Antibiotic Resistance: Development and Dissemination *in vitro* and in the Marine Environment

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Preface

The work presented in this thesis was performed in the laboratory of Dr. Peter Belenky at Brown University. I performed all of the experiments and analyses reported with the following exceptions.

Chapter 2 – Stella Ye Eun Lee, Amit K. Chakrabarti, and Ashley Choi assisted with the data collection for Figure 1A, B. Collin Ganser assisted with the data collection for Figure 3B.

Chapter 3 – Dr. Jeremy Collie and the staff of the University of Rhode Island Graduate School of Oceanography Narragansett Bay Fish Trawl assisted with the collection of the fish samples used in this study. Dr. Kathleen Duffy assisted with the collection of shark samples used in this study.

Chapter 4 – Damien Cabral performed the mouse experiments and generated the sequencing data used for this analysis.

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CHAPTER 1: INTRODUCTION

An Overview of Antimicrobial Resistance

Global Burden of Antibiotic Resistance

The global antimicrobial resistance crisis represents a critical threat to human health and our ability to prevent and control bacterial infections. In the United States alone, antimicrobial resistant infections result in an estimated 35,900 deaths and nearly three million infections annually[1]. This health burden results in significant economic loss, with resistant infections costing the United States tens of billions of dollars a year in health care costs and lost productivity[1]. Globally, it is estimated that in 2019 there were 4.95 million deaths associated with antimicrobial resistant infections[2], and this number is only projected to grow. Projections for the year 2050 estimate that antimicrobial resistant infections will account for 10 million deaths annually and become the leading cause of death globally[3]. Without changes to current antibiotic stewardship practices and treatment strategies the global healthcare system is headed towards a post-antibiotic era[4, 5]. To overcome the antimicrobial resistance crisis and ensure a future in which bacterial infections remain treatable, we must modify our current use of antibiotics and develop novel therapies.

The Rise in Resistant Infections

Without significant innovations to limit the spread of antimicrobial resistance, bacterial infections will have an extremely negative impact on global health. In 2016, 270.2 million antibiotic prescriptions were written in the United States alone. Of these, the CDC estimates that 30% were prescribed for infections

that did not require antibiotics[6]. Fluoroquinolone antibiotics are commonly misused in healthcare settings, with an estimated 5% of these prescriptions being unnecessary and roughly 20% of all fluoroquinolones prescribed in situations when the drug was not recommended as a first-line antibiotic[6, 7]. One example of an increasingly resistant bacterial pathogen is drug resistant *Neisseria gonorrhoeae*. Between 2000 and 2007 *N. gonorrhoeae* infections resistant to ciprofloxacin (a fluoroquinolone) increased by 30%, leading to ciprofloxacin being no longer recommended as a first-line therapeutic[1]. In fact, some strains of *N. gonorrhoeae* have developed resistance to nearly all antibiotic classes leading to a rise in multidrug resistant infections[8-13]. Worryingly, some other bacterial pathogens, such as totally drug resistant (TDR) *Mycobacterium tuberculosis*, have become resistant to all current antibiotics[14]. Fortunately, some studies have suggested that reducing antimicrobial usage may be effective in reducing resistant infections in a hospital setting[15-19], suggesting that proper antimicrobial stewardship is a powerful strategy to mitigate the spread of resistance[20]. In addition to misuse of antibiotics in the healthcare setting, overuse of antibiotics in agriculture and aquaculture promotes the spread of antimicrobial resistance[21, 22].

Agricultural practices, specifically livestock production, use tens of thousands of tons of antimicrobials a year and this number is only expected to grow with the increased demand for livestock[23-25]. Use of antibiotics in agriculture is known to increase antibiotic concentrations in the associated soil/water resulting in the potential selection for antibiotic resistant bacteria in the environment[26-29]. Furthermore, resistant pathogens are able to transfer

between livestock and humans through farming and consumption making it imperative to limit the unnecessary use of antimicrobials in these agriculture settings[30-33]. Evidence has shown that effective limits to antimicrobial use in livestock can reduce the abundance of associated antimicrobial resistance[34]. In a Danish study, the occurrence of glycopeptide-resistant *E. faecium* isolated from chickens was reduced from 72.7% to 5.8%, coinciding with the ban of the glycopeptide antibiotic avoparcin in Denmark[34]. This work gives hope that significant policy changes will reduce the environmental burden of antimicrobial resistance genes in the agricultural setting. In addition to curbing the misuse and overuse of antimicrobials, we must develop novel antibiotics and therapeutic strategies.

Challenges and the Future of Antibiotic Therapy

The introduction of antibiotics as therapeutics revolutionized medicine and has saved millions of lives. The identification and subsequent use of penicillin as an antimicrobial treatment marked the dawn of antibiotic discovery in the early 20th century[35-37]. Between 1930 and the 1960s, numerous broad-spectrum antibiotic classes were identified resulting in a potent arsenal of drugs targeted towards bacteria. Since this “golden era” of antibiotic discovery, there has been little innovation, and no novel classes of clinically useful antibiotics have been introduced in over 30 years[38-40]. Alongside the discovery of these new antimicrobials came the emergence of resistance to each drug[38, 41]. While the antibiotics remained potent for most of the 20th century, the rapid development and

spread of resistance combined with a lack of novel antimicrobial treatments has severely challenged our ability to treat infections. Despite the need for investment in novel drugs, most large pharmaceutical companies have abandoned antibiotic research and development due to the high cost of bringing a new drug to market, inevitable incidence of resistance, sparing use of novel antimicrobials as “last resort” drugs, and ultimately low return on investment [42-45]. Due to the resulting antibiotic resistance crisis, a number of small- and medium-sized enterprises have continued research into novel treatments that are currently in the preclinical stage with over 400 unique projects targeting direct-acting small molecules, potentiators, repurposed drugs, antibodies and vaccines, immune-modulators, antivirulence, microbiome, and phage therapies [46]. Global collaboration has led to the establishment of several non-profit agencies, including CARB-X and JPIAMR, focused on supporting the discovery of novel antimicrobials[47, 48]. In addition to developing new therapeutics, we must work to understand the process by which antimicrobial resistance develops on a molecular level in order to develop novel approaches to combat resistance.

Mechanisms and Development of Antimicrobial Resistance

Mechanisms of Antimicrobial Resistance

Soon after the discovery of penicillin, scientists had already found bacteria that could survive drug treatment, providing some of the earliest evidence of antimicrobial resistance [49, 50]. In the decades following, resistance has been identified for all antibiotic compounds in clinical use. Significant work has been

done to describe the mechanisms behind different forms of resistance, uncovering the unique strategies employed by bacteria to survive drug treatment[41]. Antibiotic resistance is separate from strategies such as 'tolerance' and 'persistence' in which bacteria are able to survive exposures to a typically lethal concentration of antibiotics without a change in minimum inhibitory concentration (MIC)[51-54]. These transient phenotypes are different from antibiotic resistance which is defined as an inheritable mechanism by which the bacteria are able to grow in high antibiotic concentrations due to an increased MIC[54].

There are several basic mechanisms of resistance that are used by many different bacterial taxa to overcome antibiotic activity. Preventing entry of antibiotics through **reduced cell permeability** is an effective strategy for resistance. While some antibiotics are able to enter the cell through outer-membrane porins, bacteria such as those in the family Enterobacteriaceae have been shown to resist carbapenems by reducing porin expression or expressing non-functional porins[55-57]. Preventing antibiotic entry is an effective strategy, however, members of Enterobacteriaceae and other bacterial families are also able to employ **antibiotic modification/degradatio**n mechanisms. Many of these bacteria, including the high priority pathogens *Klebsiella pneumoniae* and *Acinetobacter baumannii*, encode a form of β-lactamases that effectively hydrolyze β-lactam and carbapenem antibiotics rendering them inactive[58, 59]. Some resistant bacteria contain genes encoding for **drug efflux** pumps that can confer resistance to a wide range of antibiotic compounds. These efflux pumps can be fairly antibiotic specific, such as the *tet* family often found in gram-negative

bacteria[60, 61], or more general such as the multidrug resistant (MDR) efflux pumps that are found in a wide range of bacterial taxa[60, 62]. Changes in **gene expression**, such as the transcriptional activation of multidrug efflux pumps in *Salmonella enterica* serovar Typhimurium upon exposure to bile allow for transient activation of antibiotic resistance[63]. Card et al. performed a resistance evolution experiment in *E. coli* and found that all strains generally evolved resistance-conferring mutations in the transcriptional regulator *ompR* responsible for altering gene expression of the porin, OmpF[64]. Finally, **target modification** and **target protection** can result in structural changes within bacterial machinery that prevent the binding of antibiotics[60, 65, 66]. These forms of resistance are the result of genetic determinants or modifications to existing genes.

Resistance is heritable and is the result of antibiotic resistance genes (ARGs) that encode for one of the aforementioned mechanisms. Resistance genes can be found chromosomally or on mobile elements such as plasmids, like the pNDM-CIT plasmid found in *Citrobacter freundii* which contains both an metallo-β-lactamase and MDR efflux pump[60, 67]. Resistance can occur as dedicated resistance genes, duplicated genes, or result from the insertion of a novel promoter or mutational events in existing genes[41]. Mutational events can include point mutations, insertion, or deletion mutations that result in changes in bacterial phenotype.

Development of Resistance Through Mutagenesis

Mutagenesis is a vital process for introducing novel genotypes and phenotypes in bacteria. This process is especially relevant in the evolution of antimicrobial resistance in pathogenic bacteria. In fact, most commonly used antibiotics have lost their efficacy due to the introduction of resistance-conferring mutations. One of the starkest examples of this is extensively drug-resistant *Mycobacterium tuberculosis* (XDR-TB), which is resistant to both first- and second-line antibiotics due entirely to chromosomal mutations[68, 69]. Mutations in bacteria can be generated via exposure to stressors that directly mutagenize DNA or inhibit DNA repair enzymes, or stressors that activate bacterial stress responses involved in adaptive mutagenesis. MacLean et al. described these two mechanisms as stress-associated mutagenesis (SAM) and stress-induced mutagenesis (SIM), respectively[70]. When bacterial cells undergo SAM, genotoxic stressors such as UV light[71, 72], reactive oxygen species (ROS), or alkylating agents[73] physically damage DNA resulting in mutation. Additionally, it has been hypothesized that some stressors such as heavy metals may induce mutagenesis by interrupting necessary DNA-repair proteins[74-76]. In contrast, SIM mechanisms increase mutation rates via activation of mutagenic genes. These genes primarily include error-prone DNA polymerases[70, 77-80] and other "evolvability factors" such as Mfd[81]. SIM mechanisms can be induced by stressors such as antibiotics[80] and nutrient limitation[79], allowing bacteria to undergo adaptive mutation in order to increase the genetic diversity within a population and overcome stressful conditions. Stressors that can trigger both SAM

and SIM mechanisms are prevalent in bacterial environments, thus it remains imperative to study how these mechanisms contribute to the development of resistance.

The role of mutagenesis has been recognized as a potential target for intervention to slow the development of antibiotic resistance[81-83]. Previous work in fungi has shown that the protein Hsp90, a molecular chaperone, plays a key role in the evolution of adaptive traits including resistance to anti-fungal drugs[84]. Later work by Shekhar-Guturja et al. identified a small molecule that could block the activity of Hsp90, thus inhibiting the evolution of resistance through this key pathway[85]. Recently, work has identified targets to inhibit pathways of adaptive evolution in bacteria in order to prevent the development of antibiotic resistance mutations. Ragheb et al. identified the DNA translocase Mfd as having a role in promoting mutagenesis in several species of bacteria. In *Salmonela typhimurium* grown in subinhibitory concentrations of antibiotics, Mfd was shown to significantly promote the evolution of resistance to rifampicin, phosphomycin, trimethoprim, kanamycin, and vancomycin[81]. Thus, the authors propose that Mfd could be a potential target to prevent drug-resistance mutations from developing in the context of infection. Additional research found another mutagenetic pathway in bacteria, the σ^S response, led to an increase in drug resistance development in response to antibiotic-induced reactive oxygen species [80]. The researchers found that the FDA-approved drug edaravone could reduce σ^S response-dependent mutagenesis and inhibit subsequent resistance development[80]. This work shows that in addition to discovering novel antimicrobials, understanding and

targeting adaptive mutagenic pathways in bacteria is an effective way to prevent the development of resistance.

Horizontal Gene Transfer as a Mechanism to Spread Resistance

While resistance can be inherited or generated through *de novo* mutations, it can also be passed between bacteria through horizontal gene transfer (HGT). Along with mutagenesis, HGT plays a critical role in expanding the genetic diversity of bacteria, allowing DNA to be exchanged between cells. Interbacterial DNA transfer has contributed to the composition of a significant portion of bacterial genomes[86-89]. While the amount of laterally transferred DNA varies among bacterial species, some have an estimated 20% of their genome arising from HGT events[87, 89]. HGT can occur between bacteria through three main processes: conjugation, transduction, and natural transformation[90]. Conjugation occurs when chromosomal or plasmid DNA is transferred through a pilus structure physically joining two bacteria[91]. Transduction is a bacteriophage-mediated transfer of DNA between bacteria that are susceptible to the same bacteriophage[92]. Natural transformation is a process during which a bacterial cell is able to take up extracellular DNA and recombine it into its own genome[89, 90, 93]. All these mechanisms play an important role in the spread of antimicrobial resistance between bacteria[32, 91, 94-98]. Instead of having to develop a resistance gene *de novo*, HGT can provide instantaneous resistance and rapid adaptation to an antibiotic environment.

Selection for Antibiotic Resistance

Antibiotic selection is a key factor in determining whether or not resistance genes will be maintained in a given environment. Resistance is often associated with having significant fitness costs that would select against resistant bacteria in an antibiotic-free environment[99, 100]. In situations where the cost of maintaining resistance is high, such as the bla_{CMY-2} plasmid-mediated multidrug resistance found in *E. coli*, antibiotic selection is necessary for long-term maintenance of resistance[101]. In other cases, the cost of resistance mutations may be too high for resistant bacteria to establish infections. In *E. coli* responsible for urinary tract infections, fosfomycin resistance can be conferred by mutations in the *uhpT* and *glpT* genes encoding for transporters responsible for fosfomycin into the cell. While these mutations occur readily, they also incur growth defects that prevent fosfomycin-resistant *E. coli* strains from establishing colonization in the bladder and causing infection[102, 103]. Lab-based experiments in 23 drug-resistant *E. coli* strains found that evolution for 60 days under antibiotic-free conditions lead to a loss in resistance to some, but not all antibiotics[104]. This suggests that antibiotic selection is necessary for some, but not all resistance genotypes.

Further work examining antibiotic resistance and selection has found that little to no selection is needed to maintain some resistance mutations in bacterial populations. Marcusson et al. found that *E. coli* strains containing fluroquinolone-resistance mutations had fitness advantages compared to susceptible strains in both *in vitro* and *in vivo* settings[105]. There is additional evidence that antibiotic resistant strains can persist in drug free environments via the evolution of

compensatory mutations, which alleviate the fitness costs of resistance mutations[106-109]. This data suggests that antibiotic selection is not necessary for maintaining antibiotic resistance genes, making it a priority to monitor resistance even in environments where antibiotic selection pressures are very low.

Selection for Resistance in Complex Microbial Communities

Studies have found that microbial communities of the gut and the natural environment harbor diverse assemblages of resistance genes[110-112]. The total resistance gene content of a community is referred to as the "resistome"[113]. In these communities, resistance can be selected for by the introduction of antibiotics or other selective compounds. Even low, environmentally relevant concentrations of some antibiotics are sufficient to maintain resistance in bacteria[114, 115]. In the gut microbiomes of animals treated with antibiotics, there is evidence of enrichment of ARGs with activity against the antibiotic class present[27, 34, 116, 117]. This suggests that antibiotics are capable of causing selection within an organism. The process by which selection occurs in all environments is dependent on a myriad of factors, but importantly the local concentrations of antibiotic to which bacteria are exposed. For example, during therapeutic use of antibiotics in humans, there is a heterogeneous environment creating concentration gradients[118]. Baquero and Negri first put forth the concept of "selective compartments" to describe how these pockets of different concentrations have the potential to select distinct resistance genotypes[119, 120]. This is an important

consideration when studying the selection of resistance in laboratory, therapeutic, and environmental settings.

The Natural Environment and Organismal Microbiome is a Reservoir of Resistance

Antimicrobial Resistance is an Ancient Natural Phenomenon

The environment represents an important reservoir for antibiotic resistance. Resistance originated in the natural environment long before antibiotics were used as therapeutics by humans, a phenomenon which has been highlighted by numerous studies[121-125]. Bacterial strains isolated from a cave thought to be undisturbed for millions of years were shown to have resistance to 14 commercial antibiotics[122]. Beringian permafrost ice cores estimated to be 30,000 years old contained bacterial resistance genes against β-lactam, tetracycline, and glycopeptide antibiotics[123]. While antibiotics and antibiotic resistance are commonly thought of in terms of the “weapon-shield” role they perform in the clinical setting, the ancient origins of ARGs have led to alternative explanations for their existence. It has been demonstrated that at sub-clinical concentrations (levels which occur naturally in environmental microbial communities) antibiotics can act as signaling molecules between bacteria[126-130], and some bacteria even utilize antibiotics as carbon sources[131]. Despite their ubiquity and fundamental role in bacterial life, there is evidence that ARG abundance has increased in the environment in association with the widespread anthropogenic use of antibiotics. Time course sampling of soil archives from the “Pre-Antibiotic” age (before

widespread anthropogenic use of antibiotics) through modern times revealed significant increases in all 18 ARGs examined by Knapp et al. Some genes were 15 times more abundant in the most recently sampled soil (year 2008) compared to samples from the 1970s[121]. Combined, these studies illustrate the ancient origins of resistance and an association between human use of antibiotics and an increase in the abundance of ARGs in the environment.

The Gut Microbiome as a Reservoir of Resistance

Resistance is not limited to the physical environment, but also the microbiota of organisms that live there. Today, the importance of bacteria as co-habitants of humans and other animals has been widely recognized. In the past two decades, the microbiome field has made significant advances in understanding the role that bacteria play in host health and disease across the animal kingdom. Bacteria exist in and on nearly all of the body exposed to the environment and are important colonizers of the mouth, skin, and gastrointestinal tract (GIT). A majority of these microbes reside in the gut with the human GIT being home to an estimated 100 trillion bacterial cells[132, 133]. These GIT-associated bacteria provide numerous functions such as acting as a barrier against pathogen colonization[134], regulating epithelial cell and global immune function[135, 136], assisting in digestion[137], neurological function and behavior[138], and growth and development[133]. Beyond these beneficial functions, the host-associated bacteria in humans and wildlife have been shown to be diverse reservoirs of antimicrobial resistance genes[125, 139-145]. A study of the bacteria in dental

calculus from Swedish brown bears was able to provide a temporal view of antibiotic resistance associated with the bear oral microbiome over an 80-year time span, showing that human antimicrobial use has had an impact on ARGs in wildlife. The researchers found that the ARG abundance in the dental calculus bacteria of bears increased with extended anthropogenic antibiotic use over the past 80 years[145]. Further research has implicated the natural environment as a route of introduction of pathogenic bacteria and ARGs into humans and the clinical setting. Several studies have found direct transfer of antibiotic resistant bacteria between animals and humans in farm and aquaculture settings[30, 31, 33, 146, 147]. This data suggests that both the microbiome and resistome of wild animals may be impacted by human antimicrobial use and are important reservoirs of ARGs that could spread to humans.

The Marine Environment as a Reservoir of Resistance

The marine environment is one of the most expansive, species-rich, ecologically and economically important habitats on earth. As such, it is a priority to study microbes and antimicrobial resistance within this environment and the organisms that inhabit it. It is home to an estimated 200,000 catalogued species of Eukaryotes, including ray-finned fishes (Actinopterygii), the most diverse group of vertebrates on earth [148]. Marine-associated bacteria are known to harbor a diverse array of resistance genes[149]. Studying ARGs in marine wildlife will allow us to monitor how anthropogenic changes impact the resistome of wild organisms in the marine environment and monitor environment to human transmission of

ARGs. Notably, many marine organisms exhibit highly migratory behavior which has implications for these animals being vectors of ARG dispersal across great distances[150].

The microbiota of marine organisms commonly includes bacteria from potentially pathogenic genera such as *Vibrio*, *Photobacterium, Campylobacter,* and *Pseudomonas*[151]. Many pathogenic members of the genus *Vibrio*, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* which are responsible for the human diarrheal disease cholerae and seafood poisoning respectively[152], are known to be capable of HGT and thus at significant risk of gaining resistance genes. HGT has even spread ARGs from the marine environment to the clinical setting, including the quinolone resistance gene *qnrA* that originated in the marine bacteria *Shewanella algae*[153]. This gene encodes for a pentapeptide repeat protein that binds to DNA gyrase, inhibiting the negative effects of fluoroquinolones[154]. This *qnrA* gene and other marine-originating *qnr* genes have been broadly disseminated to other bacteria including the family Enterobacteriaceae, and have been described in clinical infections of the pathogenic bacteria *Klebsiella pneumoniae*[155] and *Enterobacter cloacae[156]*. The diversity of antibiotic resistant genes, potential pathogens, and evidence for the transfer of resistant bacteria from the aquaculture environment to human hosts make studying the dynamics of antibiotic resistance in the marine environment a priority.

Sequencing Tools for Detection of Antimicrobial Resistance and Studying Microbiomes

Limitations of Traditional Techniques

While antibiotic resistance has been observed in clinical isolates since the therapeutic use of antibiotics, the ability to use molecular tools to identify resistance is quite recent. Early reports of antibiotic resistance in *Bacillus coli* (now *E. coli*) occurred shortly after the introduction of penicillin as a therapeutic in 1942[157], but well before the structure of DNA, the basis for much of molecular biology, had been solved. Traditionally, clinical microbiology techniques have been used to isolate, grow, and quantify antibiotic resistance in both clinical and environmental bacterial isolates. In order to detect an antibiotic resistant isolate, it was necessary to be able to grow the specimen in culture and perform subsequent susceptibility assays[158, 159]. However, this method is limited by our ability to culture only a small fraction of all bacterial species[160]. For successfully cultured microbes, antimicrobial susceptibility/resistance is typically represented by the minimum inhibitory concentration, or MIC. This value represents the minimum amount of antibiotic needed to inhibit bacterial growth. The MIC value can be experimentally determined using a variety of methods including the broth microdilution test or disc diffusion assay[159]. Upon determining susceptibility, either targeted or whole-genome shotgun sequencing must be performed to identify the genetic basis of resistance. This process of culturing and testing isolates has been instrumental to building our current knowledge of antibiotic

resistance genes. However, it is time consuming, low throughput, and is limited by scientists' ability to culture bacteria in the lab.

Sequencing Based Methods for Studying the Resistome

The increasing accessibility of genomic sequencing and the development of powerful bioinformatic tools has revolutionized our ability to detect both known and novel antibiotic resistance genes (ARGs). New methods allow for identification of known ARGs and prediction of potential ARGs in non-culturable organisms at a high throughput scale[41, 139]. These methods are particularly effective at identifying resistance genes from shotgun sequencing of complex microbial communities such as environmental or gut microbiomes[139, 161]. D'Costa et al. coined the term "resistome" to describe the total collection of ARGs in a community[113]. In order to characterize the resistome, DNA is isolated from a sample of interest and shotgun sequenced in order to obtain untargeted sequence reads from all the bacterial genomes present. Following shotgun sequencing, bioinformatic tools such as DeepARG[162] are used to query the sequencing reads against databases containing nucleotide sequences of previously identified ARGs such as the Comprehensive Antibiotic Resistance Database (CARD)[163] and the Universal Protein Knowledgebase (UNIPROT)[162, 164]. Recent developments in these computational pipelines have implemented deep learning, a power machine learning strategy, in order to not only identify known ARGs, but also predict potential ARGs that may not otherwise be detected using the traditional strict cutoffs of traditional "best hit" approaches[162]. Using bioinformatic pipelines, it is

now possible to assemble bacterial genomes from shotgun sequencing datasets using assembly tools such as MEGAHIT[165]. This provides the opportunity to construct full-length ARG sequences and contextualize them in terms of their host phylogeny and position within the genome. While these analyses are excellent at identifying known resistance genes in complex microbial communities and provide a high throughput method to sample the resistome of clinical or environmental samples, they rely on databases of previously identified and characterized sequences and thus detection of ARGs is limited to known genes.

In addition to using shotgun metagenomic sequencing for identification of ARGs, this data can be used to further interrogate the microbiome. Traditionally, microbiome studies have primarily focused on defining bacterial taxonomy and functional potential. However, shotgun metagenomic sequencing is a powerful tool that provides an untargeted view of all the DNA in a given sample including bacterial, viral, eukaryotic parasites, host-derived sequences, and dietary signatures. Combining high throughput sequence alignment programs and databases of known sequences, it is possible to gain a more complete view of the microbiome. Beyond knowing what DNA is present in a sample, the use of metatranscriptomic sequencing of the RNA portion of a microbial community provides a view of the transcription activity within a sample[166-168]. In the context of antibiotic resistance, metatranscriptomics has the potential to show which resistance genes are being transcribed in response to external inputs such as antibiotic therapy[169]. Together, the use of modern high throughput sequencing

and bioinformatic analyses provide novel insights into complex microbial communities and associated antibiotic resistance.

Thesis Overview and Summary of Findings

Antimicrobial resistance is a significant threat to our current arsenal of antibiotics and challenges the ability of healthcare professionals to deliver effective treatments for bacterial infections. In order to combat the threat of antimicrobial resistance, we must understand (1) how antibiotic resistance develops, (2) the dynamics and prevalence of resistance in the environment, and (3) how antimicrobial therapy affects resistance gene abundance and expression in the microbiome.

Previous work has shown that antibiotic resistance can develop through the introduction of point mutations causing target modifications. Additionally, bacteria are exposed to genotoxic stressors which can cause mutations, which then undergo a process of selection. The work presented in Chapter 1 outlines the process by which stress-associated mutagenesis leads to spectinomycin resistance through a spectrum of mutations and a gradient of selection in the model organism *B. subtilis*. Using a combination of traditional microbiology and high throughput DNA sequencing methods, we are able to provide novel insights into the development and selection of spectinomycin resistance. We exposed the model bacteria *B. subtilis* to six different stressors including stationary phase stress, mitomycin C, ciprofloxacin, UV radiation, H_2O_2, and a metal $CoCl_2$. Through sequencing nearly 4,500 individual resistant isolates, we were able to

identify 69 novel ribosomal mutations in the *rpsE* and *rpsB* genes of *B. subtilis* and find that different genotoxic agents cause unique, stress-specific spectra of mutations. Some of these mutational spectra are reflective of the DNA-damaging mechanism of the stressor, while others seemed to reflect a less defined stress-induced mechanism. Finally, we grew several spectinomycin-resistant strains in competition under a range of drug concentrations in order to determine the impact of an antibiotic gradient on the selection of specific mutations within a mixed population. We show that the fitness of individual mutations and their ability to persist in a mixed population is determined by the local antibiotic concentration. Combined, this work identifies novel target mutations to spectinomycin and demonstrates that the path to resistance is determined by both the stressors present and subsequent antibiotic selection gradient.

Understanding antibiotic resistance is not limited to defining the development and selection process that shapes resistance mutations in the laboratory setting. Environmental microbiomes have been shown to be important reservoirs of resistance, with high ARG diversity, gene exchange, and the potential for transmission from environment to humans. The microbiota of marine organisms represents an understudied potential reservoir of resistance with important ecological and economic implications. In Chapter 3, I study the dynamics of antibiotic resistance within the GIT microbiomes of 7 marine fish and sharks. I collected the GIT contents from wild populations of four demersal fish species that inhabit a local estuarine habitat, Narragansett Bay, RI, including *Peprilus triacanthus* (butterfish), *Stenotomus chrysops* (scup), *Paralichthys dentatus*

(summer flounder), and *Mustelus canis* (smooth dogfish), and three large pelagic shark species, *Alopias vulpinus* (thresher shark), *Isurus oxyrinchus* (shortfin mako shark), and *Lamna nasus* (porbeagle shark) that were collected off the coast of New England. Using a shotgun metagenomic sequencing approach, I was able to define the taxonomic and resistance gene composition of these unique microbial communities. From this data I made a novel finding that the abundance of ARGs appears to increase with trophic level. Higher trophic level organisms (summer flounder, smooth dogfish, mako shark, porbeagle shark, thresher shark), defined by their piscivorous feeding behavior, tended to have higher levels of resistance genes compared to the lower trophic level species that were primarily planktivores and benthivores (butterfish and scup). Additionally, the abundance of ARGs was also significantly associated with levels of Proteobacteria within the sample. Using a computation pipeline, I assembled bacterial genomes from all samples and found that genomes from the phylum Proteobacteria had, on average, higher levels of ARGs than genomes from other bacterial phyla. Additionally, through use of DNA metabarcoding and bacterial gene function analysis, I was able to define the dietary contents of these fish species and make inferences about diet-associated carbon utilization by bacteria within the GIT microbiota. This is the first report to identify a trophic accumulation of antibiotic resistance and link it to an association between ARGs and Proteobacterial abundance.

While shotgun metagenomics is a valuable tool for defining the taxonomy, resistance gene content, and functional potential of the microbiome, it lacks the ability to determine which genes are being actively expressed. To this point, most

studies have used DNA sequencing to characterize the ARG composition of the gut resistome. In Chapter 4, I analyze resistance gene data from a dual DNA/RNA sequencing approach in order to define the impact of three antibiotics on the murine resistome. Mice were treated with either amoxicillin, doxycycline, or ciprofloxacin and the DNA and RNA of cecal contents was isolated and shotgun sequenced. I found that DNA sequencing revealed broad, untargeted changes to the resistome, but transcriptional changes were more targeted towards the antibiotic treatment. Amoxicillin and doxycycline induced an increase in abundance of β-lactamase and tetracycline resistance gene transcripts, respectively. Additionally, we observed a bloom in the bacteria *Bacteroides thetaiotaomicron* upon treatment with amoxicillin. Looking more closely at the metagenomically assembled genome of *B. thetaiotaomicron*, I found that it contains a class A β-lactamase which may have accounted for the increased abundance of β-lactamase gene transcripts and expansion of the *B. thetaiotaomicron* population during amoxicillin treatment. The data presented in this chapter demonstrates the benefits of a dual DNA/RNA sequencing methodology when studying the gut resistome in response to antibiotics. Sequencing of bacterial transcripts shows that the expression of antibiotic resistance genes is far more targeted than changes in resistance gene content would suggest.

The work presented here provides novel insights into the development of resistance mutations, the role of the marine environment as a reservoir of resistance, and the functional response of the resistome to antibiotic perturbation.

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CHAPTER 2: Genotoxic Agents Produce Stressor-specific Spectra of Spectinomycin Resistance Mutations based on Mechanism of Action and Selection in *Bacillus subtilis*

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Genotoxic Agents Produce Stressor-specific Spectra of Spectinomycin Resistance Mutations based on Mechanism of Action and Selection in *Bacillus subtilis*

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Abstract

Mutagenesis is integral for bacterial evolution and the development of antibiotic resistance. Environmental toxins and stressors are known to elevate the rate of mutagenesis through direct DNA toxicity known as stress-associated mutagenesis, or via a more general stress-induced process that relies on intrinsic bacterial pathways. Here, we characterize the spectra of mutations induced by an array of different stressors using high-throughput sequencing to profile thousands of spectinomycin resistant colonies of *Bacillus subtilis*. We found 69 unique mutations in the *rpsE* and *rpsB* genes, and that each stressor leads to a unique and specific spectrum of antibiotic resistance mutations. While some mutations clearly reflected the DNA-damage mechanism of the stress, others were likely the result of a more general stress-induced mechanism. To determine the relative fitness of these mutants under a range of antibiotic selective pressure, we used multi-strain competitive fitness experiments and found an additional landscape of fitness and resistance. The data presented here support the idea that the environment in which the selection is applied (mutagenic stressors that are present), and changes in local drug concentration, can significantly alter the path to spectinomycin resistance in *B. subtilis*.

Introduction

While maintaining the integrity of DNA is vital to survival, the introduction of mutations is equally important for the long-term success of a population as it enables continued adaptation. In bacteria, mutations that lead to even a single nucleotide change can have an extensive impact on phenotype, such as by changing host tropism[1], altering virulence[2], and introducing antibiotic resistance[3]. Point mutations can be introduced through a variety of mechanisms including the induction of mutagenic DNA repair pathways[4-7], inhibition of DNA repair[8, 9], homologous recombination[10], replication errors[7, 10-12], and direct base damage[7, 13-15]. We now understand that the rate of mutations is not static and is elevated by a variety of stimuli, and the biology, genetics, and chemistry behind these mechanisms have been extensively studied[7, 16-21].

Environmental mutagens as well as bacterial stress responses play a key role in generating mutations that drive evolution and lead to antibiotic resistance[16, 22]. Mutagens such as heavy metals[23], ultraviolet (UV) radiation[24], and antimicrobials[25-28], as well as exposure to stressful conditions such as starvation[29], have been shown to induce antibiotic resistance through mutation. Additionally, previous work has shown that different stressors can produce different types of mutations[30-34]. These types of stressors have the capacity to cause mutation by directly damaging DNA through alkylation, oxidation, or crosslinking of bases, in a process termed stress-associated mutagenesis (SAM) by MacLean et al.[16]. Bacterial stressors can also act through stress-induced mutagenesis (SIM) by regulating cellular genetic machinery that leads to

mutations, such as the induction of error-prone polymerases or inhibition of DNA repair genes [8, 9, 16, 35]. Since both of these mechanisms can act in response to a particular stressor, the spectrum of observed mutations would reflect a mixture of SAM and SIM.

Understanding the mechanisms by which bacteria develop antibiotic resistance is key to combating the current antibiotic resistance crisis, which results in millions of resistant infections and tens of thousands of deaths annually[36]. Instances of genetic mutation leading to resistance are well documented, and some of the most commonly used antibiotics have lost much of their efficacy due to the emergence of resistance-conferring mutations in a few bacterial genes. In fact, resistance to both first- and second-line drugs in *M. tuberculosis* is made possible by single point mutations in a number of genes, highlighting the great impact that single nucleotide substitutions can have on bacterial phenotypes[3].

Mutations that lead to resistance against ribosome-targeting antibiotics represent a major threat to our current medical practices. Spectinomycin is an aminocyclitol antibiotic which inhibits translocation of the peptidyl-tRNA from the A- to the P-site by binding to the 30S subunit[37-39]. This antibiotic is especially important as it is an effective option for treating *Neisseria gonorrhoeae* infections, which have become resistant to penicillin[40-42], sulfonamides[43], tetracycline[44], and worryingly, the cephalosporin, ceftriaxone[45, 46]. Spectinomycin resistance via single nucleotide mutations in ribosomal protein S5 were identified previously *N. gonorrhoeae[46, 47]* in *Escherichia coli[48, 49]* and *Bacillus subtilis[50]* as well as other bacteria[51, 52]. In *B. subtilis*, both the *rpsB*

and *rpsE* genes that encode for the S2 and S5 proteins contain multiple sites where point mutations have the potential to confer resistance. Looking at the spectrum of mutations in these two genes can be useful for understanding how environmental stressors cause spectinomycin resistance mutations through a combination of SIM and SAM.

In both experimentally and naturally selected mutations, the final product of the selection represents only a fraction of the total mutational landscape. This landscape is defined by the initial mutagenic stress such that different stressors will induce a different array of mutations[30-34] via combination of SIM and SAM mechanisms depending on the context of the stress[16]. This spectrum of mutations then undergoes various selective forces, including selection for antibiotic resistance[53-57] and bacterial fitness[16, 58-62], that shape the final mutational profile observed. Here, we utilize a high-throughput system employing traditional microbiological techniques in conjunction with next-generation sequencing to demonstrate the impact of an array of genotoxic stressors on the spectrum of spectinomycin mutations, characterizing the full mutational spectrum associated with each genotoxic agent within the *rpsE* gene of *B. subtilis*. We find that each stressor generates a unique set of mutations, some of which are reflective of their respective mechanisms of DNA toxicity and suggest SAM whereas others likely arise from SIM mechanisms. As part of this effort, we also identify novel spectinomycin resistance mutations in both the *rpsE* and *rpsB* genes of *B. subtilis*. From the full spectrum of stressor-specific mutations, selective processes narrow down the mutations to viable mutants. Utilizing a bacterial

competition assay combined with a sequencing approach; we find that, consistent with previous work, the fitness of various mutants is highly dependent on antibiotic concentration. Our results demonstrate that the full spectrum of available spectinomycin resistance mutations is narrowed down to a few mutations during multiple selection events. Thus, the final mutants that emerge are shaped by both the initial mutagen/stress and the subsequent level of antibiotic selection present.

Results

Genotoxic Agents Induce a Spectrum of Unique and Treatment-Specific Resistance Mutations

Past work indicates that exposure to mutagens and other forms of stress can cause genotoxicity and induce mutations that lead to the development of resistance. We tested the propensity of an array of genotoxic conditions, each inducing DNA damage through a different mechanism, to generate spectinomycin resistance in the wild-type (WT) background of *B. subtilis* 168 compared to no-treatment controls. As growth phase is an important factor in stress response and mutagenesis[63-65], we exposed cells to mutagenic agents for 30 minutes during logarithmic phase or for 3 hours during stationary phase growth before removing the stressing agent and plating on spectinomycin selection media (100 μg/mL) to determine the number of resistant colonies generated. The benefit of this experimental design is that the stressor is experimentally and temporally separate from the following spectinomycin selection. The short exposure of stress prevents cells from undergoing multiple rounds of division and selection. Treatment

concentrations were determined by dose titration experiments and selected based on mutagen exposure levels that were able to potentiate mutagenesis while minimizing bacterial killing. The mutational frequency of each stressor is likely the result of an agent specific mechanism of action that can also change in a concentration-dependent manner for each of the agents [21]. For example, antibiotics like ciprofloxacin may induce DNA-mutations through either the direct mechanism on DNA gyrase/topoisomerase or via ROS induced damage. Here, stressor concentrations were determined using a dose titration and selecting concentrations that induced the greatest frequency of mutants. These experiments provided both the extent of mutagenicity of the different treatments, and also generated a compendium of thousands of individual resistant colonies that we used to elucidate the spectrum of mutations associated with each treatment.

The frequency at which spectinomycin-resistant colonies were generated was dependent on both the type of mutagenic agent administered as well as the growth phase (Figure 1A). Mitomycin C, ciprofloxacin, and UV radiation all increased the frequency at which resistant colonies developed compared to untreated cells across growth phases, while hydrogen peroxide, and cobalt chloride caused an increase in resistant colony frequency only during logarithmic growth (Mann-Whitney U test, $p < 0.05$) (Figure 1A). In addition, untreated cells grown to stationary phase exhibited an increase in the formation of resistant colonies compared to untreated cells grown in logarithmic phase (Mann-Whitney U test, $p < 0.05$), likely reflecting the stress incurred by extended growth and the known phenomenon of stationary-phase mutagenesis in *B. subtilis*[63]. This data

suggests that stressors that possess different mechanisms of DNA damage are capable of inducing spectinomycin resistance in *B. subtilis* to different degrees depending on both the type and timing of exposure. The fact that stressors further elevate mutations during stationary phase compared to untreated cells likely indicates that mutations derived from SAM mechanisms are additive to SIMs related to stationary phase.

We utilized Sanger and whole-genome sequencing on a subset of colonies to determine the genetic basis of resistance in these mutagenized bacteria. These strategies identified base substitutions in two genes, *rpsE* and *rpsB,* encoding the ribosomal proteins S5 and S2, respectively (Table S1), aligning with a previous report which identified that spectinomycin resistance in *B. subtilis* can result from a single amino acid replacement in the ribosomal protein S5 [50]. More unique mutations were found in the *rpsE* gene compared to *rpsB* (Figure S1, S2), therefore we chose to examine the full spectrum of mutations in *rpsE* across all previously-generated spectinomycin resistant colonies using an amplicon-based next-generation sequencing strategy.

We used the *rpsE* gene as a model to understand how different stressors can induce spectinomycin resistance through different routes of mutation leading to unique, stress-specific spectra of mutations. Previous work in both prokaryotic and eukaryotic models have suggested that some mutagens have mutational signatures[66]. We aimed to uncover the full mutational spectrum in *rpsE* by isolating and sequencing thousands of individual spectinomycin resistant colonies derived from cultures exposed to different treatments. In order to identify the

largest possible range of mutants, we employed a low selective pressure of less than 2x MIC (spectinomycin) and grew colonies on uncrowded plates, anticipating that this would enable recovery of isolates with both common and rare resistance mutations while reducing potential negative fitness impacts of individual mutations. To identify the mutational spectrum of various stressors, we performed *rpsE* amplicon sequencing on an Illumina MiSeq to an estimated average sequencing depth of roughly 1,500 reads per colony on a library of ~5,000 individual colonies from 10 different treatment conditions (~500 colonies per condition) representing at least 6 replicates per condition.

Sequencing revealed that each treatment induced a unique set of non-synonymous mutations, most commonly resulting in single amino acid changes (Figure 1B). In all of the conditions the relative abundance of colonies with an identified *rpsE* mutation is less than 100%, indicating that mutations occur outside of this gene, and the frequency at which a colony was found to contain a *rpsE* mutation varied between conditions. For example, it appears that the *rpsE* gene is more responsible for spectinomycin resistance in mitomycin C- treated cells (71.72%) compared to those exposed to UV radiation (12.4%) (Figure 1B). It is likely that the low percentage of *rpsE* mutations in some conditions resulted from a higher frequency of mutations in other genes, such as *rpsB*. This finding highlights the fact that given multiple gene targets, different stressors will repeatably and preferentially target certain genetic loci.

Mutation Spectra Correspond to Stressor-Specific Mechanisms of Mutagenesis

Examination of the spectrum of mutation corresponding to different stressors reveals both expected patterns of mutation based on known mechanism of mutagenesis and novel, broad patterns of mutation (Figure 1B). The DNA damage mechanism of mitomycin C [15], UV [14, 67], and H_2O_2 [68, 69] has been previously described, and thus we were able to predict what mutational spectrum these stressors would produce.

Mitomycin C, an antitumor drug, induces DNA damage by forming interstrand cross-links of guanosine residues at CpG sites [15, 70, 71]. The data here reveal that the majority of mitomycin C-induced spectinomycin-resistance mutations occur at a CG (nucleotides 88,89), where it accounted for over 50% of the *rpsE* mutations during both logarithmic (68.6%) and stationary (61.41%) phase growth. However, in all other treatments the G89C mutation was only present in a maximum of 8.54% of *rpsE* mutants. The mitomycin C mutation spectrum displays how patterns of mutation caused by DNA damage can be stress-specific, and in the case of mitomycin C is reflective of the particular DNA damage mechanism of the drug. Thus, in the case of mitomycin C, SAM likely plays a greater role than SIM.

UV radiation also has a unique DNA-damaging mechanism that commonly targets adjacent pyrimidine bases leading to the C -> T base substitutions known as UV signature mutations [14, 67, 72]. A mutation in the *rpsE* gene was relatively rare among colonies generated by UV treatment (12.43% in logarithmic phase, 18.22% stationary phase). This could reflect a lack of adjacent pyrimidine bases

that could result in a productive mutation in this gene. To determine whether this site was responsible for the majority of the resistance observed in UV treated cells, we Sanger sequenced the *rpsB* gene from 90 UV-treated colonies. Roughly 68% (62 colonies) of the colonies contained a C -> T mutation characteristic of UV damage (Figure 1C). These findings illustrate that the *rpsB* gene is a more common target for spectinomycin resistance mutations in the context of UV radiation exposure, likely due to the susceptible nucleotide sequence (adjacent cytosine bases) that lends itself to the UV signature C -> T mutation.

This data suggests that bacterial evolution and development of antibiotic resistance through SAM is shaped by a combination of the mutagenic stress, the mechanism of DNA damage, the nucleotide composition of the resistance loci, and potential for the DNA damage to induce the type of mutation that will lead to resistance. Within the genome of *B. subtilis*, and even *rpsE* and *rpsB*, there are numerous sites of adjacent pyrimidines. However, out of all these sites that are subject to UV damage only the C74 site in *rpsB* is strongly selected for, likely based on its ability to provide resistance in a way that does not compromise cell viability. It is likely that some rpsE mutations were not detected due to epistatic or lethal mutations elsewhere in the genome, especially under highly mutagenic conditions. Ultimately, UV is another example where SAM is likely the predominant mechanism of mutagenesis leading to observed mutations.

We also found an observable mutation signature in the case of hydrogen peroxide, analogous to observations made regarding mutations of the *supF* gene of *E. coli*[69]. Akasaka et al. found that the mutational spectra of hydrogen

peroxide was predominantly G:C -> C:G (40%) and G:C -> T:A (37.14 %) transversions[69]. We identified a similar profile of mutations in the *B. subtilis rpsE* gene (Table S2), finding that a large portion of the mutations were G -> C (23.9%) and G -> T (23.4%) transversions; however, we also observed a substantial number of G -> A transitions (32.2%) (Figure 1B). Interestingly, when the *supF* gene was exposed to hydrogen peroxide and then passaged through simian (CV-1) cells, G:C -> A:T mutations represented 43.5% of the overall mutations[68]. The high abundance of mutations at guanine residues in both our data and previous studies is likely reflective of the mechanism of hydrogen peroxide damage, which forms hydroxyl radicals known to cause 8-oxodG lesions [73].

We also examined the mutational spectrum of stressors for which mutational spectra was unknown and could not be inferred by a distinct mechanism of DNA damage. Sequencing mutants generated by exposure to stationary phase stress, $CoCl_2$, and ciprofloxacin provided an opportunity to observe novel mutational spectra and gain insights into new mechanisms of mutagenesis.

Previous research into *B. subtilis* stationary phase mutagenesis revealed that subpopulations of stationary phase cells undergo adaptive mutagenesis [63], but there has not been significant investigation into the spectrum of mutation associated with this condition in *B. subtilis*. Overall, we found that stationary phase was not associated with an overrepresentation of a specific mutation, but rather there was a mostly even distribution among 4 different mutations. Such a distributed pattern was also observed in other treatments, such as ciprofloxacin during stationary phase. In the case of mitomycin C treatment, in both growth

phases, its contribution to the overall mutation spectrum is reduced by a signature mutation linked directly to a specific form of DNA-damage induced by the drug (Figure 1B).

Cobalt (Co) in the form of cobalt chloride ($CoCl_2$) has been shown to be mutagenic both in bacterial and mammalian cells[74, 75]. The generation of DNA-damaging reactive oxygen species (ROS) has been proposed as a method by which metal ions induce toxicity and potentially mutagenesis[76, 77], but there is not substantial evidence to link Co-induced mutagenesis to ROS in bacteria[77]. However, we observe that the most common mutation in the *rpsE* gene of *B. subtilis* treated with $CoCl_2$ is G -> T (46.2%) (Figure 1B), which could potentially be a signature of 8-oxo-dG mutations caused by ROS[78]. Interestingly, this is an even higher rate of G -> T mutations than the hydrogen peroxide treatment, which would be expected to exhibit more signatures of ROS-associated DNA damage. A potential alternate mechanism is that Co may cause toxicity through inhibition of DNA repair, independent of oxidative stress, as has been suggested to occur in *E. coli*[79]. However, based on the data presented here, we are unable to link Co mutagenesis to a distinct mechanism, though we do show that $CoCl_2$ has the potential to induce base substitution mutations that result in antibiotic resistance through a unique spectrum of mutations.

Antibiotics are known to cause mutagenesis through activation of both SOS-dependent[28] and independent pathways[80], which may be related to ROS production[25, 28]. Treatment of *B. subtilis* with ciprofloxacin led to an increase in the formation of spectinomycin resistant colonies (Figure 1A). The predominant

rpsE mutation of ciprofloxacin treatment is A67G in both log phase and stationary phase, with this mutant making up 21.94% and 17.41% of the relative abundance of resistant mutants, respectively (Figure 1B). However, while 80.61% of the *rpsE* mutant reads are A67G in log phase cells treated with ciprofloxacin, A67G was only 29.16% of *rpsE* mutations in ciprofloxacin-treated stationary phase cells (Figure 1B). This suggests that ciprofloxacin treatment has a variable mutation spectrum that depends on growth phase, and it is likely that the combined stress of stationary phase growth in addition to ciprofloxacin induced stress is responsible for a more diverse mutational spectrum during stationary phase growth. This highlights the importance of evaluating the mutational spectrum of mutagens at different growth phases and understanding the combinatorial impacts of stressors. It is also possible that the divergent signature observed between stationary and logarithmic phase cells treated with ciprofloxacin results from different rates of replication fork-stalling induced double strand breaks that may occur between dividing and non-dividing cells, potentially altering the relative contributions of SAM and SIM mechanisms.

Bacterial evolution studies have revealed that, like many other organisms, bacteria exhibit transition bias, with transition mutations overrepresented relative to the unbiased T_i:T_v [81, 82]. Of the spectinomycin resistance mutations in the *rpsE* gene, the ratio of transitions to transversions is dependent on the type of mutagenic stress administered to the bacteria (Figure S4A). While ciprofloxacin and hydrogen peroxide treatments as well as stationary phase stress induced a higher ratio of transitions to transversions in *rpsE* than expected in an unbiased

scenario (T_i:T_v > 0.5), mitomycin C, UV radiation, and cobalt chloride treatment instead all resulted in further bias towards transversions (T_i:T_v < 0.5) (Figure S4A). Interestingly, across all the possible observed mutations, there were nearly 4 times more detected (37) transversion mutations compared to transitions (9). It has been proposed that amino acid level, transversion mutations are more likely to cause a change in the biochemical properties of amino acids and that transition mutations are more conservative [83, 84]. Therefore, it may be logical to assume that a resistance conferring mutation would be a transversion. However, a more recent meta-analysis found transition mutations to be only slightly more conservative than transversions [85], and thus there may be alternative explanations for the increased abundance of transversions observed here. Due to the different mutagenic pathways in each of the treatments, not all mutational spectra reflect a transversion bias, highlighting the unique stressor-specific pathways that lead to antibiotic resistance mutations.

Location of Mutations in Ribosomal Proteins S5 and S2

Alterations of the loop 2 region of the S5 protein have been shown to impact spectinomycin sensitivity in *E. coli* by disrupting the binding of the drug to the ribosome [86, 87]. Sanger sequencing of the entire *rpsE* gene revealed that the observed mutations were strictly limited to a small region of the protein and, in agreement with previous studies, these mutations were located along the loop 2 region of the S5 protein (Figure S5A). We identified 63 unique *rpsE* mutant alleles with single, double, or triple nucleotide mutations (Figure S3). Of these 63

mutations, only 2 have been previously described in *B. subtilis* [50, 88]. We observed 6 different mutation sites in the S2 protein, but the most commonly mutated sites were at amino acid positions 22 and 25 (Figures S5B). The close proximity of these two sites and their high frequency of mutation in resistant colonies suggests that this region of the S2 protein may have an impact on the binding of spectinomycin to the ribosome. These localization patterns suggest that selection for mutations is dependent not only on the possible mutations generated by different stressors, but also that these mutations occur in sites that will provide a physiologic advantage such as resistance.

Impact of DNA Repair Genes on the Frequency and Spectrum of Mitomycin C Induced Mutagenesis

Based on the data presented above, the sequenced mutants likely result from variable combinations of SIM and SAM mechanisms. In order to ascertain the contributions of each mechanism, we decided to observe how disruption of processes known to be associated with SIM, including DNA damage repair processes and the SOS response, impacted the spectrum of spectinomycin-resistant mutants generated through mitomycin C treatment. Specifically, we tested the mutation frequency and mutational spectrum of a library of previously-validated mutants targeting processes related to DNA maintenance and repair[89]. Several mutants lacked genes involved in the SOS response including *yhaO*, *yobH*, *yozK*, *uvrX*, *uvrA*, *uvrB*, *uvrC*, *recA*, and *dinB*, which all are regulated by LexA, the transcriptional repressor of the SOS regulon[90, 91]. Other mutants

contained knockouts of genes involved in DNA maintenance and repair independent of the SOS response: *radA*, *adaA*, *sbcD*, *recN*, *radC*, *mutSB*, *exoAA, mfd*.

In WT *B. subtilis*, we had observed that the spectrum of *rpsE* spectinomycin resistance mutations arising from mitomycin C exposure was dominated by a particular mutation of a CpG site, corresponding to the interstrand cross-linking that mitomycin C induces at these sites (Figure 1A, B). Previous work has suggested that such interstrand cross-links are repaired via the nucleotide excision repair pathway (NER) or by homologous recombination [70]. Mitomycin C is also an alkylating agent capable of generating mono-adducts at the N^2 and N^7 positions of guanine, which in B. subtilis are also repaired through NER, including both the UvrABC and MrfAB pathways [70].

Utilizing the mitomycin C mutagenesis conditions used previously during logarithmic growth, we found that the deletion of genes involved in DNA maintenance and repair had vastly different effects on the frequency of formation of spectinomycin resistant colonies (Figure 2A). DNA repair is a vital part of counteracting the mutagenic impacts of various genotoxic stressors. Compared to the frequency observed in WT, the *ΔadaA*, *ΔyhaO*, *ΔrecN*, *ΔmutSB*, *ΔuvrA*, *ΔuvrB*, and *ΔuvrC* deletions all significantly increased the frequency at which resistant colonies formed as a result of mitomycin C treatment (Mann-Whitney U test, $p < 0.0001$). Surprisingly, most of the *ΔrecN*, *ΔuvrA*, *ΔuvrB*, and *ΔuvrC* colonies that formed on the spectinomycin selection plates could not be regrown in liquid media under selection, suggesting that these colonies were inviable or did not contain a

true resistance mutation. In contrast, resistant colonies from the *ΔadaA*, *ΔyhaO*, and *ΔmutSB* deletion strains had high rates of regrowth under selection compared to the *ΔrecN*, *ΔuvrA*, *ΔuvrB*, and *ΔuvrC* derived colonies, suggesting a heritable, genetically encoded resistance phenotype. Equally important are those genes that were shown to reduce the mutagenic impacts of mitomycin C treatment: strains with deletions in *radA* (a recA paralog), *sbcD*, *yobH*, *yozK*, *uvrX*, *exoAA*, *mfd*, and *dinB* all had reduced mutation frequencies compared to WT *B. subtilis* when treated with mitomycin C (Figure 2A). In fact, many of these deletion strains did not produce any spectinomycin resistant colonies after exposure to mitomycin C, nor did they not show increased sensitivity to mitomycin C. Our findings are in agreement with previous studies of *mfd* and *dinB* that have shown that these genes are necessary for mutagenesis and the development of antibiotic resistance[4, 8]. The knockout strain lacking *radC*, which encodes a protein repair homologue[92], had an increased in baseline mutation frequency, but did increase resistant mutants when exposed to mitomycin C. The decreased mutagenicity of strains lacking *radA*, *sbcD*, *yobH*, *yozK*, *uvrX*, *exoAA*, *mfd*, and *dinB* implies that these genes may play a role in the process of evolution and the development of antibiotic resistance through SIM.

The three genes whose knockouts increased mitomycin C-induced mutation rates and could be regrown under selection, *adaA*, *yhaO*, and *mutSB*, are responsible for DNA repair in ways that could reduce DNA damage by mitomycin C. The *adaA* gene encodes for a methylphosphotriester DNA methyltransferase and is part of the adaptative response to DNA alkylation in *B.*

subtilis. The AdaA protein is the transcriptional regulator of *alkA*, which encodes a 3-methyl glucosylase that can remove 7-meG lesions such as those formed by mitomycin C[90, 93, 94]. Therefore, deletion of *adaA* may lead to a higher frequency of mutant formation by shifting cells to a more mutagenic form of DNA repair. The *yhaO* gene, which is homologous with the DNA endonuclease gene *sbcD*, has been shown to interact with the nuclease SbcC and may play a role in the repair of interstrand cross-links[90, 95-97]. Finally, *mutSB* encodes the endonuclease MutS2, which may play a role in homologous recombination and potentially impact the mutational spectra in *B. subtilis*[98] through the involvement of this process in the repair of mitomycin C-induced damage[98, 99]. That increases in rates of mutant colonies appeared in *B. subtilis* strains deficient in genes thought to be directly involved in repair of mitomycin C-associated alkylation damage (*adaA*, *yhaO*, and *mutSB*) suggests that these genes are involved in limiting SAM rather than introducing mutations through SIM, which perhaps explains why the baseline mutation frequency was not increased when compared to the WT in any of the three mutants (Mann-Whitney U test, $p > 0.05$).

To determine the impact of the *ΔadaA*, *ΔyhaO*, and *ΔmutSB* deletions on the spectrum of mutations, we sequenced the *rpsE* gene of the spectinomycin resistant colonies formed by mitomycin C mutagenesis in these mutants. The frequency at which a colony was found to contain a *rpsE* mutation was decreased in each of the deletion strains compared to WT, suggesting that mutations are more likely to form in other regions of the genome, such as in *rpsB*. Consistent with observations of the WT strain, the dominant *rpsE* mutation observed in these

deletion strains was the G89C mutation, which results in an arginine to proline amino acid substitution at position 30 (Figure 2B), suggesting that direct base damage is still the chief mechanism of mutagenesis. While this dominant mutation remained the same, the complement of other mutations appeared to differ from the WT strain. Compared to the WT, the *ΔadaA*- and *ΔyhaO*-derived colonies had a higher proportion of mutated reads containing double mutants with both a transition and transversion mutation in the same copy of *rpsE*: 0.67% for WT versus 11.92% and 7.26% for *ΔadaA* and *ΔyhaO*, respectively (Figure S4B). These data suggest that lacking *adaA*, *yhaO*, and *mutSB* increase mutagenicity during mitomycin C exposure, and while the mutation spectrum was still dominated by a particular G -> C mutation, the array of other mutations appeared to be changed by the lack of these DNA-repair genes. Thus, these DNA repair systems play an important role in mutagenesis and the overall spectrum of mutations.

Growth Phenotypes of Selected Spectinomycin Resistance Mutations

Determining the functional impacts of the spectrum of mutations is critical for understanding their impacts on fitness and ultimately how they are selected for. Before a mutation results in a viable and antibiotic-resistant cell, the bacterium must be able to tolerate the mutation and overcome physiological barriers to survival and proliferation. In addition, relative fitness of the mutation is critical to the establishment of the newly-developed mutation in microbial environments with and without antibiotic selection. We aimed to quantify the potential fitness effects of different mutations to find out whether different mutations might be more suitable

for the development of resistance and which mutations would introduce physiological barriers to success. To better understand the physiologic significance of the spectinomycin resistance mutations observed in our data we isolated 11 unique mutants containing either single or double mutations in the *rpsE* gene (Table 1), which included 7 of the top 10 observed *rpsE* mutations in Figure 1B.

We then determined that the mutations had varying impacts on spectinomycin resistance, growth rate, and delay in onset of logarithmic growth phase (length of time it took a culture to reach OD_{600} = 0.2) (Table 1). The MIC_{90} of spectinomycin for WT *B. subtilis* was 62.5 µg/mL while the various mutant strains had MIC_{90} values ranging from 250 µg/mL to >2,000 µg/mL (Table 1. The doubling time of WT *B. subtilis* was determined to be 27.53 minutes, while the doubling times of the mutant strains ranged from 28.06 up to 51.16 minutes (Table 1). In general, growth rates were fairly similar across mutants, but there were significant differences in the time it took strains to exit lag phase and enter logarithmic growth. We found that most strains had a significant delay in reaching OD_{600} = 0.2 compared to WT (ANOVA, $p < 0.05$). These findings show that within the spectrum of possible resistance mutations, some may be better suited for growth under non-limiting conditions by having higher growth rates or the ability to reach exponential growth in less time, while others would be well-suited to survive higher antibiotic exposures due to increased MIC_{90} values. Previous reports of ribosomal protein S5 spectinomycin resistance mutations in *E. coli* noted that cold sensitivity was associated with these mutations [86, 87, 100, 101]. Indeed, we

found that one of the mutations, G27D, was unable to grow at 20°C, indicating cold sensitivity (Table 1). Taken together, these physiological differences in growth and resistance may play a role in shaping the spectrum of mutations observed under different conditions or environments.

Determining the Fitness of Mutations through Competition Assays under a Gradient of Spectinomycin Concentrations

When we initially conducted the mutagenesis assays, we noticed that the colonies that formed on spectinomycin selection plates were variable in size, suggesting that mutations may impact the relative fitness of the bacteria. To model the potential selective pressures or benefits provided by altered growth kinetics, we utilized bacterial competition assays in the presence and absence of spectinomycin. Twelve strains of *B. subtilis* - the 11 unique spectinomycin resistant mutants that were previously isolated and the WT background strain - were chosen for this experiment. Competition experiments were performed at spectinomycin concentrations that matched the MIC of the WT background (62.5 µg/mL, or "low"), the lowest MIC of the mutant backgrounds (250 µg/mL, or "medium"), and below the highest MIC of the mutant strains (1,000 µg/mL, or "high"). To initiate the experiment, multiple replicates of each strain were grown overnight and then mixed at approximately equal proportions. During initial growth, the microbial mixture was sampled at 0, 3, 6, 12, and 24 hours. Cultures were passaged every 24 hours, and the mixture was again sampled at the 48 and 72-hour timepoints. Using the Nanopore MinION sequencing technology, we were able to rapidly identify the

relative abundance of each strain at the various timepoints to determine how the level of antibiotic selection impacts the fitness of various mutant strains during competition.

While all the mutations tested were shown to confer some level of spectinomycin resistance, their ability to outcompete other strains was dependent on the level of selection they encountered. Over a short time period of 48 hours, with no antibiotic selection, the relative abundance of mutations did not change drastically, although the abundance of the strains containing the G83A, A67G, and G83A-G151T double mutation were significantly decreased at the 48-hour timepoint compared to the 3-hour timepoint (Stepdown Bonferroni, $p < 0.0001$) (Figure 3A). All three of these decreased mutants were shown to have a decreased growth rate compared to WT in the absence of drug (Table 1). Additionally, the A67G and G83A-G151T mutants were shown to have significantly lengthened exits from lag phase, possibly explaining their decrease in abundance (ANOVA, $p < 0.01$) (Table 1).

At the 62.5 μg/mL spectinomycin concentration, reflective of the MIC for the WT *B. subtilis* strain, we found that there were substantial shifts in the relative abundance of the mutants (Figure 3A). At 48 hours, the first timepoint after the first passage, there was a significant expansion of the strain containing the G70A-G82A double mutation (Stepdown Bonferroni, $p < 0.0001$) and a significant reduction in all other mutants (Stepdown Bonferroni, $p < 0.0001$) - except those containing the G64C or A78C mutations, which did not significantly change in relative abundance (Stepdown Bonferroni, $p > 0.05$) (Figure 3A). This trend was

again observed at the 72-hour timepoint where the G70A-G82A strain had expanded further to dominate the population, making up roughly 81% of the sequence reads (STDEV = ± 1.77%). While there was a separate mutant strain containing the G82A mutation included in the competition, there was none containing G70A alone. The double mutant outcompeted the G82A mutant strain, indicating that this double mutation is likely beneficial under these conditions.

While we saw the sole dominance of the G70A-G82A double mutant strain at the low antibiotic concentration, this trend did not hold true for the medium concentration of 250 μg/mL. Under these conditions, the relative abundance of most strains began to diminish at 3 hours and continued until the point of extinction by 72 hours in all strains except those containing the A78C, G64C, G70A-G82A, and A67G mutations (Stepdown Bonferroni, $p < 0.0001$) (Figure 3A). By 48 hours the community had undergone a significant shift to mainly the A78C strain and G70A-G82A double mutant. This trend was sustained at 72 hours, although the relative abundance of the A67G and G64 strains still persisted at near baseline levels.

At the highest antibiotic concentration of 1,000 μg/mL, we observed a further change in the outcome of the competition experiment. Several of the strains, including those containing the G83A, G82A, G89C, A78T, G82T, and G83T mutations as well as the wild type strain, experienced a significant reduction in abundance within the first 24 hours of growth at this high concentration of spectinomycin (Stepdown Bonferroni, $p < 0.0001$). Of these strains, five of the seven had MIC^{90} values that were below the 1,000 μg/mL selective concentration

suggesting they were unable to compete due to an insufficient level of resistance (Figure 3A). However, two of the lost strains, G89C and G83T, had MIC^{90} values of at least 2,000 μg/mL, suggesting that a lack of ability to grow under the high drug concentration was not the key factor, but instead an inability to outgrow the other strains. This possibly suggests a growth defect under antibiotic stress compared to the dominant mutants. On the other hand, during the first 24 hours of growth, the A78C and G64C strains expanded to become a majority of the population. The first passaging after the 24-hour timepoint proved to have a significant effect on the population, allowing for further expansion of the A78C strain that neared significance (Stepdown Bonferroni, $p = 0.064$) followed by a reduction in the G64C strain (Stepdown Bonferroni, $p < 0.0001$). The final 72-hour timepoint marked both continued prevalence of the A78C strain as well as a significant expansion of the A67G strain (Stepdown Bonferroni, $p = 0.0061$), which had maintained a foothold in the total population throughout the previous timepoints. This final timepoint also marked a final reduction of the G64C and G70A-G82A double mutant (Stepdown Bonferroni, $p < 0.0001$) resulting in a population that consisted nearly entirely of the A78C and A67G strains. The fitness of the A78C mutation at this high concentration mirrors its activity in the medium drug concentration suggesting fitness of this mutation is enhanced at increased levels of spectinomycin selection. Spectinomycin dependence derived from a mutation in the *rpsL* gene was previously reported by Henkin et al. [102], however whole genome sequencing of the strains used in our competition assay did not reveal any mutations in the *rpsL* gene or other regions of the genome.

In order to understand the fitness of different mutants across spectinomycin concentrations, we performed growth assays at each antibiotic concentration used in the competition experiment (Figure 3B). Antibiotic concentration had a dramatic effect on doubling time in the mutant and WT strains. However, within an antibiotic concentration the doubling of time of mutants was fairly similar, with the exception of G83A-G151T at no treatment, G82A at 250 μg/mL, and G82T, G83T, and G83A at 1,000 μg/mL, which all had significantly decreased doubling times compared to other mutants (ANOVA, $p < 0.05$) (Figure 3B, Table S3). Since the doubling time of most mutants was not able to explain the differences in fitness during the competition assays, we next looked at other growth parameters. We found that the most successful mutants at each antibiotic concentration were able to enter logarithmic phase growth more quickly than those they outcompeted. When we compared the time it took strains to exit lag phase, determined by the time it took strains to reach an $OD_{600} = 0.2$, we found that at the 0 μg/mL drug concentration those strains with the greatest latency in exit from lag phase times performed the poorest in the competition experiment (Figure 3A, B). Strains G83A-G151T, G83T, A67G, G70A-G82A all had significantly extended times to exit lag phase compared to their more successful counterparts (ANOVA, $p < 0.05$) (Figure 3B, Table S3). The addition of 62.5 μg/mL spectinomycin challenge during growth increased the time it took many of the mutants to exit lag phase, but the most successful mutant, G70A-G82A, did not experience any increase in the time it took to exit lag phase Figure 3B, Table S3). At the medium drug concentration of 250 μg/mL spectinomycin, we observed that the highly successful A78C and G70A-G82A

strains had the shortest times to exit from lag phase (Figure 3B, Table S3). At the highest spectinomycin concentration of 1,000 µg/mL the A78C mutant was one of the most successful and again had the shortest time to exit lag phase (Figure 3B, Table S3). Interestingly, the other mutant that was able to successfully compete at 1,000 µg/mL, A67G, did not have a significant advantage in time to exit to lag phase. However, A67G did have one of the fastest doubling times at 1,000 µg/mL suggesting that the time to exit from lag phase was not the only determining factor in fitness at higher concentrations of spectinomycin. These growth assays suggest that a combination of factors including MIC^{90}, doubling time, and the length of time to exit lag phase contributes to the fitness of mutations at different drug concentrations.

The initial strategy employed to observe the full mutational spectrum of different mutagens provided insight into the frequency of different mutations at a relatively low level of selection (100 µg/mL spectinomycin) and little to no competition between isolates. While this strategy allowed us to gain a more complete picture of the mutational landscapes (Figure 1B), it does not reflect the process of selection that occurs in natural bacterial populations that are exposed to stressors in which mutants must compete. Using nanopore sequencing, we introduce a high-throughput, rapid method to measure the fitness of different antibiotic resistant bacterial strains at different selection concentrations. While both the A78C and G70A-G82A double mutant had MIC^{90} values of at least 2,000 µg/mL, well above all the selection concentrations used, they had vastly different population trajectories at different concentrations of spectinomycin. A78C grew to

the highest relative abundance of any strain by the 48 and 72-hour timepoints at the two higher levels of selection while it was unable to increase in abundance at the lowest level of selection where G70A-G82A displayed greater fitness (Figure 3A). Using growth assays with spectinomycin pressure, we find that both doubling time and the length of time it took each mutant strain to exit lag phase likely impacted their success at different antibiotic concentrations (Figure 3B), with those strains that more rapidly entered logarithmic phase growth and were able to grow more rapidly in the presence of drug having greater success in the competition experiments. This finding is in agreement with Lenski et al. who previously demonstrated that a shortened lag phase plays a key role in fitness and success during bacterial evolution[103]. Additionally, some mutations that had comparatively high MIC^{90} values and were the predominant mutants that emerged from the mutagenesis assays, such as G89C, were unable to compete at any level of antibiotic selection. This may be due to the drastic structural change of the G89C mutation, which resulted in an amino acid change of arginine to proline in the S5 ribosomal protein, which could have had detrimental effects on ribosome function. Taken together, the results of this competition experiment suggests that the fitness of individual mutations and their ability to persist in mixed populations is dependent on the concentration of spectinomycin selection.

Discussion

In this work, we define the distinct mutational spectra of several genotoxic stressors in the context of spectinomycin resistance in *B. subtilis*. Separate studies

have reported that stressors such as starvation[29], UV radiation[24], mitomycin C[15], and ROS[104] can generate specific and distinct types of mutations. Specific antibiotic resistance mutations have been shown to be the result of different types stress [30-34]. We sequenced thousands of individual colonies in order to uncover how genotoxic stress is linked to mutational signature and resulting spectinomycin resistance derived from ribosomal mutations. In doing so, we identified novel spectinomycin resistance mutations in both the S2 and S5 proteins of *B. subtilis*, and further analyzed individual strains containing base substitutions to characterize the impacts of these mutations on bacterial fitness. Utilizing competition assays we add to existing evidence that shows the concentration of antibiotic pressure shapes which mutations emerge from a mixed population. This work describes the process by which spectinomycin resistance develops in *B. subtilis* through a stress-specific pattern of base substitutions, which are narrowed down to a unique spectrum of resistance mutations by a series of selective processes.

Stress-Associated Mutagenesis Leads to Spectinomycin Resistance Through a Spectrum of Mutations and Gradient of Selection

Figure 4 illustrates a process in which *B. subtilis* exposed to various stressors may undergo mutagenesis that leads to spectinomycin resistance through an initial generation of a stressor-specific spectrum of mutations followed by selection for viable and resistant mutants. Initially, we show that different forms of stress increase the formation of antibiotic resistant colonies (Figure 1A). Next,

we show that each stressor is associated with a unique spectrum of mutations (Figure 1B), in some cases reflecting the DNA-damaging mechanism of the mutagen. This suggests that for certain genotoxic agents, SAM likely plays a greater role in the observed spectrum. It is important to state that in this experiment we are only capturing the fraction of the total induced mutations that were selected by two criteria of cell viability followed by spectinomycin resistance. Thus, while the spectrum of all possible mutations is unique for each stressor, the observed mutations are quite limited. In order for a base substitution mutation to be generated, it must be possible in the context of the spectrum of mutation for a given stress condition. For example, mitomycin C-treated colonies often exhibit a G89C mutation that is never observed in the ciprofloxacin-treated colonies, as it is not part of the mutational repertoire of ciprofloxacin. Therefore, development of a resistance through a G89C mutation is only possible given a stress (mitomycin C) that has the potential to generate such a mutation.

There are also clearly fundamental barriers to the development of antibiotic resistance through base substitution mutations, such as the ability of mutated cells to tolerate the mutation and proliferate. While our experimental methods only allow us to identify non-lethal mutations under optimal growth conditions, we do illustrate that the A67G mutation in *rpsE* results in a growth defect at 20^{o}C (Table 1). The ability to grow at given environmental conditions, such as a certain temperature, represents an additional selective hurdle that a mutated cell must cross to become a viable antibiotic resistant bacterium. Once a bacterium undergoes a mutagenic process that results in viable antibiotic resistance, there is then a potential for

competition with other mutants that further narrows the spectrum of antibiotic resistance mutations (Figure 4). Resistant bacteria containing different mutations will be more or less competitive depending the antibiotic concentration present, and thus the encountered antibiotic concentration will further narrow the mutational spectrum (Figure 3A).

Each Genotoxic Agent Causes a Unique Spectrum of Mutation that Reflect Mechanisms of DNA-Damage

We provide a comprehensive view of spectinomycin resistance base substitution mutations in *B. subtilis* that allow us to demonstrate the stressor-specific mutational signatures caused by various genotoxic agents. Using a low level of selection (< 2x MIC), uncrowded plates, collection of thousands of individual colonies, and next-generation sequencing allowed us identify stressor-specific signatures of mutation. This novel approach allowed us to capture a wide range of base substitutions, including mutations that were both rare and potentially less fit. We have shown that each genotoxic stress applied causes a unique signature of antibiotic resistance mutations, and that mechanisms of SAM result in mutational spectra that are often reflective of the mutagens mechanisms of DNA damage (Figure 1A, B). For example, in stationary phase cells treated with UV or mitomycin C, the mutational spectrum was highly divergent from untreated stationary phase cells suggesting, that direct base damage characteristic of these mutagens plays a stronger role in the final mutational spectrum than stationary phase-induced stress (Figure 1B). While stationary phase stress has been

previously shown to be mutagenic[63], we show the additional role that external mutagens play in the mutational spectrum and development of resistance.

DNA Maintenance and Repair Genes Contribute to the Frequency and Spectrum of Stress-Associated Spectinomycin Resistance Mutations in B. Subtilis

Using a set of *B. subtilis* gene knockouts, each deficient in a single gene involved in DNA maintenance or repair, we show that the presence of certain genes can limit or exacerbate the generation of antibiotic resistance through mitomycin C-induced mutagenesis (Figure 2A). Our data show that the *radA*, *sbcD*, *yobH*, *yozK*, *uvrX*, *exoAA*, *mfd*, and *dinB* genes are essential to the mutagenic activity of mitomycin C in *B. subtilis.* Interestingly, some of these genes are involved in the SOS response of *B. subtilis* while others are not, suggesting that mutagenesis may be dependent on the activity of multiple DNA repair pathways[90, 91]. On the other hand, strains deficient in *adaA*, *yhaO*, or *mutSB* had an increased frequency of spectinomycin-resistant colony formation when exposed to mitomycin C. Understanding which DNA repair genes are involved in altering the rate of mutagenesis could be valuable in limiting the development of antibiotic resistance.

Impact of a Spectinomycin Selection Gradient on the Fitness of Resistant Strains

The development of antibiotic resistance is highly complex and involves a myriad of factors. The concentration of the antibiotic selection is one such factor that plays a key role in determining the evolutionary trajectory of a bacterial population when selecting for resistance [105]. While using a fixed selection

concentration allowed us to understand the spectrum of mutations caused by different mutagens, this homogenous selective force is not representative of antibiotic concentrations in nature and is often a constraint on studies of the evolution of resistance [106]. In therapeutic and naturally-occurring settings, antibiotics are part of heterogeneous environments, resulting in concentration gradients [107, 108]. It is vital to understand the role of these gradients in the selection of antibiotic resistance mutations. Here we build on the idea of "selective compartments" put forth by Baquero & Negri by studying the selection of different mutations across a gradient of antibiotic concentrations, by assessing mutational spectra and mutant fitness in the context of no, low, medium, and high antibiotic selection concentrations. In competition experiments using spectinomycin concentrations varying by less than one order of magnitude, we obtained highly disparate final populations with certain mutations showing a distinct fitness advantage at different drug concentrations (Figure 3A). Furthermore, these results show that the strength of antibiotic selection modulates the fitness of individual mutations based not on MIC alone. Instead, the fitness of mutations under selection is likely determined by growth dynamics, namely growth rate and the length of time to exit lag phase as well as MIC (Figure 3A, B, Table S3).

Limitations

This work has several limitations intrinsic to the system and methodologies that were used. While we were able to identify unique mutation spectrums for various bacterial stressors, this study only looked at the mutations from a single

concentration of a handful of stressors at a set timepoint. Different lengths of exposure could significantly alter the mutational spectrum of each stressor. In addition, our exposures were selected to limit the number of bacterial division cycles to approximately one division, and thus we would not detect additive mutations acquired through cycles of mutation and replication. While we could infer the mechanism behind mutagenesis for stressors that exhibited a signature mutation, we do not provide direct evidence for the molecular basis of DNA damage associated with each stressor, although in many cases these mechanisms have been thoroughly validated in the literature. Additionally, we mainly focus on the spectrum of mutations in a single gene, *rpsE*, in which resistance develops primarily through base substitution mutations, excluding the potential for studying insertion, deletion, or frameshift mutations. Therefore, we are unable to capture the "full" mutational spectrum, which also includes regions outside of *rpsE* as well as any deleterious mutations that did not make it through the selection process. Further work is necessary to fully define the array of deleterious mutations to understand the mutational spectrum of each stressor tested here, which would require whole-genome sequencing of both viable and unviable cells. Furthermore, we utilized the model organism *B. subtilis*, but it is possible that these stressors exhibit different spectra of mutation in other organisms based on taxa-specific stress responses. Finally, in terms of selection, we examined a fraction of the selection factors that shape which mutations are able to manifest viable, antibiotic-resistant bacteria. There are many more drug concentrations, growth conditions, and other selective forces that are important for the development

of antibiotic resistance in a population. Thus, future studies are needed to explore the role of other stressors and selective factors in shaping the development of antibiotic resistance.

Material and Methods

Bacterial Strains

The wild-type strain of *Bacillus subtilis* was *B. subtilis* 168 obtained from the Bacillus Genetic Stock Center (Columbus, OH, USA) (http://www.bgsc.org/index.php). All spectinomycin-resistant mutant strains were derived from this strain with the exception of those resistant isolates derived from the DNA-repair gene knockout strains in Figure 2. The DNA-repair knockout strains were originally generated by Koo et al. [89] and were obtained from the Bacillus Genetic Stock Center (Columbus, OH, USA) (http://www.bgsc.org/index.php).

Stress Conditions

Growth for all experiments occurred in Lysogeny Broth (LB) and at 37°C unless otherwise specified. Doses of each stress were determined by dose titration experiments with the goal of inducing mutagenesis and minimizing cell mortality or growth inhibition. The following concentrations/doses of each drug were used: 100 ng/mL mitomycin C, 600 ng/mL ciprofloxacin, 118.5 μg/mL $CoCl_2$, 0.3125 mM hydrogen peroxide, and 500 J/m^2 UV administered using a FB-UVXL-1000 UV Crosslinker from the Spectronics Corporation (Westbury, New York).

Spectinomycin Mutant Generation Experiments

Logarithmic Phase Growth

During logarithmic phase growth experiments, cells were grown overnight in SpC media (Table S7) for ~16 hours to an OD_{600} of at least 0.8. From the overnight cultures, subcultures were seeded 1:333 (WT strain) or 1:143 (Δ strains) into LB.

Cultures were grown at 37°C, shaking at 250 rpm, to an OD_{600} ~ 0.5 and stressors were then administered. Cells were grown with stressors for 30 minutes at 37°C, shaking at 300 rpm; exceptions were for UV treatments, involving a short exposure followed by 30 minutes of growth, and $CoCl_2$, which was administered for 60 minutes. Following the exposure to stressors, cells were washed with LB and grown in an equal volume of LB for 30 minutes at 37°C, shaking at 300 rpm. Following outgrowth, 40 µL of cells were serially diluted 10-fold in PBS and plated on LB agar (without selection) for counting of CFUs. The remainder of the cells were bead-spread on LB plates containing 100 µg/mL spectinomycin. Plates were grown for 16 hours at 37°C at which point CFU counts were taken from LB plates. Selection plates were grown for an additional 24 hours at room temperature and colonies were counted.

Stationary Phase Growth

For the stationary phase growth experiments, cells were grown overnight in SpC media (Table S7) for ~16 hours to an OD_{600} of at least 0.8. From the overnight cultures, subcultures were seeded 3:7 (WT strain) into LB. Cultures were grown at 37°C, shaking at 250 rpm to an OD_{600} ~ 1.8, at which point stressors were administered. Cells were exposed to stressors for 3 hours at 37°C, shaking at 300 rpm, except for UV treatments which involved a short exposure followed by 3 hours of incubation. Following the exposure to stressors, cells were washed with LB and incubated in an equal volume of LB for 30 minutes at 37°C, shaking at 300rpm. Following outgrowth, 40 µL of cells were serially diluted 10-fold in PBS and plated on LB agar without selection for counting CFUs. The remainder of the cells were

bead spread on LB plates containing 100 µg/mL spectinomycin. Plates were grown for 16 hours at 37°C, at which point CFU counts were taken from LB plates. Selection plates were grown for an additional 24 hours at room temperature and colonies were counted.

Isolation, Growth, and DNA Extraction of Spectinomycin Resistant Mutants

Individual colonies were manually isolated from LB spectinomycin selection plates using pipette tips. Each colony was grown in individual wells of a 96-well plates containing 200 µL of LB media with 100 µg/mL spectinomycin. Plates were grown for 16 hours at 37°C, shaking at 250 rpm and adjusted to have equal cell density in each well. 100 µL from each well was taken and pooled for DNA extraction. DNA extraction was performed using the ZymoBIOMICS DNA Miniprep Kit from Zymo Research (Irvine, CA, USA) following manufacturer's instructions with final elution in 100 µL of molecular grade H_2O. Total DNA concentration was measured using the SpectraMax M3 microplate reader from Molecular Devices LLC (San Jose, CA, USA) using the DNA Quantitation with the SpectraDrop Micro-Volume Microplate protocol.

Illumina Sequencing Amplicon Library Preparation and Sequencing

The *rpsE* gene was amplified from DNA derived from ~500 colonies from each condition. Primers used for generating amplicon libraries are listed in Table S5. A 300 bp region of the *rpsE* gene was amplified using a common reverse primer and barcoded forward primer (Table S5), with primers for each condition using a unique

barcode. PCRs for each condition were performed in 25 µL triplicate reactions in a T100 thermal cycler from BioRad (Hercules, CA, USA) under the following conditions: 180 seconds at 98°C, 45 seconds at 98°C, 60 seconds at 60°C, 90 seconds at 72°C, steps 2-4 repeated 34x, and a final step of 600 seconds at 72°C. PCR products of the triplet reactions of each condition were combined and purified using the NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel Inc. (Düren, Germany). 240 ng of purified amplicon from each condition were pooled together for the final sequencing library, which was sequenced at the Rhode Island Genomics and Sequencing Center at the University of Rhode Island (Kingston, RI, USA). Amplicons were paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform using a 600-cycle kit with standard protocols.

Illumina Amplicon Sequencing Analysis

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp). Reads were quality filtered, trimmed, de-noised and merged using DADA2[109]. Representative sequences (unique mutations) were determined and extracted using QIIME2 (version 2020.8)[110]. Processed reads were matched to representative sequences using the QIIME2 “Closed-reference clustering” tool with vsearch[111] and the percent identity set to 1.00 (--p-perc-identity 1.00) to only identify exact matches to representative sequences. Protein models of the mutations were generated using VMD version 1.9.3[112].

Generation of Spectinomycin Resistant Base Substitution Mutant Strains

To generate single-nucleotide *rpsE* mutant strains, PCR-amplified DNA of individually-isolated spectinomycin resistant colonies was used for natural transformation into the WT *Bacillus subtilis 168* background strain. Natural transformation allowed us to transfer only the region of DNA containing the select S2 base substitution mutations we wanted to test, thus excluding any other possible sites of mutation that might have occurred in the generation of the initial spectinomycin-resistant colonies. DNA from 11 different colonies that were previously Sanger sequenced was used to amplify the *rpsE*. The same protocol used for generating the Sanger sequencing amplicons was used to generate the DNA used for transformation into the WT strain. Details of this protocol can be found in the "Sanger Sequencing" section of these methods and materials. The purified *rpsE* amplicons were then incorporated into the WT background using the following procedure for natural transformation. First, WT *B. subtilis 168* was grown overnight (~16 hours) in LB at 37°C, shaking at 300 rpm. Second, the overnight culture was diluted 1:100 into 5mL of freshly prepared MNGE media and grown for 7 hours at 37°C, shaking at 250 rpm, to an OD_{600} ~ 0.5. Third, 300 – 500 ng of the amplified *rpsE* gene from the previous PCR step was added to 400 µL aliquots of the cells, which were then grown for 60 minutes at 37°C, shaking at 300 rpm. Fourth, 100 µL of expression mix was added to each 400 µL culture and grown for an additional 60 minutes at 37°C, shaking at 300 rpm. Finally, the cells from each culture were pelleted, the supernatant was removed, the pellet was resuspended in 200 µL of LB and spread onto LB plates containing 100 µg/mL spectinomycin using glass beads. Plates were grown at 37°C for 24 hours and individual colonies

were picked and grown up in 2 mL of LB broth containing 100 µg/mL spectinomycin for selection. Frozen stocks of these mutants were generated and a portion of the cells was used to perform Sanger sequencing as described in the "Sanger Sequencing" section of these methods and materials to confirm the mutations present in the strains in Table 1 and Figure 3. Upon sequencing the isolates to confirm mutations, we identified 7 isolates with the expected mutation, and 4 containing mutations (A78C, G83A, G83A-G151T, G70A-G82A) that developed spontaneously and did not correspond to the DNA added to the natural transformation assay.

Competition Experiments between Spectinomycin-Resistant Base Substitution Mutant Strains

The strains used for the competition experiment were generated as described above. Using the eleven mutants listed in Table 1, as well as WT *B. subtilis* 168, we performed growth competition experiments with no antibiotic selection or in media containing 62.5 µg/mL, 250 µg/mL, or 1,000 µg/mL of spectinomycin. These drug concentrations correspond to the MIC of the WT strain (62.5 µg/mL), the lowest MIC of the 11 isolates tested (250 µg/mL), and the second highest MIC of all the isolates (1,000 µg/mL). For each of the conditions, there were four biological replicates. Four cultures of each of the 12 strains used for this competition were grown overnight (~16 hours) at 37°C, shaking at 300rpm in LB broth with 100 µg/mL selection added to the 11 spectinomycin resistant isolates. No selection was added to the WT cultures. The OD_{600} of overnight cultures was taken and all twelve

strains were added in equal proportions into a new culture containing LB broth with 0, 62.5, 250, or 1,000 µg/mL spectinomycin with a final combined OD_{600} of 0.1. A 2mL sample of cells was taken from the initial no antibiotic replicates immediately before starting incubation and was used for the T:0 timepoint for all concentrations. Cultures were then grown at 37°C, shaking at 250 rpm, and 500 µL of cells were taken at 3, 6, 12, and 24 hours. Immediately after the 24-hour timepoint, the cultures were passaged into fresh media (maintaining antibiotic selection) to an OD_{600} of 0.01. Additional samples were taken from cultures at 48 hours, immediately followed by another passaging of cells, and 72 hours after the initial T:0 timepoint. DNA from cells collected at each timepoint were extracted using the ZymoBIOMICS DNA Miniprep Kit from Zymo Research (Irvine, CA, USA) immediately after sampling. The extracted DNA was quantified using the Qubit High Sensitivity reagent with a Qubit 3.0 Fluorometer from Thermo Fisher Scientific (Waltham, MA, USA). The 24-hour, 250 µg/mL, replicate D sample had an issue with the extraction and could not be used for downstream processing.

Nanopore MinION Sequencing Amplicon Library Preparation and Sequencing

The samples from the competition experiment were prepared for sequencing on the Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK). Due to the limitation of having only 96 barcodes and 100 samples, the 72-hour, no-antibiotic samples were not sequenced. The *rpsE* gene was amplified from the DNA of each sample taken during the competition experiment. PCRs for each sample were performed in 25 µL triplicate reactions in a T100 thermal cycler from

BioRad (Hercules, CA, USA) under the following conditions: 180 seconds at 98°C, 45 seconds at 98°C, 60 seconds at 60°C, 90 seconds at 72°C, steps 2-4 repeated 34x, and a final step of 600 seconds at 72°C. PCR products of the triplicate reactions of each condition were combined and purified using the NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel Inc. (Düren, Germany) and DNA was quantified using a Qubit 3.0 Fluorometer from Thermo Fisher Scientific (Waltham, MA, USA).

Cleaned, quantified amplicons were then prepared for sequencing using the Native Barcoding Expansion 96 (EXP-NBD196) and Ligation Sequencing Kit (SQK-LSK109) from Oxford Nanopore Technologies (Oxford, UK). The protocol was followed according to the manufacturer instructions with any modifications detailed here. The protocol began with 240 fmol (50ng of 320bp amplicon) of DNA for each reaction. End prep was performed using the NEBNext Ultra II End repair/dA-tailing Module (E7546) from New England Biolabs (Ipswich, MA, USA) and the end repair reaction was performed in a T100 thermal cycler from BioRad (Hercules, CA, USA) at 20°C for 20 mins and 65°C for 20 mins. The native barcode ligation was performed using the Native Barcoding Expansion 96 (EXP-NBD196) (Oxford Nanopore Technologies, Oxford, UK) in conjunction with NEB Blunt/TA Ligase Master Mix (M0367) from New England Biolabs (Ipswich, MA, USA) according to manufacturer instructions. The barcoded DNA was quantified using the Qubit High Sensitivity reagent with a Qubit 3.0 Fluorometer from Thermo Fisher Scientific (Waltham, MA, USA). Adapter ligation was performed using the Ligation Sequencing Kit (SQK-LSK109) from Oxford Nanopore Technologies (Oxford

Nanopore Technologies, Oxford, UK) and NEBNext Quick Ligation Module (E6056) from New England Biolabs (Ipswich, MA, USA) according to manufacturer instructions. The final library was quantified using the Qubit High Sensitivity reagent with a Qubit 3.0 Fluorometer from Thermo Fisher Scientific (Waltham, MA, USA). Priming and loading the SpotON Flow Cell was performed using the Flow Cell Priming Kit (EXP-FLP002) (Oxford Nanopore Technologies, Oxford, UK) according to manufacturer instructions. Performed sequencing on the Oxford Nanopore MinION using MinKNOW (MinION Mk1B software) (Oxford Nanopore Technologies, Oxford, UK) with the default protocol and live basecalling turned OFF. The run was terminated after ~48 hours as none of the pores had shown sequencing activity for several hours.

Nanopore MinION Sequencing Analysis

Raw sequences were processed using the Guppy (version 4.0.11) software (Oxford Nanopore Technologies, Oxford, UK) via the command line interface. Basecalling was performed using the guppy_basecaller command with the default settings including use of the high accuracy (HAC) model. Basecalled sequences were then demultiplexed using the guppy_barcoder command with default settings. Demultiplexed reads were then filtered using the Filtlong command with the following parameters --min_length 400 --min_mean_q 9 --trim. Filtered reads were then mapped to a reference of the *rpsE* sequences from the 12 strains used in the competition experiment using the minimap2 version 2.17 mapping

software[113]. Finally, only mapped reads with 100 percent identity to the reference sequences were used for downstream analyses.

Statistical Methods for Competition Experiments

Generalize Estimating Equations were used for all hypothesis testing. Observations drawn from within a particular tube (and its subsequent passage tube) were nested as having correlated residual error. Relative abundance was modeled as binomial, with each strain's count per total count across all strains. Adjusted counts were modeled as Poisson with the natural log of the absolute amount of starting DNA added to the library preparation as an offset in the model (creating dependent variable units as counts per ng of DNA input into the Nanopore Library Prep). All models also implemented classical sandwich estimation to adjust for how empirical variances may have differed from model assumptions. Comparisons were made between all timepoints for the no-drug selection group, and all timepoints except T:0 for the groups in which spectinomycin was added. A total of 163 hypothesis tests for relative changes between aliquots were carried out as orthogonal linear estimates (4 means) for each strain, maintaining an alpha of 0.05 using the Holm test to adjust each p-value. The above statistical analyses were performed using Statistical Analysis Software, SAS (Cary, NC, USA).

Whole Genome Sequencing

Four spectinomycin-resistant isolates with MICs of >1,000 µg/mL were selected for whole-genome sequencing. DNA from these selected isolates were extracted using the ZymoBIOMICS DNA Miniprep Kit from Zymo Research (Irvine, CA, USA) and quantified using a Qubit 3.0 Fluorometer from Thermo Fisher Scientific (Waltham, MA, USA). The metagenomic library was prepared using the NEB Next Ultra II DNA Library Prep Kit from New England Bio Labs (Ipswich, MA). Metagenomic libraries were sequenced on a NovaSeq 6000 or ISeq100 instrument (San Diego, CA).

Whole Genome Sequencing Analysis

Reads were trimmed with Trimmomatic (version 0.36) with SLIDINGWINDOW set at 4:20, MINLEN set at 50, and ILLUMINACLIP: TruSeq3-PE.fa:2:20:10[114]. Trimmed reads were assembled and single nucleotide variants were searched for using the Variation Analysis tool with BWA-mem/FreeBayes setting of the PATRIC webserver (version 3.5.38)[115].

Sanger Sequencing

A portion of individual colonies used for Sanger sequencing of the *rpsE* and *rpsB* genes were boiled at 100^{o}C for 15 minutes in 20 µL of TE buffer to lyse cells. A 1 µL aliquot of a 1:10 dilution of boiled cell lysate was then used as template for PCR of the genes of interest. Primers pairs #388, 389 and #553, 554 were used to amplify the *rpsE* and *rpsB* genes, respectively (Table S4). PCRs for each gene were performed in 25 µL reactions in a T100 thermal cycler from BioRad (Hercules,

CA, USA) under the following conditions: 180 seconds at 98°C, 45 seconds at 98°C, 60 seconds at 55°C, 90 seconds at 72°C, steps 2-4 repeated 34x, and a final step of 600 seconds at 72°C. PCR products were then purified using the NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel Inc. (Düren, Germany) and DNA was quantified on the SpectraMax M3 microplate reader from Molecular Devices LLC (San Jose, CA, USA) using the DNA Quantitation with the SpectraDrop Micro-Volume Microplate protocol. Amplicons were sequenced at Eurofins Genomics. Alignments of the Sanger sequencing data was performed using Unipro UGENE (version 1.32.0)[116].

Minimal Inhibitory Concentration Determination

Minimal inhibitory concentrations (MICs) were determined using the broth dilution method [117]. *Bacillus subtilis* strains were grown overnight in LB. Overnight cultures were diluted 1:10,000 and added to a 96-well plate. Spectinomycin was added to a concentration of 1 mg/mL to cell culture media and serially diluted two-fold across the plate. Cells were then incubated at 37°C, shaking at 300rpm for ~20 hours and the OD_{600} was taken to measure growth using the SpectraMax M3 microplate reader from Molecular Devices LLC (San Jose, CA, USA). MIC_{90} was recorded for all MIC experiments.

Growth Rate Determination & Time to Exit Lag Phase

Overnight cultures of *B. subtilis* strains grown in LB were diluted to an OD_{600} ~ 0.05 and then grown in LB in triplicate at 37°C, shaking at 300rpm, for 390 minutes for

no drug conditions or 510 minutes with spectinomycin present, with OD_{600} measurements taken every 30 minutes using the SpectraMax M3 microplate reader from Molecular Devices LLC (San Jose, CA, USA). The growth assays were performed with 0, 62.5, 250, and 1,000 μg/mL spectinomycin with 6 – 12 replicates in each group. To determine the doubling time of each strain, the growth curves from OD_{600} ~ 0.15 – 0.4 were fitted to an exponential growth function using default settings in Prism (version 8.0). The time to exit lag phase was determined by fitting a simple linear regression to estimate the time at which cultures reached OD_{600} = 0.2. An ANOVA with an alpha of 0.05 was used to compare the doubling time and time to exit lag phase for each strain.

Cold Sensitivity Determination

Selected spectinomycin-resistant strains were grown up in LB with spectinomycin (100 μg/mL) in triplicate. Cultures were serially diluted 10-fold in PBS and 5 μL of each diluted was spot plated on LB plates with two sets of plates for each replicate. One set of replicates was grown for 18 hours at 37°C and the other for 84 hours at 20°C.

Data Availability

Raw Illumina *rpsE* amplicon sequencing reads were deposited in the NCBI Sequence Read Archive under the BioProject number PRJNA703389. The base called Nanopore *rpsE* amplicon sequencing reads were deposited in the NCBI Sequence Read Archive under the submission number PRJNA704934. Raw Illumina whole genome sequencing reads from the twelve strains used for the competition experiment were deposited in the NCBI Sequence Read Archive under the submission PRJNA748029.

Acknowledgments

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Author Contributions:

Conceptualization: B.J.K, P.B. Methodology: B.J.K, P.B. Formal Analysis: B.J.K, J.T.M. Investigation: B.J.K., S.Y.E.L., A.K.C, A.H.C., C.G., P.B. Data Curation: B.J.K., P.B. Writing – Original Draft: B.J.K., P.B., J.T.M. Writing – Review and Editing: B.J.K., P.B., C.G. Visualization: B.J.K, J.T.M. Supervision: P.B. Funding Acquisition: P.B., J.T.M, B.J.K

Figures

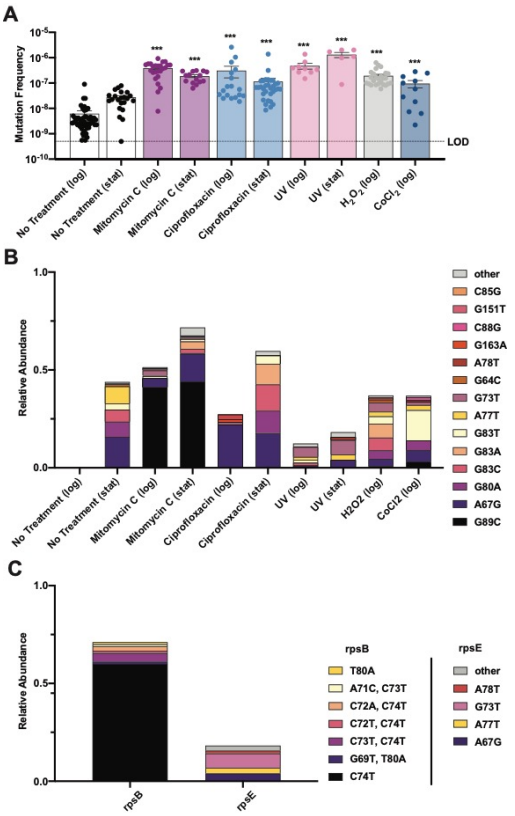

Figure 1. Genotoxic Agents Induce a Spectrum of Unique, Treatment-Specific Resistance Mutations

(A) Mutation frequency of spectinomycin resistance in WT *B. subtilis* treated with various stressors during logarithmic or stationary phase growth. Mutation frequency calculated by dividing the number of spectinomycin resistant colonies by the total number of cells. Significant differences between untreated and treated cells determined using Mann-Whitney U test ($p \leq 0.0001$ = ***). Horizontal LOD line represents the limit of detection.

(B) Spectrum of nucleotide mutations in the *rpsE* gene of the spectinomycin-resistant mutant colonies of WT *B. subtilis*. Notably, there were no mutations identified in the no treatment condition during logarithmic phase as this condition produced very few or no resistant colonies. In lieu of the resistant colonies, logarithmic phase cells were plated on non-selective media and 500 of these non-resistant isolates were sequenced.

(C) The relative abundance of nucleotide mutations in the *rpsB* gene from 90 spectinomycin-resistant mutant colonies compared to the relative abundance of mutations from the *rpsE* gene from 480 colonies (including the 90 colonies displaying the *rpsB* mutations on the left), all of which were from UV-treated cells.

(B-C) Each treatment represents ~500 colonies collected from 3 to 12 biological replicates.

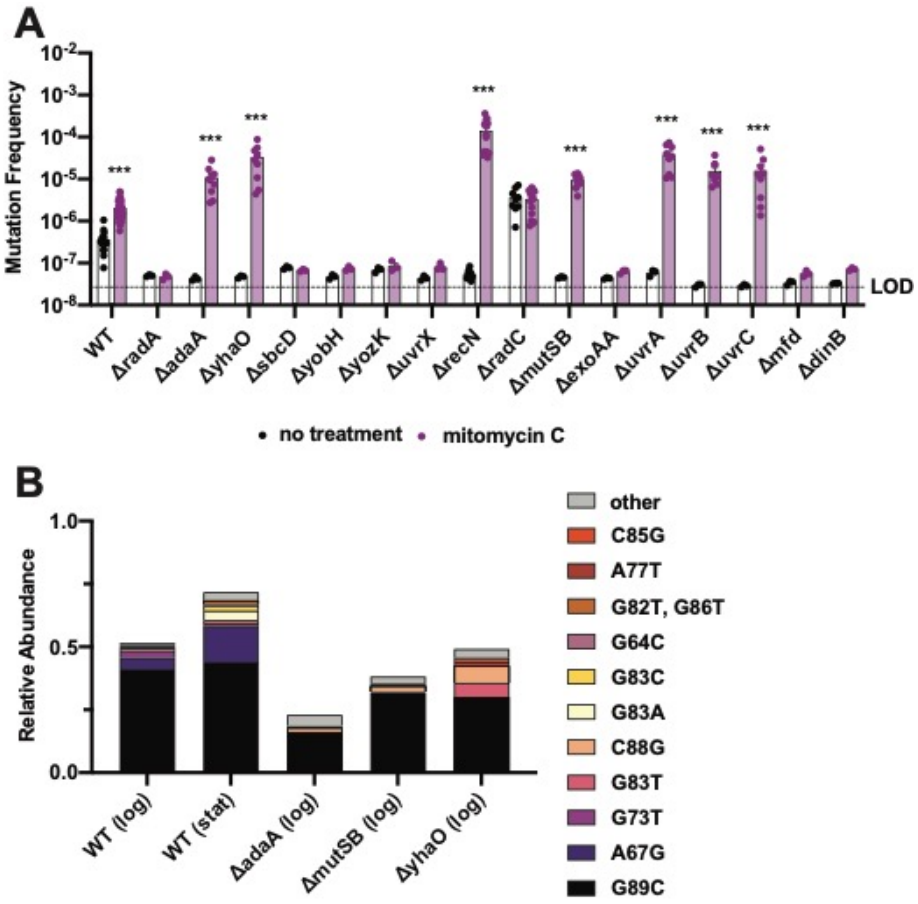

Figure 2. Impact of DNA Repair Genes on the Frequency and Spectrum of Mitomycin C-induced Mutants

(A) Mutation frequency of spectinomycin resistance in DNA repair-deficient *B. subtilis* treated with 100 ng/mL mitomycin C. Mutation frequency calculated by dividing the number of spectinomycin resistant colonies by the total number of cells. Bars represent mean $\pm$ SEM (n=3 minimum). Significance between mitomycin C-treated and untreated WT, and between mitomycin C-treated WT and mitomycin C-treated DNA knockouts; determined using Mann-Whitney U Test ($p \leq 0.0001$ = ***). Horizontal LOD line represents the limit of detection.

(B) Spectrum of nucleotide mutations in the *rpsE* gene of the mutant colonies from WT and DNA repair-deficient strains of *B. subtilis* treated with mitomycin C. *Each treatment represents ~500 colonies collected from 3 to 12 biological replicates.*

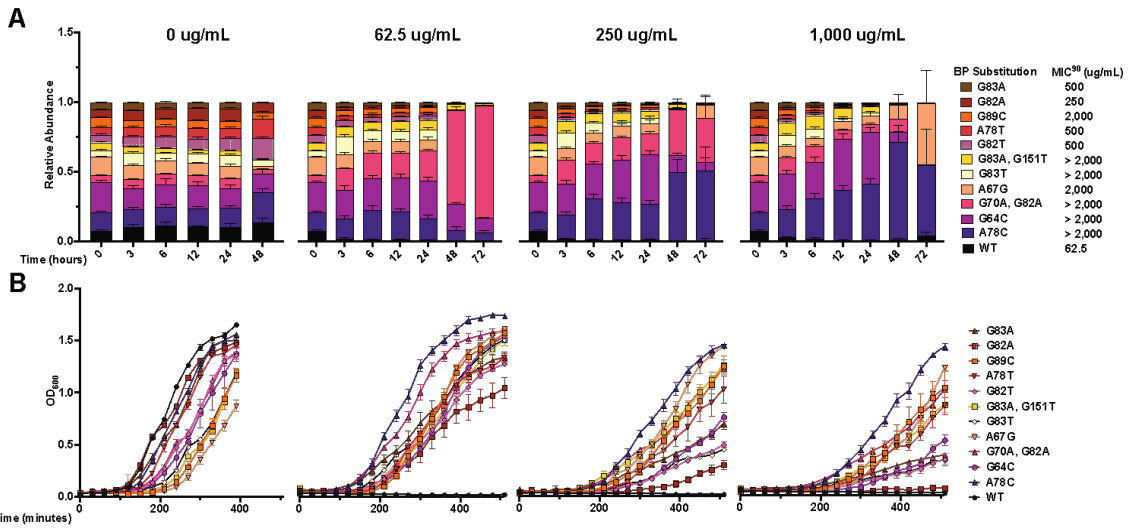

Figure 3. Competition of Base Substitution Mutants over a Gradient of Selection

(A) Stacked bar plots of the relative abundance of each strain within a mixed community at times 0, 3, 12, 24, 48, 72 hours under spectinomycin selection concentrations of 0, 62.5, 250, 1,000 µg/mL. Cultures were passaged to an OD_{600} of 0.01 immediately after sampling of the 24- and 48-hour timepoints. Plots represent the average relative abundance ± SEM (n=4). Determination of statistical differences is described in the Methods section.

(B) Growth rates of the twelve strains used in the competition experiments performed at each of the concentrations shown above in panel A (0, 62.5, 250, 1,000 µg/mL spectinomycin). Points represent mean ± SEM (n=6 minimum). Cell growth over time determined by measuring OD_{600} at 30 min time intervals.

(A-B) The relative abundance plots in (A) are separated by drug concentration, and the line plots (B) correspond to the drug concentrations in the relative abundance plots shown directly above each plot.

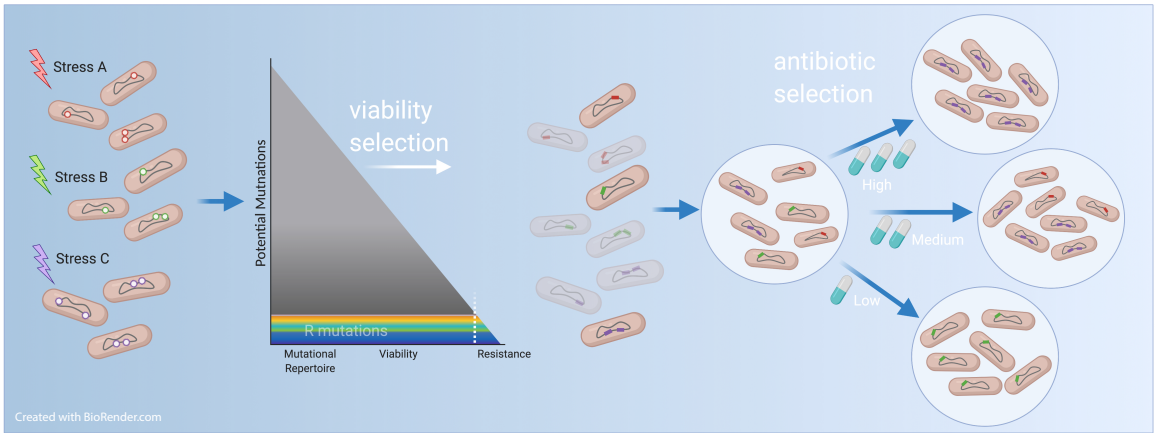

Figure 4. Stress-Associated Mutagenesis Leads to Spectinomycin Resistance Through a Spectrum of Mutation and a Gradient of Selection in *B. subtilis*

A graphical representation of the process by which stress-associated mutagenesis in *B. subtilis* leads to spectinomycin resistance through the generation of base substitution mutations and subsequent selection. The initial stress causes a stress-specific pattern of mutations, but of all these mutations most are deleterious or do not impact antibiotic susceptibility (represented by the grey portion of the triangle). Deleterious or growth-inhibiting mutants are lost as they are unable to produce viable cells. Of the viable mutations (denoted by the rainbow in the bottom portion of the pyramid), only a portion of the possible mutations will be able to manifest a resistance phenotype based on fundamental selection criteria such as that mutation being in the mutational repertoire of the stressor and not impacting cell viability. Transparent cells represent those that were unable to meet the selection requirements for resistance. Finally, the level of antibiotic selection pressure will ultimately determine the final spectrum of base substitution mutations in a mixed population.

Table 1. Growth Dynamics of Competition Strains

Strain		MIC^{90}	Cold Sensitivity	Doubling Time			Time to Exit Lag Phase		
AA	BP	MIC (ug/mML)		Time (min)	95% CI	Significance	Time (min)	95% CI	Significance
WT	WT	62.5	No	27.53	22.28 - 34.93	-	136.58	135.4 - 137.8	-
R30P	G89C	2,000	No	36.18	22.88 - 65.57	ns	254.72	240.0 - 269.5	****
K26N	A78C	>2,000	No	33.02	27.65 - 40.00	ns	158.86	154.0 - 163.7	*
K26N	A78T	500	No	26.55	20.67 - 34.56	ns	171.15	165.7 - 176.6	***
G28C	G82T	500	No	35.05	26.30 - 48.83	ns	194.03	185.4 - 202.6	****
A22P	G64C	>2,000	No	39.69	32.34 - 83.55	***	211.81	185.9 - 237.7	****
G28S	G82A	250	No	28.06	25.31 - 31.20	ns	133.37	131.3 - 135.4	ns
G28D	G83A	500	No	44.48	35.07 - 58.87	***	148.64	138.7 - 158.6	ns
K23E	A67G	2,000	**Yes**	40.37	32.34 - 51.49	*	268.2	258.4 - 278.0	****
G28V	G83T	>2,000	No	33.87	31.36 - 36.69	ns	223.74	221.3 - 226.2	****
G28D, G51C	G83A, G151T	>2,000	No	51.16	41.28 - 65.95	****	239.16	236.1 - 242.3	****
V24I, G28S	G70A, G82A	>2,000	No	34.40	28.99 - 41.40	ns	190.62	186.7 - 194.6	****

Growth curves were used to calculate both doubling time and the length of time for stationary phase cells to exit lag phase and enter into logarithmic growth. Significant differences in doubling time and time to exit lag phase between each strain and WT was determined using an ANOVA (*p < 0.05, ***p < 0.001, ****p < 0.0001). The minimum inhibitory concentration is the MIC^{90} of spectinomycin and cold sensitivity was determined by measuring growth at 20°C.

Supplementary Information

Figure S1: Nucleotide alignment of *rpsE* gene sequences from sanger sequenced spectinomycin resistant colonies
Figure S2: Nucleotide Alignment of *rpsB* gene sequences from sanger sequenced spectinomycin resistant colonies
Figure S3: Nucleotide alignment of all unique *rpsE* sequences identified from MiSeq sequencing of the compendium of resistant isolates from Figure 1B.
Table S1: Mutations from select whole genome sequenced spectinomycin resistant colonies.
Table S2: Spectrum of H_2O_2 - Induced Mutations. A comparison of the spectrum of hydrogen peroxide induced mutations in the *supF* gene of *E. coli* (Akasaka et al. 1992) and *rpsE* from *B. subtilis* 168 (this work).
Table S3: Growth rate and exit from lag phase of strains used in competition experiment at 0, 62.5, 250, or 1,000 µg/mL spectinomycin.
Table S4: Sanger Sequencing Primers for rpsE and rpsB
Table S5: *rpsE* Illumina Sequencing primers
Table S6: Raw read counts and relative abundance from Illumina sequencing
Table S7: Growth media used in this study

Figure S1. Alignment of *rpsE* gene sequences from sanger sequenced spectinomycin resistant colonies

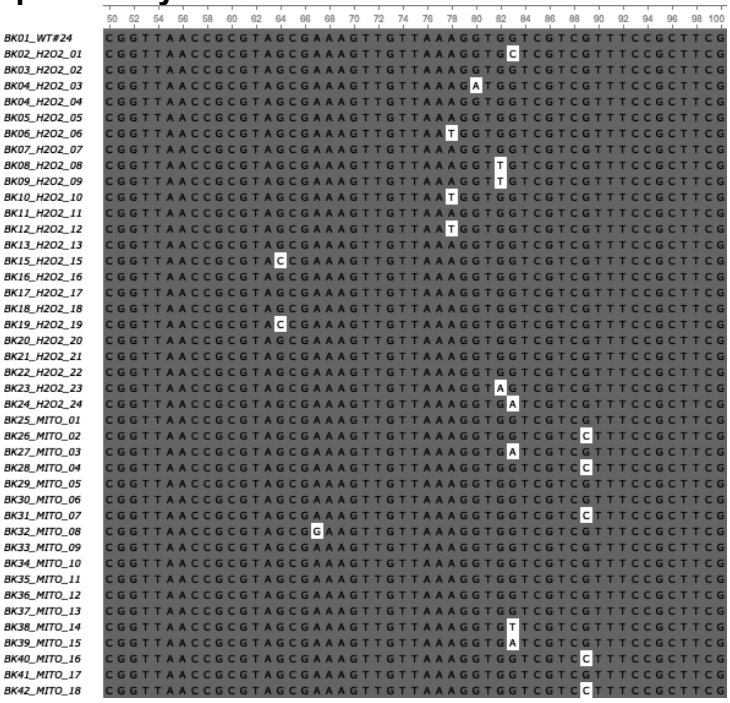

Figure S2. Alignment of *rpsB* gene sequences from sanger sequenced spectinomycin resistant colonies

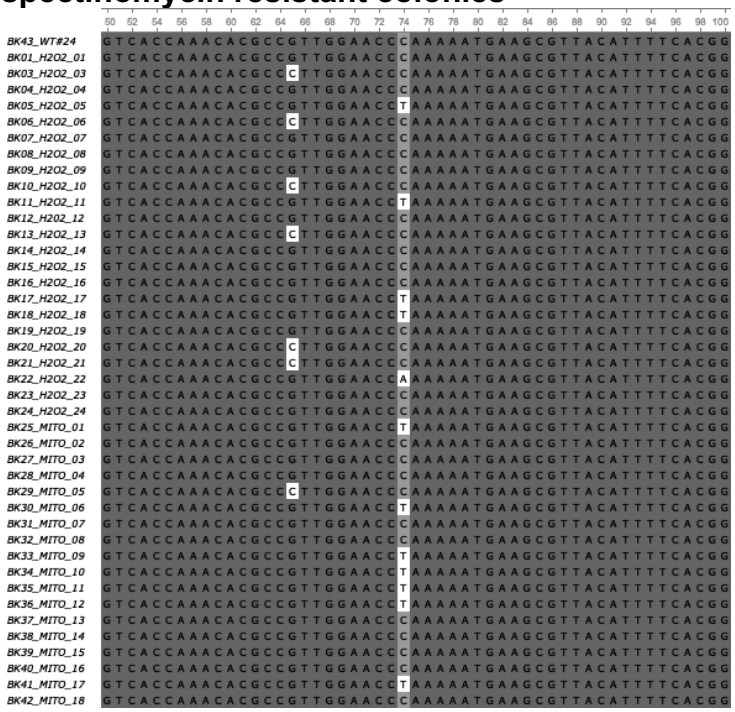

Figure S3. Alignment of all unique *rpsE* sequences identified from MiSeq sequencing of the compendium of resistant isolates from Figure 1B.

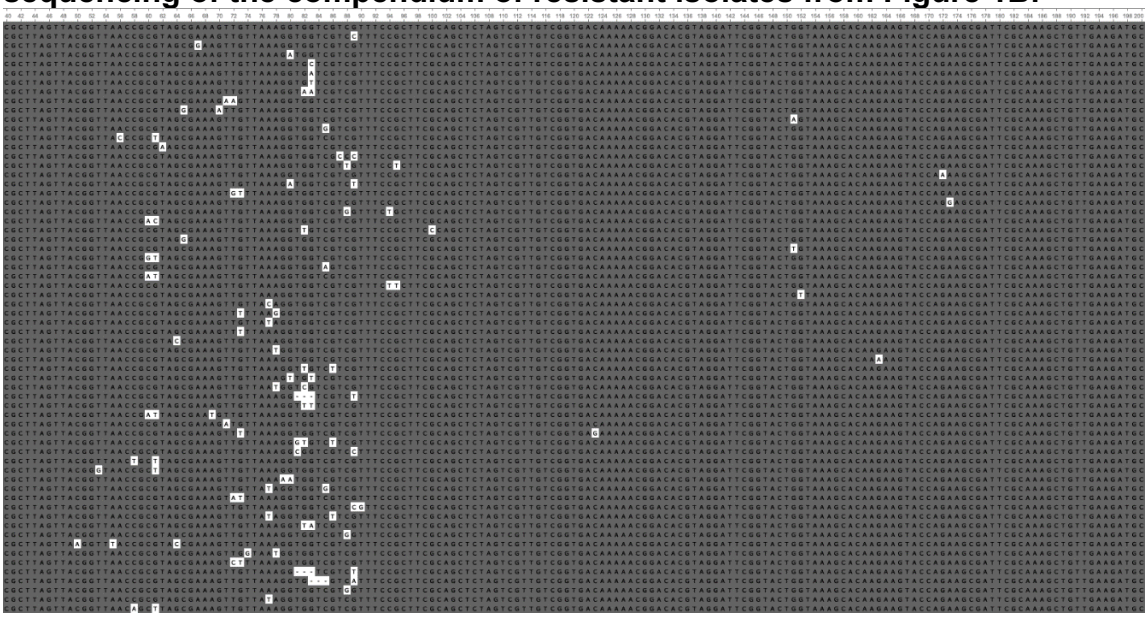

Figure S4. Relative Abundance of Transitions and Transversions in Spectinomycin Resistant Colonies

(A) Relative abundance of transversions and transitions in the *rpsE* gene mutations from of the spectinomycin-resistant mutant colonies of WT *B. subtilis*. The TI:TA ratio is displayed above each bar.

(B) Relative abundance of transversions and transitions in the *rpsE* gene mutations from WT and DNA repair-deficient strains of *B. subtilis* treated with mitomycin C. The TI:TA ratio is displayed above each bar.

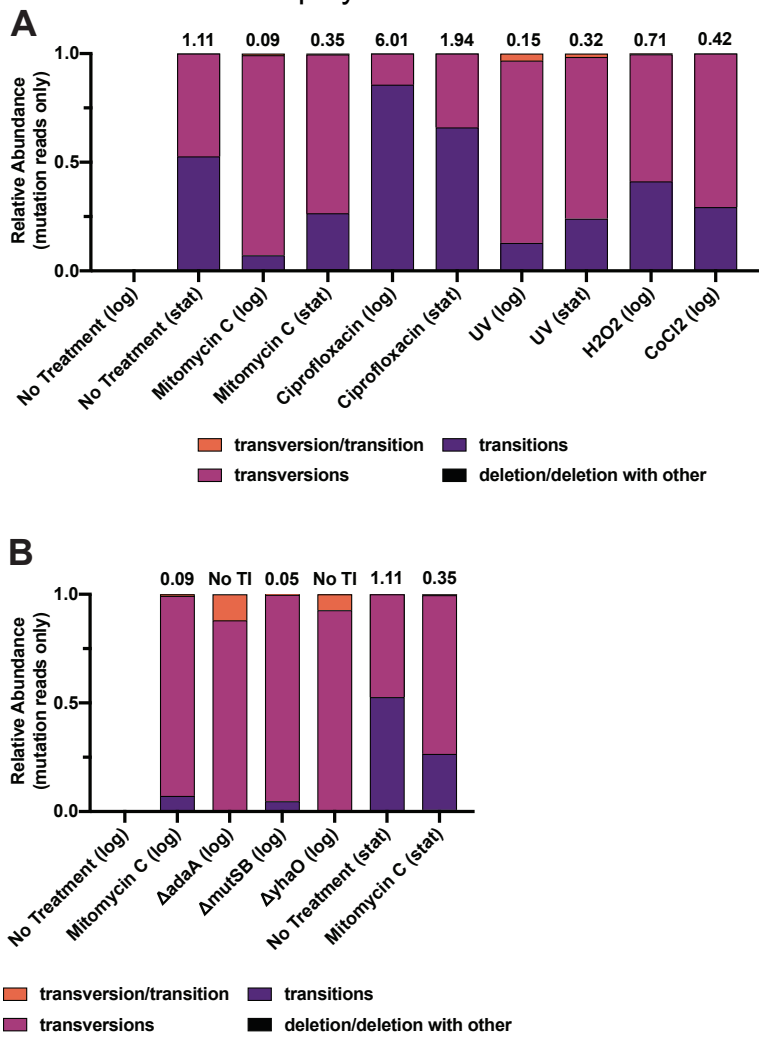

Figure S5. Mutagenesis-Associated Changes in the Structure of Ribosomal Proteins S5 and S2

(A) Protein structure of the S5 protein, with red highlighting the positions modified in the top 10 most common mutations (top), which are displayed in the amino acid alignment (bottom).

(B) Protein structure of the S2 protein, with red highlighting the positions modified in the top 2 most common mutations (top), which are displayed in the amino acid alignment (bottom).

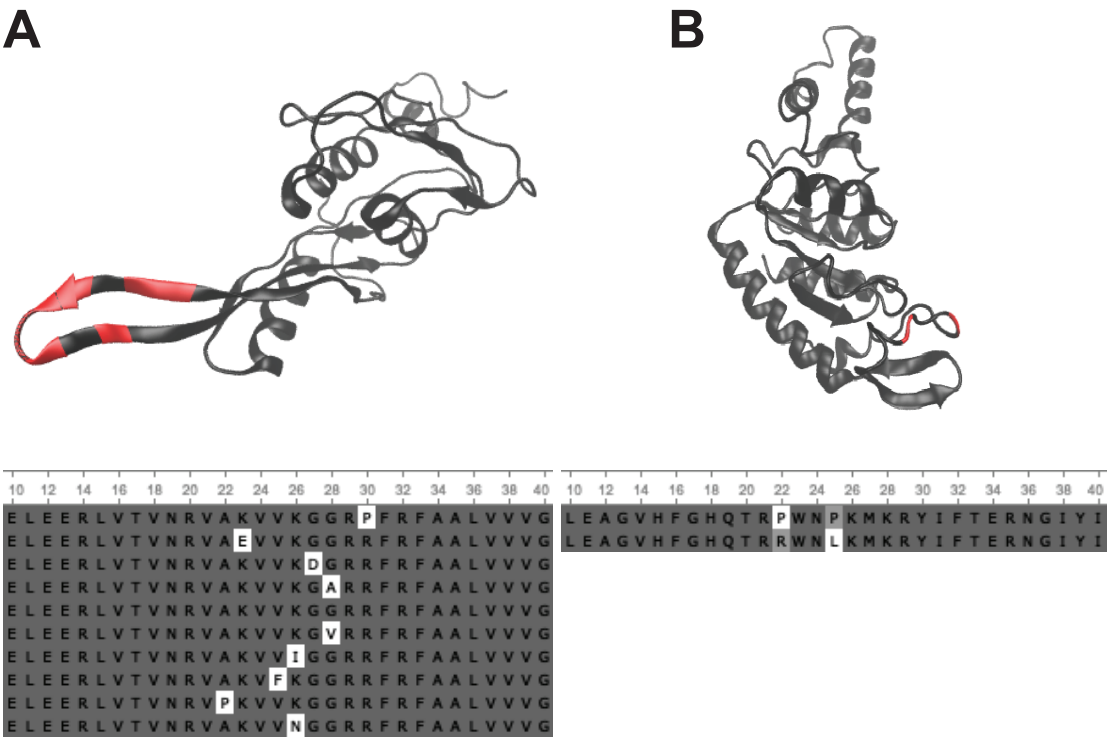

Table S1. Mutations from Select Spectinomycin Resistant Colonies Mutations found in ribosomal genes through whole genome sequencing of four spectinomycin resistant colonies. There were two more mutations found in isolate #17 that resulted in single base substitutions in the *yhcT* and *gcvT* genes.

Colony ID	Mutated Gene	Nucleotide Substitution	Amino Acid
#1	*rpsB*	C74T	Pro > Leu
#6	*rpsB*	C74T	Pro > Leu
#17	*rpsB*	C74T	Pro > Leu
#4	*rpsE*	G89C	Arg > Pro

Table S2. Spectrum of H_2O_2 – Induced Mutations
A comparison of the spectrum of hydrogen peroxide induced mutations in the *supF* gene of *E. coli* (Akasaka et al. 1992) and *rpsE* from *B. subtilis* 168 (this work). Data here represent the percent of total mutations.

	(Akasaka et al. 1992)	(Korry et al. 2021)
Transition		
G:C -> A:T	12.9%	31.7%
G:C -> A:T	4.3%	10.0%
Transversion		
G:C -> A:T	37.1%	23.0%
G:C -> C:G	40.0%	23.9%
A:T -> T:A	4.3%	11.4%
A:T -> C:G	1.4%	0.0%

Table S3. Doubling time and exit from lag phase of competition experiment strains grown with 0, 62.5, 250, and 1,000 ug/mL of spectinomycin. Doubling time (DT) and exit from lag phase (EfL) values are represented as mean (± 95% confidence interval) in minutes (n= 6 -12).

Spectinomycin	0ug/mL		62.5ug/mL		250ug/mL		1,000ug/mL	
	DT (95% C.I.)	EfL (95% C.I.)	DT (95% C.I.)	EfL (95% C.I.)	DT (95% C.I.)	EfL (95% C.I.)	DT (95% C.I.)	EfL (95% C.I.)
WT	27.53 (22.3 - 34.9)	136.58 (135.4 - 137.8)						
G89C	36.18 (22.9 - 65.6)	254.72 (240.0 - 269.5)	45.25 (31.8 - 71.5)	241.56 (230.0 - 253.2)	68.26 (44.7 - 121.9)	294.96 (268.0 - 322.0)	104.2 (81.2 - 138.4)	320.95 (277.6 - 364.4)
A78C	33.02 (27.7 - 40.0)	158.86 (154.0 - 163.7)	31.34 (24.5 - 40.5)	154.5 (145.1 - 163.9)	48.51 (38.5 - 63.1)	225.66 (214.7 - 236.7)	55.14 (41.5 - 77.9)	241.41 (219.6 - 263.2)
A78T	26.55 (20.7 - 34.6)	171.15 (165.7 - 176.6)	32.06 (24.7 - 42.8)	227.19 (220.6 - 233.7)	71.62 (51.2 - 110.0)	301.8 (275.4 - 328.2)	61.52 (52.9 - 72.6)	307.32 (300.1 - 314.6)
G82T	35.05 (26.3 - 48.8)	194.03 (185.4 - 202.6)	43.29 (35.7 - 54.0)	213.48 (202.9 - 224.3)	103.5 (84.1 - 132.7)	344.8 (323.0 - 366.6)	149.1 (94.7 - 312.1)	431.69 (348.4 - 515.0)
G64C	49.69 (32.3 - 83.6)	211.81 (185.9 - 237.7)	55.66 (41.9 - 80.1)	227.38 (200.9 - 253.9)	88.8 (73.3 - 111.1)	337.38 (322.3 - 352.5)	86.84 (77.2 - 97.8)	407.37 (381.6 - 433.1)
G82A	28.06 (25.3 - 31.2)	133.37 (131.3 - 135.4)	104.8 (78.9 - 148.0)	243.54 (209.8 - 277.3)	146 (75.6 - 1184)	410.67 (308.5 - 512.8)		
G83A	44.48 (35.1 - 58.9)	148.64 (138.7 - 158.6)	69.03 (52.9 - 94.0)	168.3 (144.7 - 192.0)	120.3 (102.2 - 144.5)	269.38 (242.5 - 296.3)	167.8 (156.0 - 181.0)	324.27 (292.8 - 355.7)
A67G	40.37 (32.3 - 51.5)	268.2 (258.4 - 278.0)	47.89 (35.1 - 71.0)	215 (202.7 - 227.3)	57.02 (40.9 - 87.2)	260.58 (242.9 - 278.3)	77.91 (75.0 - 80.9)	312.5 (302.6 - 322.4)
G83T	33.87 (31.4 - 36.7)	223.74 (221.3 - 226.2)	74.06 (54.5 - 112.5)	191.05 (177.5 - 204.6)	86.73 (63.6 - 131.7)	345.55 (338.8 - 352.4)	146.7 (128.3 - 168.7)	393.47 (321.0 - 465.9)
G83A, G151T	51.16 (41.3 - 66.0)	239.16 (236.1 - 242.3)	65.73 (52.3 - 85.6)	227.92 (213.6 - 242.2)	69.99 (42.7 - 155.3)	248.45 (201.1 - 295.8)	108.9 (100.4 - 118.2)	283.12 (252.8 - 313.4)
G70A, G82A	34.4 (29.0 - 41.4)	190.62 (186.7 - 194.6)	65.44 (50.0 - 92.6)	154.27 (140.6 - 168.0)	62.35 (52.3 - 76.1)	225.01 (214.1 - 235.9)	120.6 (111.4 - 130.6)	242.6 (217.8 - 267.4)

Table S4. Sanger Sequencing Primers for *rpsE* and *rpsB*

Primer	Sequence
rpsE upstream FWD *B. subtilis*	CTCGTGAAGCTGGACTTAAA
rpsE downstream REV *B. subtilis*	TTACACTTCGTTTGAGGGTA
rpsB upstream FWD *B. subtilis*	AAAAATGACCTAAGCGGAGG
rpsB downstream REV *B. subtilis*	TCCCTCTTATCACCTTTTGAATAGG

Table S5. Illumina Sequencing primers for *rpsE*

Primer	Primer Sequence 5' Illumina Adapter – Barcode – Pad – Linker – FWD Primer
FWD01	AATGATACGGCGACCACCGAGATCTACACGCTAGCCTTCGTCGCTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD02	AATGATACGGCGACCACCGAGATCTACACGCTTCCATACCGGAATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD03	AATGATACGGCGACCACCGAGATCTACACGCTAGCCCTGCTACATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD04	AATGATACGGCGACCACCGAGATCTACACGCTCCTAACGGTCCATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD05	AATGATACGGCGACCACCGAGATCTACACGCTCGCGCCTTAAACTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD06	AATGATACGGCGACCACCGAGATCTACACGCTTATGGTACCCAGTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD07	AATGATACGGCGACCACCGAGATCTACACGCTTACAATATCTGTTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD08	AATGATACGGCGACCACCGAGATCTACACGCTAATTTAGGTAGGTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD09	AATGATACGGCGACCACCGAGATCTACACGCTGACTCAACCAGTTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD10	AATGATACGGCGACCACCGAGATCTACACGCTGCCTCTACGTCGTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD11	AATGATACGGCGACCACCGAGATCTACACGCTACTACTGAGGATTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD12	AATGATACGGCGACCACCGAGATCTACACGCTAATTCACCTCCTTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD13	AATGATACGGCGACCACCGAGATCTACACGCTCGTATAAATGCGTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD14	AATGATACGGCGACCACCGAGATCTACACGCTATGCTGCAACACTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD15	AATGATACGGCGACCACCGAGATCTACACGCTACTCGCTCGCTGTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD16	AATGATACGGCGACCACCGAGATCTACACGCTTTCCTTAGTAGTTCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD17	AATGATACGGCGACCACCGAGATCTACACGCTCGTCCGTATGAATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD18	AATGATACGGCGACCACCGAGATCTACACGCTACGTGAGGAACGTCTGGCGG CTATCTCGTGAAGCTGGACTTAAA
FWD19	AATGATACGGCGACCACCGAGATCTACACGCTGGTTGCCCTGTATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
FWD20	AATGATACGGCGACCACCGAGATCTACACGCTCATATAGCCCGATCTGGCGGC TATCTCGTGAAGCTGGACTTAAA
Reverse	CAAGCAGAAGACGGCATACGAGAT AGCATGCTAG GC CGTGTGGAATTGTAGTTCCA
Sequencing FWD	TCTGGCGGCT AT CTCGTGAAGCTGGACTTAAA
Sequencing REV	AGCATGCTAG GC CGTGTGGAATTGTAGTTCCA

Table S6. Raw read counts and relative abundance from Illumina sequencing

Table S7. Growth media used in this study	
T base	0.2% $(NH_4)_2SO_4$; 0.6% KH_2PO, 1.83% K_2HPO_4; 0.1% $Na_3C_6H_5O_7$; in molecular water
SpC (5mL)	5mL T base; 50uL of 50% glucose; 75uL of 1.2% $MgSO_4$, 100µL of 10% yeast extract; 125µL of 1% casamino acids
10x MN	13.6% K_2HPO_4; 6.0% KH_2PO_4; 1.0% $Na_3C_6H_5O_7$; in molecular water
MNGE (10mL)	9.2mL 1 x MN; 1ml 20% glucose; 50µl 40% $C_5H_8KNO_4$; 50uL 2.2mg/mL $C_6H_8O_7{\cdot}Fe^{3+}{\cdot}NH_3$; 100uL 5mg/mL tryptophan, 30uL 1M $MgSO_4$
Expression Mix (1.05mL)	500µl 5% yeast extract; 250µl 10% casamino acids; 250µl H2O; 50µl 5mg/mL tryptophan

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CHAPTER 3: Trophic Level and Proteobacteria Abundance Drive Antibiotic Resistance Levels in Fish from Coastal New England

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Trophic Level and Proteobacteria Abundance Drive Antibiotic Resistance Levels in Fish from Coastal New England

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Abstract

The natural marine environment represents a vast reservoir of antimicrobial resistant bacteria. The wildlife that inhabits this environment plays an important role as the host to these bacteria and in the dissemination of resistance. The relationship between host diet, phylogeny, and trophic level and the microbiome/resistome in marine fish is not fully understood. To further explore this relationship, we utilize shotgun metagenomic sequencing to define the gastrointestinal tract microbiomes of seven different marine vertebrates collected in coastal New England waters. We identify inter and intraspecies differences in the gut microbiota of these wild marine fish populations. Furthermore, we find an association between antibiotic resistance genes and host dietary guild, which suggests that higher trophic level organisms have a greater abundance of resistance genes. Additionally, we demonstrate that antibiotic resistance gene burden is positively correlated with Proteobacteria abundance in the microbiome. Lastly, we identify dietary signatures within the gut of these fish and find evidence of possible dietary selection for bacteria with specific carbohydrate utilization potential. This work establishes a link between host lifestyle/dietary guild, and microbiome composition and the abundance of antibiotic resistance genes within the gastrointestinal tract of marine organisms.

Introduction

Fish are the most diverse group of vertebrates on earth with over 34,000 species inhabiting aquatic environments ranging from freshwater streams to the deep oceans [1]. They are essential to the ecosystems they inhabit, as well as the global food supply with fish providing over 3 billion people with 20% of their average protein consumption[2]. The global fishing industry is worth an estimated US$400 billion and employs nearly 60 million people worldwide making the health of the world's fisheries of great economic importance[2, 3]. In order to ensure the future of this ecologically and environmentally invaluable group of organisms we must understand fish biology including their associated microbial communities. Fish harbor a large number of bacterial symbionts in their gastrointestinal tract (GIT), and these microbes have been shown to play a role in growth, development, and disease [4, 5]. They have coevolved with their microbial symbionts for over 400 million years [6], yet despite their antiquity and diversity, the microbiota of fish remain understudied compared to those of mammals. To date most studies have utilized 16s sequencing to understand how dietary supplementation impacts growth, development, and health in the context of the microbiome in commercial fish species raised in aquaculture [4, 5, 7-13]. These methods are limited to broad taxonomic changes in fish that are raised in captive settings. Less is known about the microbiota of wild fish populations and few studies have implemented shotgun sequencing technologies to gain a broader perspective on the functional gene content of fish microbiota.

The use of shotgun metagenomic sequencing on the gastrointestinal tract (GIT) contents of wild fish populations provides not only the taxonomic structure of the gut microbiota, but it's functional potential, detection of dietary signatures and parasites [14-16], and identification of antimicrobial resistance genes (ARGs). The gut microbiomes of several wild marine fish have been sequenced and data suggests that habitat, diet, and host phylogeny play a role in shaping the GIT microbiome [14, 17-23]. Fewer studies have looked into antimicrobial resistance genes (ARGs) harbored by fish associated microbes [24-26]. Antimicrobial resistance is an increasing threat to human health with resistant organisms leading to 2.8 million infections and more than 35,000 deaths a year in the United States alone. The natural environment is known to be a reservoir of ARGs with wildlife playing a role in the dissemination of resistant microbes [27]. Utilizing databases of ARGs[28-31] and traditional microbiology culturing techniques we are beginning to see that ARGs are widespread throughout different environments and organisms. Studies have found ARGs in humans [32-34], animals [35-38], soils [33, 39], caves [40], ice cores [41], and marine environments [37, 42, 43] demonstrating that resistance can be found wherever bacteria live. Understanding the role of marine environments as a reservoir of antimicrobial resistance is crucial due to widespread aquaculture and seafood consumption and resulting interactions between humans and marine bacteria.

Narragansett Bay is the largest estuary in New England and provides an essential habitat for numerous commercially and ecologically important species [44]. Demersal fish species that inhabit the bay, including *Peprilus triacanthus*

(butterfish), *Stenotomus chrysops* (scup), *Paralichthys dentatus* (summer flounder), and *Mustelus canis* (smooth dogfish), each occupy different trophic positions based on previously defined dietary guilds – planktivore (butterfish), benthivore (scup), and predatory crustacivores/piscivores (summer flounder, smooth dogfish) [45]. These four species of interest represent a unique model in which to study the gut microbiome as they inhabit the same environment, differ in lifestyle and physiology, occupy specific trophic positions, and have direct predator/prey interactions. The waters off the coast of New England are also important fisheries and are home to larger migratory predators including *Alopias vulpinus* (thresher shark), *Isurus oxyrinchus* (shortfin mako shark), and *Lamna nasus* (porbeagle shark). Due to their position as apex predators, unique physiology, and highly migratory behavior, it remains a priority to better understand the shark GIT microbiome. In this work we aim to use shotgun metagenomic sequencing to define the microbiome composition and relationship between host, microbiota, and antimicrobial resistance in the GIT of four demersal marine species as well as three large migratory shark species. We find inter and intraspecies differences in the GIT microbiome based on host species and GIT sampling location, and that higher trophic level organisms with piscivorous diets have an increased abundance of ARGs. Additionally, this abundance of ARGs is positively correlated with the abundance of Proteobacteria. Using a barcoding approach to identify non-host/bacterial DNA signatures in the shotgun sequencing data combined with a functional assessment of the microbiome, we are able to infer dietary habits and bacterial carbohydrate utilization. These habits play a role in

determining the composition of the gut microbiota and, in turn, the levels of Proteobacteria and resulting abundance of detected ARGs.

Results

Sampling and Collection

Sampling of four demersal fish species, butterfish, scup, summer flounder, and smooth dogfish, was performed using an otter trawl in Narragansett Bay, RI, USA (Figure 1). Samples were collected at two sampling locations, Fox Island (upper bay) and Whale Rock (lower bay), during the months of May, June, July, August, and September between 2017 and 2021. During each sampling session, benthic water samples were also obtained using a niskin flask. The spiral valve contents of three large offshore sharks, thresher, mako, and porbeagle sharks, were obtained from specimens caught for recreational shark tournaments in the offshore waters from Rhode Island to Maine during July 2018 and July 2019.

Microbial Diversity of the GIT of Wild Marine Fish

Utilizing a whole genome shotgun sequencing approach followed by read filtering and taxonomic assignment via the Kraken2/Braken pipelines we were able to define the microbiome composition of seven fish/shark species and their seawater environment. We find that the gut microbiota of all species is predominantly composed of Proteobacteria (50.4%), Firmicutes (20.8%), and Bacteroidetes (10.0%) (Figure 2A). This finding matches those of previous studies that have identified Proteobacteria and Firmicutes as being the major constituents of the gut microbiota of marine fish[4, 5, 17, 23, 46-49]. Within the phylum

Proteobacteria, *Photobacterium* (5.6%), *Vibrio* (4.5%), *Alivibrio* (3.5%), and *Edwardsiella* (3.2%) were the most abundant genera found in the fish samples collected in the bay (Figure 2B). Here we observe significantly increased levels of Proteobacteria in benthivorous/piscivorous (scup, summer flounder, smooth dogfish) species compared to a plankivorous species (butterfish) (Mann-Whitney U test, $p < 0.0001$). Previous studies have also identified an increased abundance in Proteobacteria in omnivorous and carnivorous organisms compared to herbivores[5, 19, 49, 50] suggesting trophic level and dietary guild play a role in the level of Proteobacteria present in the gut microbiota. Due to a high degree of variability within sample types, fish species did not group together significantly within a principal coordinate analysis of Bray-Curtis Dissimilarity (Figure 2 C). However, the microbiomes of species clustered more separately when samples were separated by site and sampling time, suggesting that there may be significant temporal and spatial variability within the microbiome of fish (Figure S1 B, D). Between the samples collected at the two Narragansett Bay sampling locations there were notable differences in the microbiome composition of the summer flounder at the genus level; *Photobacterium* was significantly increased in the Fox Island population (padj < 0.05) and six less prominent genera were significantly increased in the Whale Rock population (padj < 0.05) (Figure S1 A, B Table S2). Surprisingly, no significant differences in taxonomy were found between the two sites in the butterfish and scup populations. This suggests that the microbiota of summer flounder may have characteristics unique to either the upper or lower bay locations, while the butterfish and scup populations are more homogeneous.

Shark Spiral Valves Harbor Species-Specific Microbiota

The gut microbiota of sharks has only been characterized in a few reports[20, 21, 47, 51, 52], and represents an understudied area of shark physiology, which likely plays a major factor in host health. Here, we define the microbiota of four shark species, the mako shark, thresher shark, porbeagle, and smooth dogfish. Sharks have unique digestive architecture defined by the spiral valve, an organ that maximizes absorption and minimizes the length of GIT by increasing surface area through a corkscrew-like arrangement of intestinal tissue (Figure 3A). The spiral valve of all sharks was dominated by Proteobacteria (53.9%) and Firmicutes (18.0%), with *Photobacterium* (17.5%), *Campylobacter* (6.0%), and *Dickeya* (5.7%) the most prominent genera (Figure 3B, 2B). Analysis of the Bray-Curtis Dissimilarity metric revealed a significant difference between the microbiota of each species (PERMANOVA, $p = 0.001$), defined by distinct clustering in a principal coordinate analysis (Figure 3C). Previous studies of Elasmobranchii have also found an abundance of *Photobacterium* as well as *Campylobacter* in the spiral intestine of sharks[47, 52], but to date only one study utilizing 16s sequencing has examined the taxonomic differences between regions of the shark GIT[51].

Here, we compare the microbiota of the spiral valve (SV) to the distal intestine (DI) in smooth dogfish. The principal coordinate analysis plot of the Bray-Curtis Dissimilarity metric displays the significantly distinct clustering of the SV and DI microbial communities (PERMANOVA, $p = 0.018$) (Figure 3E). These disparate communities are defined by a significantly greater abundance of Proteobacteria in

the DI (63.3%) compared to the SV (39.1%) (p = 4.55E-05), and a significantly reduced abundance of Actinobacteria in the DI (1.8%) compared to the SV (8.0%) (p = 1.89E-08) (Figure 3D, 3F, 3G). The most differentially abundant species between GIT sites was *Photobacterium damselae*, which was significantly more abundant in the DI compared to the SV (log2FC = 9.84, padj = 4.52E-74) (Figure 3E). Such differences in microbial composition were not found in the GIT of the previously studied bonnethead shark (*Sphyrna tiburo*)[51], suggesting this may not be a universal phenomenon among sharks. Our findings suggest that the SV and DI represent unique ecological niches for commensal microbes, and that perhaps nutrient availability, host immunity, or oxygen levels may act as selective factors for bacterial colonization in these regions of the smooth dogfish GIT.

The GIT Microbiota of Marine Fish act as a Reservoir of ARGs which are Associated with Proteobacteria

Environmental reservoirs of antimicrobial resistance play an important role in the selection, proliferation, and transfer of resistance genes [53]. We used the computation tool DeepARG [54] to identify resistance genes and find that the gut microbiota of marine fish represent one such reservoir of ARGs. Across all fish GIT samples we detected 518 different resistance genes covering 27 antibiotic resistance classes (Table S3). The most abundant resistance gene classes were multidrug (34.3%), macrolide, lincosamide, streptogramin (MLS) (16.1%), tetracycline (16.0%), and beta-lactam (4.6%) (Figure 4C). Recently, Collins et al. found multidrug and beta-lactam resistance genes in the microbiota of deep-sea fish [24], and similarly a study of ocean waters around the globe found tetracycline,

beta-lactam, and multidrug resistance genes to be the most prevalent resistance gene types in seawater [55]. These findings suggest resistance mechanisms may be conserved across bacteria that inhabit the marine environment and fish GIT.

An increase in antibiotic resistance gene abundance was associated with certain fish/shark species, specifically in higher trophic level organisms (Figure 4A, top). In general, those fish that exhibited piscivorous feeding behavior, occupying a higher trophic level, had a greater burden of antibiotic resistance. Rowan-Nash et al. found a significant correlation between Gammaproteobacteria and ARGs in human gut microbiota samples suggesting that the presence of certain bacteria may be driving levels of resistance in host-associated microbial communities[34]. Expanding on this idea, we examined the relationship between ARGs and Proteobacteria in the GITs of fish and found that samples from piscivores with a higher relative abundance of Proteobacteria harbored an increased abundance of ARGs compared to planktivorous/benthivorous species with less Proteobacteria (Figure 4A, bottom, Figure S2). A correlation analysis between ARG abundance and Proteobacteria relative abundance in fish within Narragansett Bay showed a significant positive correlation ($r = 0.7971$, $R^2 = 0.6353$, $p < 0.0001$, Pearson's correlation) (Figure 4B). When we factored in the large offshore shark species, we find that this trend generally holds true with the exception of the thresher shark, which despite having high levels of Proteobacteria had relatively low levels of ARGs (Figure S3). These findings show that fish with high levels of Proteobacteria are likely to have an increased level of detectable ARGs. Furthermore, this may

suggest that higher trophic level organisms with a more carnivorous diet and Proteobacteria rich gut microbiota will have a greater resistance gene burden.

In order to determine the bacterial hosts of these resistance genes, metagenomically assembled genomes (MAGs) were assembled using the MetaWRAP assembly pipeline [56] and subsequently queried for ARGs. From all metagenomic reads across fish and water samples, we assembled 267 MAGs covering 9 bacterial phyla (Figure S4). We found that the MAGs from Firmicutes (n = 8), Fusobacteria (n = 2), and Proteobacteria (n = 121) had the highest prevalence of ARGs, and had significantly more resistance genes than MAGs from Bacteroidetes, Verrucomicrobia, Spirochaetes, Planctomycetes, and Tenericutes (n = 104) (Mann-Whitney U test, $p < 0.05$) (Figure 4D). Notably, the second most ARG-rich MAG was identified as *Photobacterium damselae*, which occurred at a high abundance in all the piscivorous fish and shark gut microbiota supporting the theory that higher trophic level organisms may harbor more ARGs (Figure 2C).

Inferring Diet Through Metabarcoding of GIT Shotgun Metagenomic Data

The levels of Proteobacteria were highly correlated with the relative abundance of ARGs in the fish microbiome. This relationship may be driven in part by the dietary inputs. Dietary analysis provides insight into the trophic structure and predator/prey relationships within a community and is a driving factor in shaping the gut microbiome. Traditionally techniques to study diet in wild animals, such as direct observation or stomach content analysis, have been low throughput and time consuming and are unable to identify phenotypically indistinguishable or rapidly digested prey items [57]. The use of DNA-barcoding methods circumvents

these issues by providing molecular level resolution that reduces the need for human identification of physical dietary components [57]. Additionally, molecular methods provide a high throughput alternative that can detect not only dietary items, but also potential parasites. Here, we utilize DNA-metabarcoding targeting the cytochrome c oxidase subunit I (COI) [58], elongation factor TU (*tufA*), and ribulose-1,5-bisphosphate carboxylase (*rbcL*) genes [59] to identify the diet and potential GIT parasites of seven marine species.

Of the seven species examined in this study, four occupy a shared demersal habitat in Narragansett Bay, RI providing an opportunity to detect interspecies predation and differential dietary preferences within a habitat. The planktivorous butterfish had a diet primarily consisting of diatoms (Bacillariophyta), algae (Chlorphyta, Ochrophya, Haptophyta), and to a lesser extent arthropods (Arthropoda), characteristic of an organism occupying a low trophic level (Figure 5A, B). The benthivorous scup occupies a higher trophic level than the butterfish, characterized by dietary signatures of diatoms (Bacillariophyta), arthropods (Arthropoda), and segmented worms (Annelida) which were known to be a major prey source for this benthic species (Figure 5A, B) [60]. At the order level we find that the Metazoan portion of the scup diet is derived from amphipods (Figure 5E). Previous dietary studies of both the summer flounder and smooth dogfish in New England waters identified these species as high trophic level predators preying on fish, squid, and crabs [61, 62]. It is notable that due to the feeding patterns of these species they were sometimes captured with empty stomachs and intestinal tracts resulting in an absence of detectable DNA markers making diet identification

impossible (Figure 5A). We find that these highly carnivorous species prey primarily on chordates in the class Actinopterygii (ray-finned fishes) as well as arthropods (Figure 5A, B, E). In summer flounder the Metazoan derived diet came from primarily Decapoda (crustaceans) and Clupeiformes (herring and anchovy family) (Figure 5E). The smooth dogfish DI contained Metazoan signatures of Stromatopoda (mantis shrimp) and fish across several orders (Figure 5E). DNA markers corresponding to butterfish and scup were found in the GIT of the high trophic level predators (summer flounder and smooth dogfish) suggesting that predation occurs within this benthic food web and represents a possible route of bacterial and ARG transfer from lower- to higher-trophic level organisms. We also obtained dietary signatures from three large migratory shark species that play an important role in the food web as apex predators. All three sharks exhibited piscivorous diets based on metabarcoding (Figure 5A, B, D, E). A closer look at order level taxonomy revealed that each shark had a fairly specialized diet with DNA from only one or two different prey species (Figure 5E). The COI dietary signatures for the thresher, mako, and porbeagle sharks were primary from Clupeiformes, Scombriformes, and Perciformes, respectively (Figure 5E). Using this metabarcoding approach for dietary contents we confirmed that the summer flounder, smooth dogfish, mako, thresher, and porbeagle sharks all had highly piscivorous diets compared to the butterfish and scup. Furthermore, these each species harbored a significantly distinct diet that was host specific (PERMANOVA, $p = 0.006$) (Figure 5C). These trends in prey preference likely influence the microbial communities inhabiting the GIT as diet is a strong modulator of the

microbiome. From a metabarcoding analysis of wild marine fish GIT samples we were able to infer diet, trophic interactions, and gain insights into the role of host diet in shaping the microbiota through nutrient availability and potential bacterial transfer between diet and host.

Functional Differences in the Microbiota Linked to Host Diet and Trophic Level

The gut microbiota plays an important role in host digestion, increasing nutrient availability and uptake [4, 19, 63]. Host diet in turn plays a key role in determining the makeup of the gut microbiome, and evidence shows that dietary modulation and macronutrient availability can drastically alter the composition and function of the intestinal flora [12, 64, 65]. Through previous stomach content analyses [61, 62], and our own metabarcoding analysis (Figure 5), we are able to gain an understanding into the role of diet in shaping the gut microbiome of these marine fish. Investigation into the carbohydrate-active enzymes (CAZymes) known to play a role in metabolism of dietary polysaccharides revealed 120 differentially abundant CAZymes between the piscivorous and planktivorous/benthivorous species suggesting that the divergent diets of these groups may have an impact on the functional capacity of the microbiome (Figure 6A).

Glycosaminoglycans, including chondroitin, are a group of diverse polysaccharides that are components of a variety of tissues including cartilage derived from mammals, marine fish, squid, and other organisms [66-71]. The diet of piscivorous fish, such as those studied here, include a number of organisms known to contain chondroitin (Arthropoda and Chordata). Thus, the piscivorous fish and sharks occupying a higher trophic level would likely have greater dietary

intake of this polysaccharide compared to the butterfish and scup, whose prey is less rich in chondroitin. We find that several CAZymes linked to chondroitin metabolism are significantly enriched in the piscivores compared to planktivores/benthivores (log2fc > 1.5, padj < 0.05) (Figure 6A, B). Interestingly, these genes were predominantly detected in MAGs isolated from the piscivorous species, summer flounder, smooth dogfish, thresher, mako, and porbeagle sharks (Table S4). This data suggests that host diet, associated with trophic level and dietary guild, may select for bacteria with particular carbohydrate utilization patterns. In this case, piscivorous fish and sharks likely have a more chondroitin rich diet and the abundance of chondroitin could provide an ecological niche for bacteria with chondroitin lyase and hydrolase enzymes. Chitin is one of the most abundant polysaccharides in nature and makes up the exoskeletons of many arthropods [72, 73]. Several chitinases were detected across nearly all the gut microbiota samples collected (Figure 6C), suggesting that the ability to utilize chitin may be a widespread trait among marine associated microbiomes likely due to the fact that chitin is ubiquitous in this environment. Overall, our evaluation of carbohydrate active enzymes within the fish gut microbiota suggests that the availability of dietary polysaccharides associated with different trophic levels may have a role in selecting for certain bacteria based on polysaccharide utilization. This finding has the potential to link host trophic level and related prey consumption with selection for specific microbes.

Discussion

Studying the microbiota of wild marine fish is important for monitoring the health of populations, understanding fundamental fish biology, and evaluating their role as an environmental reservoir of antimicrobial resistance. Here, we utilize shotgun metagenomic sequencing to define the microbial taxonomic composition, ARG burden, and dietary DNA signatures from GIT samples of seven marine fish/sharks. Across all GIT microbiota samples we find a predominance of Proteobacteria and Firmicutes, which is consistent with previous reports of marine fish gut microbiota [4, 5]. Each species harbored unique taxonomic profiles, which remained consistent between two sampling locations within the bay. The exception was that of the summer flounder, which had seven differentially abundant genera between the two sites including a significant increase in *Photobacterium* in the Fox Island samples (Figure 2A, S1). This is particularly interesting given that previous research of summer flounder within Narragansett Bay has found sex-based differences between the two sampling locations. Data suggests that the inshore habitat (Fox Island) has a higher proportion of females during the months our collections took place (May – September), whereas lower in the bay the Whale Rock location has a lower proportion of females[74]. Combined with other work showing that female summer flounder exhibit a faster growth rate[75], and that sex has an effect on microbiome composition in fish[76], future studies could examine whether the changes in summer flounder microbiota observed between the two sites are sex dependent. While the butterfish and smooth dogfish appeared to have consistent microbiome profiles across individuals, the scup and summer flounder

samples appeared to have greater intra-species microbiome variability highlighted by samples with high abundances of Proteobacteria (Figure S2). It is unclear the driving factors behind this variability, nor is it unique to our data set [76], but it should be taken into consideration when evaluating the data shown here and in future studies of fish microbiota.

The GIT microbiota of sharks has been studied in only a few species to date [20-22, 47, 51, 52], despite their important role as apex predators within the marine trophic structure. We define the microbiota of four shark species including three highly migratory pelagic species. These organisms shared core bacterial taxa at the class level (Figure 3B), while still having species- specific microbiome profiles (Figure 3C). The universal presence of *Photobacterium* across all shark samples presented here, as well as previously published shark GIT microbiomes[47, 52] suggests that this genus is an essential part of the microbiota in these animals. Interestingly, *Campylobacter* seems to be a significant member of the GIT microbiota of sharks in the Lamnidae family, of which representative species from all three extant members of this family (*Carcharodon* [52], *Isurus* [this study], *Lamna* [this study]) have shown an abundance of *Campylobacter* (Figure 2B). This is in contrast to sharks across almost all other Elasmobranchii families (Triakidae [this study], Alopiidae [this study], Carcharhinidae [20, 52], Rhincodontidae [20, 52], Sphyrnidae [21, 51], Ginglymostomatidae [20]) that did not report significant levels of this bacterial genus in their GIT, suggesting that this may be evidence of phylosymbiosis [77], though more work would be needed to substantiate this theory. Additionally, we observe significant differences between the spiral valve

and distal intestine within smooth dogfish specimens (Figure 3D, E, F, G). This is in contrast to 16s sequencing studies of the bonnethead shark which observed no differences between these two sites along the GIT [51], thus providing novel evidence that there may be spatial differentiation of the microbiota within the elasmobranch GIT. Our finding suggests that sampling method (cloacal swab vs direct sampling of spiral valve contents) and location along the GIT have significant impact on the detected microbiome profile.

The position of Proteobacteria as a commensal in the microbiome of marine organisms is well established. Our data is consistent with this finding, and we also show that the genus *Photobacterium* is associated with piscivorous fish/sharks. While *Photobacterium damselae* is known to cause pathogenesis in both fish and humans [78, 79], ours and several other studies have recently found it in the GIT microbiome of marine fish suggesting it is likely a member of the natural gut flora. The *Photobacterium* MAGs assembled from both summer flounder and smooth dogfish contained genetic regions assigned to four different CAZymes related to chitinase activity (CMB50, GH18, GH19, and GH23) (Table S4) [80]. Due to the abundance of chitinous prey sources (Arthropoda) identified in the gut of many of these samples, perhaps as a commensal *Photobacterium* play a role in the utilization of dietary derived chitin. Future studies should focus on strain level analyses of *Photobacterium* to characterize potential genomic and phenotypic differences between pathogenic and commensal strains.

Environmental microbiomes act as reservoirs of bacteria harboring AMR. Here, we define the resistome of seven wild marine fish/shark species and find

that multidrug, MLS, tetracycline, and beta-lactam resistance genes are prevalent among these bacteria (Figure 4C). Interestingly, we identify a positive association between the abundance of Proteobacteria and level of ARGs within fish GIT microbiomes (Figure 4B). Proteobacteria abundance was found to be higher in piscivorous species compared to planktivores, likely leading to a greater ARG burden in higher trophic level species (Figure S3B). This study represents the first known report linking trophic level to ARG abundance. These findings are critical to understanding the dynamics of resistance in the context of marine food webs as well as the prevalence of resistant bacteria (especially pathogens) in highly migratory species such as the mako, thresher, and porbeagle sharks which have the ability to disperse such bacteria across great distances [81]. A recent study by Collins et al. identified a sparsity of resistance genes in the GIT of deep-sea fish that presumably experience a low level of anthropogenic impacts compared to the coastal species presented here [23]. The proximity to humans could be one factor leading to the much greater number of ARGs recovered from the samples presented here compared to those collected in the deep-sea. Previous work has shown that marine sediments with greater proximity to human activity have significantly higher abundances of ARGs compared to those in the less anthropogenically impacted waters [43, 82, 83].

The composition of the microbiome is greatly affected by host diet, though interactions between diet and microbiota composition in wild fish populations remain less well understood. Here we utilize a metabarcoding approach to identify non-host/bacterial DNA in the GIT of marine fish and detect dietary signatures and

potential host parasites. Using several marker genes, we were able to discern prey items from GIT contents, representing a potentially less invasive alternative to traditional stomach content analyses. Interestingly, while squid has been reported to be a significant portion of the diet of summer flounder and smooth dogfish [61, 62], we did not observe any dietary signatures indicative of the longfin squid native to Narragansett Bay. This could be due to the rapid degradation of this type of prey item in the stomach, and if so, would be an important caveat to using this approach for diet detection. The fact that dietary signatures could not be discerned for all samples may be a factor of gut transit time and that only a single feeding period is detected at one time using these metabarcoding techniques. While this is a possible limitation, this short time frame of detection is also a strength as it provides a snapshot of recent dietary activity. Additionally, we were able to identify a known parasitic Platyhelminthes worm of the genus *Clistobothrium* in the spiral valve contents of one of the mako shark specimens [84]. Metagenomic assembly allowed us to assembled a full COI sequence for this parasite displaying the power of shotgun metagenomic sequencing in identification of GIT parasites in wild animals (data not shown). Overall, the use of molecular barcoding techniques from shotgun metagenomic data provided insights into host dietary habits and trophic interactions between species in a complex marine ecosystem. This information is valuable to understanding the nutrient availability driving microbial selection within the GIT and ultimately shaping the gut microbiome.

While this study effectively uses molecular techniques to define the microbiome, resistome, and dietary signatures, there are several limitations. As

with any study assigning taxonomy or gene identifications to sequencing data, the results are limited by the completeness of existing databases. This is evidenced here by the fact that a high proportion of the host-filtered reads (up to 90%) remained unclassified after taxonomic assignment. Further characterization of microbes from understudied environments is needed to improve databases in order to better characterize these unique microbial communities. As well as taxonomic assignment, our ability to identify antibiotic resistance genes is limited by the available sequence databases. For an ARG to be present in a database, it must be previously characterized. The characterization of resistance disproportionately occurs in pathogens due to the importance of resistance in clinical microbiology samples, and many human pathogens are Proteobacteria. Thus, there is a potential for existing ARG databases to be biased towards Proteobacterial ARGs, a fact that must be examined further to obtain a true picture of resistomes. Additionally, the data presented here is derived from shotgun DNA sequencing, thus it is only able to infer the functional potential of the genes identified. Without RNA sequencing and proteomics, we are unable to make strong conclusions regarding the activity of the microbes that inhabit the GIT. While these microbiome samples represent unique, previously unstudied species, there were limitations in obtaining more samples and thus it is possible some microbiome differences were not observed due to a low number of individuals sampled from each species. Despite these limitations, we are able to provide valuable insights into the microbiota and resistomes of wild marine fish occupying diverse dietary guilds and ecological niches.

Materials and Methods

Sample Collection

All Narragansett Bay fish samples, butterfish (n= 22), scup (n = 31), summer flounder (n = 20), smooth dogfish spiral valve (n = 5), smooth dogfish distal intestine (n = 6), were collected in the months of May, June, July, August, and September during 2017 – 2021 from the fish trawl surveys conducted by the University of Rhode Island Graduate School of Oceanography. Specimens were collected according to the IACUC protocols covering both this study as well as the work of the collection vessel. Fish trawl for samples was approved and permits were obtained from the Rhode Island Department of Environmental Management. The trawl was conducted by the R/V *Cap'n Bert* which utilized an otter trawl net with an effective opening of 6.5 m and towed at 2 knots for 30 minutes. Trawling was performed at two sites in Narragansett Bay, Rhode Island: Fox Island and Whale Rock (Figure 1). After the trawl was emptied on the deck, target fish were humanely euthanized and dissected and the intestinal contents were emptied into Zymo Research bashing bead lysis tubes (Irvine, CA, USA) containing 750uL of ZymoBIOMICS Lysis Solution (Irvine, CA, USA), shaken, and stored on ice until extraction. Water samples were collected ~1 m above the seafloor using a niskin flask. For each individual seawater sample (n = 12), one liter of seawater was filtered through a 0.22 µm membrane from which a 3x3 cm section was added to a Zymo Research bashing bead lysis tubes (Irvine, CA, USA) containing 750uL of ZymoBIOMICS Lysis Solution (Irvine, CA, USA), shaken, and stored on ice until extraction. The three large offshore shark species, thresher (n = 4), mako (n = 4),

and porbeagle shark (n = 5), were collected from specimens caught as part of recreational shark tournaments in Massachusetts and Rhode Island. The samples were caught in the offshore waters from Rhode Island to Maine. All shark samples were collected postmortem from sharks collected by licensed recreational fishermen. The sharks were dissected and contents from the spiral valve were transferred into Zymo Research bashing bead lysis tubes (Irvine, CA, USA) containing 750uL of ZymoBIOMICS Lysis Solution (Irvine, CA, USA), shaken, and stored on ice until they could be frozen and subsequently extracted.

Permits and IACUC

Narragansett Bay trawl samples were collected under the 2020 RIDMF Scientific Collector's Permit #527. Fish were handled according to the Brown University IACUC Protocol Number AN2021-005.

DNA Extraction

DNA was extracted with the ZymoBIOMICS DNA miniprep kit from Zymo Research (Irvine, CA, USA) following the manufacturer's instructions, with final elution in 100 µl of molecular grade H2O. Extracted DNA was quantified using a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States).

Library Preparation and Sequencing

Metagenomic libraries for samples BK001 – BK072 were prepared using the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina (NEB) (Ipswich, MA, USA) and libraries were sequenced on the NovaSeq 6000 with v1.5 reagents. Metagenomic libraries for samples BK073 – BK114 were prepared using the

iGenomeX Riptide High Throughput Rapid DNA Library Prep (Twist Bioscience, San Francisco, CA, United States).

Metagenomic Analysis

Read Processing and Filtering

Raw reads from metagenomic sequencing were processed using the KneadData wrapper script[85]. Reads were then trimmed using Trimmomatic (version 0.36) with SLIDINGWINDOW set at 4:20, MINLEN set at 50, and ILLUMINACLIP: TruSeq3-PE.fa:2:20:10 [86]. Sequences from contaminating host were filtered out using Bowtie2 [87]. Since fully sequenced genomes of the host species used in this study have not yet been sequenced, the next most phylogenetically similar fish with sequenced genomes were used as a reference during read filtering; *Paralichthys olivaceus* (PRJNA344006), *Spondyliosoma cantharus* (PRJEB12469), *Pampus argenteus* (PRJNA240272), *Scyliorhinus canicular* (PRJEB35945), and *Carcharodon carcharias* (PRJNA725502). In addition to this preprocessing, bacterial ribosomal reads were removed from the datasets using the SILVA 128 database[88].

Taxonomic Identification

Taxonomic classification of metagenomic reads was performed using Kraken2 (version 2.1.2) [89]. The taxonomic output was analyzed in R (version 4.1.2) using the phyloseq package (version 1.38.0) to calculate alpha and beta diversity[90]. The PCoA analysis was performed using the Bray-Curtis dissimilarity metric[91].

Identification of Antimicrobial Resistance Genes

Processed reads were joined using the fastq-join function of the ea-utils package[92] and queried for antibiotic resistance genes using DeepARG (version 2)[54] using the default settings (0.8 minimum coverage of alignment, E-value cutoff 1e-10, 50% minimum percentage of identity). Assembled genomes were queried for resistance genes using DeepARG (version 2) using the --genes flag and the default settings (0.8 minimum coverage of alignment, E-value cutoff 1e-10, 50% minimum percentage of identity).

Identification of Functional Genes

Additionally, using the SAMSA2 pipeline[93] clean reads were merged using Paired-End Read Merger (PEAR) (version 0.9.10)[94] and aligned to the RefSeq, CAZy, and SEED subsystems databases using DIAMOND (version 0.9.12)[80, 93, 95-97].

Metabarcoding for Diet Detection

The origins of non -host/bacterial DNA content in the gut was determined by using blastn to align cleaned, merged reads to a custom database containing the cytochrome C oxidase subunit I gene (COI) sequences in the database generated by the CO-ARBitrator algorithm developed by Heller et al. [58] and all unique sequences from NCBI Gene search of the genes *tufA*, encoding for elongation factor TU, and *rbcL*, encoding for ribulose-1,5-bisphosphate carboxylase. Non host/bacterial DNA content alignments were filtered based on alignment length of $\geq$ 100bp and percent identity $\geq$ 97% with any singletons removed.

Metagenomic Assembly, Binning, and Taxonomic Identification

Metagenomic assembly was conducted using the metaWRAP pipeline[56] with the --megahit flag. Binning was conducted using the metaWRAP binning module employing metabat2, maxbin2, and CONCOCT binning softwares. Final bins with completion $\geq$ 50% completeness and < 5% contamination were used for downstream analysis. The Bin Annotation Tool (BAT) (version 5.2.3) was used for taxonomic classification of metagenome-assembled genomes [98].

Generation of Phylogenetic Tree

The phylogenetic tree of MAGs was generated using PhyloPhlAn 3.0 using the "--diversity high" flag[99].

Statistical Analyses and Figure Generation

Differential abundance of sequence annotations was determined using DESeq2 (version 1.34.0) [93]. Beta diversity was analyzed with a PERMANOVA via the ADONIS function within the vegan R package (version 2.5-7). All figures were generated with GraphPad Prism (version 8.0) (GraphPad Software, La Jolla, CA, United States).

Figures

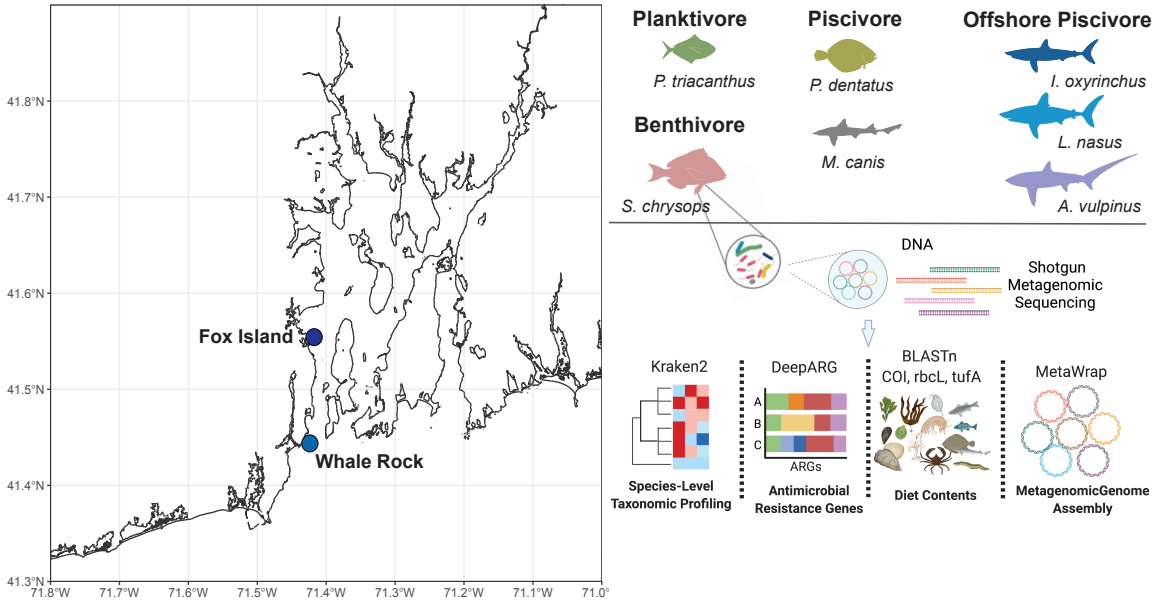

Figure 1. Sample Collection and Experimental Overview
The collection locations within the Narragansett Bay – Fox Island and Whale Rock. Trophic guild characterization of 7 fish and shark species examined in this study. Workflow of highlighting metagenomic analyses performed on DNA sequencing data.

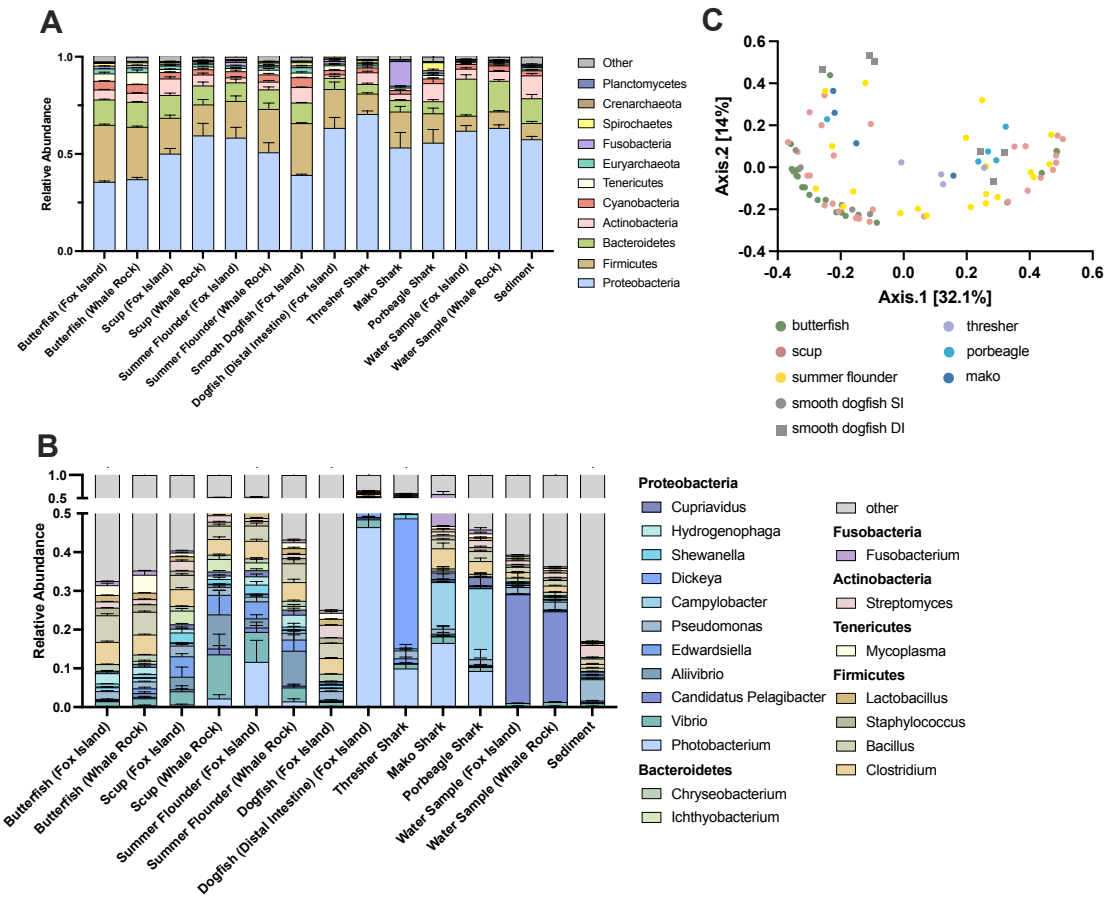

Figure 2. Taxonomy and Diversity of Fish and Environmental Samples
Relative abundance of bacterial phyla (A) and genera (B) averaged across samples within groups and error bars representing standard error of the mean. Principal coordinate analysis of Bray-Curtis Dissimilarity of all fish microbiota samples (excluding water samples) (C).

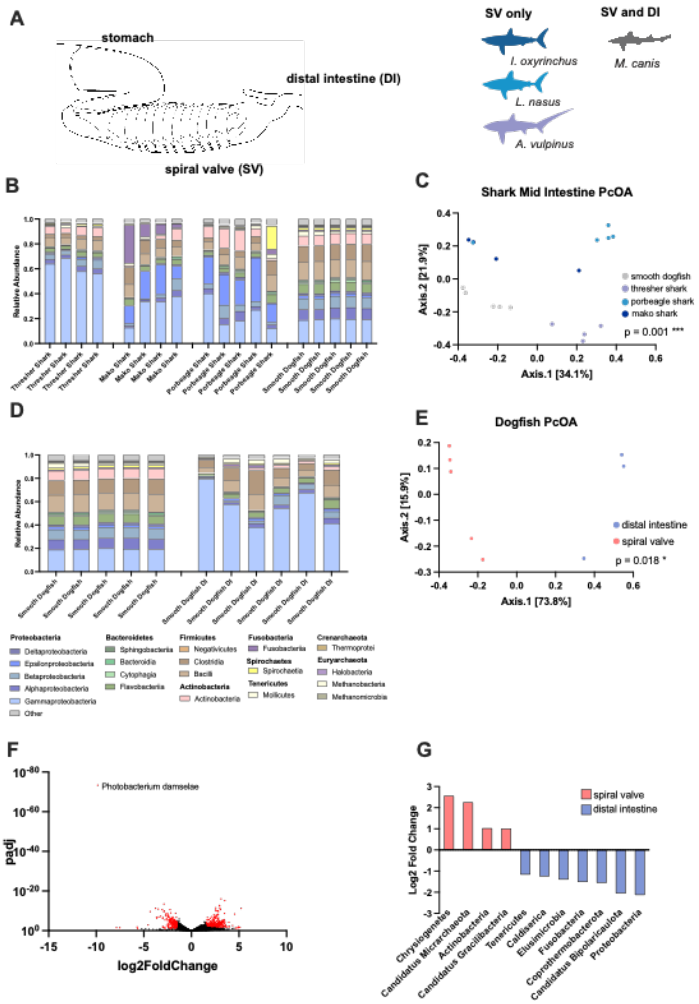

Figure 3. Taxonomy of Four Shark Species and Divergent Microbiota of Spiral Valve and Distal Intestine

Diagram of the elasmobranch GIT with the stomach, spiral valve, and distal intestine labeled (adapted from De luliis and Pulerà 2019), and list of the four shark species included in this study (A). Relative abundance of bacterial classes across individual spiral valve contents from four shark species (B). Principal coordinate analysis of Bray-Curtis Dissimilarity of spiral valve microbiota cluster by species and are significantly different from one another (PERMANOVA, p = 0.001) (C). Relative abundance of bacterial classes across individual spiral valve and distal intestine contents isolated from smooth dogfish (D). Principal coordinate analysis of Bray-Curtis Dissimilarity of smooth dogfish spiral valve and distal intestine microbiota cluster by GIT location and are significantly different from one another (PERMANOVA, p = 0.018) (E). Volcano plot of differentially abundant species between the smooth dogfish spiral valve and distal intestine. Points in red represent significantly different species with an adjusted p-value of < 0.05 and log2 fold change of >1.5 (F). Significantly differentially abundant phlya with an adjusted p-value of < 0.05 and log2 fold change of >1.5. Phyla more abundant in the spiral valve are shown in red and those in more abundant in the distal intestine are blue (G).

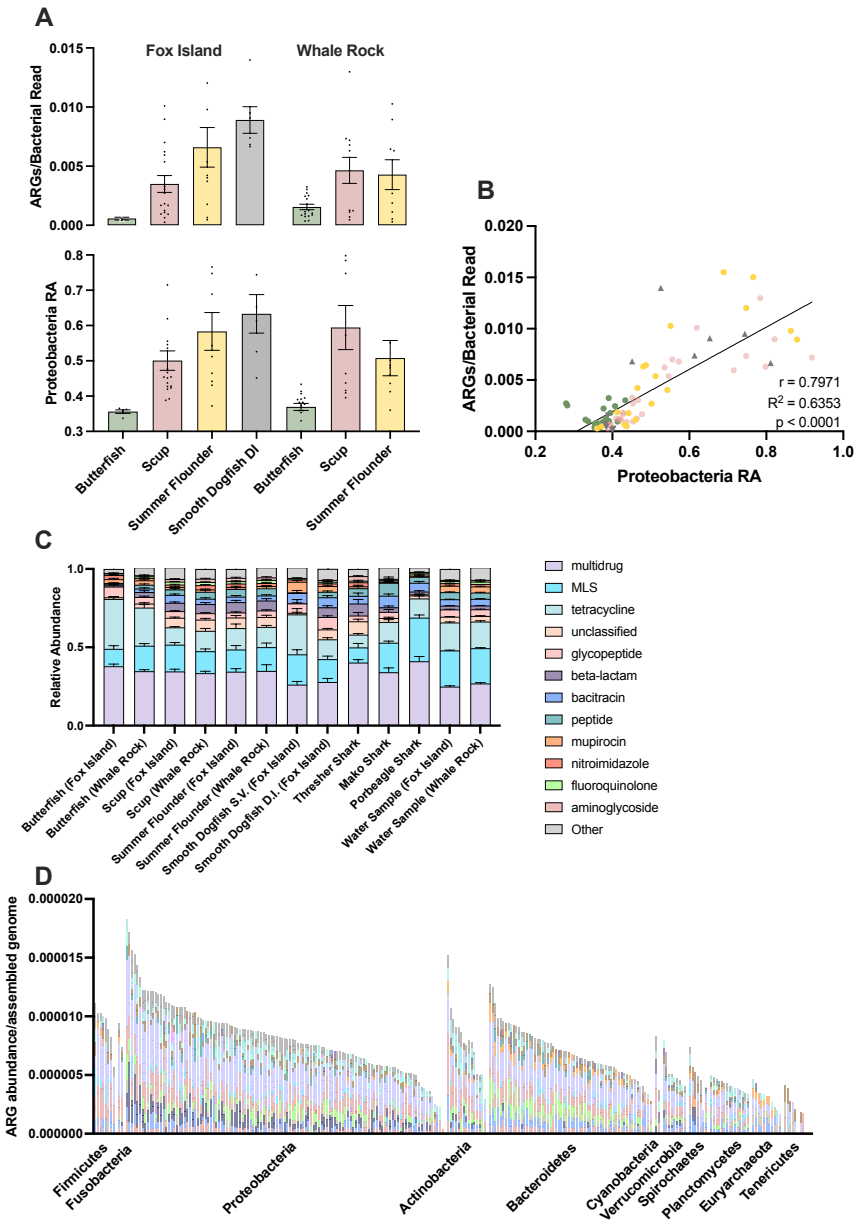

Figure 4. Antimicrobial Resistance and Association Between ARGs and Proteobacteria

(Bottom) Relative abundance of Proteobacteria in each species at the Fox Island (left) and Whale Rock location (right) with bars representing mean ± standard error of the mean (A). (Top) ARGs normalized to bacterial reads in each species at the Fox Island (left) and Whale Rock location (right) with bars representing mean ± standard error of the mean (A). The bars in A (top) correspond to those in A (bottom). Correlation between ARGs (y-axis) and Proteobacteria relative abundance (x-axis) ($r = 0.7971$, $R^2 = 0.6353$, $p < 0.0001$) (B). Relative abundance of ARG classes averaged across samples for each species at each location with error bars representing standard error of the mean (C). Abundance of ARGs in each MAG with 50% ≥ completeness and ≤ 5% contamination (D). The colors of the stacked bars in this plot correspond to those in the legend above.

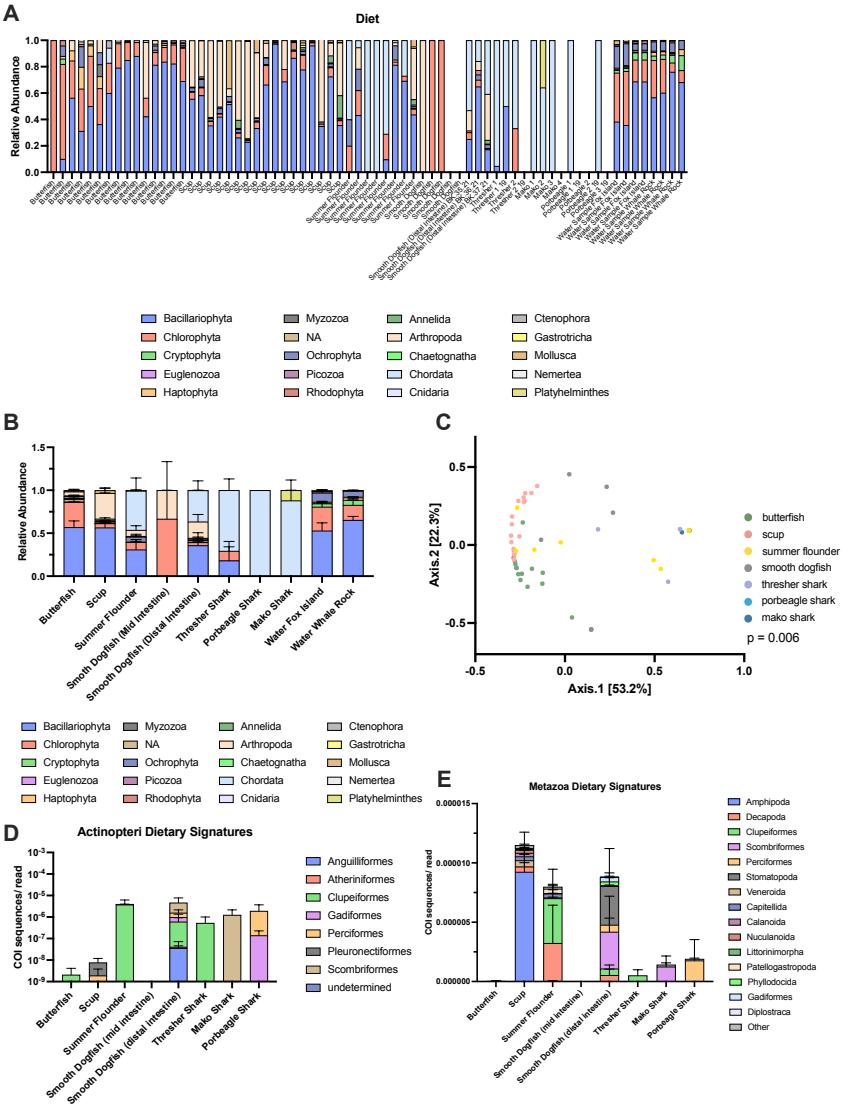

Figure 5. Dietary Signature Identification Through GIT Metabarcoding
Relative abundance of dietary components at the phylum level determined through metabarcoding of shotgun metagenomics using the *tufA*, *rbcL*, and *COI* genes (A). Relative abundance of dietary components at the phylum level averaged across samples with error bars representing standard error of the mean (B). Principal coordinate analysis presenting the Bray-Curtis Dissimilarity analysis of the dietary components of seven fish species. Each species' diet profile grouped separately (PERMANOVA, p = 0.006) (C). Normalized abundance of Actinopteri dietary signatures determined by using the *COI* reads from shotgun metagenomic sequencing of the fish GIT contents (D). Bars represent the average across samples within species and error bars represent standard error of the mean (D). Normalized abundance of Metazoa dietary signatures determined by using the *COI* reads from shotgun metagenomic sequencing of the fish GIT contents (E). Bars represent the average across samples within species and error bars represent standard error of the mean (E). (All data here are from the 69 samples collected in 2021 and do not include previous collections)

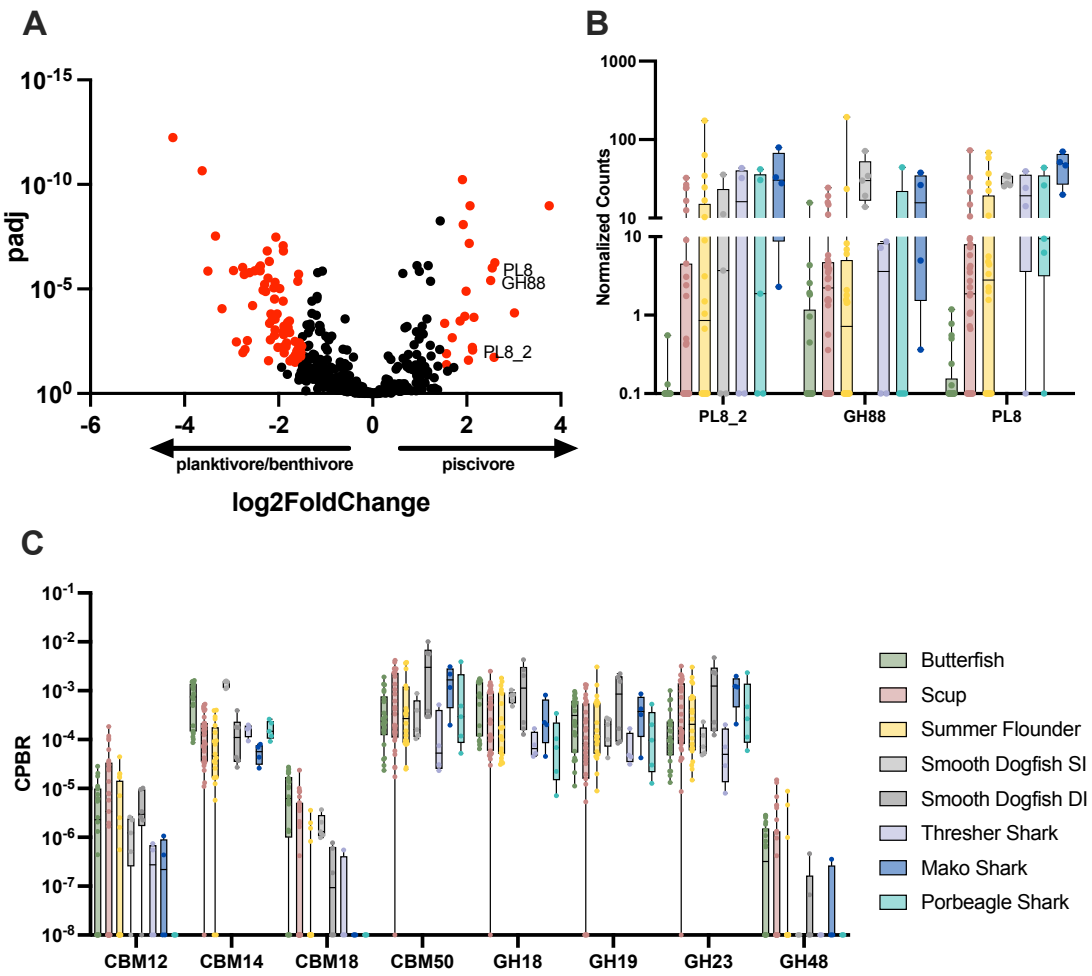

Figure 6. Functional Differences in the Microbiota Linked to Host Diet and Trophic Level

Volcano plot of differentially abundant CAZymes between planktivores/benthivores (butterfish and scup) and piscivores (summer flounder, smooth dogfish, thresher shark, porbeagle shark, mako shark) with three chondroitin metabolism genes highlighted (A). Points in red represent significantly different species with an adjusted p-value of < 0.05 and log2 fold change of >1.5. Normalized counts of three chondroitin metabolism genes in each sample type (B). Normalized counts of eight CAZymes related to chitin metabolism across all samples (CPBR represents Copies per Bacterial Read) (C).

Supplementary Information

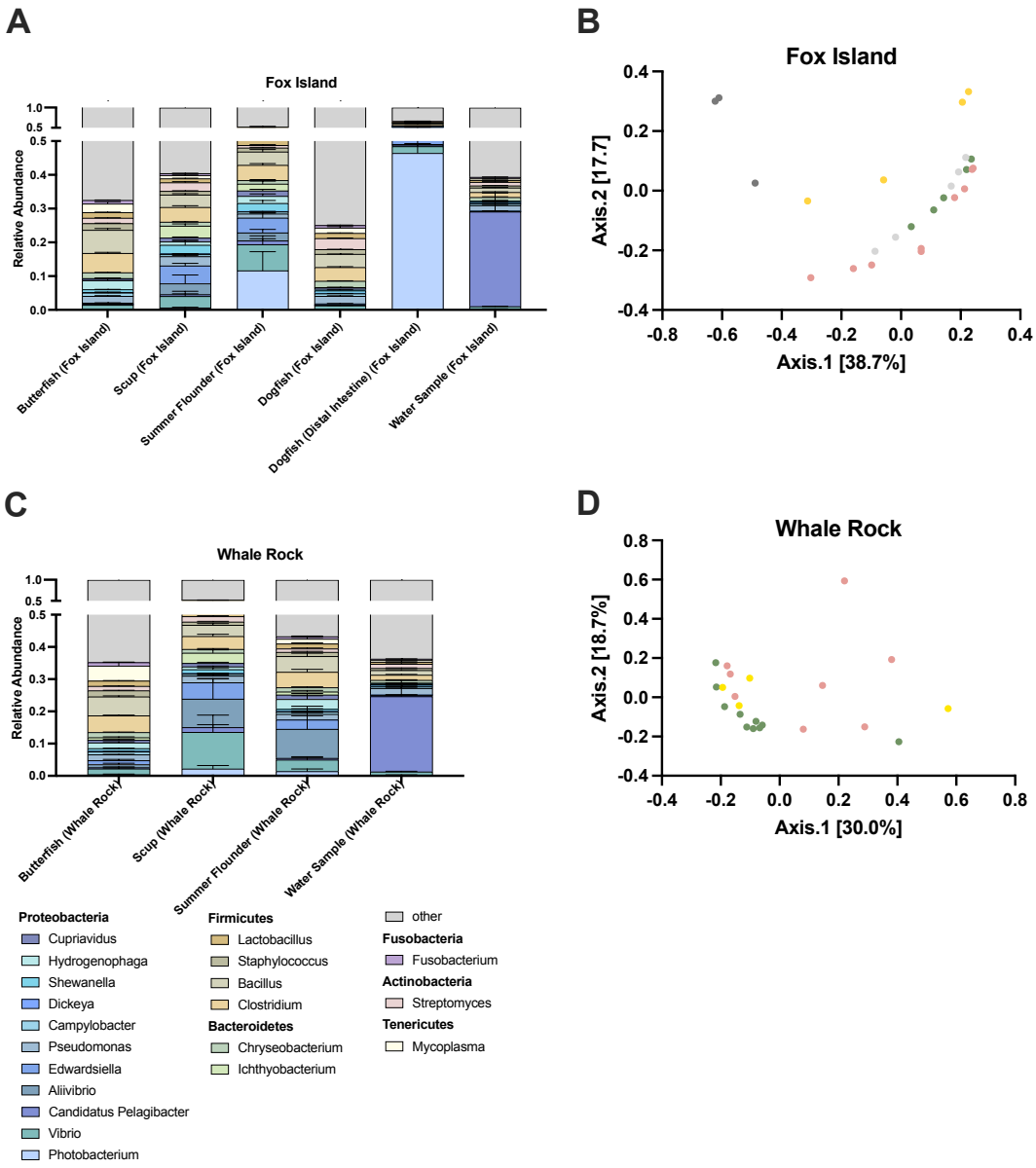

Figure S1. Intra Species Microbiota Differences Between the Inner and Upper Bay Locations

Genus level relative abundance (A) and Principal coordinate analysis of Bray-Curtis Dissimilarity of Fox Island fish samples collected in 2021 (B). Genus level relative abundance (C) and Principal coordinate analysis of Bray-Curtis Dissimilarity of Whale Rock fish samples collected in 2021 (D).

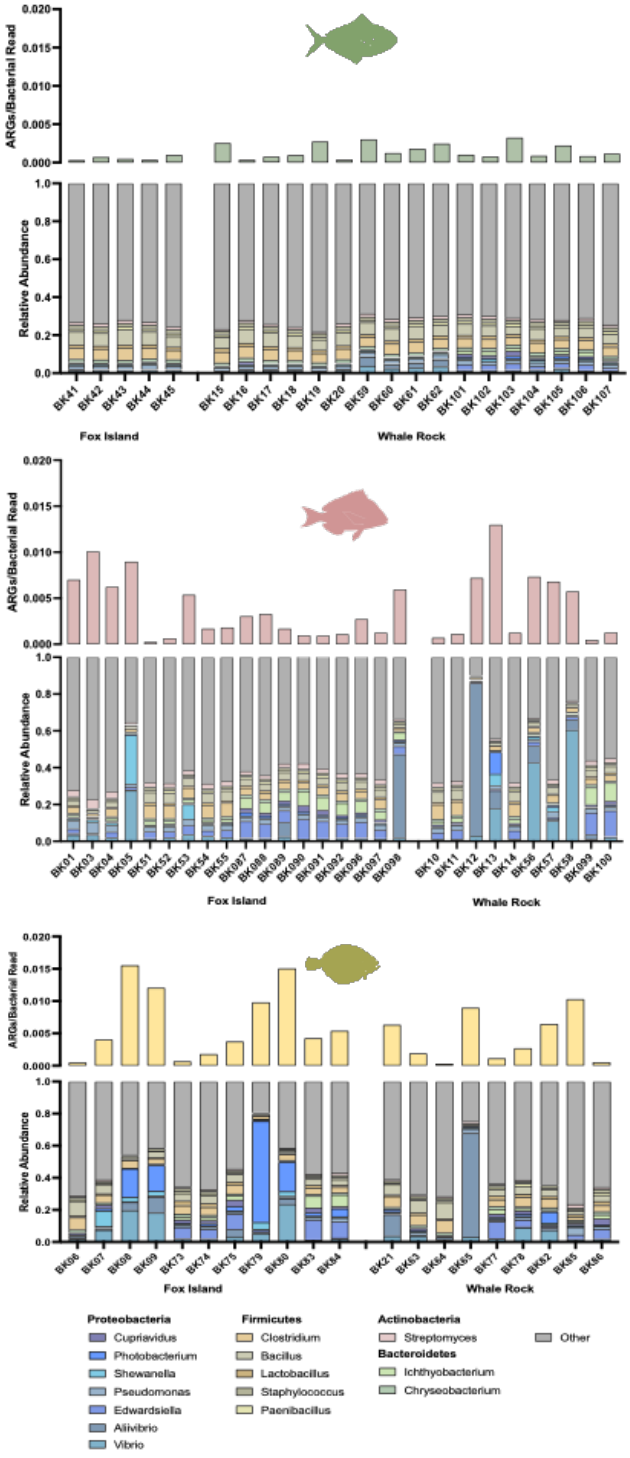

Figure S2. Heterogeneity in Proteobacteria Blooms Associated with ARG Abundance

Abundance of ARGs normalized to bacterial reads plotted for each sample separated by species. Below the normalized ARG abundance for each species is the genus taxonomy plot for the corresponding sample.

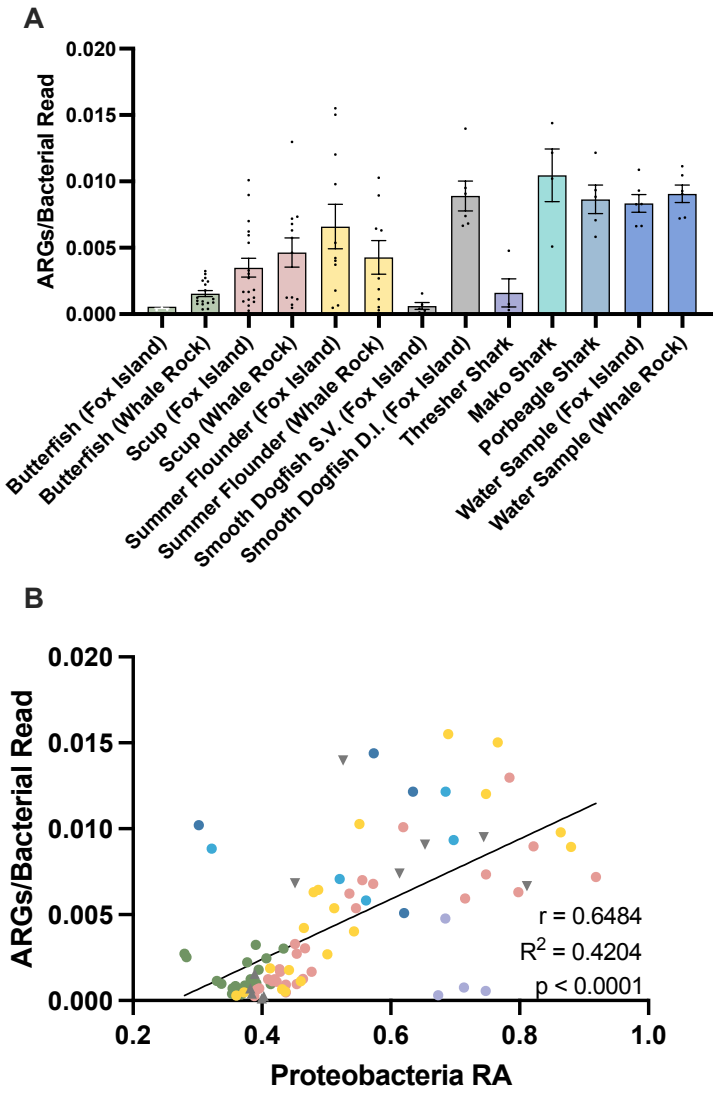

Figure S3. ARG Abundance and Association between ARGs and Proteobacteria (All Samples)

ARGs normalized to bacterial reads in each species and water samples with bars representing mean ± standard error of the mean (A). Correlation between ARGs (y-axis) and Proteobacteria relative abundance of all fish/shark samples (x-axis) ($r = 0.6484$, $R^2 = 0.4204$, $p < 0.0001$) (B).

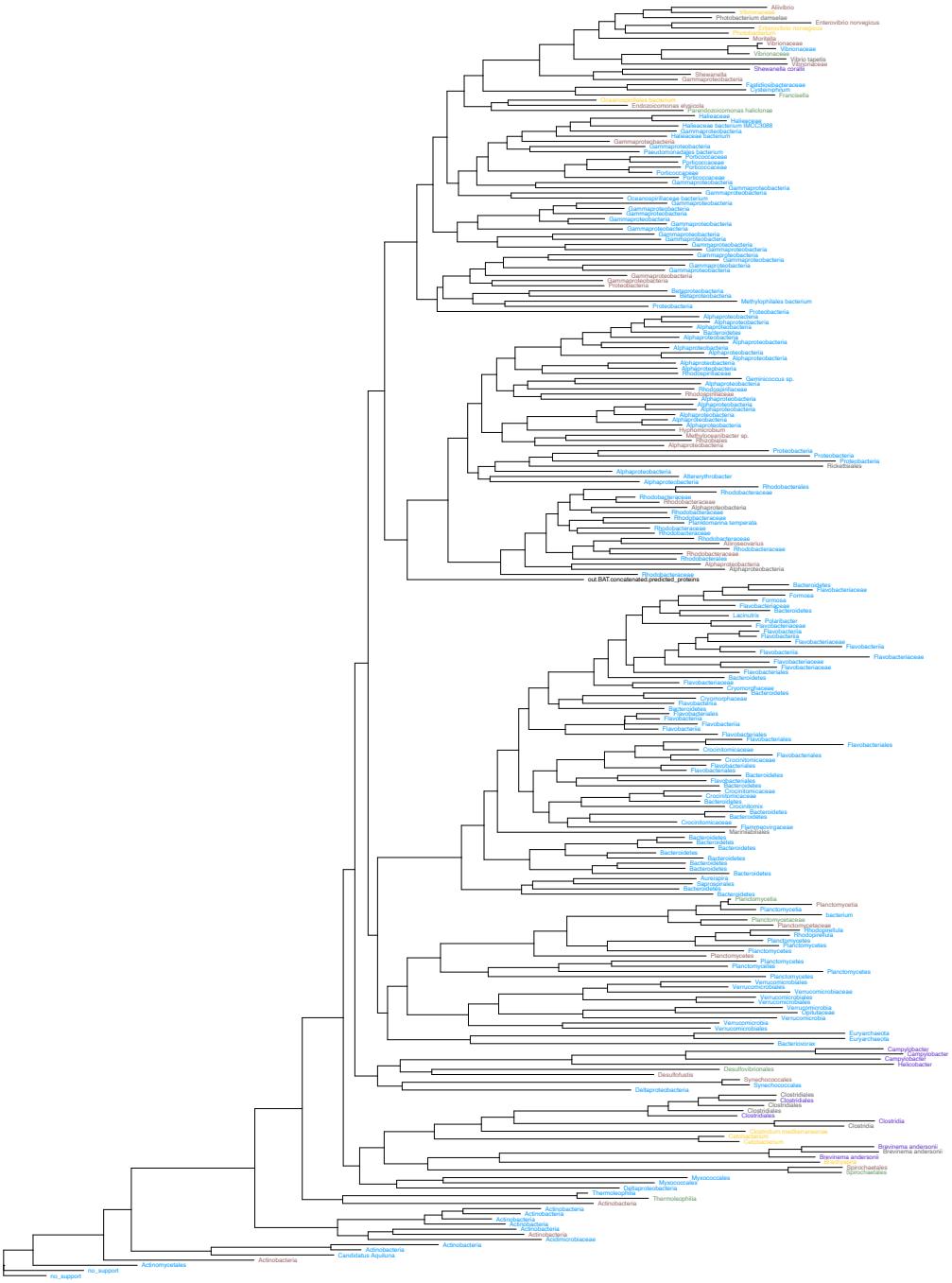

Figure S4. Phylogenic Tree of Assembled Bins
Phylogenetic tree generated using PhyloPhlAn 3.0 with nodes displaying the taxonomy. The color of the node labels corresponds to the sample from which they originated – blue: water, green: butterfish, red: scup, yellow: summer flounder, grey: smooth dogfish, purple: shark.

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CHAPTER 4: Metatranscriptomics Reveals Antibiotic-Induced Resistance Gene Expression in the Murine Gut Microbiota

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Metatranscriptomics Reveals Antibiotic-Induced Resistance Gene Expression in the Murine Gut Microbiota

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Abstract
Antibiotic resistance is a current and expanding threat to the practice of modern medicine. Antibiotic therapy has been shown to perturb the composition of the host microbiome with significant health consequences. In addition, the gut microbiome is known to be a reservoir of antibiotic resistance genes. Work has demonstrated that antibiotics can alter the collection of antibiotic resistance genes within the microbiome through selection and horizontal gene transfer. While antibiotics also have the potential to impact the expression of resistance genes, metagenomic-based pipelines currently lack the ability to detect these shifts. Here, we utilized a dual sequencing approach combining shotgun metagenomics and metatranscriptomics to profile how three antibiotics, amoxicillin, doxycycline, and ciprofloxacin, impact the murine gut resistome at the DNA and RNA level. We found that each antibiotic induced broad, but untargeted impacts on the gene content of the resistome. In contrast, changes in ARG transcript abundance were more targeted to the antibiotic treatment. Doxycycline and amoxicillin induced the expression of tetracycline and beta-lactamase resistance genes, respectively. Furthermore, the increased beta-lactamase resistance gene transcripts could contribute to an observed bloom of *Bacteroides thetaiotaomicron* during amoxicillin treatment. Based on these findings, we propose that the utilization of a dual sequencing methodology provides a unique capacity to fully understand the response of the resistome to antibiotic perturbation. In particular, the analysis of transcripts reveals that the expression and utilization of resistance genes is far narrower than their abundance at the genomic level would suggest.

Introduction

Antibiotic resistance has emerged as a major threat to human health. In the United States, millions of people suffer from infections caused by antibiotic resistant bacteria, and tens of thousands die as a result[1]. Although antibiotic resistance is recognized to be an ancient phenomenon predating the therapeutic use of antibiotics[2-4], recent misuse and overuse of antibiotics has led to an increase in the selection for antibiotic resistance genes (ARGs) and has contributed to the spread of infections caused by antibiotic resistant bacteria. Thus, it is important to understand how antibiotic exposure impacts the abundance and expression of resistance genes in the host. Culture-independent methods of profiling entire microbial communities for ARGs have greatly expanded our ability to detect and track resistance elements utilizing high-throughput sequencing techniques[5-9]. This important development in detection has led to the discovery of ARGs in gut colonizing microbes.

The gut microbiota is now recognized as an important element in human health and disease[10-13], and can serve as a reservoir of antibiotic resistant bacteria[14]. Research into the collection of ARGs within the microbiome, termed the “resistome”[15, 16], has begun to explore resistance genes within the gut. Studies have characterized the gut resistome in terms of composition[6, 14, 17], life history[7, 18, 19], geographic location[17, 20, 21], antibiotic perturbation[22, 23], and other factors. In addition, horizontal gene transfer of ARGs between bacteria in the gut has been theorized to contribute to the spread of resistance[24-29]. Since the gut microbiome is a reservoir of antibiotic resistance and has the

potential to promote HGT of ARGs, it is of particular importance to understand the role of antibiotics in shaping the landscape of antibiotic resistance in this microbial environment.

Antibiotic therapy has been shown to have a dramatic impact on the microbiome, playing a role in gut dysbiosis[30-32], increasing susceptibility to infection[33, 34], and altering the composition of the gut resistome[23, 35, 36]. Less is known about how antibiotics promote ARG selection *in vivo*[22, 37], and the impact of antibiotics on the expression of resistance genes in the host. In response to antibiotic treatment, changes in the resistome may be stochastic, induced by the underlying changes in population structure, or more directed and targeted towards the drug administered. Here we utilize a dual sequencing methodology that employs both metagenomics and metatranscriptomics to reveal broad, untargeted changes in the resistome at the DNA level and a narrower, drug-specific response at the RNA level.

Materials and Methods

Mouse Experiments

In a previous study, we obtained total cecal DNA and RNA from six-week-old female C57BL/6J mice that were treated with amoxicillin (0.1667 mg/mL) for 12 hours, or ciprofloxacin (0.0833 mg/mL), or doxycycline hydrochloride (0.067 mg/mL) for 24 hours[38]. All antibiotic treatments were administered in drinking water, which was provided *ad libitum*. Based on the estimate that a healthy mouse drinks 150 mL/kg of water per day, these concentrations were selected to administer a dosage of 25 (amoxicillin), 12.5 (ciprofloxacin), or 10 (doxycycline)

mg/kg/day[39]. Control mice were provided with pH-matched water. Each group had four mice that were split into at least two cages per group to account for cage effects. After the 12- or 24-hour treatments, mice were sacrificed and cecal contents were collected and stored in DNA/RNA Shield Collection and Lysis Tube from Zymo Research (Irvine, CA, USA). Samples were kept on ice prior to being transferred to -80°C for permanent storage. Mouse experiments were carried out at the Brown University mouse facility with approval from the Institutional Animal Care and Use Committee of Brown University.

Nucleic Acid Extraction and Quantification

DNA and RNA were extracted from cecal samples using the ZymoBIOMICS DNA/RNA Miniprep Kit from Zymo Research (Irvine, CA, USA), eluted in nuclease-free water, and stored at -80°C. Extracted DNA and RNA were quantified using the dsDNA-BR and RNA-HS kits on a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, U.S.).

Metagenomic and Metatranscriptomic Library Preparation

Metagenomic libraries were prepared using the Ovation® Ultralow System V2 kit from NuGEN (San Carlos, CA, USA). DNA was sheared to a median fragment size of 300 bp using a Covaris S220 High Performance Ultrasonicator (Woburn, MA, USA), and used to prepare metagenomic libraries following the manufacturer's protocol. Metatranscriptomic libraries were prepared using Ovation® Complete Prokaryotic RNA-seq Library System from NuGEN. Extracted RNA was first treated with DNA rDNase I to remove contaminating DNA. Next, host mRNA and bacterial ribosomal RNA was reduced using the MICROBEnrich

and MICROBExpress kits from Invitrogen (Carlsbad, CA, USA). This processed RNA was then used to generate metatranscriptomic libraries following the manufacturer's protocol with the addition of AnyDeplete probes designed to specifically remove fragments originating from murine osteosarcoma virus, a known source of contamination sequences.

Sequencing

Metagenomic and metatranscriptomic libraries were sequenced on an Illumina HiSeqX using paired-end, 150 bp reads. Sequencing yielded an average of 26,113,145 (±11,436,616) and 85,599,941 (± 11,674,614) raw reads per metagenomic and metatranscriptomic libraries, respectively. All reads were deposited in the NCBI Short Read Archive under BioProject numbers PRJNA504846 (metagenomics) and PRJNA515074 (metatranscriptomics). This data set was previously published by Cabral et al., 2019[38]. The DNA, RNA, and subsequent libraries and sequencing data were the same as those initially published in Cabral et al. Here, we reanalyze this data using a different set of pipelines and with a focus on the detection of antibiotic resistance genes.

Processing of Raw Reads

Raw reads from both metagenomic and metatranscriptomic sequencing were processed using the kneadData wrapper script[40]. Reads were trimmed with Trimmomatic (version 0.36) with SLIDINGWINDOW set at 4:20, MINLEN set at 50, and ILLUMINACLIP: TruSeq3-PE.fa:2:20:10 [41]. Sequences from contaminating C57BL/6NJ mouse genome and two murine retroviruses (murine osteosarcoma virus (accession NC_001506.1) and mouse mammary tumor virus (accession

NC_001503)) were filtered out using Bowtie2[42]. In addition to this preprocessing, bacterial ribosomal reads were removed from the datasets using the SILVA 128 database[43]. Based on the PCoA analysis of the metatranscriptomic derived resistomes we determined that doxycycline sample1was an outlier and it was removed from further analysis (Figure S1). Doxycycline sample 1 had roughly 10 times the number of ARG hits relative to all other samples despite it being sequenced to a similar depth (Table S1).

Taxonomic Analysis of Metagenomic Reads

Cleaned metagenomic forward reads were classified against a database containing all prokaryotic genomes downloaded from NCBI RefSeq using Kaiju (version 1.7.0) using the MEM run mode and the default cutoffs for E-value and minimum required match length[44] (full relative abundance tables can be found in Table S2). The taxonomic output table was analyzed in R (version 3.5.2) using the phyloseq package (version 1.24.2) to calculate alpha and beta diversity metrics[45]. Principle coordinate analysis (PCoA) was performed using the Bray-Curtis Dissimilarity metric[46].

Metagenomic Assembly and Binning

The PATRIC webserver (3.5.43)[47] was utilized to bin and assign taxonomy to contigs assembled using metaSPAdes within SPAdes (3.13.0)[48].

Antibiotic Resistance Gene Analysis

Processed reads were joined using the fastq-join function of the ea-utils package[49]. The joined reads were then queried for antibiotic resistance using DeepARG (version 1)[8] using the default settings (0.8 minimum coverage of

alignment, e-value cutoff 1e-10, 50% minimum percentage of identity) and excluding “predicted” resistance genes (full counts tables of ARGs and ARG classes can be found in Table S3). Antibiotic resistance genes at the DNA level from the metagenomic data were identified by running cleaned, merged PE reads through the DeepARG pipeline. On average 45,768 (± 10,213) ARG hits were obtained from an average 26,281,760 (± 5,410,439) cleaned paired-end (merged forward and reverse read files). Using the same method to identify ARGs at the RNA level from metatranscriptomic sequencing, we found an average of 14,576 (± 6,147) ARG hits from an average 49,338,997 (± 10,392,548) cleaned paired-end (merged forward and reverse read files) reads.

Statistical Analyses and Figure Generation

Differential abundance of ARGs and ARG classes between treatments and controls was determined using DESeq2 (version 1.20.0)[50] in R (version 3.5.2) using default parameters. Statistical differences between alpha diversities were calculated in GraphPad Prism (version 8.0) (GraphPad Software, La Jolla California USA). All figures were generated using Prism 8.0, except supplementary Figure S4 which was generated using the Clustal Omega tool[51] on the EMBL-EBI web server[52].

Results & Discussion

Microbial Diversity

Antibiotic treatment was administered for either 12 hours for amoxicillin or 24 hours for ciprofloxacin and doxycycline with untreated time-matched controls. The microbiome of the amoxicillin treated mice displayed a marked reduction in bacterial alpha diversity ($p < 0.05$, Mann-Whitney U test), however, no change in diversity was observed in mice treated with ciprofloxacin or doxycycline (Figure 1A). Shifts in beta diversity were observed between each treatment and their respective controls indicating that these antibiotics elicit unique changes to the murine gut microbiome at a taxonomic level ($p < 0.05$, PERMANOVA) (Figure 1B). Perhaps the most drastic taxonomic change was the expansion of *Bacteroides thetaiotaomicron* in the microbiota of amoxicillin treated mice (Figure 1C, Figure S2). Overall, we found that out of the three antibiotics tested, amoxicillin has the most profound impact on the murine cecal microbiome community in terms of diversity and species relative abundance, while ciprofloxacin and doxycycline exhibit less drastic changes. A more detailed description of the taxonomic shifts is detailed in Cabral et al. 2019[38], while in this study we focus on the ARGs.

ARG abundance

While metagenomic analysis is commonly used to characterize the resistome, it can only report the genes found in the community but does not provide information about the actual expression of those genes. In the metagenomic data, we determined the average number of ARGs relative to total reads to calculate the relative abundance of ARGs in the community. There were 1.74E-03 (± 1.62E-04)

ARGs per read detected in the metagenomic data (Table S1), with an average of 336 (± 14) unique ARGs found in each metagenomic sample (Figure S3A). In contrast to the metagenomic data, metatranscriptomics cannot describe the structure of the community, but it can identify the portion of the total genes actively transcribed by the microbiome. In the metatranscriptomic data, there were 3.1E-04 (± 1.67E-04) ARGs per read (Table S1), with an average of 161 (± 40) unique ARGs found in each metatranscriptomic sample (Figure S3B). This data shows that there are more unique ARGs found in the DNA than in the RNA despite the higher sequencing depth used for metatranscriptomics. This discrepancy could indicate that many of the ARGs encoded in the microbiome are not actively transcribed with or without drug pressure.

Resistome Diversity

Two of the antibiotics examined, amoxicillin and ciprofloxacin, had unique impacts on the taxonomic composition of the microbiome resulting in corresponding shifts in the resistome diversity at the DNA level. Metagenomic data showed that compared to time-matched controls there was a significant increase in the Shannon diversity of the resistomes with amoxicillin ($p < 0.05$, Mann-Whitney U test), and a decrease in the Shannon diversity with ciprofloxacin ($p < 0.05$, Mann-Whitney U test), while doxycycline treatment had no impact (Figure 2A). Analysis of the Bray-Curtis beta diversity revealed significant differences in amoxicillin and ciprofloxacin groups compared to their time-matched controls ($p < 0.05$, PERMANOVA), but not in the doxycycline treated group ($p = 0.2$, PERMANOVA) (Figure 2C). However, these shifts in ARG diversity profiles did not necessarily

reflect a drug specific selection but rather resulted from the overall shift in microbiome composition. We found that while treatment induced changes in alpha diversity of the resistome at the DNA level, it did not impact resistome alpha diversity at the RNA level (Figure 2B). The lower and more stable alpha diversity of the RNA reads compared to the DNA reads likely stems from the fact that many of the genes detected in the metagenomics are not actively transcribed under vehicle or antibiotic treatment. We also found that amoxicillin and ciprofloxacin did not significantly impact Bray-Curtis ARG diversity at the RNA level ($p = 0.128$, $p = 0.397$, PERMANOVA). Additionally, in the metatranscriptomic data, doxycycline did induce a significant Bray-Curtis shift from the 24-hour controls ($p < 0.05$, PERMANOVA) (Figure 2D). This shift in beta-diversity may be driven by the induction of drug targeted ARG transcripts observed in the doxycycline treated samples.

ARG Class Level Changes in Response to Antibiotics

Antibiotic-induced shifts in ARG classes were determined using DESeq2 and considered significant with a $\log_2FC \geq 1.5$ or ≤ -1.5 and an adjusted p-value < 0.05 (Figure 3A). At the DNA level, we did not find any changes in ARG classes that directly corresponded to antibiotic treatment. For example, the beta-lactam resistance class was not increased with amoxicillin. Instead, we report that the only significant changes in ARG classes were an increase in kasugamycin class ARGs in response to amoxicillin treatment, decreases in the fosmidomycin and trimethoprim classes in response to ciprofloxacin treatment, and a decrease in the fosmidomycin class in response to doxycycline treatment (Figure 3A). As a whole,

none of the treatments led to an induction of ARG classes targeted towards the drug administered.

In contrast to the lack of drug targeted changes at the DNA level, metatranscriptomic sequencing showed an induction of ARG classes targeted to two of the treatments at the RNA level (Figure 3B). Overall, there were a number of significant changes in ARG classes against beta-lactams, fosmidomycin, polymyxin, and trimethoprim in response to amoxicillin treatment, triclosan in response to ciprofloxacin treatment, and fosfomycin, rifampin, and tetracycline in response to doxycycline treatment. Most interestingly, amoxicillin significantly increased the abundance of the beta-lactam ARG class with log_2FC 2.67 (*padj* = 2.50E-04), and doxycycline increased the abundance of the tetracycline ARG class with log_2FC 1.81 (*padj* 3.10E-21) in the RNA (Figure 3B). Thus, in contrast to the metagenomic data, metatranscriptomics shows a significant increase in ARG classes that are targeted to the antibiotic treatment and have the potential to provide a fitness advantage to members of the gut microbiota.

ARG Level Changes in Response to Antibiotics

Results from the differential abundance analysis show that the antibiotics tested have variable impacts on the abundance of AR genes and transcripts. At the metagenomic level, we found a set of differentially abundant genes that appeared general and unrelated to the antibiotic utilized. In contrast, the transcriptional response was much narrower and in the case of amoxicillin and doxycycline, it appears that antibiotic therapy promoted genes directly targeted to the drug utilized. This dichotomy between DNA and RNA level responses could

not have been detected without using a dual sequencing approach. Overall, there were fewer differentially abundant ARG transcripts (21 transcripts) found in the metatranscriptomic analysis compared to the number of differentially abundant ARGs (116 genes) in the metagenomic data (Figure 4A-F). This is a reflection of the fewer ARG reads found in the metatranscriptomic data, as well as the more specific response of the microbiome at a transcriptional level compared to the broad metagenomic changes. This is best exemplified by changes in ARGs targeted to antibiotic treatments, specifically amoxicillin and doxycycline.

While the observations made at the ARG class level were fairly broad, the gene level data provided more insights into the direct impact of antibiotic administration on specific ARGs. The differential expression tool DESeq2 was used to analyze the antibiotic-induced changes in AR gene and transcript abundance (Figure 4A). We found 56 significantly elevated or reduced ARGs after amoxicillin treatment. Of these 56, two beta-lactamase genes of interest, *cepA* and *bl2e_cepA*, had significant increases in gene abundances of $\log_2$FC 4.96 (*padj* = 4.10E-20) and $\log_2$FC 4.73 (*padj* = 6.72E-16), respectively. In addition to drug targeted genes, we also found increases in a much larger set of untargeted genes (42 genes). It is possible that these changes are the result of taxonomic shifts in bacteria that encode these genes, rather than a direct selection promoted by the induced resistance genes. The beta-lactamase genes increased in the metagenomic data are also increased at the RNA level, highlighted by significantly higher transcript abundances of $\log_2$FC 5.12 (*padj* = 2.40E-05), 4.93, (*padj* = 2.01E-3), for *cepA* and *bl2e_cepA*, respectively (Figure 4B). This may suggest that

in response to amoxicillin the community increased transcription of beta-lactamase genes leading to increased bacterial fitness. Conversely, it is also possible that this change merely reflects a bloom in the bacterium encoding these transcripts rather than a direct transcriptional response.

To identify the bacterial origin of the beta-lactamase genes found in our dataset, bacterial genomes were assembled from metagenomic data. Within the *B. thetaiotaomicron* metagenomically assembled genome (MAG), we identified a region corresponding to a class A beta-lactamase gene with 100% protein sequence homology to a subclass A2 beta-lactamase. Due to its high degree of sequence similarity, this gene likely corresponds to the reads assigned to the *cepA* and *bl2e_cepA* genes (Figure S4). The relative bloom *of B. thetaiotaomicron* after amoxicillin treatment may account for the increase in both the *cepA* and *bl2e_cepA* gene abundance and transcript level abundance. It is possible that the survival of this taxa during amoxicillin treatment may be promoted by these genes, although we cannot make a definitive conclusion without more evidence. Various laboratory strains and patient isolates of *Bacteroidales* have been shown to exhibit high levels of resistance to beta-lactams including amoxicillin[53-55]. Previous research into *B. thetaiotaomicron* found that this bacterium produces outer membrane vesicles (OMVs) containing cephalosporinase enzymes that protect neighboring bacteria from beta-lactam antibiotics [55]. Because *B. thetaiotaomicron* is a common human commensal[56, 57], this work has interesting implications for the complex microbial environment of the gut microbiome where *B. thetaiotaomicron* could have

a role in modulating antibiotic activity across many taxa through OMV-secreted beta-lactamase enzymes.

Metagenomic data showed that ciprofloxacin treatment induced significant changes in ARG abundance, most notably an increase in the relative abundance of several chloramphenicol, aminoglycoside, and MLS class genes, $\log_2FC > 1.5$ (*padj* < 0.05), and a decrease in the abundance of genes related to multidrug and fosmidomycin resistance $\log_2FC < -1.5$ (*padj* < 0.05) (Figure 4C). No significant increases in fluoroquinolone resistance genes were found in this dataset, however, it should be noted that point mutations conferring resistances were not included in the ARG database used. Thus, fluoroquinolone resistance mutations in *gyrA*, *gyrB*, or *parC* could not be identified from this analysis and might be present in the resistome. No ARG transcripts corresponding to fluoroquinolone resistance were significantly elevated in the ciprofloxacin treated samples. The transcripts of *tetC*, *muxC*, and *mdtC*, all genes encoding efflux system components, were significantly increased by ciprofloxacin treatment ($\log_2FC \geq 1.5$, *padj* < 0.05) (Figure 4D). Although none of these genes have been shown to directly efflux ciprofloxacin, the fact that fluoroquinolone treatment exclusively increased transcription of efflux type ARGs remains interesting.

At the DNA level, no genes were increased in abundance during doxycycline treatment; however, several were decreased in abundance. Although genes known to confer doxycycline resistance were detected in the metagenomic data (Table S3), they were not significantly changed due to doxycycline treatment (Figure 4E). While doxycycline did not increase the abundance of any tetracycline

class ARGs in the metagenomic data set, we did detect changes in the metatranscriptomic data. At the RNA level, doxycycline appears to have a targeted effect on the transcript abundance of tetracycline resistance genes with significant increases in the abundance of *tet*32, *tet*44, and *tetW* with log_2FCs of 5.06, 4.63, and 4.07, respectively (*padj* = 1.6E-2, 1.2E-2, 2.2E-2, respectively) (Figure 4E). This distinct difference in gene versus transcript abundance of these tetracycline class ARGs suggests that while short-term treatment with doxycycline may not select for bacteria encoding these resistance genes, it may induce their expression. All three tetracycline resistance genes with increased transcript abundance, *tet*32, *tet*44, and *tetW* have been shown to offer protection to tetracycline antibiotics through ribosomal protection mechanisms[58-60]. The increased transcript abundance of several *tet* genes in response to doxycycline, combined with unchanged levels of these same *tet* genes in the metagenomic dataset, suggests that their elevated transcriptional activity may be providing protection and enabling the population of *tet*-carrying bacteria to remain stable during treatment. The doxycycline-induced expression of several tetracycline resistance genes highlights the need for increased transcriptional profiling of ARGs. Relying solely on metagenomics without utilizing metatranscriptomic sequencing, we would have missed this ARG activity that may contribute to bacterial survival during antibiotic pressure.

Conclusion

In this study, we show that short-term antibiotic pressure leads to the expression of specific ARGs within the microbiome, but alters the metagenomic landscape in a less targeted way. We use both shotgun metagenomics and metatranscriptomics to profile how three antibiotics, amoxicillin (beta-lactam), doxycycline (tetracycline), and ciprofloxacin (fluoroquinolone), impact the diversity, composition, and transcriptional response of the murine gut resistome. We found that combining these two sequencing methods provides unique perspectives on ARGs in the microbiome that would have been missed by using metagenomics exclusively. For example, we found that at the RNA level a majority of the induced ARGs were targeted against the administered antibiotic, while at the DNA level we found more changes overall, but we did not find a drug-specific pattern. Our results show that both bactericidal and bacteriostatic antibiotic treatment alters the resistome at both the RNA and DNA level, but that these changes may be more specific to the drug administered at the transcript abundance level.

This work highlights the impacts of three antibiotics on the murine cecal resistome as well as the importance of both metagenomic and metatranscriptomic profiling of ARGs. However, due to the current methodology, there are several limitations to this work that must be considered. Experiments were done in mice in a closed mouse facility with reduced exposure to environmental bacteria and the antibiotic exposure period was fairly short. Both of these factors will reduce the opportunity for new ARGs to enter the microbiome and for selection to act on existing ARGs. In addition, due to limitations in strain level identification from

current metagenomic methodologies, we are unable to predict whether or not there was selection of specific ARG containing strains following antibiotic treatment. Additionally, the computational pipeline utilized to detect ARGs is unable to identify point mutations and their contributions to the resistome. These aspects limit our ability to detect selective events and the evolution of resistance. Finally, these experiments were conducted with a limited sample size (n = 4), and no samples were collected at time 0. These two factors may limit our ability to detect smaller shifts in ARG levels with sufficient significance and may hinder the detection of baseline changes to the microbiota of the control mice over the course of the experiment.

Despite these limitations, we believe that the dual sequencing approach has real benefits over purely DNA-based approaches. The widespread use of common antibiotics such as those tested in this study contributes to the dissemination of resistance genes in the human population and our microbiomes. However, as demonstrated here, the presence of an ARG in the metagenome may not necessarily indicate that it will be transcribed at baseline or in response to antibiotics. Thus, as we continue to develop strategies to monitor resistance in patient populations it will be important to track both gene presence and expression.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

BK, DC, PB contributed to the conception and design of the study. DC performed the experiments and sample processing. BK performed the data analysis and drafted the manuscript. BK, DC, PB all contributed to editing drafts of the manuscript.

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Data Availability Statement

Raw metagenomic and metatranscriptomic reads were deposited in the NCBI Short Read Archive under the BioProject number PRJNA504846.

Figures

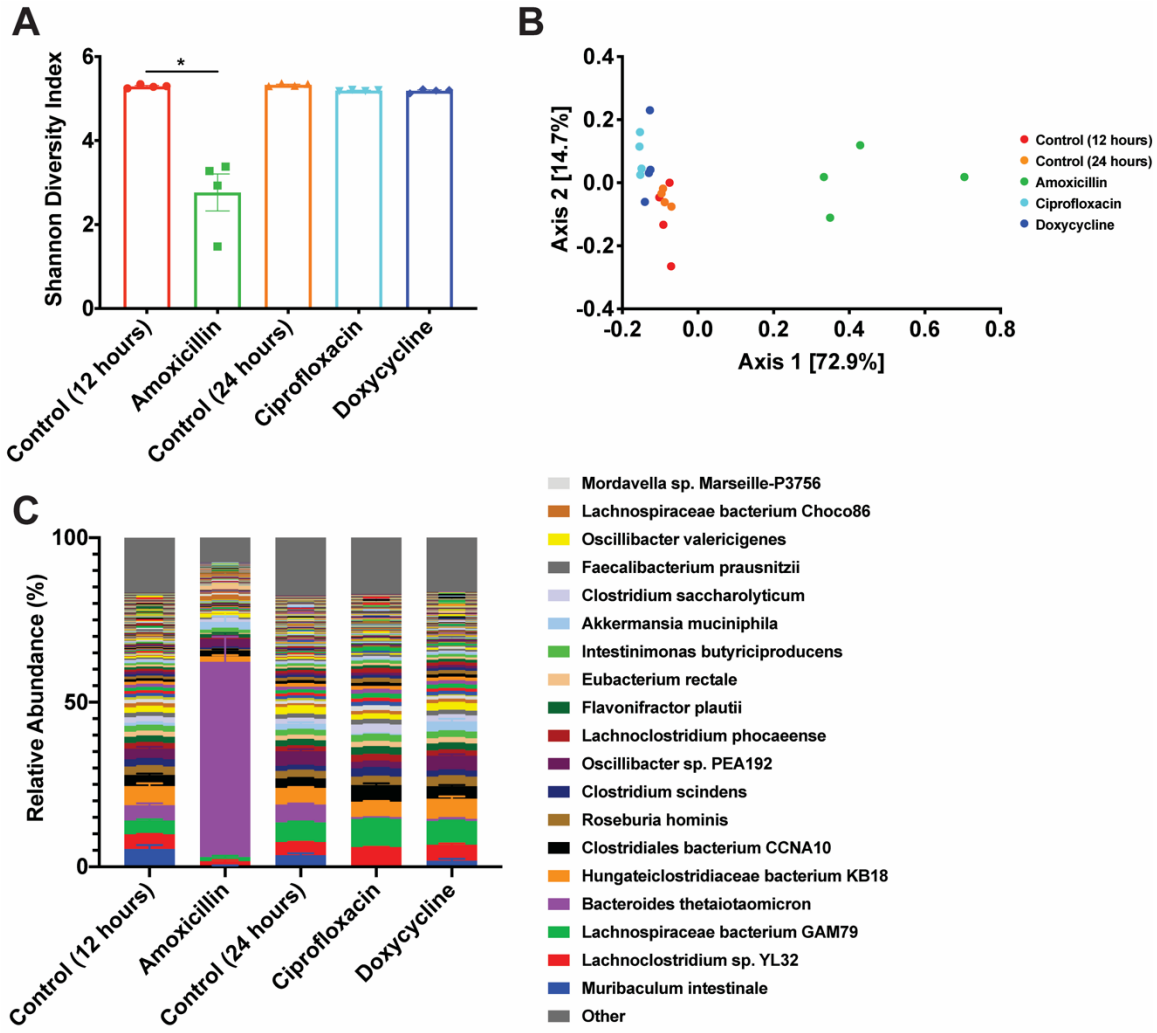

Figure 1: Antibiotic treatment has variable impacts on the diversity and taxonomic structure of the microbiome. (**A**) Shannon diversity index displayed as mean ± SEM ($p < 0.05$ Mann-Whitney U test, n = 4). (**B**) PCoA based on Bray-Curtis beta diversity. (**C**) Relative abundance of bacterial species displayed as mean ± SEM (n = 4, top 250 most abundant species colored, full relative abundance table available in Table S2).

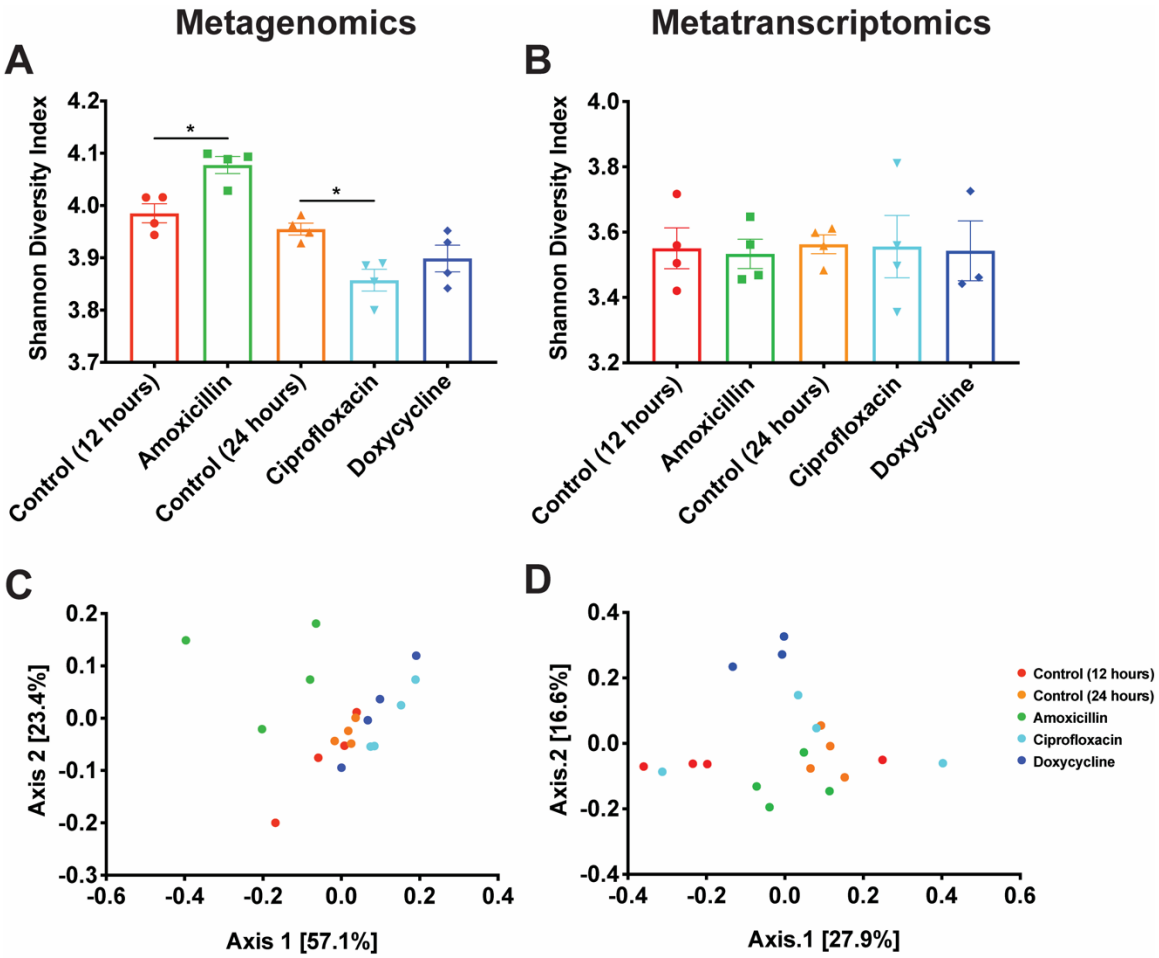

Figure 2: Antibiotics have variable impacts on the diversity and structure of the resistome. (**A-B**) Shannon diversity index based on resistance gene counts displayed as mean ± SEM for both metagenomic and metatranscriptomic data ($p < 0.05$ Mann-Whitney U test). (**C-D**) PCoA based on Bray-Curtis of resistance gene counts for both metagenomic and metatranscriptomic data.

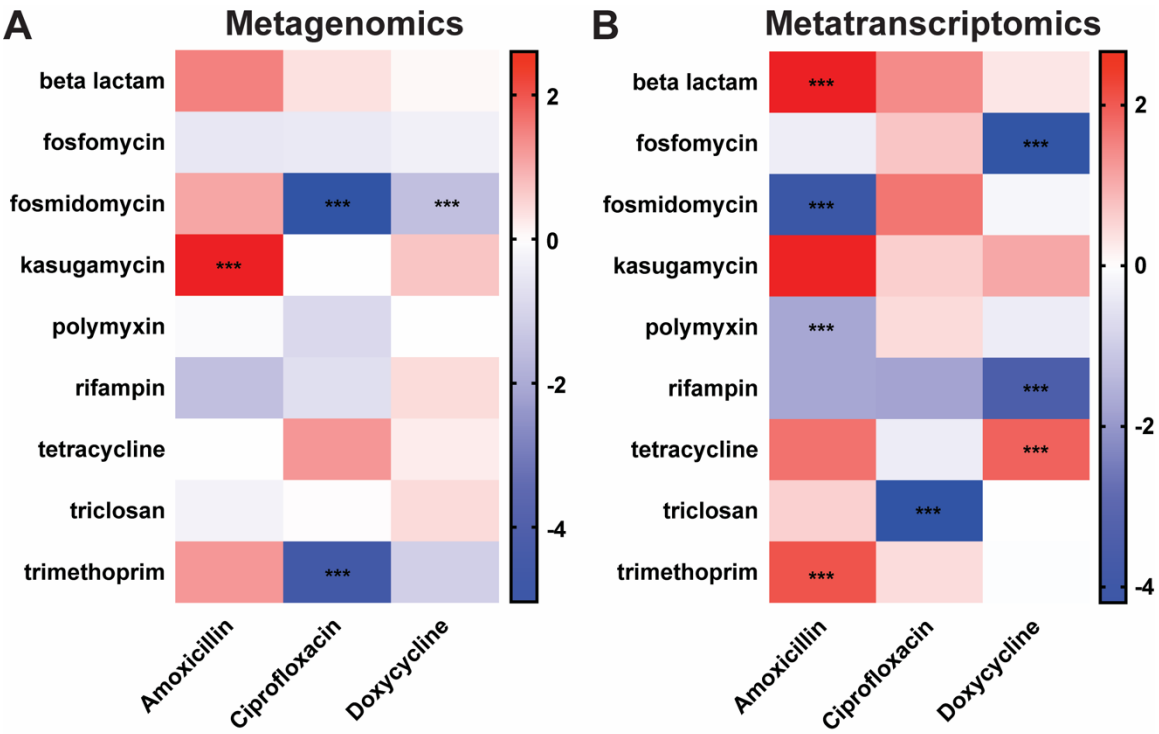

Figure 3: Differential abundance of antibiotic resistance gene classes. Changes in ARG class abundances after antibiotic treatment observed in (**A**) metagenomic and (**B**) metatranscriptomic data. The color scale represents $\log_2$ fold change ($\log_2$FC $\geq$ 1.5/ $\leq$ -1.5 and *padj* < 0.05) (Full ARG class counts available in Table S3).

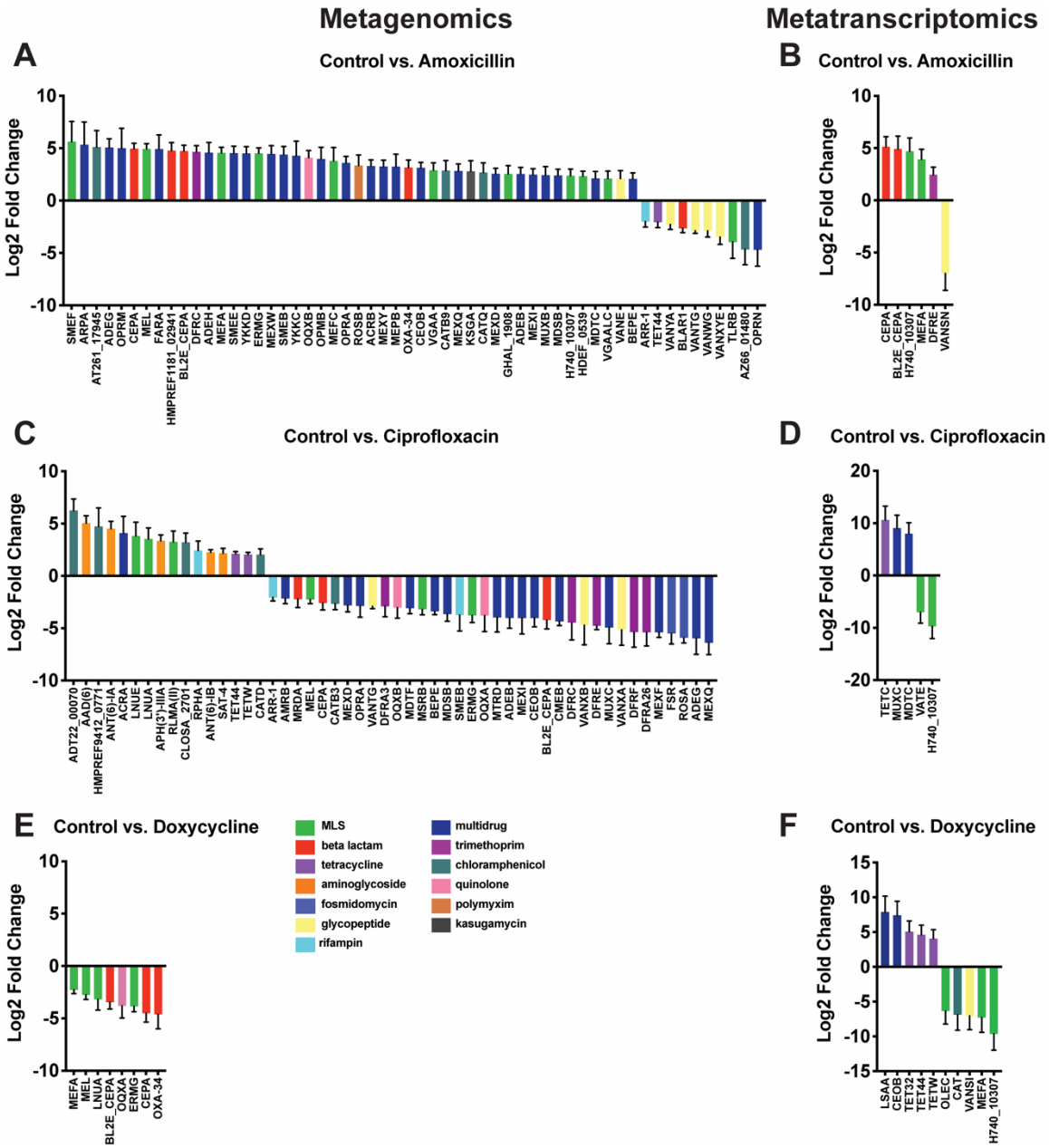

Figure 4: Differential abundance of antibiotic resistance genes. Changes in ARG abundances after antibiotic treatment observed in metagenomic (**A**, **C**, **E**) and metatranscriptomic data (**B**, **D**, **F**). Bars represent change in gene/transcript abundance after exposure to antibiotics displayed as $\log_2$ fold change $\pm$ standard error ($\log_2$FC $\geq$ 1.5/ $\leq$ -1.5 and *padj* < 0.05) (Full ARG class counts available in Table S3).

Supplementary Data

Table S1 – Number of cleaned merged reads and ARG hits detected per sample in the metagenomic and metatranscriptomic data. The numbers in parenthesis next to the "control" in the treatment column represent the timepoint at which the control sample was collected (in hours). The numbers after the treatment name (1 - 4) represent the numbering of the four samples in each treatment group.

Treatment	Clean Reads Metagenomic	ARG Hits Metagenomic	Clean Reads Metatranscriptomic	ARG Hits Metatranscriptomic
Control (12) 1	22,551,814	38,184	54,135,535	5,368
Control (12) 2	14,987,819	25,446	55,616,863	7,874
Control (12) 3	27,926,253	47,348	67,241,907	8,087
Control (12) 4	23,628,173	41,221	34,474,417	27,594
Amoxicillin 1	27,113,593	36,827	42,340,472	14,427
Amoxicillin 2	28,594,334	47,139	41,967,886	14,324
Amoxicillin 3	36,967,463	57,183	48,806,177	11,350
Amoxicillin 4	21,918,209	34,402	59,681,015	16,113
Control (24) 1	24,380,522	42,049	76,421,879	18,847
Control (24) 2	25,042,390	40,697	41,795,407	14,231
Control (24) 3	28,390,347	46,533	40,949,940	16,494
Control (24) 4	26,005,446	44,284	43,928,662	16,298
Ciprofloxacin 1	21,915,351	43,161	42,954,907	6,257
Ciprofloxacin 2	27,536,934	54,808	46,399,664	15,004
Ciprofloxacin 3	30,780,514	61,164	59,725,060	13,521
Ciprofloxacin 4	23,470,889	44,813	48,628,764	29,332
Doxycycline 1	26,777,060	48,068	40,546,403	108,037

Doxycycline 2	20,214,974	37,301	44,226,004	14,729
Doxycycline 3	39,177,796	71,030	44,420,451	11,237
Doxycycline 4	28,255,309	53,699	43,725,928	15,858

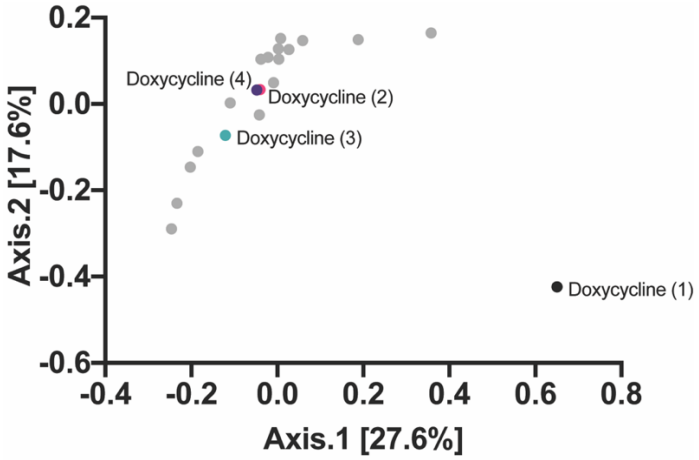

Figure S1 - PCoA based on Bray-Curtis of resistance gene counts for metatranscriptomic data with the doxycycline samples highlighted.

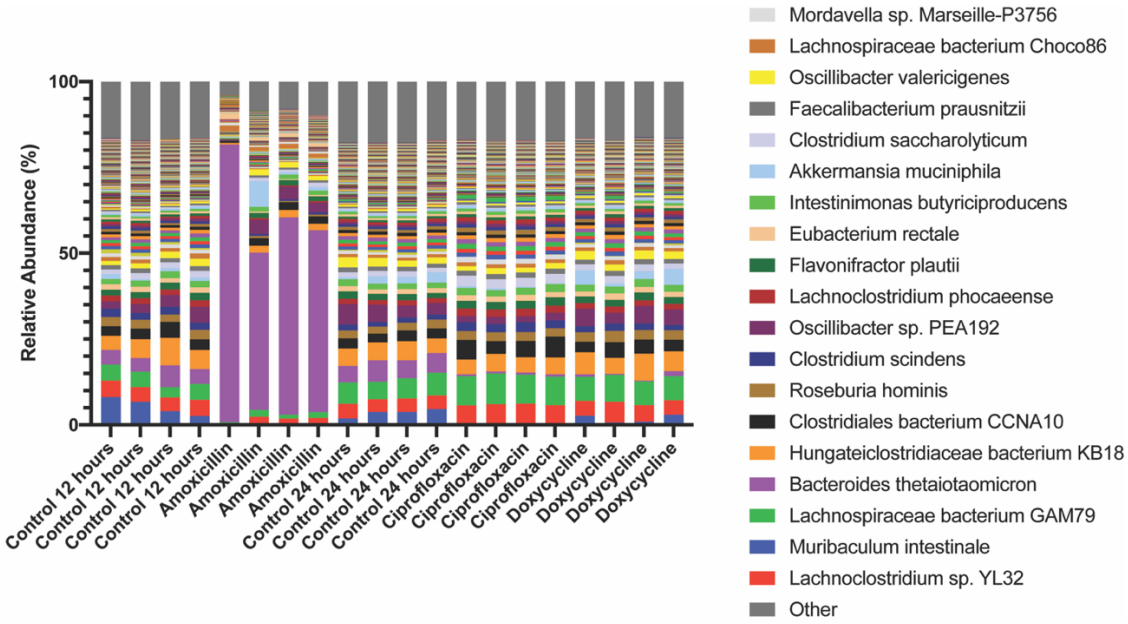

Figure S2 - Relative abundance of bacterial species displayed for each metagenomic sample (top 250 most abundant species colored, full relative abundance table available in Table S2).

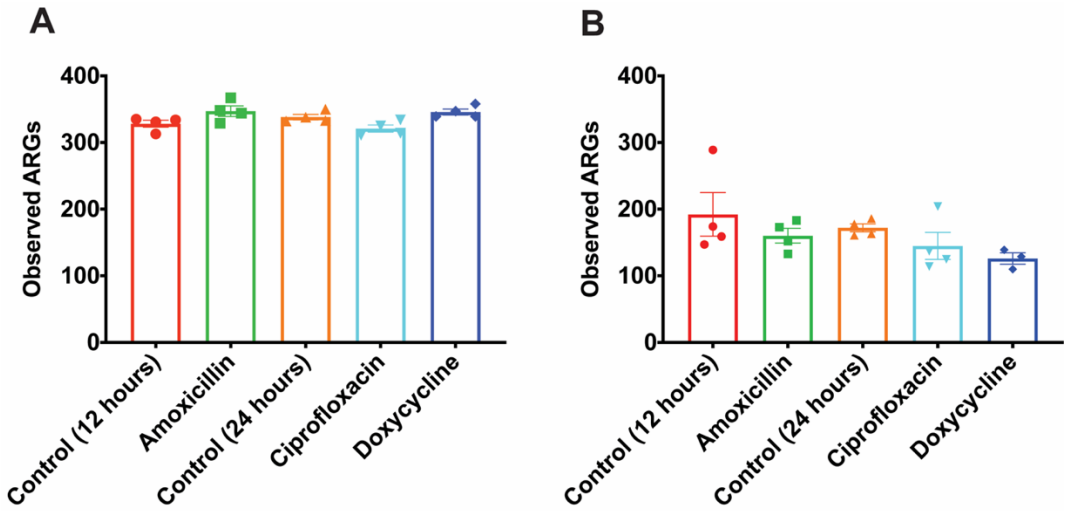

Figure S3 - Number of unique ARG types observed in both (**A**) metagenomic and (**B**) metatranscriptomic datasets ($p < 0.05$ Mann-Whitney U test).

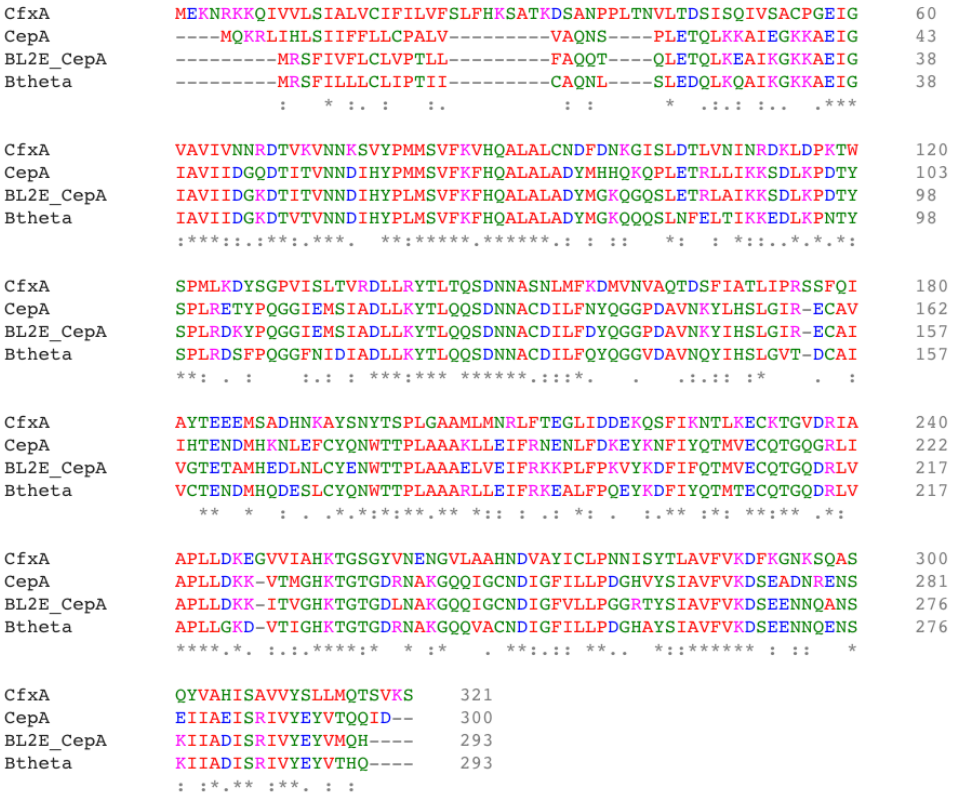

Figure S4 - Multiple sequence alignment of *cftxA*, *cepA*, and *bl2e_cepA* protein sequences from the DeepARG V1 database, as well as the subclass A2 beta-lactamase found in the *B. thetaiotaomicron* MAG in this study (labeled Btheta). The alignment was generated using Clustal Omega (1.2.4).

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CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

Summary

The development and spread of antimicrobial resistance over the past 80 years has weakened our current antibiotic arsenal and hindered our ability to treat bacterial pathogens. Through SAM and SIM mechanisms bacteria are able to rapidly develop resistance mutations. Additionally, the natural environment represents a vast reservoir of antimicrobial resistant (AMR) bacteria from which resistance and pathogens can be transmitted to humans. In order to combat the current antibiotic resistance crisis, it is necessary to gain a greater understanding of how resistance develops, where it occurs naturally in the environment, and improve strategies to track antibiotic resistance selection and development. My thesis provides novel insights into the dynamics of antibiotic resistance both at the molecular level and a broader environmental perspective. A discussion of these findings and future directions for these projects are addressed in the following section.

In Chapter 2, I present work that illustrates the process by which novel ribosomal target modification mutations arise through genotoxic stress. Notably, we find that genotoxic agents cause stressor-specific signature mutations in which some mutational profiles clearly reflect the DNA-damaging mechanism of the stress. When resistant strains containing unique target mutations are grown together, as would be the case in a natural microbial community, we demonstrate that the final mutational spectrum is determined by the level of antibiotic selection. Together, these findings suggest that mutations arise from a combination of unique

stress-associated DNA damage, and subsequent fitness across a gradient of selection.

Resistance is not unique to laboratory or clinical settings, though. To investigate the dynamics of antibiotic resistance in local wildlife, I studied the GIT microbiota and associated resistomes of seven marine fish species. The work in Chapter 3 represents one of the most extensive sequencing-based studies of marine fish microbiomes to date. Here, I report the first association between trophic level and ARG burden. Furthermore, through metagenomic genome assembly, we are able to find evidence that this increase in resistance genes is driven by an abundance of Proteobacteria. Finally, we are able to utilize a metagenomically based DNA barcoding approach to discern host dietary habits from GIT contents and link dietary carbohydrate availability to bacterial colonization within the fish gut. This work presents a novel look into the gut microbiota of wild fish and has implications for their role as a reservoir of antibiotic resistance.

Recently, shotgun sequencing approaches have been used to characterize the resistomes of complex microbial communities. In Chapter 4 we expand on the metagenomic sequencing approach (DNA based) by implementing a metatranscriptomic analysis (RNA based) that focuses on resistance gene transcripts. In order to test this dual sequencing approach, we utilized data collected from the gut microbiota of mice treated with three different antibiotics. Our analyses suggest that using a dual sequencing approach uncovers antibiotic induced, targeted shifts in the expression of β-lactam and tetracycline resistance genes within the murine gut microbiota. Additionally, we find that the transcriptional

activity of β-lactam resistance genes may be directly involved in the expansion of *B. thetaiotaomicron* within the gut microbiome during amoxicillin treatment. Together these findings demonstrate the strength of a dual sequencing approach in studying antimicrobial resistance.

DNA Repair and Mutagenic Pathways as Novel Antimicrobial Targets

In order to combat the development of stress-induced resistance mutations, previous work has examined so called “anti-evolution” drug targets. Recent work has described bacterial pathways that increase rates of adaptive mutagenesis leading to the development of resistance mutations[1, 2]. In Chapter 2, we utilize a gene-knockout library of *B. subtilis* and find that mitomycin C associated mutagenesis is attenuated by the absence of certain genes involved in DNA repair and mutagenesis such as *dinB* and *mfd* (Chapter 2, Figure 2A). These and six other genes identified, including Y-family polymerases involved in translesion synthesis repair, represent possible targets for inhibiting the development of resistance in bacteria. Additionally, blocking bacterial DNA-repair mechanisms may be a way to increase the efficacy of current antibiotics. This approach is used in cancer chemotherapy. Orthologous DNA-repair mechanisms in tumor cells are known to reduce the efficacy of alkylating agents during chemotherapy of gliomas [3]. The development of poly(ADP-ribose)polymerase (PARP-1) inhibitors has been shown to increase the effectiveness of alkylating chemotherapeutics by preventing repair of alkylation damage in tumor cells[3]. A similar approach could be taken to potentiate antibiotic efficacy in bacteria. Our results identify potential

targets for inhibiting translesion synthesis repair of antibiotic-induced damage, which may have the potential to increase antibiotic efficacy. A future direction for this chapter of my thesis would be to test the mutagenicity and lethality of a broad range of antibiotics against the select DNA-repair gene knockouts used in this study. This would help elucidate which pathways of repair and mutagenesis are important to bacterial survival and resistance development in response to antibiotic stress.

Understanding the Role of Mutagenesis in Antibiotic Resistance Development in Vivo

While the work in Chapter 2 examines the role of mutagenesis and selection in the development of resistance *in vitro*, it would be interesting and clinically relevant to study this process *in vivo*. The *in vitro* study presented here utilized standardized culturing methods and defined antibiotic concentrations. While this is useful at minimizing variables to answer fundamental microbiology questions, it is not able to recapitulate the process of mutagenesis and selection in the host. Within a host environment –either in the gut or site of infection– there are innumerable factors that cannot be re-created in the lab. Host immune response and antibiotic, oxygen, and nutrient concentration gradients are all key factors in shaping bacterial metabolic state and mutagenic response to stressors. Understanding how mutagenesis occurs in the host is vital to preventing the development of resistance in clinical settings such as the lungs of cystic fibrosis patients in which drug resistance mutations develop in the pathogen

Pseudomonas aeruginosa leading to treatment failure [4]. Future studies in the lab could undertake this question by using the mouse GIT as a model in which to test the development of resistance in enteric pathogens. Administering oral treatments of genotoxic agents and subsequently plating fecal samples on selective antibiotic media to isolate resistant bacteria would allow one to track the development of resistance in an *in vivo* setting. In order to gain temporal data and make this approach higher throughput, the fecal pellets could be sequenced daily to track changes in the mutational profile of the gut bacteria over time. This could provide further insights into the selection process within the GIT environment.

Implications for the Trophic Accumulation of ARGs

Wildlife plays a significant role as a reservoir of resistance with the potential to transfer pathogens and antibiotic resistance to humans. In Chapter 3 we examined the dynamics of antibiotic resistance within the microbiota of wild marine vertebrates. Our finding that ARGs are more abundant in higher trophic level organisms with piscivorous diets has implications for potential trophic accumulation of resistance in the GIT of predatory animals. We found that this high abundance of ARGs was associated with an increased relative abundance of Proteobacteria. This finding could suggest that organisms that harbor high Proteobacterial abundance in their GIT may be more significant reservoirs of ARGs. Proteobacteria are known to be prominently involved in the horizontal gene transfer of antibiotic resistance determinants [5]. Proteobacteria of the genus *Vibrio* were commonly found in the marine samples studied in Chapter 3 of this thesis,

and this genus is known for its ability to undergo HGT [6], which increases its potential to acquire resistance genes. Additionally, the gut environment is densely populated by bacteria, making it an optimal environment for cell-cell contact and resulting HGT [7]. Combined, this information implicates Proteobacteria rich GIT environments as reservoirs of ARG, and also as sites of enriched resistance gene transfer. To understand the role of HGT in the spread of ARGs within the gut microbiome, future studies could investigate the association of resistance genes with mobile genetic elements such as integrases, phages, or plasmids within assembled bacterial genomes.

Our sampling was restricted to a limited number of species from a narrow geographic range in the North Atlantic. In order to understand if the association between trophic levels and ARGs is conserved across the animal kingdom we must look at the microbiota of a wide range of wildlife that occupy different ecological niches. Recently, Levin et al. published a paper regarding the gut microbiota of 184 different species from across the globe [8]. This represents a valuable trove of microbiome sequence data that could be utilized to further test the relationship between levels of ARGs, Proteobacteria abundance, and host trophic level.

Utilizing Narragansett Bay Wildlife to Monitor Anthropogenic/Environmental Impacts on Antibiotic Resistance

The University of Rhode Island Graduate School of Oceanography fish trawl is a valuable scientific resource that has decades of sampling data from

Narragansett Bay. From this weekly trawl survey, we were able to obtain the fish samples used in Chapter 3 of this thesis. Due to the high frequency of sampling and collaboration formed with members of the URI GSO, samples from the bay could be used for a long-term monitoring effort. While we collected samples over a short time period, it would be interesting to answer questions about the long-term stability of fish GIT microbiota, as well as how environmental changes –such as climate changes or prey availability– alter the bacterial flora of the bay. Additionally, monitoring this benthic community could answer questions about how host diet alters the microbiome of wild marine fish as there are temporal fluctuations in fish populations and prey availability within the bay [9]. By continuing annual sampling and microbiome sequencing we can monitor levels of ARGs within Narragansett Bay fish populations and understand how environmental or anthropogenic disturbances impact the bay microbiome and resistome.

Highly Migratory Species as Vectors of ARG and Pathogen Dissemination

We show in Chapter 3 that the GITs of wild marine fish act as reservoirs of antibiotic resistance. Some of these species, especially the large pelagic sharks (mako shark and thresher shark), exhibit highly migratory behavior. In fact, mako sharks are known to migrate thousands of kilometers a year from the North Atlantic to the Southern Caribbean Sea [10]. Throughout this migration, these sharks will be dispersing bacteria via fecal deposition and thus spreading bacteria and ARGs across great distances. Interestingly, previous work has shown that gulls laden with AMR bacteria were able to travel thousands of kilometers in a matter of days

and suggested this as a route of long-distance dispersal [11]. Further research found that gulls feeding at a landfill likely acquired resistant *E. coli* strains which they then dispersed at a separate breeding location [12]. These studies provide a precedent for wildlife-mediated dispersal of AMR bacteria, and due to the highly migratory nature of the mako shark and other marine species it would be interesting to investigate their role in the dispersal of AMR bacteria.

Role of Tetracycline Genes during Doxycycline Therapy Revealed by Metatranscriptomics

The findings in Chapter 4 suggest that previous intervention studies of the microbiome that rely solely on DNA metagenomic sequencing may not be getting a complete picture. This is especially true for potential antibiotic resistance activity. We found that mice treated with doxycycline (a tetracycline class antibiotic), did not display a significant increase in genes conferring resistance to tetracycline class antibiotics (Chapter 4, Figure 3A). However, gene transcripts associated with tetracycline resistance were significantly increased in doxycycline treated mice (Chapter 4, Figure 3B). Moreover, these tetracycline resistance genes, *tet32*, *tet44*, and *tetW*, all exhibit a target protection mechanism of resistance[13, 14]. Curiously, no efflux pump encoding *tet* resistance genes increased in transcript abundance as a result of doxycycline treatment. This suggests that *tet* efflux pumps may have a narrow range of efflux activity and are not effective against certain types of tetracycline class antibiotics such as doxycycline. Thus, target protection of the ribosome may be a more broadly acting resistance mechanism in

this class of antibiotics. Comparative *in vitro* studies of mechanistically different tetracycline resistance genes are needed in order to elucidate the breadth of resistance provided by either target protection or efflux mediated resistance mechanisms. More importantly, this is a strong example of the power of metatranscriptomic sequencing in revealing ARG activity that was unseen when looking at DNA sequencing alone.

Limitations of Databases when Analyzing Microbiome Sequencing Data

One important caveat when considering the data presented in Chapters 3 and 4 of this thesis is the use of databases for defining both microbial taxonomy and antibiotic resistance. When studying microbial communities one of the most fundamental questions is “what’s there?”. To answer this question, we use unbiased shotgun sequencing approaches to sequence microbial DNA. In Chapters 3 and 4 of this thesis we used current bioinformatic pipelines[15, 16] and the most comprehensive bacterial sequence databases available[17] to identify what bacteria species inhabited murine GIT and marine environmental samples. Despite decades of effort culturing, classifying, and sequencing bacterial species, our existing databases remain limited to those species that have already been defined. A vast majority of bacterial species and genomes remain uncharacterized and make up what is referred to as “microbial dark matter”[18-21]. Furthermore, the limitations of taxonomic databases pose greater challenges to some datasets compared to others. Microbes from well-studied sources such as the GIT of lab mice are highly characterized and well represented in current taxonomic

databases. In contrast, microbes from less clinically relevant and under-studied samples such as the natural environment and the GIT of wild organisms are not as numerous in these databases. This is particularly evident in the datasets analyzed here. When looking at the percent of sequence reads that received a taxonomic annotation, we found that roughly 90 – 95% of sequences derived from the murine GIT in Chapter 4 were classified. In contrast, many of the fish GIT samples from Chapter 3 only had 10 – 20% read classification, meaning that a majority of these sequences could not be identified taxonomically. Further work is needed to illuminate the proverbial “microbial dark matter” that is prevalent within environmental microbial communities.

In addition to the challenges facing taxonomic annotation, antibiotic resistance gene databases also have significant limitations. The Comprehensive Antibiotic Resistance Database (CARD) is an extremely valuable resource containing thousands of experimentally validated resistance gene sequences[22, 23]. Using this resource, we were able to detect known resistance determinants from our sequencing data in Chapters 3 and 4 of this thesis. However, due to the fact that resistance is of particular interest in the clinical setting, and that antibiotic susceptibility testing is most common in clinical microbiology, the majority of resistance genes are likely described from human or livestock pathogens. Additionally, many of these pathogens such as those in the genus *Neisseria, Shigella*, *Escherichia, Salmonella*, *Yersinia*, *Vibrio*, and *Acinetobacter* are members of the phylum Proteobacteria [24]. Thus, it is likely that most of the ARGs that have been classified originate in Proteobacteria, making the current ARG

databases biased towards Proteobacterial resistance genotypes. This idea is reinforced by the findings of a recent study on mobile resistant genes. Researchers found that all the resistance genes in their study originated in Proteobacteria, and almost all of those Proteobacteria were associated with human or domestic animal infections[5]. Thus, it is likely that there are many more forms of resistance that might be found in non-pathogenic bacteria. Expanding the search for novel resistance genes across a wider taxonomic range of bacteria through techniques such as functional metagenomics will help us gain a more complete view of the resistome [25, 26].

Conclusion

The work presented in this thesis contributes novel findings to the field of antibiotic resistance. Through the use of traditional microbiology techniques and modern high-throughput sequencing technology, we were able to gain insights into the development, distribution, and activity of antibiotic resistance genes. The work here will provide the foundation for future projects examining the dynamics of resistance in the laboratory and the environment.

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