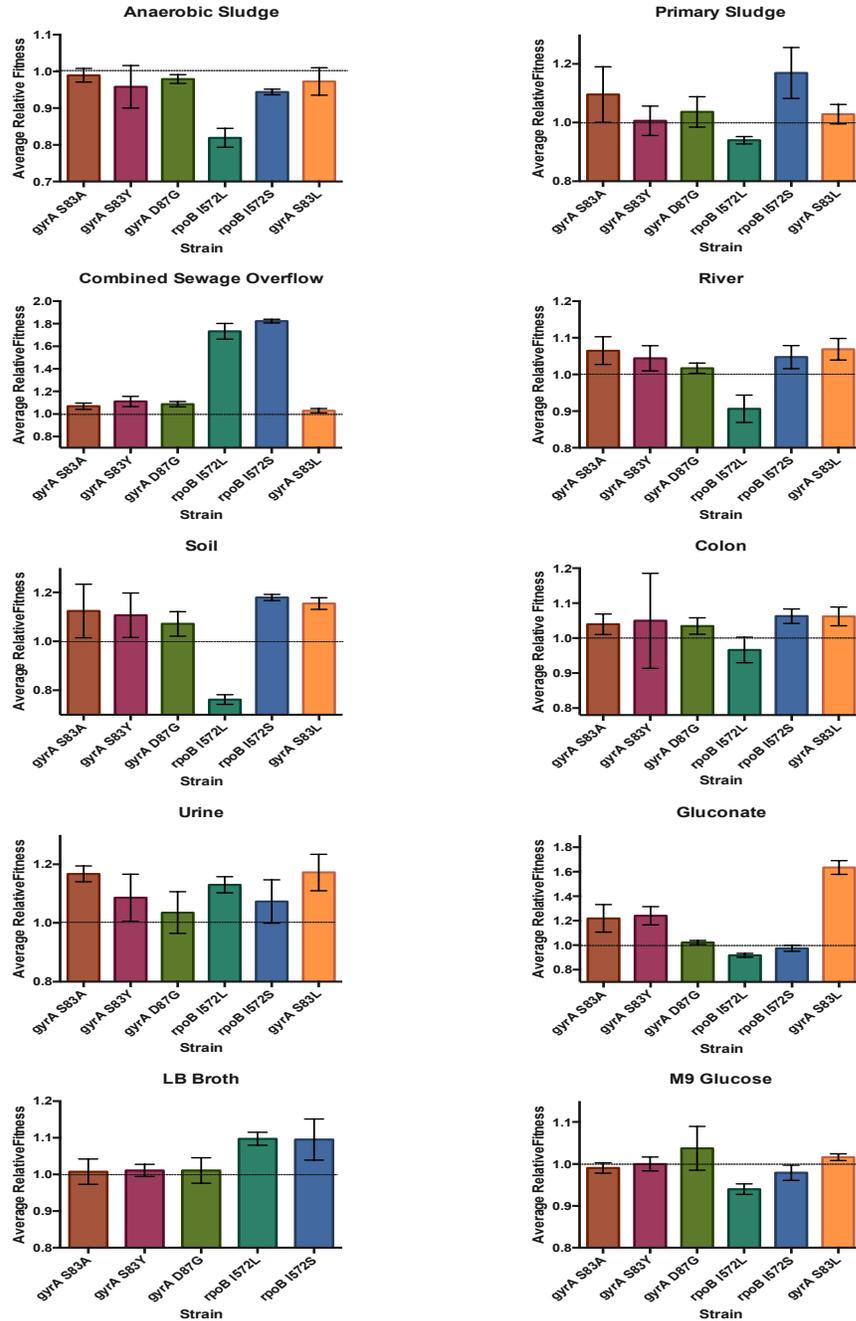


Figure 2. Average relative fitness of AMR *E. coli* in 10 different environments. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, MG1655, in the media of interest. Average relative fitness was calculated by sequencing the area where the AMR mutation was located and calculating change in frequency over 24 hours. A two-factor ANOVA (Fitness ~ Environment* Genotype) found significant effects of environment ($P = < 2e-16$), genotype ($P = 1.21e-06$), and their interaction ($P = 2e-16$). Mean values from 4 replicates of each strain and +/- S.E.M. are shown. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Fitness of gyrA S83L in LB is not included.

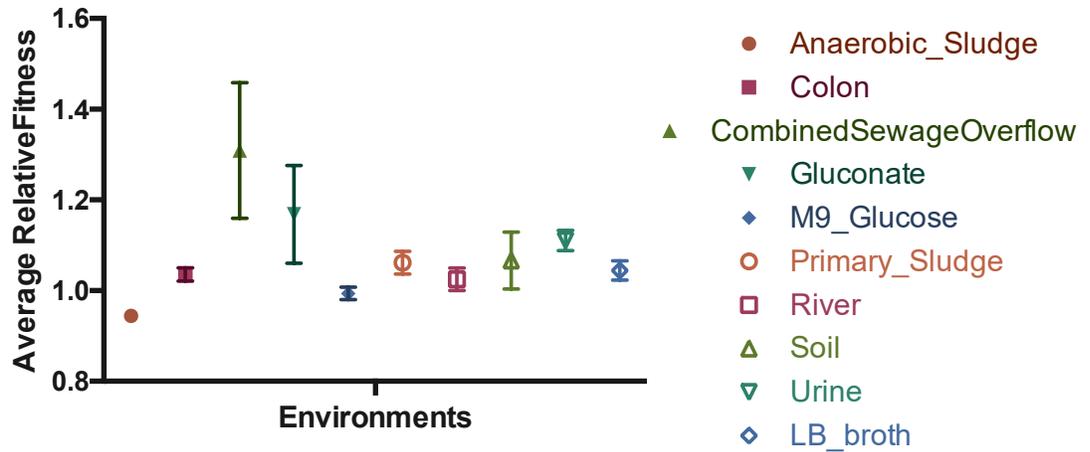


Figure 3. Average relative fitness of all AMR strains by environment. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, MG1655, in the media of interest. Average relative fitness was calculated by sequencing the area where the AMR mutation was located and calculating change in frequency over 24 hours. All fitness values for all strains were averaged by environment. Mean of mean fitness for 6 strains in 10 environments is shown, with S.E.M. +/- . Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Fitness of *gyrA* S83L in LB is not included.

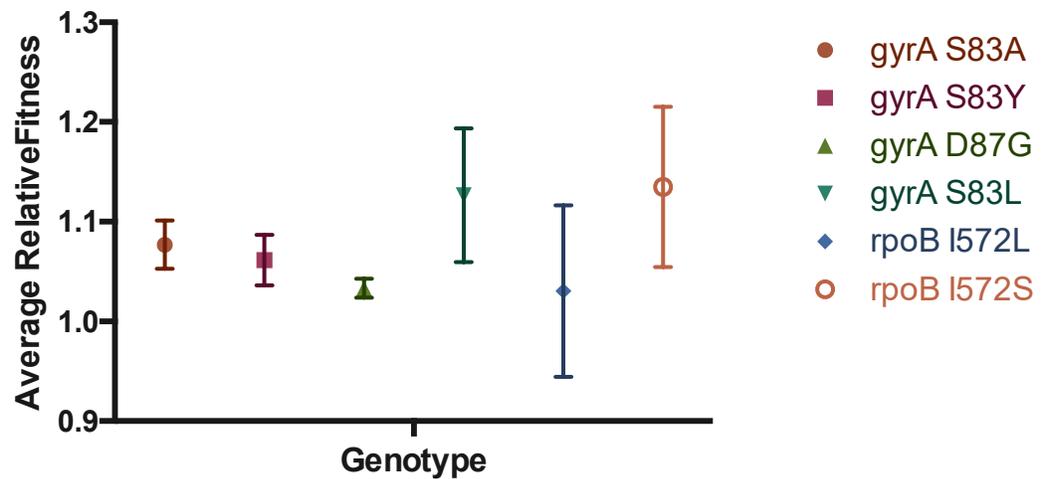


Figure 4. Average relative fitness of all AMR strains by genotype. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, MG1655, in the media of interest. Average relative fitness was calculated by sequencing the area where the AMR mutation was located and calculating change in frequency over 24 hours. All fitness values for all strains in all environments were averages. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Mean of mean fitness for 6 strains in 10 environments is shown, with S.E.M. +/- . Fitness of *gyrA* S83L in LB is not included

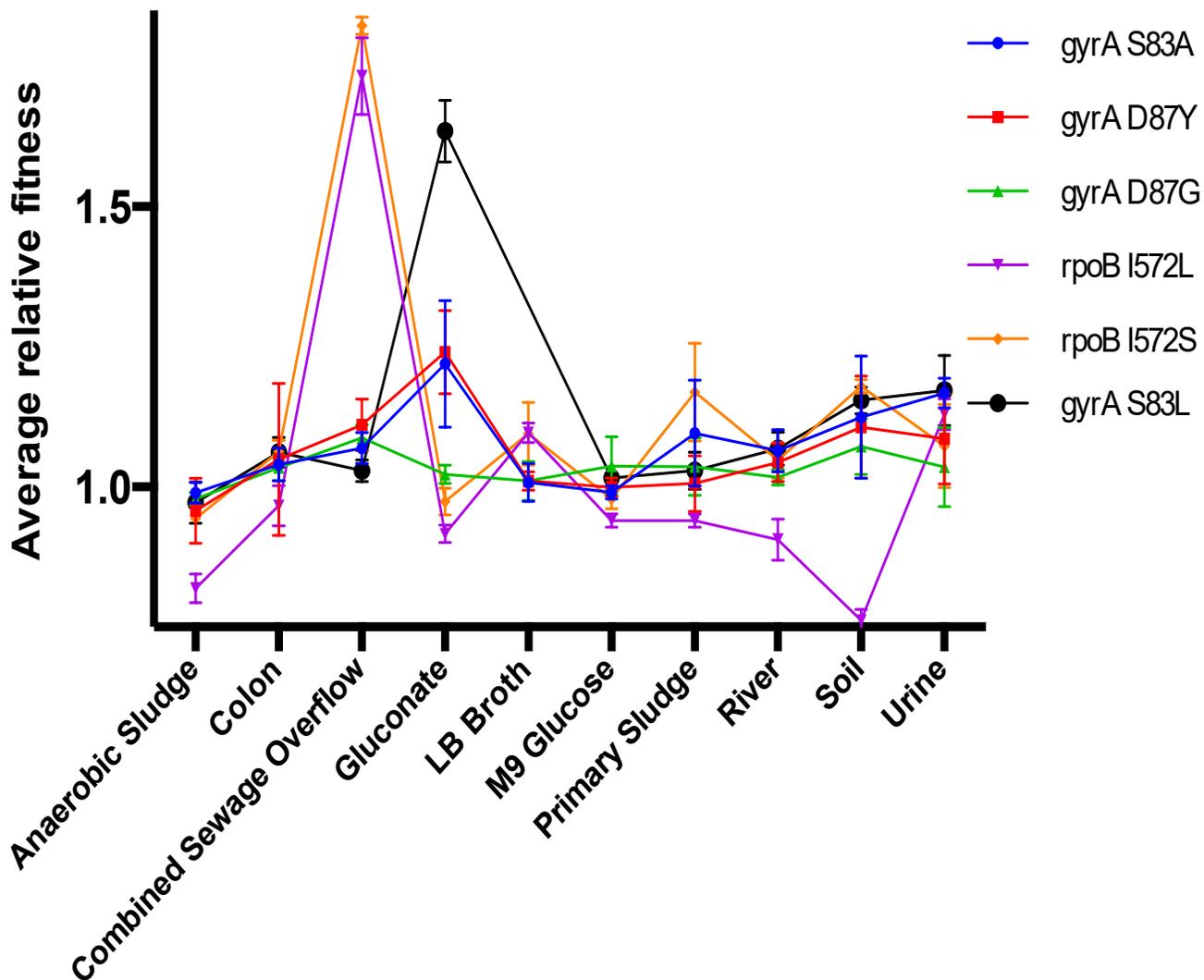


Figure 5. Average relative fitness of AMR genotypes (legend) by environment. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, MG1655, in the media of interest. Average relative fitness was calculated by sequencing the area where the AMR mutation was located and calculating change in frequency over 24 hours. Average relative fitness of all 6 genotypes (legend) was plotted against environment. Mean of 4 replicate fitness measurement for each genotype in each environment is shown, with S.E.M. +/- error bars. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Change in rank order of fitness of genotypes between environments can be seen. Fitness of *gyrA* S83L in LB is not included.

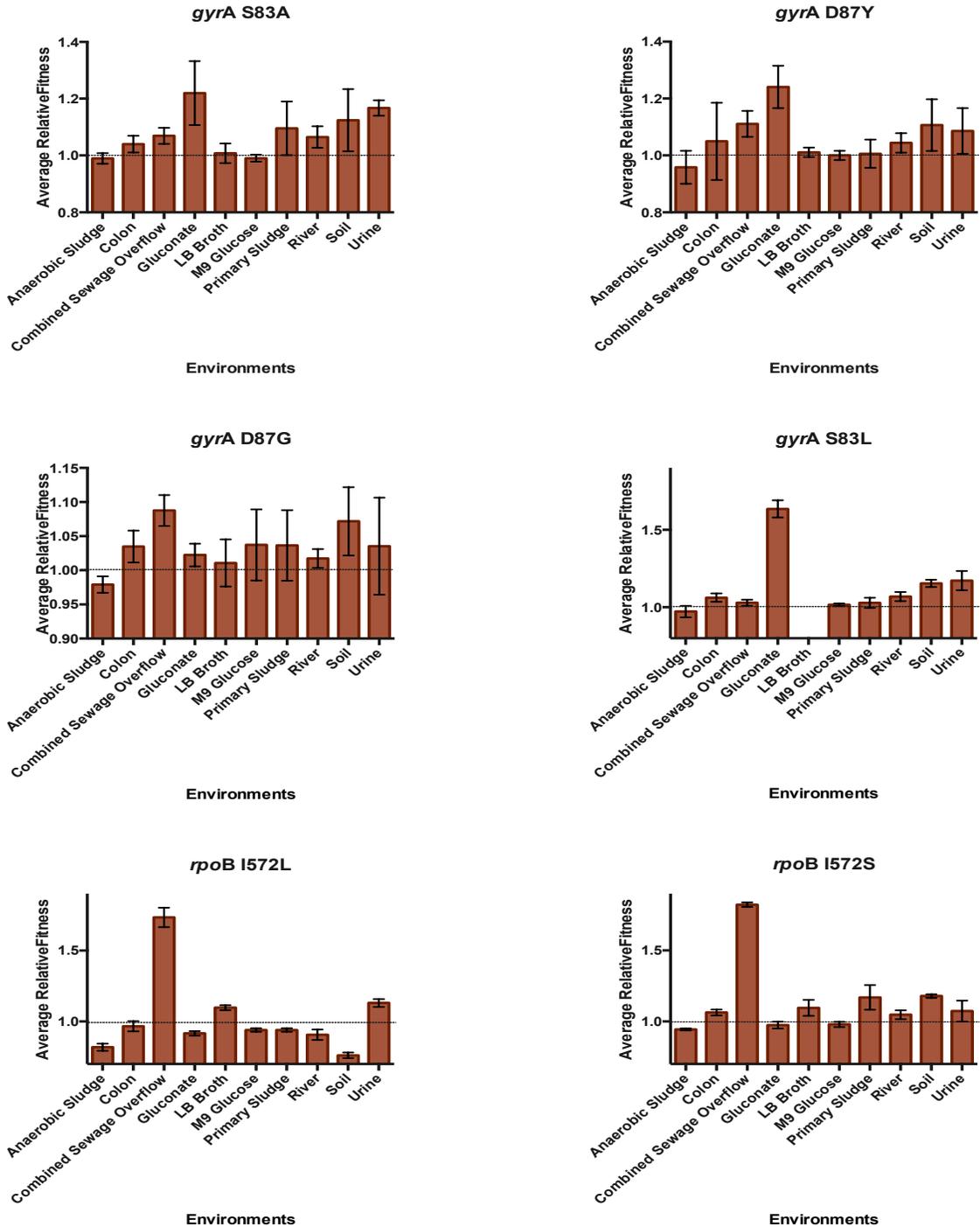


Figure 6. Average relative fitness of genotypes in each environment, by genotype. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, MG1655, in the media of interest. Average relative fitness was calculated by sequencing the area where the AMR mutation was located and calculating change in frequency over 24 hours. Mean of 4 replicate fitness measurement for each genotype in each environment is shown, with S.E.M. +/- error bars. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Fitness of *gyrA* S83L in LB is not included.

3.2 Predicting the fitness of AMR mutants with limited data

Although we found a significant interaction between genotype and environment (ANOVA Table 5), this does not rule out the possibility that some environments closely resemble each other. As such, we asked to what extent fitness is correlated between environments. Pearson's r was calculated between environments for the 6 AMR strains, using fitness estimates from sequencing. Figure 7 shows a heat map of pairwise correlations between environments for the sequencing data, with stronger correlations in deep red (positive) or blue (negative). Looking at the heat map for the sequencing data, Soil and River have a strong positive correlation ($r \approx 0.83$, $P < 0.04$), which means that fitness estimates in these two environments are similar. From looking at this Figure, only 8 interactions seem to be significant and they are dark red in colouring (indicating a positive correlation). Overall, strong correlations between environments are few, and no single medium is representative of overall fitness.

A principal component analysis was completed on the fitness estimates from the sequencing data in order to summarize the data set by reducing the dimensionality of the data without losing important information. Data for *gyrA* S83L in LB was not included. A biplot was created to visualize this PCA analysis with individual variances and variable variances, shown in Figure 8. Loadings are indicated by the arrows. The variables that are close together on the PCA are the ones that have similar fitness profiles – for example, the *gyrA* mutants cluster closely together.

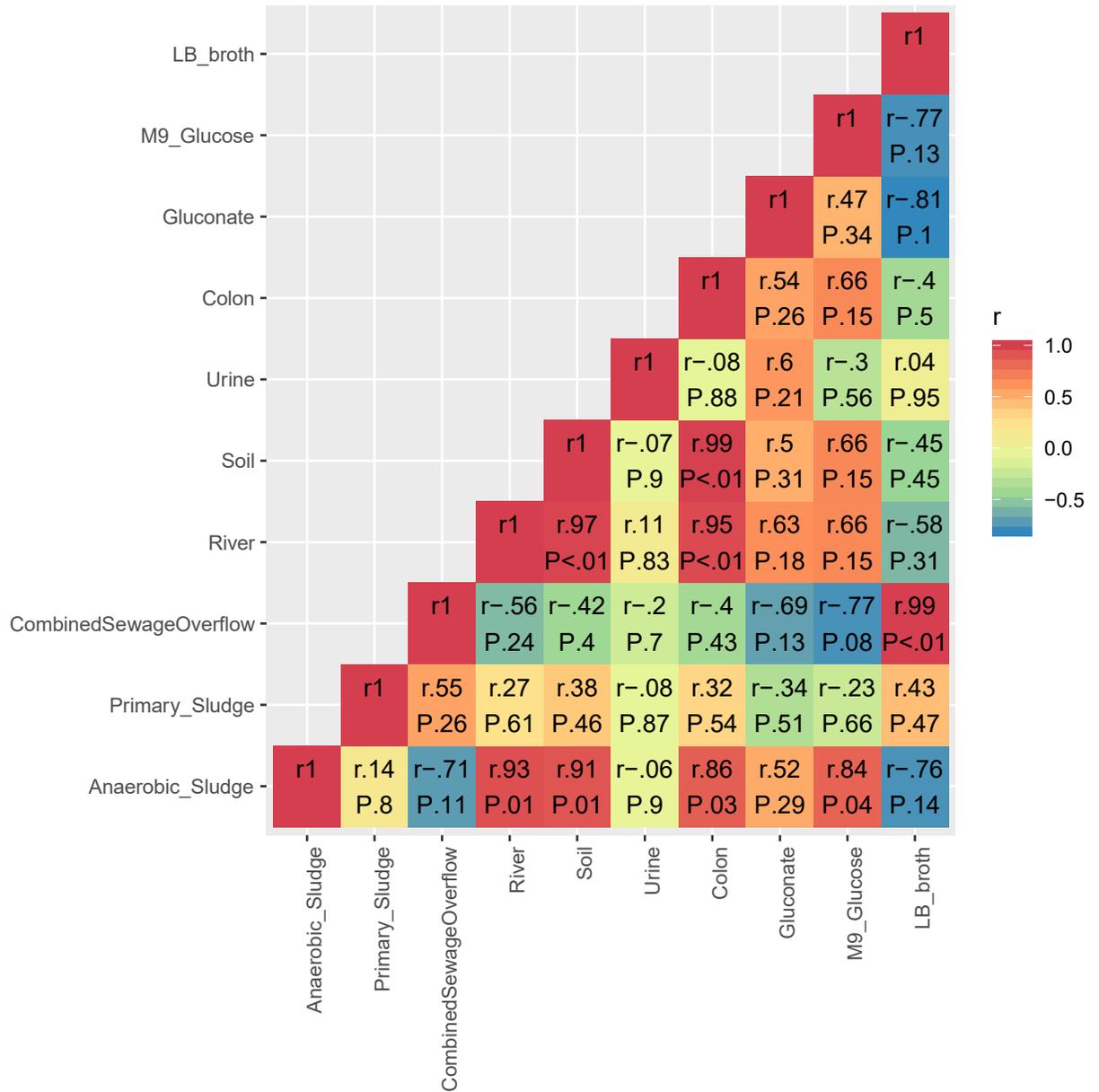


Figure 7. Pearson correlation heatmap with correlation coefficient and significance levels based on the mean fitness value of 4 replicates of each strain (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in each environment (LB broth, Glucose, Gluconate, Urine, River, Anaerobic Sludge, Primary Sludge, Colon, Combined Sewage Overflow and Soil) measured by sequencing competitive fitness assays. Data for *gyrA* S83L in LB was not included. An interaction was considered significant when P is <0.05, and P values are indicated in text on the plot. The colour coordinated legend (“r” for Pearson’s rho) indicates the value and sign of Pearson’s correlation coefficient, which is also indicated as a value on the plot (r).

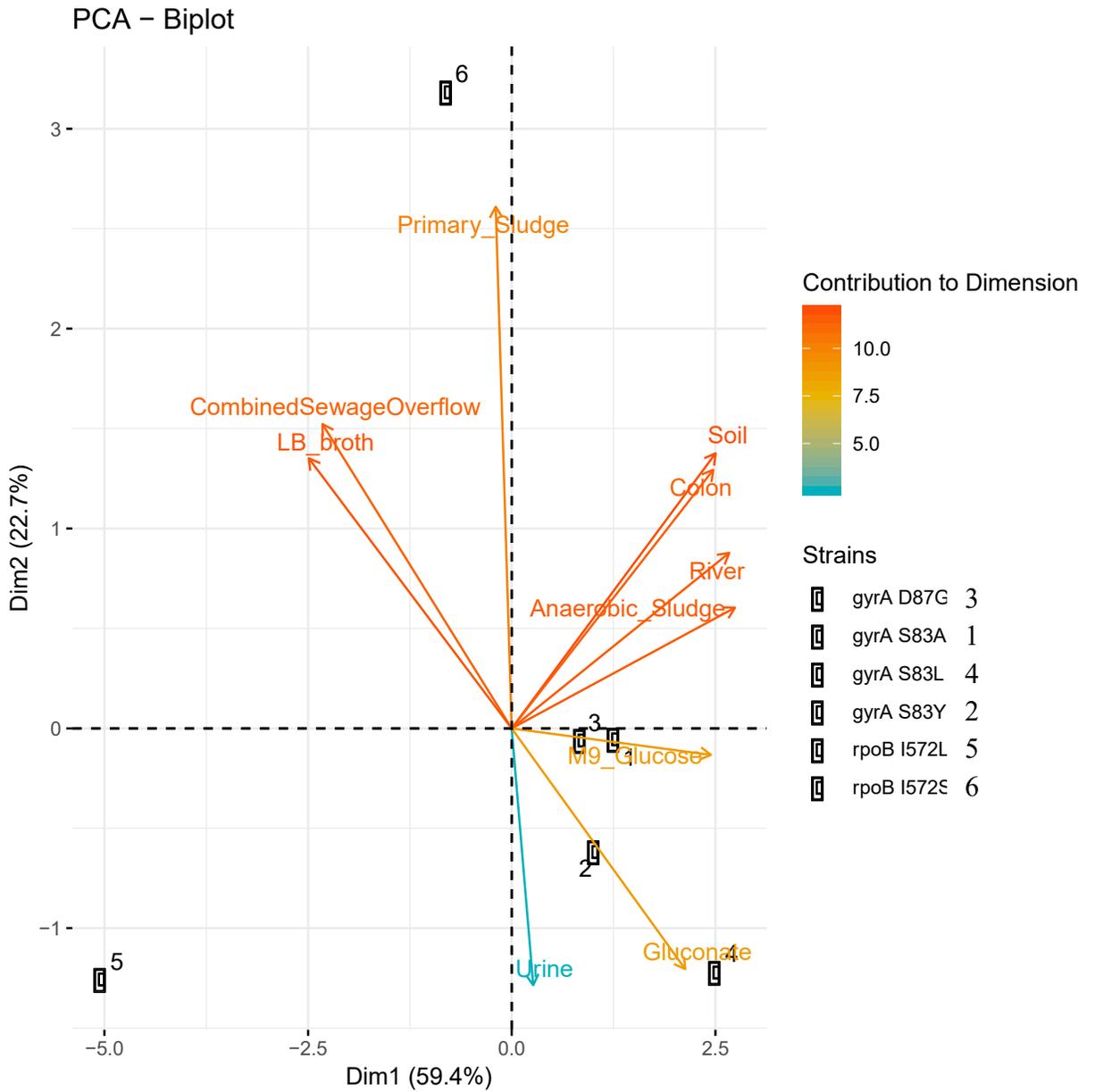


Figure 8. Principal component analysis on mean fitness values 6 AMR strains (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in 10 different media (Lb broth, Glucose, Gluconate, Urine, River, Anaerobic Sludge, Primary Sludge, Colon, Combined Sewage Overflow and Soil) measured by sequencing. Data for *gyrA* S83L in LB was not included. Variable colours were colour-coded according to their contribution (“Contribution” legend) to the principal axis. Individuals are labeled according to legend (“Strains”).

3.3 Relationships between productivity and fitness

In order to test for a relationship between productivity and fitness costs, I carried out yield assays on 4 replicates of 6 AMR strains of *E. coli* (and 2 wildtype strains (MG1655 and NCM520) in 7/10 (Soil, River, Urine, Colon, Gluconate, LB broth, and Glucose) environments by plating dilutions of overnight culture onto LB agar plates and counting colonies. Every strain grew to over 10^6 CFU/ml, as shown in Figure 9.

Next, a linear regression was performed in order to see whether yield values can predict fitness and is shown in Figure 10. All available yield and fitness (sequencing method) data was used. The coefficient of determination, R^2 , was very low (0.03382) indicating that yield and fitness values are not well correlated. In addition, the P-value is over 0.05, indicating that the slope of the regression line is not significantly different from 0. This suggests that there is no relationship between the fitness and yield values, positive or negative. Overall it appears that yield is not an ideal predictor of fitness.

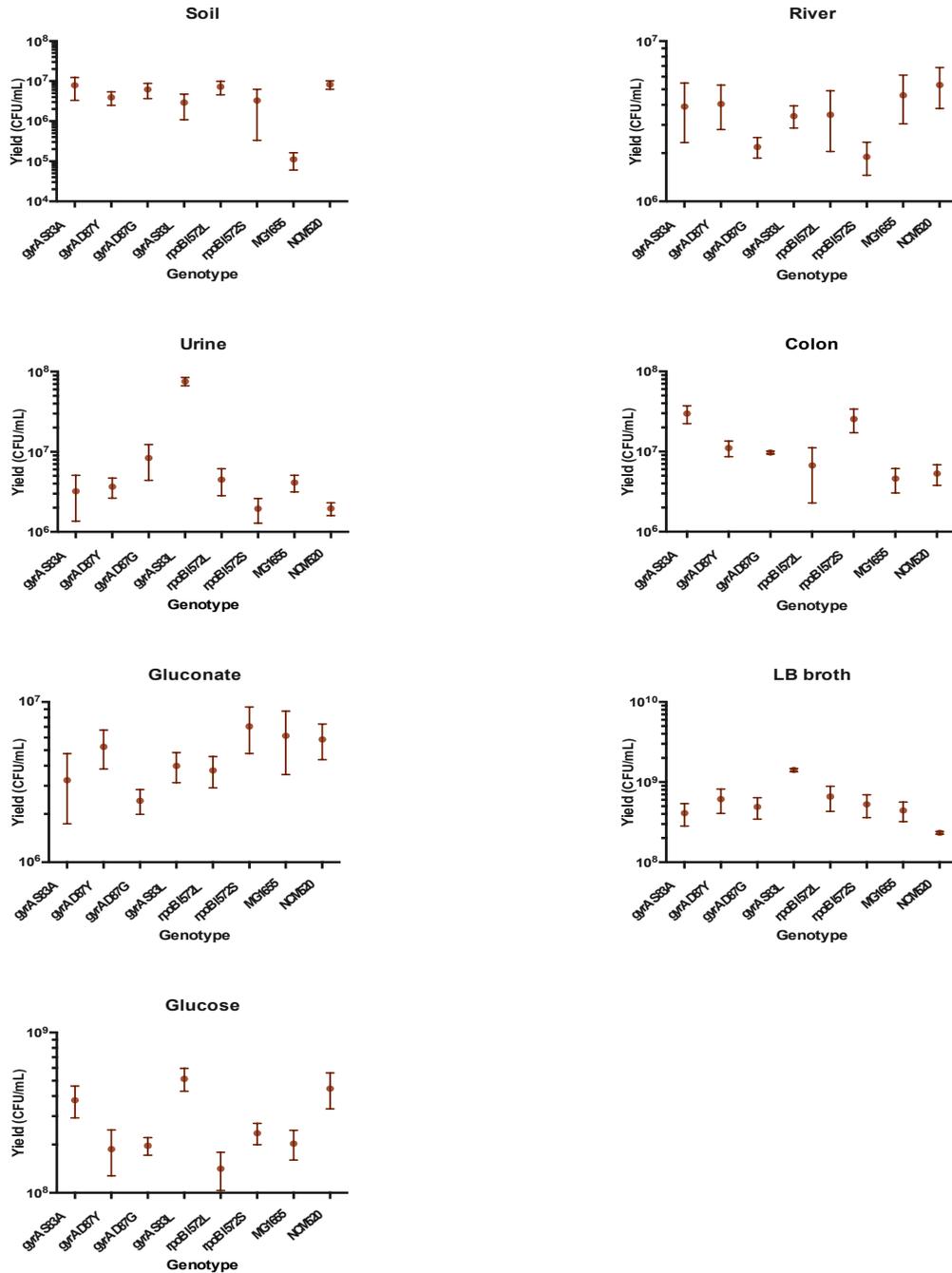


Figure 9. Yield assays on 4 replicates of 6 AMR strains of *E. coli* (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) and 2 wildtype strains (MG1655 and NCM520) in 7/10 (Soil, River, Urine, Colon, gluconate, LB broth, and Glucose) environments measured by plating dilutions of overnight culture onto LB agar plates and counting colonies. Data for *gyrA* S83L in Colon was not obtained. Mean of 4 replicates per strain in each environment is shown, with S.E.M. +/- error bars.

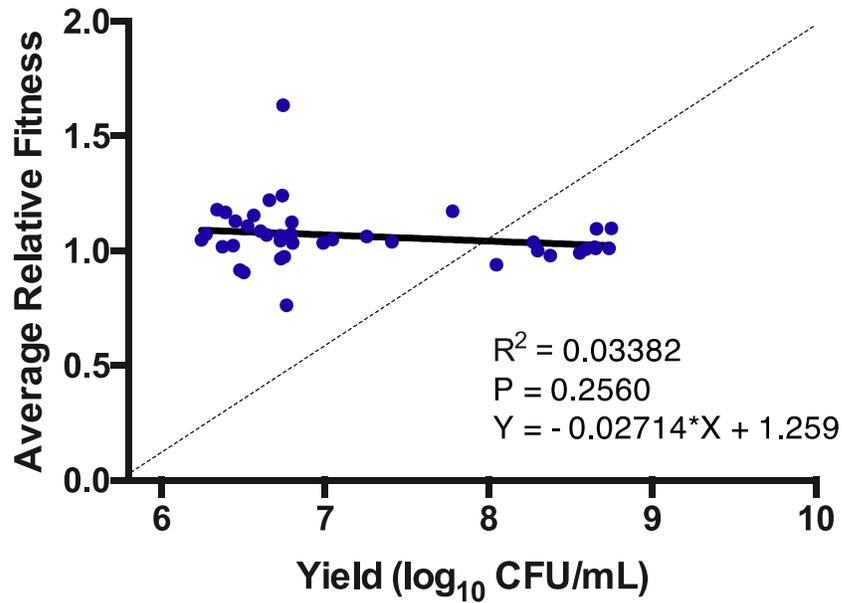


Figure 10. Yield values (log₁₀ CFU/mL) versus average relative fitness of 6 AMR strains of *E. coli* (*rpoB* I572L, *rpoBI*572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in 7 environments (Soil, River, Urine, Colon, Gluconate, LB broth, and Glucose). Yield was measured by plating dilutions of overnight culture onto LB agar plates and counting colonies. Fitness was estimated by sequencing competitive fitness assay cultures and counting wildtype and AMR reads. Fitness data is missing for S83L in LB (no reads) and s83L yield assay data in Colon media. The mean value for yield and fitness of a certain strain in a certain environment is shown. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type.

3.4 Reproducibility of fitness assays

Head-to-head competitive fitness assays were performed on the 6 AMR strains versus the *lac*⁻ wildtype strain, NCM520, in 4 replicates in 5 environments (LB broth, Glucose, Gluconate, Urine, and River). Average relative fitness was calculated by plating competition cultures on IPTG+X-gal agar plates, in order to compare this traditional method of measuring fitness to our sequencing method. A linear regression was performed in order to see if there is a linear relationship between these two methods (Figure 11). I found a moderate correlation between fitness estimates obtained by these two methods (Figure 11; $R^2 = 0.3158$, $P = 0.0023$). For example, in the Glucose media, both the plating method and the sequencing methods seem to have similar fitness values, as can be seen visually in Figure 2 and Figure 13. Notably, *rpoB* suffers a fitness cost with its fitness being around 0.9. In the synthetic Urine media, both the plating method and the sequencing methods also have similar fitness values. For example, *gyrA* S83A and *gyrA* S83L both have fitness values around 1.2 and *gyrA* D87G has the lowest fitness in both methods. Notably, fitness estimates obtained from sequencing tended to be higher than those from plating, with most data points falling below the one-to-one line on Figure 11. This suggests that one of the methods may be systematically biased in its fitness estimates. In addition, when 4 visually apparent outliers were removed (Figure 12), this greatly improved the R^2 value, increasing it to 0.7279. Given, this improvement, it would be interesting to see if more data points would increase the R^2 value and decrease the influence of the outliers. In Figure 11 and 12, it looks like the outliers have a large effect on R^2 .

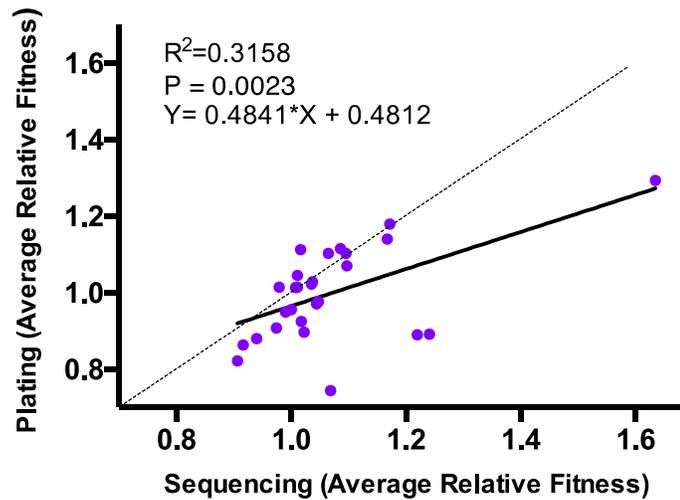


Figure 11. Average Relative fitness of plating method (y) versus average relative fitness of sequencing method (x) for 6 AMR strains (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in 5 environments (Glucose, Gluconate, LB, Urine, and River). Data for *rpoB* I572L and *rpoB*I572S in Urine media and *gyrA* S83L in LB was not included. Mean values of 4 replicates for each strain were used. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. The correlation coefficient (R^2) is 0.3158 and the equation of the linear regression is $y = 0.4841x + 0.4812$.

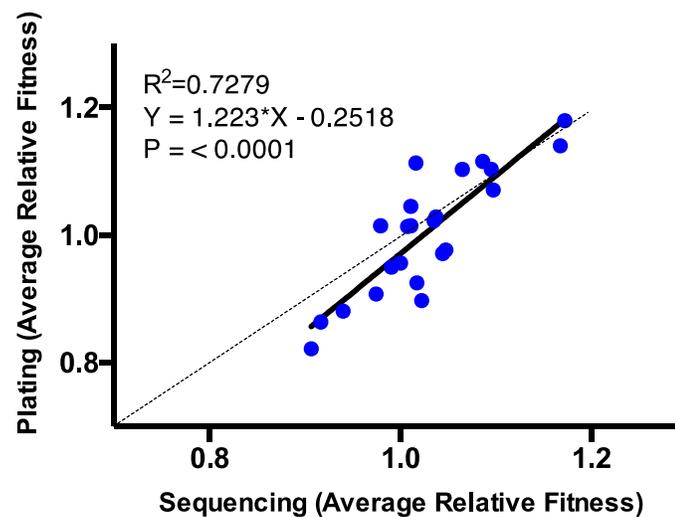


Figure 12. Average Relative fitness of plating method (y) versus average relative fitness of sequencing method (x) for 6 AMR strains (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in 5 environments (Glucose, Gluconate, LB, Urine, and River) with 4 major outliers removed. Mean values of 4 replicates for each strain were used. Data for *rpoB* I572L and *rpoB*I572S in Urine media and *gyrA* S83L in LB was not included. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. The correlation coefficient (R^2) is 0.7279 and the equation of the linear regression is $y = 1.223x + 0.2518$.

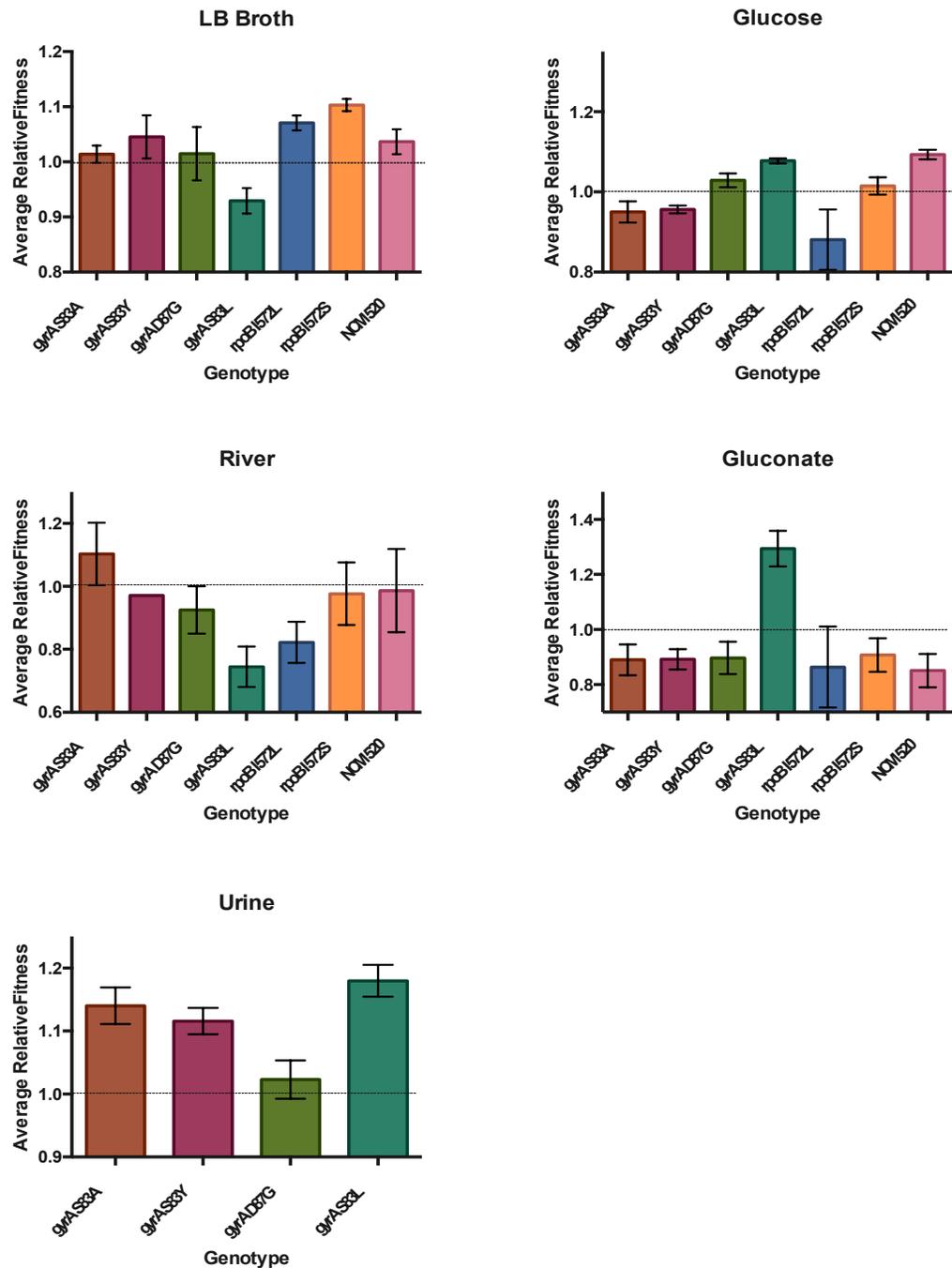


Figure 13. Average Relative Fitness of AMR *Escherichia coli* in 5 different environments. Head-to-head competitive fitness assays were performed on the AMR strain of interest versus the wildtype strain, NCM520, in the media of interest. Average relative fitness was calculated by plating competition cultures on IPTG+X-gal agar plates counting colonies. Mean values from 4 replicates of each strain and +/- S.E.M. are shown. Fitness above the value “1” means that a strain has a higher fitness than the wild type, and fitness below “1” means that a strain has lower fitness than the wild type. Data for *rpoB* I572L, *rpoB*I572S, and NCM520 in Urine is not shown.

Broad trends that were observed using the sequencing method were also seen with plating (Figure 13). A two-way ANOVA on the plating data found main effect and interaction terms (Table 6), much like the sequencing data (Table 5). Similar to the sequencing data, I found few significant correlations between environments using the plating method when a Pearson correlation was performed (Figure 14). Thus, regardless of the method used to measure allele frequencies, we find substantial and unpredictable variation in fitness between environments and genotypes.

Table 6. A two-way ANOVA on the Average Relative Fitness data from counting colonies on IPTG + X-gal LB agar plates (Average Relative Fitness ~ Environment and Genotype).

Dependent Variable	Df	Sum Sq	Mean Sq	F	P (*<0.05)
Environment	4	0.4133	0.10332	8.23	1.19e-05
Strain	5	0.1484	0.02968	2.364	0.0466
Environment: Strain	18	0.9703	0.05391	4.294	2.33e-06

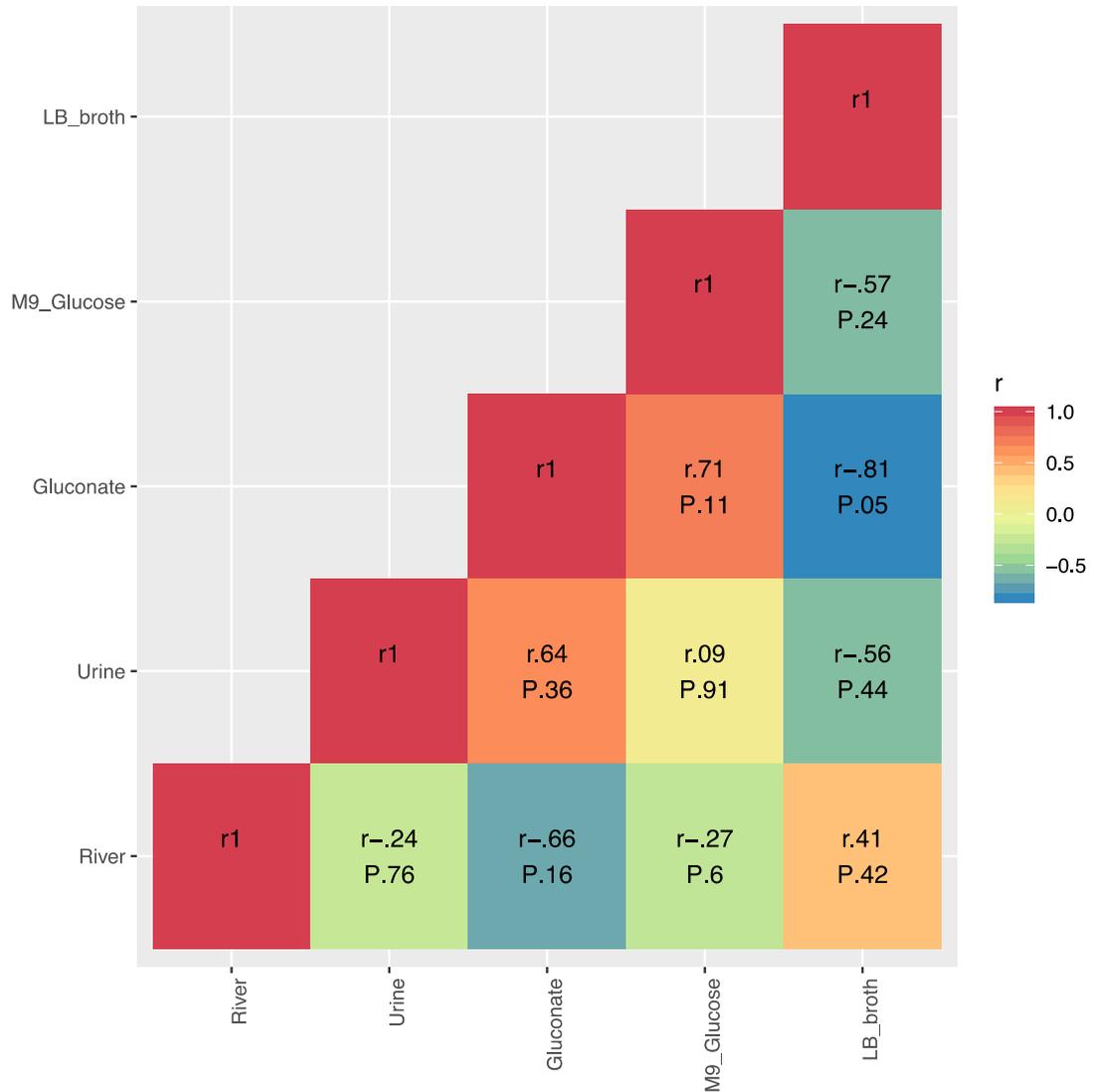


Figure 14. Pearson correlation heatmap with correlation coefficient and significance levels based on the mean fitness value of 4 replicates of each strain (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L) in 5 environments (LB broth, Glucose, Gluconate, Urine, River) measured by plating competitive fitness assays onto agar plates. Data for *rpoB* I572L, *rpoB*I572S in Urine was not included. An interaction was considered significant when P is <0.05, and P values are indicated in text on the plot. The colour coordinated legend (“r”) indicates the value and sign of Pearson’s correlation coefficient, which is also indicated as a value on the plot (r).

4 Chapter: Discussion

4.1 The costs of antimicrobial resistance

“It is not the strongest of the species that survives, nor the most intelligent, but rather the one most responsive to change.”

Charles Darwin

The fate of an AMR pathogen in a given environment depends on its fitness – how well it can pass on its genes – in that environment (Hall, 2013). Mutations or plasmids that confer AMR to bacteria may cause them to grow better, the same, or worse than they did in the environment that selected for AMR. For example, an AMR bacterial strain that was selected for in ciprofloxacin and evolved a mutation in an enzyme or efflux pump may have a fitness cost in a natural environment like soil. In the absence of antibiotic, resistant genotypes may have lower growth rates than their sensitive ancestor. Resistance mutations usually disrupt a normal physiological process in the cell, which can cause side-effects that result in a change in fitness. In the case of plasmid encoded resistance functions, bacteria must synthesize additional nucleic acids and proteins; this synthesis imposes an energetic burden (da Silva & Bailey, 1986) that causes the bacteria to allocate more energy to this instead of replication.

The fitness cost of a mutation is the reduction of competitive ability of a mutant relative to the wildtype. The fitness costs of antibiotic resistance mutations have been reviewed previously (Lenski, 1998; Melnyk et al., 2015; Vogwill & Maclean, 2015). In the literature, most fitness estimates are obtained by growing the bacterium in a single environment, usually a rich medium such as LB broth, and on a single genetic background (Björkman & Andersson, 2000; Melnyk et al., 2015). However, these fitness estimates in rich media may not be representative of what actually happens in nature, as most environments in the natural world are not rich in nutrients. According to a meta-analysis by Melnyk *et al.* (2015), the literature on the cost of antimicrobial resistance mutations and organisms is sparse, and even in model organisms such as *Escherichia coli* costs have only been measured for a handful of resistance mutations in very few media. This is of concern because environmental variation could act as an important factor in adaptive trajectories if the fitness effects of resistance mutations are dependent on environment (Gifford et al., 2016). Hall *et al.* (2013) found that it was nearly impossible to predict the evolutionary fate of resistant bacteria based on fitness measurements in a single environment, because when these strains undergo subsequent adaptation in the absence of antibiotics, they are moved into uncharted areas of phenotype space. It is also possible that some AMR mutations may have no costs in a certain environment, allowing them to take environmental refuge (Levy, 2002).

Once antibiotics are used and consumed by animals and humans, they are excreted into the environment along with bacteria (O'Neill J., 2014). They may end up in a sewage system that leads to a treatment plant and a river, or farm crop fields and

overflow ditches. These places where AMR bacteria disseminate to may become environmental reservoirs for AMR if their conditions are suitable for the bacteria to grow (Levy, 2002). This is concerning because if they are reintroduced to humans or animals and cause an infection, this infection may be resistant to antibiotics.

An aim of this study was to measure the fitness of several strains of AMR *E. coli* in environments in which they may reside in in the natural world. Such environments include wastewater, sewer water, river water, soil, and the gastrointestinal tract. For this study, a selection of wild environments, host-like environments, and two lab media were chosen. Anaerobic sludge, Primary sludge, Combined Sewage Overflow, Soil, and River water were collected from real-world sources and processed such that they could be easily used in the laboratory experiment. In addition, synthetic Colon media, synthetic Urine media, and Gluconate media were made in the laboratory and represented host-like environments. Notably, Gluconate (gluconic acid) is a primary source of carbon for *E. coli* in the lower intestine (Bleibtreu et al., 2013) and this is why this media was included. LB broth and Glucose media are common laboratory media and were used as a comparison to these natural environment media.

I estimated the relative fitness of 6 AMR strains by competitive fitness assays in 10 different media. Fitness data were gathered by sequencing the locus of the AMR mutation of the competition cultures and counting the reads associated to the wildtype and AMR mutant. This fitness data can be seen in Figures 2-5.

When a two-factor ANOVA testing the effect of environment and genotype on fitness, it was found that the main effect of environment ($P = 2e-16$) was significant and this means that fitness values are significantly different from each other by environment (Table 5). Since fitness values are differing by environment this suggests that different environments offer the bacteria different nutritional content, in which they have different fitness because of the way these nutrients are used. The main effect of genotype ($P = 1.21e-06$) was significant and this indicates that fitness values are significantly different across genotypes. This implies the fitness of the bacteria is associated with what mutation they have, suggesting that there could be fitness costs associated with the mutations. The Genotype by Environment Interaction can also be visualized in Figure 5, which shows the fitness of the AMR genotypes by environment, such that change in rank order of fitness of the genotypes between environments can be seen. This finding is especially relevant to AMR research because this means that measuring fitness of AMR pathogens in one environment such as LB broth may not represent what their fitness may be in another environment in the natural world. This finding is consistent with a study done by Gifford and colleagues (2016), where competitive fitness of 3 *Pseudomonas aeruginosa rpoB* mutants had significant variation by genotype, environment, and their interaction (two-way ANOVA, $p < 0.001$). This indicates that fitness is different in each environment, but it does not test whether fitness values in each environment are correlated in any way. The section below talks about whether fitness in one environment can predict another (Section 4.2)

Similarly, results from another study found that genotype by environment interactions generated by AMR mutations made it difficult to predict the evolutionary fate of AMR bacteria based on fitness measurements in one environment (Hall, 2013). In this study, the growth of 9 rifampicin resistant strains of *E. coli* was measured in 31 antibiotic free environments. It was concluded that G*E interactions generated by antibiotic resistance mutations are more pronounced after adaptation to other types of environmental variation, making it difficult to predict selection on resistance mutations from fitness effects in a single environment (Hall, 2013). In addition, a study by Remold and Lenski in which the fitness of 26 genotypes of *E. coli* with a single random insertion mutation were measured in four environments differing in resource and temperature (Glucose, 28°C; maltose, 28°C; Glucose, 37°C; and maltose, 37°C) also found a highly significant interaction between genotype and environment (Remold & Lenski, 2001). Significant G*E interactions were also found in a study by Sabarly and coauthors (2016) in which 5 strains of *E. coli* were grown in human urine, LB, Glucose, and Gluconate media.

In general, the results from this study and other studies (Bataillon, Zhang, & Kassen, 2011; Remold & Lenski, 2001), imply that bacterial strains perform differently in different environments, and both genotype and environment contribute to this performance. This implies that mutations that are beneficial in one environment can have different fitness outcomes in other environments (Bataillon et al., 2011; Hall, 2013). A study done in 2011 found that AMR mutations that are beneficial in one environment are did not have much cost in other environments (Bataillon et al., 2011). Traditionally,

fitness experiments that measure the costs of resistance are done in rich lab media such as LB broth. The data from these studies and this thesis suggests that doing this may have limited inferential value because fitness in one environment is different than fitness in another.

4.2 Can we predict how well an AMR genotype will do in a new environment?

Prediction of fitness using sequencing data

Mutations that are beneficial in one environment can have different fitness effects in other environments. Genotype-by-environment interactions potentially make selection on resistance unpredictable in heterogeneous environments in the context of antibiotic resistance (Hall, 2013). To be able to predict the fitness of AMR genotypes in realistic environments using measurements from a small number of laboratory media would be useful to have to better understand the evolution of AMR. As such, we asked whether fitness is correlated between environments. Although we found a significant interaction between genotype and environment (ANOVA Table 5), this does not rule out the possibility that some environments closely resemble each other. Figure 7 shows a heat map of pairwise correlations (using Pearson's r) between environments, with stronger correlations in deep red (positive) or blue (negative). The Pearson correlation coefficient, r , can be used to examine the strength and direction of the linear relationship between two variables. The coefficient value can range from +1 (red in the Figure) and -1 (blue in the

Figure), where 1 is total positive linear correlation, 0 is no linear correlation, and -1 is a total negative linear correlation.

On this heatmap the only significant correlation LB broth has is with Combined Sewage Overflow ($r \approx 0.99$, $P < 0.01$), and the only significant correlation the minimal Glucose medium has is with Anaerobic Sludge ($r \approx 0.84$, $P = 0.04$). This suggests that these common laboratory media do not have any strong significant correlations with the other media in this study, which means that they do not predict fitness in these other natural-like environments. This has implications in AMR research, where LB broth and Glucose media are commonly used. These findings suggest that using these media to predict fitness in the natural environment would not work because there is no correlation between many of them. In addition, Colon medium has a significant positive correlation with 3 of the natural environment media (Soil $r \approx 0.99$, $P < 0.01$, Anaerobic Sludge $r \approx 0.86$, $P = 0.03$, and River $r \approx 0.95$, $P < 0.01$) and this indicates that in this study, it performed better than the common lab media (LB broth and Glucose) in predicting the fitness of AMR *E. coli* in natural environments.

Interestingly, it seems that some of the natural environments are good predictors of fitness for each other such that they have strong, positive, and significant correlation coefficients. Soil has a strong positive correlation with River ($r \approx 0.97$, $P < 0.01$), Anaerobic Sludge ($r \approx 0.91$, $P = 0.01$), and Colon medium ($r \approx 0.99$, $P < 0.01$), which means that fitness estimates in these four environments are similar. River also has a

positive correlation with Anaerobic Sludge ($r \approx 0.93$, $P = 0.01$), and Colon medium ($r \approx 0.95$, $P < 0.01$).

It should be noted that the Pearson correlations should be corrected for multiple comparisons. For Bonferroni corrections, the significance cut-off for the P-value is $0.05 /$ the number of tests. Since there were 50 tests, the new cut-off would be $P = 0.001$. This would leave us with 3 significant interactions instead of 8 (Soil and River water with Colon medium, and LB broth with Combined Sewage Overflow). However, all tests are viewed with a grain of salt, and the Pearson correlation map still shows some general trends that are useful. In addition to correcting Pearson correlations for multiple comparisons – future work will examine whether some modelling applications may use this data to predict fitness, and an analysis of plasticity and rank change in fitness can be done.

Next, a principal component analysis was completed on the fitness estimates from the sequencing data in order to summarize the data set by reducing the dimensionality of the data without losing important information (Figure 8). A biplot was created to visualize this PCA analysis with individual variances and variables. The direction that the loadings point corresponds to the environments that have similar response profiles. For example, in the heatmap of Pearson correlations Soil, Colon, River, and Anaerobic Sludge all have positive significant correlations to each other, and on this biplot they are also grouped together in the top right corner and seem to strongly influence PC1 and mildly influence PC2. The PCA in Burghardt's study (Burghardt et al., 2018) also

showed that environments that were more similar to each other and had significant Pearson correlations grouped together in PCA (Figure 3 B, (Burghardt et al., 2018)). The variables (AMR mutants, legend) that are close together on the PCA are the ones that have similar fitness profiles. For example, it seems as though the *gyrA* mutants have similar fitness profiles as they are grouped in the bottom right quadrant. The *rpoB* mutants seem to have very different fitness profiles from both each other and the *gyrA* mutants because they are spread far apart. The *rpoB* mutants may spread far apart because they respond very differently across resources – this has been shown previously in *E. coli* (Maharjan & Ferenci, 2017) and *P. aeruginosa* (Hall, Iles, & MacLean, 2011).

This PCA may be underpowered, because mean values were used for the fitness of the 4 replicates of the 6 mutants in each environment. 6 data points is not a lot to make inferences from, but we can observe general patterns that were already discerned from the Pearson correlation heatmap and the fitness graphs.

Previous work in a variety of taxa has similarly found that correlations in fitness between environments are often weak or absent. An analysis done by Hereford on a collection of reciprocal transplant data across several taxa found a small negative correlation between a population's relative fitness in 2 different environments, indicating weak trade-offs associated with local adaptation (Pearson's $r = -0.14$) (Hereford, 2009). Similarly, an analysis done by Bennet and Lenski on several strains of *E. coli* found that there was no correlation between fitness in two environments ($r = 0.006$, $P > 0.50$) (2007). Recently a study was done on measuring the fitness of a rhizobia community in two plant

hosts, soil, and laboratory media by sequencing whole genomes of the community before and after competition and measuring change in allele frequency (Burghardt et al., 2018). This study is similar to what we have done but instead used whole genomes to track allelic frequency and rhizobial bacteria instead of AMR bacteria. Strain fitness in the hosts was significantly positively correlated, although fitness in one host was not strongly predictive of fitness in the other host ($R^2 = 0.27$) (Burghardt et al., 2018). The correlations between rhizobial fitness in each host and in each of the free-living environments were weak (all $R^2 < 0.05$); however, there was a significant negative correlation between rhizobial fitness in soil and in nutrient-rich liquid media ($R^2 = 0.43$, Pearson's $r = -0.66$, $p < 0.001$) (Burghardt et al., 2018). In general, like this thesis, this study also found that fitness in one environment does not strongly predict fitness in another environment although there were a few positive correlations.

In another model organism, *Arabidopsis thaliana*, similar trends have been observed. A recent review warns against extrapolating results of plants grown in a controlled chamber or a greenhouse to those grown in the field because of the large G*E effect (El-Soda, Malosetti, Zwaan, Koornneef, & Aarts, 2014). This is supported by several studies that have indicated that there is a poor correlation between fitness traits in field experiments and greenhouse conditions (Brachi et al., 2010; Hancock et al., 2011; Méndez-Vigo, Gomaa, Alonso-Blanco, & Xavier Picó, 2012). This means that fitness traits under natural conditions is influenced by environmental cues that are likely to be absent under controlled greenhouse conditions. Another example reported significant differences in leaf size, shape, and pigment composition when comparing field- and

climate chamber-grown *Arabidopsis* under different light conditions (El-Soda et al., 2014). In addition, major differences were found in adjusting the functions of individual proteins involved in the photosynthetic apparatus when shifting *Arabidopsis* from the climate chamber to the field (Jankanpaa, Mishra, Schroder, & Jansson, 2012.) El Soda and colleagues concluded that to fully understand fully G*E and its role in shaping adaptive variation under natural conditions, and to extrapolate such knowledge to plant breeding and ecological studies, it is vital to consider plants grown in the field or similar conditions rather than to focus solely on plants grown under climate chamber conditions (El-Soda et al., 2014). This is similar to what we found in this study concerning G*E, AMR strains, and environment.

Most notably from both the Pearson correlation heatmap and the PCA, it looks like some of the real environmental media group together and the synthetic lab media are correlated. It would be interesting to see if more data points would support these observations. In the literature, it has also been found that AMR resistomes cluster by ecology such that strains of different ecologies cluster together in a PCA (Gibson, Forsberg, & Dantas, 2015). In conclusion, this Pearson correlation and PCA of my data suggests that measuring fitness in one environment does not predict fitness in another environment very well, and this is also supported by several studies in AMR research, plant microbiome research, and also across several levels of taxonomic groups (Bennett & Lenski, 2007; Burghardt et al., 2018; El-Soda et al., 2014; Gifford et al., 2016; Hall, 2013; Maharjan & Ferenci, 2017; Remold & Lenski, 2001)

Prediction of fitness using yield data

It has been previously shown that the costs associated with resistance could be influenced by environmental variation. For example, it has been suggested that productivity may impact costs, with higher nutrient environments imposing a higher cost for efflux over-expression mutants of *Pseudomonas aeruginosa* (Lin et al., 2018). It was shown that ciprofloxacin resistant *P. aeruginosa* strains had low productivity (CFU/ml) and lower fitness compared to the wild type strain in high nutrient conditions, and fitness and productivity increased as nutrient levels in the media decrease (Lin et al., 2018).

We set out to test whether yield could predict fitness. A linear regression was performed in order to see whether yield data can predict fitness (Figure 10). The correlation coefficient of the linear regression, R^2 , was very low, indicating that yield and fitness values are not well correlated. This indicates that yield is not an ideal predictor of fitness. In addition, the P-value is over 0.05, indicating that the slope of the regression line is not significantly different from 0. This suggests that there is no relationship between the fitness and yield values, positive or negative.

4.3 Competitive fitness assays: sequencing versus plating

Fitness is a complex trait that varies with environmental and competitive conditions. Competitive fitness assays are based on plating, incubation time, and colony counting and are time consuming. Several authors have developed new methods that

automatically count large numbers of cells, saving time and increasing the power of the experiment. Such methods include flow-cytometry based methods (Bleibtreu et al., 2013; Gifford et al., 2016) and sequencing methods (Burghardt et al., 2018; Hietpas, Bank, Jensen, & Bolon, 2013; Hietpas, Jensen, & Bolon, 2011) . A benefit of using the sequencing method is that the competitor strain does not have to be genetically modified to be visually distinguishable from the other strain, as it would have to be in plate counting or flow cytometry. The sequencing method of counting the frequency of the AMR strain versus the wild type made it possible to increase the power of the experiment and reduce sampling error by using the data of several hundred reads.

More competitive fitness assays were completed in order to see how plating on agar plates compares to the sequencing method used in this thesis. It was found that fitness estimates were visually comparable, and that broad trends and G*E are similar (Figure 11, 12, 13). A Pearson correlation heatmap also gave the same inference as the sequencing data – that fitness in one environment is not a good approximation for another (Figure 14).

A linear regression was also performed such that each fitness value for one strain in one environment measured by sequencing was plotted against the same strain in the same environment measured by plating on agar plates in order to see if there is a linear relationship between these two methods (Figure 12). It was found that the correlation coefficient (R^2) for this relationship is 0.3158 (Figure 12). In addition, another linear regression was performed in which 4 apparent outliers were removed (Figure 13). This

greatly improved the R^2 value, increasing it to 0.7279. This means that the fitness values for the sequencing method and the plating method are well correlated and similar.

It was noted that sequencing fitness values were slightly higher than the plating method overall. This could mean that the sequencing method could be overestimating fitness, or the plating method could be underestimating fitness. In the sequencing method four replicates were used to measure fitness, however within the four sequencing replicate files are hundreds of reads generated by the sequencing machine. The number of reads gives this method more inferential power in numbers. In addition, there could be bias in this PCR-based method, such that fitness could be overestimated because DNA from dead cells could be amplified – it cannot differentiate DNA from live or dead cells.

In conclusion, it was found that plating results were similar to the sequencing methods, and that the plating method also found that fitness in one environment does not predict fitness in another (Figure 14). In the literature there have been several instances of using sequencing technology to do some form of fitness measurement (Bank, Hietpas, Wong, Bolon, & Jensen, 2014; Burghardt et al., 2018; Hietpas et al., 2013, 2011; Wetmore et al., 2015), and this seems to be the trend in doing these sorts of studies now. Most of these studies have also provided examples of how they have compared their results with traditional methods, such as in Hietpas's study in 2013 (Hietpas et al., 2013) and Wetmore's in 2015 (Wetmore et al., 2015).

4.4 Future Implications of AMR and G*E

The fate of an AMR mutation in a population is determined in part by its fitness. Mutations that suffer little or no fitness cost are more likely to persist in the absence of antibiotic treatment. Resistance mutations may have a fitness cost because they target important biological functions in the cell. If costs are as widespread as they seem to be (Melnyk et al., 2015; Vogwill & Maclean, 2015) then we expect that resistance should be selected against in antibiotic-free environments. Yet, resistance persists in the clinic (Enne, Livermore, Stephens, & Hall, 2001) and in the environment (Finley et al., 2013).

For example, clinical studies have shown that in some cases, resistant bacteria remained abundant in the population (Enne et al., 2001; Sundqvist et al., 2010) or even increased in frequency (Arason et al., 2002) despite the absence of drug, while in others the proportion of resistant bacteria within the population declined (Bergman et al., 2004; Gottesman, Carmeli, Shitrit, & Chowers, 2009), as expected. It has been shown that reducing the use of antibiotics usually leads to a reduction in resistant strains, but it rarely succeeds in eliminating them (Andersson, 2003; Enne, 2010; Johnsen et al., 2011).

Two meta-analyses of costs of resistance showed that resistance mutations were generally costly in laboratory studies, although several drug classes and species of bacteria on average did not show a cost (Melnyk et al., 2015; Vogwill & Maclean, 2015). The authors acknowledged that a limitation of this analysis and previous studies on the costs of resistance is that most of the fitness estimates for a given mutation are gathered

in single environment (most often a common laboratory media such as LB) on a single genetic background - which is not representative of nature (Melnyk et al., 2015).

To address this, I set out to measure the fitness of several AMR mutations in several different media representing environmental, host-like, and laboratory medium. In general, I found that fitness in one environment is different from fitness in another environment, and that fitness in one environment does not strongly correlate to fitness in another environment. Notably, I found that fitness in common laboratory media that I used in the experiment, LB broth and Glucose media, does not strongly predict fitness in the environmental media or the host-like media. This means that fitness estimates in laboratory media are unlikely to be representative of fitness costs more widely – such as in nature or in hosts.

My study is supported by data in the recent literature, such as a study by Hubbard et. al. (2018) in which they measured the fitness costs of AMR mutations in *E. coli* in several media using a competitive fitness assay and identifying frequencies of each mutant from sequencing reads (Hubbard, 2018). They observed within media-type variability in the fitness costs associated with resistance, and the study showed that in some cases the fitness is dependent on the media in which the assay is carried out (Hubbard, 2018) . There are also several studies mentioned earlier that support this work (Bennett & Lenski, 2007; Burghardt et al., 2018; El-Soda et al., 2014; Gifford et al., 2016; Hall, 2013; Hall et al., 2011; Hereford, 2009; Maharjan & Ferenci, 2017; Melnyk et al., 2015; Remold & Lenski, 2001). Our data highlights the importance of media

consideration when interpreting the results of evolutionary studies which will ultimately be taken into consideration by policy makers.

Future research should focus on: 1) the contribution of different sources of antibiotics and antibiotic resistant bacteria in the environment; 2) the role of the environment on the evolution of resistance; 3) the overall human and animal health impacts caused by exposure to resistant bacteria from the environment; and 4) the efficacy of technological, social, economic and behavioral interventions to mitigate environmental antibiotic resistance (Larsson et al., 2018) and surveillance and identification of high risk environments for the evolution and emergence of resistance. One of the prerequisites for translation of these ideas to the clinic in the form of antibiotic prescription rules is robust and reproducible data which can convince clinicians and policy makers that what is seen in the research is likely to occur in the natural world and harm patients. Therefore, there is a need to analyse the consequences of different experimental conditions thoroughly. The creation of a database with fitness data for different environments would be a start on these research aims.

4.5 Limitations of this study

It is acknowledged that head to head competitive fitness assays are not entirely representative of what may occur in nature. In the natural world, myriads of microbial strains are competing for resources in any given environment – not just two. However, the benefit of head to head competitive fitness assays is that from the aforementioned

complex web of competitions, we may disentangle one interaction and measure it quantitatively. This method is preferred over alternatives such as the measurement of population growth rates in pure culture because it is an integrated measure involving all phases of the growth cycle and can capture aspects of competition such as lag times, exponential growth rates, and stationary phase dynamics that may not be reflected in pure culture assays (Wiser & Lenski, 2015). Using growth rate as a proxy for fitness only incorporates a single component of bacterial fitness and only gives absolute fitness (Wiser & Lenski, 2015). Competitive fitness assays give relative fitness, which is more important than absolute fitness when considering the evolutionary fate of a particular genotype (Wiser & Lenski, 2015). In the future, more studies may be performed like Burghardt's (2018) study in which they measured the fitness of a microbial community by tracking allelic frequency before and after competition by whole genome sequencing (Burghardt et al., 2018).

In addition, because real environmental media were used there is a possibility that the experiment could have been contaminated by bacteriophage, bacteriocins, plasmids, or other environmental bacteria in the media. Before the competitive fitness assays were sequenced trial runs were completed and plated. Most of the plates from the "environmental" media such as River water, Soil, and Combined Sewage Overflow were very strange. Some had very small colonies, some had light yellow-toned colonies with a filamentous, convex morphology, and some did not grow at all. At this time, I was not autoclaving these media, just filtering them with a 0.22 μm filter. When I added the autoclaving step, these problems went away (Appendix B.1).

Another limitation is the number of strains used for this study. It was originally planned to use 30 strains with diverse mutations in different genes and to measure fitness over several time points in 24hrs. However, we realized the cost to do this would have been astronomical, so it was decided that using a few strains to measure fitness at 0 hours and 24 hours would do. If more strains were added to the study, it would have more inferential power and the PCA and correlations would give us more convincing data. Due to the time limitations, only 6 strains were able to be sequenced and analyzed at both time points in the 10 environments.

Sequencing consistency and quality is also a limitation. Each file for each replicate in each environment varies in both number of reads and quality of the reads. Two additional strains (*marR* mutants) were sequenced and not included in this thesis because poor sequencing quality would not allow them to pass through the same quality control filters that the other reads went through. Another downside is that while the cost of sequencing has decreased, it is still really expensive in comparison to plating methods. Future studies using similar methodology would benefit from finding a way to keep consistency and quality while maintaining a high number of reads.

5 Chapter: Conclusion

The cost of antimicrobial resistance (AMR) is the reduction of fitness of a resistant mutant relative to a susceptible strain in the absence of drug. Most of the fitness

estimates for a given mutation are gathered in single environment (most often a common laboratory media such as LB) on a single genetic background - which is not representative of nature (Melnyk et al., 2015). I measured the fitness of AMR *E. coli* strains in different environments, including medically and ecologically relevant ones. To address this, I set out to measure the fitness of several AMR mutations in several different media representing environmental, host-like, and laboratory medium. Overall, I found that fitness in one environment is different from fitness in another environment, and that fitness in one environment does not strongly correlate to fitness in another environment. We found environments in which resistance alleles suffered no cost, suggesting that these mutants may persist for long periods of time. I also found that fitness in common laboratory media that I used in the experiment, LB broth and Glucose media, does not strongly predict fitness in the environmental media or the host-like media. This means that fitness estimates in laboratory media are unlikely to be representative of fitness costs more widely – such as in nature or in hosts. Our results indicate that environmental settings strongly affect whether drug resistance is a cost or a benefit in the absence of selection pressure of antibiotics.

My study is supported by data in the recent literature, such as a study by Hubbard in which they measured fitness costs of AMR mutations in *E. coli* in several media using a competitive fitness assay and identified frequencies of each mutant from sequencing reads (Hubbard, 2018). They observed within media-type variability in the fitness costs associated with resistance, and the study showed that in some cases the fitness is

dependent on the media in which the assay is carried out (Hubbard, 2018). Many other studies have drawn similar conclusions (Bennett & Lenski, 2007; Burghardt et al., 2018; El-Soda et al., 2014; Gifford et al., 2016; Hall, 2013; Hall et al., 2011; Hereford, 2009; Maharjan & Ferenci, 2017; Melnyk et al., 2015; Remold & Lenski, 2001). This data highlights the importance of media consideration when interpreting the results of evolutionary studies which will ultimately be translated into the clinic. Future research should focus on the contributions of different sources of antibiotics and antibiotic resistant bacteria into the environment, the role of the environment on the evolution of resistance, health impacts caused by exposure to AMR bacteria from the environment; and the efficacy of technological and behavioral programs to mitigate the spread and persistence of environmental antibiotic resistance.

Appendices

Appendix A - Additional Data

A.1 Yield ANOVA and Correlation data

In order to test for a relationship between productivity and fitness costs, I carried out yield assays on 4 replicates of 6 AMR strains of *E. coli* (and 2 wildtype strains (MG1655 and NCM520) in 7/10 (Soil, River, Urine, Colon, Gluconate, LB broth, and Glucose) environments by plating dilutions of overnight culture onto LB agar plates and counting colonies. Data for *gyrA* S83L in Colon were not obtained. A two-factor ANOVA (Yield ~ Environment* Genotype, Table 7) was calculated on this yield data. Similar to the fitness data, it found significant effects of all terms.

Table 7. Two-way ANOVA analyzing Yield across Environment and Genotype.

Dependent Variable	Df	Sum Sq	Mean Sq	F	P
Environment	5	9.237e+18	1.847e+18	104.722	< 2e-16
Strain	7	1.000e+18	1.429e+17	8.102	2.87e-08
Environment*Strain	34	3.094e+18	9.101e+16	5.159	1.85e-12

Pearson' r was calculated between environments using yield estimates. As Figure 15 shows, it is apparent that yield in one environment does not strongly predict another. There is only one significant interaction between LB and Urine ($r=0.94$, $P<0.01$). This implies that using yield values in one environment to predict productivity in another would not be accurate.

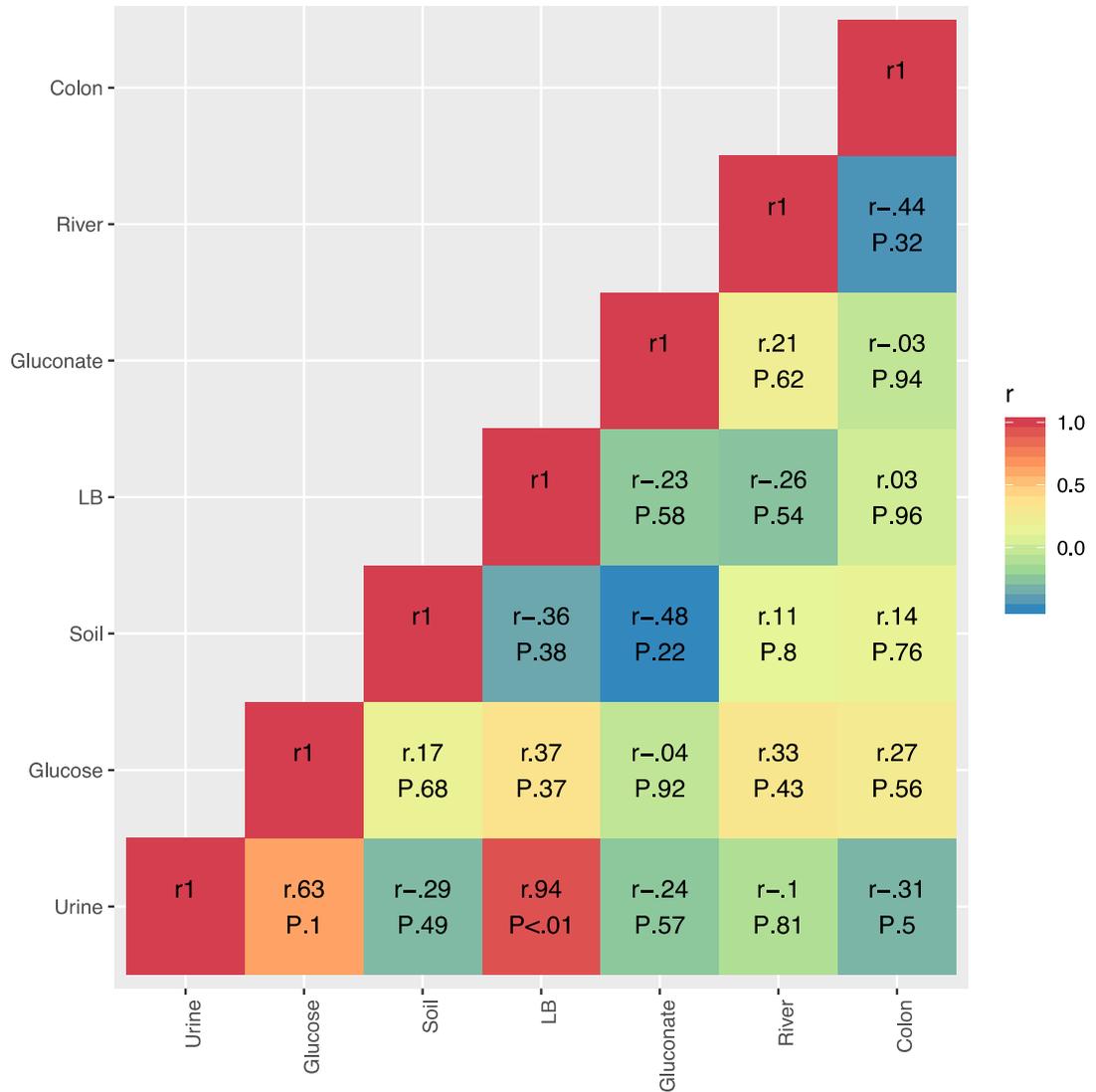


Figure 15. Pearson correlation heatmap with correlation coefficient and significance levels based on the mean yield value of 4 replicates of each strain (*rpoB* I572L, *rpoB*I572S, *gyrA* S83A, *gyrA* S83Y, *gyrA* D87G, *gyrA* S83L, NCM520, MG1655) in 7 environments (LB broth, Glucose, Gluconate, Urine, River, Soil, Colon) measured by plating yield assays onto agar plates. Data for *rpoB* I572L, *rpoB*I572S in Urine was not included. An interaction was considered significant when P is <0.05, and P values are indicated in text on the plot. The colour coordinated legend (“r”) indicates the value and sign of Pearson’s correlation coefficient, which is also indicated as a value on the plot (r).

Appendix B - Protocol Procedures

B.1 Environment Ingredients & Instructions

Table 8. Environment ingredient list and preparation instructions.

Media	Ingredients	Temperature	Reference
LB Broth	<p>Bio-Rad brand LB Broth LB Broth Miller – from Bioshop Canada, Burlington, ON</p> <p>Tryptone 10g/L Yeast extract 5g/L Sodium chloride 10g/L</p> <p>Preparation: 25g/L in water - Autoclave at liquid 20</p>	37°C	
Minimal Media (M9) + Glucose	<p><i>1x Min Salts</i> 6.78g/L Na₂HPO₄ Anhydrous Dibasic 3g/L KH₂PO₄ 0.5g/L NaCl 1g/L NH₄Cl</p> <p>- Autoclave at liquid 20</p> <p><i>Then add (to 1X Salts):</i> 2ml 1M MgSO₄ (1M MgSO₄: 12g anhydrous/ 100mL or 24.6g heptahydrous)</p> <p>- Autoclave at liquid 20</p> <p>100µL 1M CaCl₂ (1M CaCl₂: 14.7g/100mL)</p> <p>- Autoclave at liquid 20</p> <p>0.8 % (w/v) glucose final concentration (autoclave the stock solution at liquid 20)</p>	37°C	
Lower intestine environment (Minimal Media (M9) + gluconate)	<p>Preparation modified from Bleibtreu et al. 2013:</p> <p><i>1x Min Salts</i> 66.78g/L Na₂HPO₄ Anhydrous Dibasic 3g/L KH₂PO₄ 0.5g/L NaCl</p>	37°C	(Bleibtreu et al., 2013) According to this reference, this environment

	<p>1g/L NH₄Cl</p> <ul style="list-style-type: none"> - Autoclave at liquid 20 <p><i>Then add (to 1X Salts):</i> 2ml 1M MgSO₄ (1M MgSO₄: 12g anhydrous/ 100mL or 24.6g heptahydrous)</p> <ul style="list-style-type: none"> - Autoclave at liquid 20 <p>100μL 1M CaCl₂ (1M CaCl₂: 14.7g/100mL)</p> <ul style="list-style-type: none"> - Autoclave at liquid 20 <p>20mM gluconate final concentration (monosodium glutamate)</p> <ul style="list-style-type: none"> - Autoclave the stock solution at liquid 20 		is similar to lower intestine environment.
Synthetic Urine media	<p>Preparation was modified from Laube et al. 2013:</p> <p>400μL 1M CaCl₂ (more gives precipitate) 0.73g NaCl 0.56g NaSO₄ 0.35g KH₂PO₄ 0.4g KCl 0.25g NH₄Cl 6.25g Urea 0.28g Creatinine</p> <ul style="list-style-type: none"> - in 250mL H₂O total, - pH was adjusted to 6.0 using NaOH/HCl - filter sterilize with 0.22μm filter 	37°C	(Laube et al., 2001)
Soil Media	<p>Collected on the shore of the Ottawa River on Carleton University on 25/9/17. It had not rained the week previously. Samples were dried out in aerated container until use.</p> <p>Preparation:</p> <p>3g soil in tea bag (David's tea, empty tea bags), in 50mL H₂O. Covered beaker with tinfoil and allowed to steep for 48 hours.</p> <p>Supplemented with 10g/mL glycerol (0.5mL glycerol into 50mL total)</p>	25°C	Adapted from (Kraemer & Kassen, 2015)

	Filter sterilize with 0.22µm filter		
River water	<p>Collected on the shore of the Ottawa River on Carleton University on 25/9/17. It had not rained the week previously. Samples were frozen at -80°C until time of use.</p> <p>Preparation:</p> <p>The water was passed through a 0.22µM filter to remove particles, then autoclaved for 10 minutes (liq10) to kill bacteriophage present in the water. Glucose was added to a final concentration of 0.2% (w/v).</p>	25°C	Adapted from (Chai, 1983)
Synthetic Colon media	<p>Preparation was modified from Polzin et al. 2013:</p> <p>6.25g/L Biotryptone 0.88g/L NaCl 2.7g/L KHCO₃ 0.43g/L KHPO₄ 1.7g/L NaHCO₃</p> <p>- Solution was autoclaved liquid 20 cycle</p> <p><i>Then add:</i></p> <p>4.0g/L bile salts #3* 2.6g/L D-glucose (0.26% w/v)*</p> <p>- Solutions were filter sterilized (0.22µm) separately</p>	37°C	Adapted from (Polzin et al., 2013)
Combined Sewage Overflow	<p>Collected at the Main Street Combined Sewage overflow site (Ottawa, ON) on 1/10/17. It had rained the previous day. Samples were frozen at -80°C until time of use.</p> <p>Preparation:</p> <p>The water was passed through a 0.22µM filter to remove particles, then autoclaved for 10 minutes (liq10) to kill bacteriophage present in the water. Glucose was added to a final concentration of 0.2% (w/v).</p>	25°C	Adapted from (Chai, 1983)

<p>Primary Sludge</p>	<p>Primary Sewage Sludge was collected from Robert O. Pickard Environmental Centre (ROPEC) by a student in Banu Ormeci's Laboratory, Carleton University, Ottawa, ON. The primary sludge was collected from an outlet in a pipe transporting the primary sludge. The Primary Sludge has not been treated yet. Samples were frozen at -80°C until time of use.</p> <p>Preparation: The sludge was first passed through circular filter paper with large porosity, then medium porosity, then fine porosity in order to remove solids (GE Whatman circular filter papers).</p> <p>The sludge was passed through a 0.22µM filter to remove particles, then autoclaved for 20 minutes (liq20) to kill microorganisms.</p> <p>Note: Wear appropriate face mask (PPE).</p>	<p>25°C</p>	<p>Adapted from (Chai, 1983)</p>
<p>Anaerobic Sludge</p>	<p>Anaerobic Sewage Sludge was collected from Robert O. Pickard Environmental Centre (ROPEC) by a student in Banu Ormeci's Laboratory, Carleton University, Ottawa, ON. It was collected from an anaerobic digester and has been treated. Anaerobic digestion reduces pathogens, reduces biomass quantity and produces a usable gas as a byproduct (methane). Samples were frozen at -80°C until time of use.</p> <p>Preparation: The sludge was first passed through circular filter paper with large porosity, then medium porosity, then fine porosity in order to remove solids (GE Whatman circular filter papers).</p> <p>The sludge was passed through a 0.22µM filter to remove particles, then autoclaved for 20 minutes (liq20) to kill microorganisms.</p> <p>Wear appropriate face mask (PPE).</p>	<p>25°C</p>	<p>Adapted from (Chai, 1983)</p>

B.2 Amplicon PCR Protocol Adapted from Illumina 16S protocol

Adapted from Illumina's 16S Protocol (Illumina, 2013).

General advice:

- Pipettes and counter space were cleaned well to reduce contamination from the environment and previous samples
- Many steps called for centrifugations of 96-well plates. These steps are important for consistency across wells with small volumes of liquid.
- The potential for contamination of samples and primers is high. Filter tips were used to reduce this risk and were changed every time between samples.

Materials and equipment used throughout the protocol

- Sterile DNase free water (Hyclone)
- Filter tips (Rainin p20, p200, and p1000)
- Manual multichannel pipettes (Rainin)
- DNase free microcentrifuge tubes
- 96-well PCR plates and Microseal
- Centrifuge capable of spinning 96-well plates
- Thermocycler (Bio-rad T1000 or T100)
- Plate reader with fluorescence capabilities
- Illumina MiSeq machine, cartridge, and reagents
- Ice bucket

STEP 1. Amplicon PCR

Firstly, primers were made to amplify a 150 bp region, with the AMR mutation at the center. Reverse and forward primers were made for each AMR strain using NCBI's PrimerBLAST program (Ye et al., 2012). To these optimum sequences, Illumina adapter overhang nucleotide sequences were added (Illumina, 2013). Out of the queries returned, the optimum one was selected and then ordered from IDT DNA as a dried oligo. These primers were tested and proved to be specific and robust, providing a single solid band on an agarose gel. A gradient PCR was performed to determine optimum annealing temperature of all primers.

The following PCR protocol was optimized for use for this experiment from Illumina's 16S Metagenomic Library Prep Guide (Illumina, 2013), but instead of amplifying the 16S region, the region where the mutation is in the AMR strain was amplified with region-specific primers. Notably, in this experiment pure culture from the frozen competitive fitness assay samples was used instead of genomic DNA and half the volume of reagents were used in this PCR reaction than Illumina's Protocol (Illumina, 2013), to be more cost effective. The PCR reaction was set up as follows in the Table below, in a 96-well PCR plate:

Table 9. Amplicon PCR reaction using pure microbial culture

Item	Volume
Microbial pure culture	1 μ L

Amplicon PCR forward primer, 1 μ M final concentration	0.5 μ L
Amplicon PCR reverse primer, 1 μ M final concentration	0.5 μ L
NEB Q5 Hot Start High-Fidelity 2X Master Mix	5 μ L
Water (molecular grade, sterile)	3 μ L
Total	10 μ L

The plate was sealed using Thermofisher Microseal 'A' film, centrifuged at 1000 x g at 20°C for 1 minute, and put in a Bio-RAD T100 or T1000 using the following program:

- 1) 95°C for 10 minutes
- 2) 35 cycles of:
 - a. 95°C for 30 seconds
 - b. Primer-specific annealing temperature for 30 seconds
 - c. 72°C for 30 seconds
- 3) 72°C for 5 minutes
- 4) Hold at 12°C

STEP 2: PCR Clean up #1

This step is a basic AMPure XP protocol with some modifications. It uses AMPure XP beads to purify the PCR product away from extra primer and primer dimers.

Table 10. PCR Clean Up #1 Consumables

Item	Volume
10 mM Tris pH 8.5	52.5 μ L per sample
AMPure XP beads	20 μ L per sample
Freshly prepared 80% Ethanol	400 μ L per sample
96-well 200 μ L PCR plate	
Thermofisher Microseal 'B'	
96-well MIDI plate (deep)	

1. Bring AMPure XP beads to room temperature
2. Centrifuge PCR plate at 1000 x g at 20°C for 1 minute to collect condensation.
3. Remove seal and transfer entire Amplicon PCR product from the PCR plate to the MIDI plate using a multichannel pipette. Change tips between samples.
4. Vortex AMPure XP beads for 30 seconds and add them to sterile trough depending on the amount of samples.
5. Add 20 μ L of AMPure XP beads to each well of the PCR Amplicon product. Change tips between samples.
6. Seal the plate with Microseal and place MIDI plate in shaker at 1800 rpm for 2 minutes.
7. Centrifuge plate at 1000 x g at 20°C for 1 minute.
8. Incubate MIDI plate at room temperature for 5 minutes without shaking.
9. Put MIDI plate on magnetic stand for 3-5 minutes or as long as it takes for the supernatant to clear.

10. With the MIDI plate on the magnetic stand, remove and discard the supernatant with a multichannel pipette, changing tips between samples. Place the tip of the pipette on the sidewall of the MIDI plate and suck up the liquid, making sure not to disturb the beads.
11. With the MIDI plate on the magnetic stand, wash the beads with the ethanol:
 - a. Add 200 μL of ethanol to each sample well, do not mix up and down, changing tips between samples.
 - b. Incubate the plate on the magnetic stand for 1 minute.
 - c. Remove and discard the supernatant with a multichannel pipette, changing tips between samples.
12. With the MIDI plate on the magnetic stand, wash the beads a second time with the ethanol:
 - a. Add 200 μL of ethanol to each sample well, do not mix up and down, changing tips between samples.
 - b. Incubate the plate on the magnetic stand for 1 minute.
 - c. Remove and discard the supernatant with a multichannel pipette, changing tips between samples.
 - d. Using a P20 multichannel pipette, carefully try to remove every last drop of ethanol.
13. With the MIDI plate on the magnetic stand, allow the beads to air-dry for 10 minute or until you can see fine hairline cracking on the bead droplet with a matte finish. If it looks like disintegrated powder, you have waited too long.

14. Remove the MIDI plate from the magnetic stand and add 52.5 μL 10 mM Tris pH 8.5 to each well that contains PCR product, changing tips between samples. Mix up and down with the pipette to re-suspend the beads.
15. Seal the plate with Microseal and place MIDI plate in shaker at 1800 rpm for 2 minutes.
16. Centrifuge the plate at 1000 x g at 20°C for 1 minute.
17. Incubate at room temperature for 2 minutes
18. Place the MIDI plate on the magnetic stand for 2 minutes or until the supernatant is clear
19. Transfer 50 μL of the supernatant from MIDI plate to a new 96-well PCR plate using a multichannel pipette. Change tips between samples.

This is a safe stopping point. The PCR plate can be sealed and stored at -20°C for 2 weeks. Always change tips between samples to avoid cross contamination.

STEP 3: Index PCR

This step is performed to add Nextera XT Index Primers. The reaction volume was scaled down from Illumina's (Illumina, 2013) to be more cost effective.

Table 11. Index PCR reaction

Item	Volume
DNA	1 μ L
Index 1 Nextera XT primer (N7xx)	1 μ L
Index 2 Nextera XT primer (S5xx)	1 μ L
NEB MasterMIX	5 μ L
Water (molecular grade, sterile)	2 μ L
Total	10 μ L

1. Arrange the Index 1 and Index 2 primers in a TruSeq Index Plate fixture, or at least make sure that every sample has a unique pair of Index primers. Make sure to record what samples have what Index primers.
2. Using a multichannel pipette, transfer 1 μ L of the cleaned PCR product into a new plate and set up the reaction according to Table 11
3. Mix the reaction mix with a pipette. Cover and seal the plate with a Microseal.
4. Centrifuge PCR plate at 1000 x g at 20°C for 1 minute.
5. Put in a Bio-RAD T100 or T1000 using the following program:
 - 1) 95°C for 3 minutes
 - 2) 8 cycles of:
 - a. 95°C for 30 seconds
 - b. 55°C for 30 seconds
 - c. 72°C for 30 seconds

- 3) 72°C for 5 minutes
- 4) Hold at 12°C

STEP 4: PCR Clean up #2

This step is a basic AmpureXP protocol with some modifications. It is to clean up the final library before quantification.

Table 12 . PCR Clean Up #2 Consumables

Item	Volume
10 mM Tris pH 8.5	27.5 µL per sample
AMPure XP beads	25 µL per sample
Freshly prepared 80% Ethanol	400 µL per sample
96-well 200 µL PCR plate	
Thermofisher Microseal 'B'	
96-well MIDI plate (deep)	

1. Bring AMPure XP beads to room temperature
2. Centrifuge Index PCR plate at 1000 x g at 20°C for 1 minute to collect condensation.
3. Remove seal and transfer entire Index PCR product from the PCR plate to the MIDI plate using a multichannel pipette. Change tips between samples.
4. Vortex AMPure XP beads for 30 seconds and add them to sterile trough depending on the number of samples.

5. Add 25 μL of AMPure XP beads to each well of the PCR product. Change tips between samples.
6. Seal the plate with Microseal and place MIDI plate in shaker at 1800 rpm for 2 minutes.
7. Centrifuge the plate at 1000 x g at 20°C for 1 minute.
8. Incubate MIDI plate at room temperature for 5 minutes without shaking.
9. Put MIDI plate on magnetic stand for 3-5 minutes or as long as it takes for the supernatant to clear.
10. With the MIDI plate on the magnetic stand, remove and discard the supernatant with a multichannel pipette, changing tips between samples. Place the tip of the pipette on the sidewall of the MIDI plate and suck up the liquid, making sure not to disturb the beads.
11. With the MIDI plate on the magnetic stand, wash the beads with the ethanol:
 - a. Add 200 μL of ethanol to each sample well, do not mix up and down, changing tips between samples.
 - b. Incubate the plate on the magnetic stand for 1 minute.
 - c. Remove and discard the supernatant with a multichannel pipette, changing tips between samples.
12. With the MIDI plate on the magnetic stand, wash the beads a second time with the ethanol:
 - a. Add 200 μL of ethanol to each sample well, do not mix up and down, changing tips between samples.
 - b. Incubate the plate on the magnetic stand for 1 minute.

- c. Remove and discard the supernatant with a multichannel pipette, changing tips between samples.
 - d. Using a P20 multichannel pipette, carefully try to remove every last drop of ethanol.
13. With the MIDI plate on the magnetic stand, allow the beads to air-dry for 10 minute or until you can see fine hairline cracking on the bead droplet with a matte finish. If it looks like disintegrated powder, you have waited too long.
 14. Remove the MIDI plate from the magnetic stand and add 27.5 μL 10 mM Tris pH 8.5 to each well that contains PCR product, changing tips between samples. Mix up and down with the pipette to re-suspend the beads.
 15. Seal the plate with Microseal and place MIDI plate in shaker at 1800 rpm for 2 minutes.
 16. Incubate at room temperature for 2 minutes
 17. Place the MIDI plate on the magnetic stand for 2 minutes or until the supernatant is clear
 18. Transfer 25 μL of the supernatant from MIDI plate to a new 96-well PCR plate using a multichannel pipette. Change tips between samples.

This is a safe stopping point. The PCR plate can be sealed and stored at -20°C for 2 weeks. Always change tips between samples to avoid cross contamination.

STEP 5: Normalize concentrations and pool

This step is to normalize all of the DNA samples to 4nM before pooling. A fluorometric quantification assay is recommended by (Illumina, 2013) and in this study PicoGreen (Invitrogen) was used. Below are the instructions for the PicoGreen Assay:

DNA Quantification Protocol – Pico green Assay

A) Preparing your DNA:

1. Get 200x PicoGreen (Invitrogen) stock from supplier
2. Dilute this 200x stock to 2X with 10mM Tris pH8.5 in an Eppendorf or falcon tube
3. Diluted the DNA samples in a black 96-well plate: 5 μ L DNA + 45 μ L 10mM Tris pH 8.5
4. Next, add 50 μ L of the 2x Pico stock to the 50 μ L diluted DNA in the black 96 well plate, mix them well

B) Preparing the Standard Curve:

- The Stock lambda phage DNA is – 100 μ g/mL from Invitrogen, unless otherwise specified on tube – then you will have to adjust curve dilutions accordingly
- You will need to make the following 5 dilutions to create your standard curve, you can do this with a serial dilution as specified below.

- Make the dilutions in a clear 96 well plate and then transfer them into the black 96 well plate alongside your samples
- Make sure that they are in descending order in the black 96 well plate (highest to lowest)
 1. 2 $\mu\text{g}/\text{mL}$ – 2/100 dilution, 2ul stock DNA into 98 μL 10mM Tris pH 8.5
 2. 0.2 $\mu\text{g}/\text{mL}$ – 10 μL of 2ug/ml dilution into 90ul 10mM Tris pH 8.5
 3. 0.02 $\mu\text{g}/\text{mL}$ – 10 μL of 0.2ul/ml dilution into 90ul 10mM Tris pH 8.5
 4. 0.002 $\mu\text{g}/\text{mL}$ - 10 μL of 0.02ul/ml dilution into 90ul 10mM Tris pH 8.5
 5. 0 $\mu\text{g}/\text{mL}$ – 100 μL 10mM Tris pH 8.5
 6. Put 50 μL of these standard curve solutions combined with 50 μL 2x pico into the black 96 well plate, mix them well

C) Preparing the plate to be read by fluorescence spectrophotometer:

1. Set up standards and samples into black 96 well plate
2. Let the plate incubate in dark drawer for 5 minutes, this ensures Pico adheres to DNA
3. Turn on plate reader and choose “Pico green protocol”
4. Create new experiment with the protocol
5. Fill in the sample sheet to include your standard curve and samples
6. Export data to excel

- Using the standard curve, calculate DNA concentration in nM, based on the size of the DNA amplicon:

$$\left(\frac{\text{concentration in } \frac{\text{ng}}{\mu\text{L}}}{\left(\frac{660\text{-g}}{\text{mol}} \right) (\text{length of DNA amplicon})} \right) (10^6) = \text{concentration in nM}$$

- Use this information to dilute the DNA to 4nM with 10mM Tris pH 8.5 for sequencing
- Take 5 μL from each library and pool into new Eppendorf tube.

STEP 6: Right before sequencing

This step is to denature and dilute the pooled library before injecting the machine with a library sample.

Table 13 . Consumables for library denaturation and sample loading

Item	Volume
10 mM Tris pH 8.5	27.5 μL per sample
HT1 (Hybridization buffer)	1540 μL , on ice
0.2 N NaOH (fresh)	10 μL
PhiX control kit	
MiSeq reagent cartridge	

A) Preparation

- Set a heat block that would fit a 1.7mL Eppendorf tube to 95°C
- Remove MiSeq cartridge from -20°C freezer and thaw at room temperature
- Get ice bucket

B) Denature and Dilute DNA

1. Combine 5 μL of the pooled final DNA library and 5 μL of fresh 0.2 N NaOH in an Eppendorf tube.
2. Vortex gently to mix.
3. Centrifuge at 280 x g at 20°C for 1 minute.
4. Incubate at room temperature for 5 minutes to denature the DNA into single strands.
5. Add 990 μL of HT1 buffer to the 10 μL denatured DNA. This gives a 20 pM library in 1 mM NaOH.
6. Dilute with pre-chilled HT1 buffer to desired loading concentration.
 - a. For this study, 180 μL of the 20 pM library was added to 420 μL of HT1 buffer for a final loading concentration of 6 pM
7. Invert the DNA several times to mix and pulse centrifuge.
8. Place the DNA on ice until you are ready to proceed

C) Denature and Dilute PhiX DNA

1. Combine 2 μL of the 10 nM PhiX with 3 μL of 10 mM Tris pH 8.5 to dilute the PhiX library to 4 nM.
2. Combine 5 μL of the 4 nM PhiX library to 5 μL of 0.2 N NaOH
3. Vortex briefly to mix and then incubate for 5 minutes at room temperature to denature the dsDNA

4. Add 990 μL of chilled HT1 buffer to the 10 μL PhiX to make 20 pM PhiX library.
5. Invert the DNA several times to mix and pulse centrifuge.
6. Place the DNA on ice until you are ready to proceed

D) Combine Library and PhiX

1. Combine 30 μL of denatured and diluted PhiX control to 570 μL of denatured and diluted amplicon library in a micro-centrifuge tube.
 - a. For this study, 30 μL of 20 pM PhiX was added to 570 μL of 6 pM amplicon library
 - b. Some things that were learned:
 - It is worth using 10-20% PhiX to increase the diversity of the library
 - Don't load the sequencer with more than 10pM final library concentration. A lower than recommended loading concentration can help significantly with amplicon/low diversity libraries, and there will still be plenty of reads.
2. Set the sample library aside on ice until you are ready to heat denature the mixture immediately before loading it into the MiSeq v3 reagent cartridge.
3. Place the combined library on a heat block for 2 minutes at 95°C.
4. Then invert the tube to mix, and place on ice.
5. Keep the tube on ice for 5 minutes
6. Follow to Step 7.

STEP 7: Sequencing

1. Open reagent box that comes with the v3 MiSeq cartridge and clean the flow cell off with 70% Ethanol and a Kimwipe. Make sure that there are no smudges on the glass.
2. Load the cartridge with 600 μ L of the combined library sample on the indicated foil section.
3. Open the MiSeq program on the machine and follow the MiSeq on-screen prompts.

Appendix C - Scripts

C.1 Read Processing Script

```
#!/bin/bash

# ARGV 1 - data dir (where *.fastq files reside)
# ARGV 2 - output_prefix (make this unique between trials)
# Usage: Make new directory and put this script in it, and put a "data" directory in it that
contains the fastq.gz files, NAME THIS DIRECTORY "DATA"
# Usage: sh read_process_v6-final.sh "data" "trial", where data is the data directory and
trial is whatever name you want to call this run (ex: gyrA)
# Uncomment the following if your name is Leah:
fastqc="/home/leahclarke3/nas/leahclarke/programs/FastQC/fastqc"
flash="/home/leahclarke3/nas/leahclarke/programs/FLASH-1.2.11/flash"
trimmomatic="/home/leahclarke3/nas/leahclarke/programs/Trimmomatic-
0.36/trimmomatic-0.36.jar"

leading="10"; #minimum quality score for leading bases in trimmomatic
trailing="10"; #minimum quality score for trailing bases in trimmomatic
window_length="4"; #window length for sliding window in trimmomatic
window_qual="10"; #required quality for sliding window
min_length="36"; #minimum read length

min_overlap="20"; #minimum overlap for merging
max_overlap="250"; #maximum overlap for merging

suffix_for_paired="for_paired.fq";
suffix_rev_paired="rev_paired.fq";
suffix_for_unpaired="for_unpaired.fq";
suffix_rev_unpaired="rev_unpaired.fq";

ls -l ${1}/*.fastq.gz | sed 's,data/,g' | sed 's,_,\t,g' | cut -f 1 | sort -n | uniq >
${2}_read_LIBs.list

mkdir ${2}_trimmed_dir
mkdir ${2}_merged_dir
mkdir ${2}_qc_dir
mkdir ${2}_MultiQC_pretrimming
```

```

mkdir ${2}_MultiQC_posttrimming

for i in `cat ${2}_read_LIBs.list` ; do \
echo "#####"; \
echo "##### Starting process for strain ${i}"; \
echo "#####"; \

read_1_file="$(ls -l ${1}/${i}_*_R1*fastq.gz)";\
read_2_file="$(ls -l ${1}/${i}_*_R2*fastq.gz)";\

echo "#####"; \
echo "##### Make directories"; \
echo "#####"; \

mkdir ${2}_qc_dir/${i}_strain; \
mkdir ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.pre; \
mkdir ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.pre; \
mkdir ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.post; \
mkdir ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.post; \

echo "#####"; \
echo "##### Quality check raw reads with FastQC"; \
echo "#####"; \

${fastqc} --extract -o ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.pre $read_1_file; \
${fastqc} --extract -o ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.pre $read_2_file; \

echo "#####"; \
echo "##### Quality check FastQC zip files with multiqc"; \
echo "#####"; \

cp ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.pre/*fastqc.zip
${2}_MultiQC_pretrimming; \
cp ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.pre/*fastqc.zip
${2}_MultiQC_pretrimming; \

echo "#####"; \
echo "##### Trimming reads with Trimmomatic"; \
echo "#####"; \

```

```

java -jar ${trimmomatic} PE -threads 2 -phred33 $read_1_file $read_2_file
${2}_trimmed_dir/${i}_${suffix_for_paired}
${2}_trimmed_dir/${i}_${suffix_for_unpaired}
${2}_trimmed_dir/${i}_${suffix_rev_paired}
${2}_trimmed_dir/${i}_${suffix_rev_unpaired} LEADING:${leading}
TRAILING:${trailing} SLIDINGWINDOW:${window_length}:${window_qual}
MINLEN:${min_length}; \

```

```

echo "#####"; \
echo "##### Quality check the trimmed reads with FastQC"; \
echo "#####"; \

```

```

${fastqc} --extract -o ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.post
${2}_trimmed_dir/${i}_${suffix_for_paired}; \
${fastqc} --extract -o ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.post
${2}_trimmed_dir/${i}_${suffix_rev_paired}; \

```

```

echo "#####"; \
echo "##### Quality check FastQC zip files with multiqc"; \
echo "#####"; \

```

```

cp ${2}_qc_dir/${i}_strain/${i}_strain.r1_qc.post/*fastqc.zip
${2}_MultiQC_posttrimming; \
cp ${2}_qc_dir/${i}_strain/${i}_strain.r2_qc.post/*fastqc.zip
${2}_MultiQC_posttrimming; \

```

```

echo "#####"; \
echo "##### Trimmed, now merging with FLASH"; \
echo "#####"; \

```

```

${flash} -m $min_overlap -M $max_overlap -o $i -d ${2}_merged_dir
${2}_trimmed_dir/${i}_${suffix_for_paired}
${2}_trimmed_dir/${i}_${suffix_rev_paired}; \

```

```

echo "#####"; \
echo "##### Process complete for strain ${i}"; \
echo "#####"; \

```

```

done;

```

```

echo "#####"; \
echo "##### MutliQC"; \
echo "#####"; \
multiqc -o ${2}_MultiQC_pretrimming ${2}_MultiQC_pretrimming
multiqc -o ${2}_MultiQC_posttrimming ${2}_MultiQC_posttrimming

```

C.2 Fastq to Frequency Script

```
#!/bin/bash
```

```

#install a tool called dos2unix, you should be able to get it with:
#sudo apt-get install dos2unix

```

```

#Before running the script I did:
#cat SampleIDs-Nov9-gyrA.csv | sed 's, ,_g' | sed 's/,/t/g' > SampleIDs-Nov9-gyrA.tab
#SampleID file contains this information: Plate, PlateID, strain, replicate, timepoint,
medium, derived from Illumina Sample Sheet
#dos2unix SampleIDs-Nov9-gyrA.tab

```

```

# Usage: sh Fastq_to_Freqs_v2.sh SampleIDs-Nov9-gyrA.tab | column -t > counts.tsv
# ARGV 1 - TAB file of sample sheet with only samples to process
#$2=merged_reads_dir # make arg
merged_reads_dir="./good_merged_dir"
allele_file="mutants-short.unix.txt"

```

```

echo "Strain Sample Medium Replicate Timepoint wt_count mut_count sum total" | sed
's, ,\t,g'
samplelist="$(tail -n +2 "${1}" | cut -f 1)"

```

```
for i in $samplelist; do \
```

```

plate="$(grep -E "^${i}[:space:]" "${1}" | cut -f 2); \
plateid="$(grep -E "^${i}[:space:]" "${1}" | cut -f 3); \
strain="$(grep -E "^${i}[:space:]" "${1}" | cut -f 4); \
replicate="$(grep -E "^${i}[:space:]" "${1}" | cut -f 5); \
timepoint="$(grep -E "^${i}[:space:]" "${1}" | cut -f 6); \
medium="$(grep -E "^${i}[:space:]" "${1}" | cut -f 7); \

```

```

total="$(awk -F\t 'BEGIN{n=0} { n++ }END{print n/4}'
$merged_reads_dir/"${i} ".extendedFrag.fastq"); \

mut="$(grep -E "^${strain}" $allele_file | awk '{print toupper($2)}'); \
wt="$(grep -E "^${strain}" $allele_file | awk '{print toupper($3)}'); \

mutc="$(grep -c "$mut" $merged_reads_dir/"${i} ".extendedFrag.fastq); \
wtc="$(grep -c "$wt" $merged_reads_dir/"${i} ".extendedFrag.fastq); \

echo "$strain $i $medium $replicate $timepoint $wtc $mutc $total" | sed 's, ,\t,g' | awk
'{print $1, $2, $3, $4, $5, $6, $7, $6+$7, $8}' | sed 's, ,\t,g'; \

done;

```

Appendix D - Miscellaneous

D.1 Table of Abbreviations

Table 14. Abbreviations

<i>Abbreviation</i>	<i>Explanation</i>
AMR	Antimicrobial Resistance
Cip	Ciprofloxacin
Rif	Rifampicin
HGT	Horizontal Gene Transfer
WHO	World Health Organization
LB	Lysogeny Broth
MIC	Minimum Inhibitory Concentration
MDR	Multidrug resistant
MBE	Mobile Genetic Element
G*E	Genotype-by-Environment interaction
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. X-gal is an analog of lactose, and can be hydrolyzed by the β -galactosidase enzyme which cleaves the β -glycosidic bond in D-lactose. X-gal, when cleaved by β -galactosidase, yields galactose and 5-bromo-4-chloro-3-hydroxyindole - 1. The latter then dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo - 2, an intensely blue product.
IPTG	Isopropyl β -D-1-thiogalactopyranoside. IPTG binds to the lac repressor and releases the repressor from the lac operator, thereby allowing the transcription of genes in the lac operon, such as the gene coding for beta-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of β -galactosides into monosaccharides.
Sewage Sludge	Sludge is whatever is removed from the wastewater in order to ensure the effluent (treated wastewater) meets effluent

	guidelines.
Anaerobic digestion	In an anaerobic digester, initially there are complex organics within the sludge that are broken down to soluble organics by enzymes through a process known as hydrolysis. These soluble organics are then converted to organic acids by acid producers. Finally, methanogens (a type of bacteria) use the organic acids and convert them into methane and carbon dioxide.

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