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A Novel Assessment of Antibiotic Resistance

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Stacy Ann Suarez

Dissertation Committee:
Professor Adam Martiny, Chair
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2023

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

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ABSTRACT OF THE DISSERTATION

A Novel Assessment of Antibiotic Resistance

by

Stacy Ann Suarez

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2023

Professor Adam C. Martiny, Chair

It is important to understand the evolution and prevalence of antibiotic resistance as it is considered a major threat to global public health. Predicting where new antibiotic resistance (AR) genes will rise is a challenge and especially important when new antibiotics are developed. Adaptive resistance allows sensitive bacterial cells to become transiently resistant to antibiotics through changes in gene expression. This provides an opportune time for cells to develop more efficient resistance mechanisms and permanent resistance to higher antibiotic concentrations. Intraspecific genomic diversity may be a driving force in the emergence of adaptive antibiotic resistance. Here, I use an amplification assay adapted from functional metagenomics to investigate cryptic antibiotic resistance, an adaptive resistance mechanism, across eight micro-diverse *Escherichia coli* from clinical and laboratory origins. Cryptic (unclassified) AR genes primarily conferred resistance within clinical strains as opposed to known AR genes as hypothesized. Most genes conferring resistance within multiple strains were classified AR genes. Cryptic AR genes are highly variable as most conferred resistance in only

one strain. Hydrophilic antibiotics are more likely to induce cryptic resistance as resistance occurred to all hydrophilic antibiotics tested. These studies may help detect novel AR genes that confer resistance when upregulated.

Additionally, it is important to study antibiotic resistance in the environment, including coastal water. At the beach, people may ingest bacteria harboring AR genes which can lead to infection and/or transfer of genes to commensal and opportunistic pathogens resident in the human microbiome. The ingestion of seafood potentially harboring antibiotic resistant bacteria can lead to indirect contact with antibiotic resistance. Through a ten-year time series, I used metagenomics to provide a comprehensive understanding of the AR genes present within Newport Beach, CA seawater, the temporal distribution of these genes, and the factors driving their frequencies. I found that seasonal and interannual trends of AR genes vary by gene and the taxa carrying them as opposed to a general increase of most resistance genes during specific seasons. However, I found that precipitation and *Enterococcus* levels may be accurate indicators for total AR gene levels in Newport beach coastal water. Mostly marine taxa carry AR genes in Newport Beach coastal water, but there are also terrestrial taxa and opportunistic pathogens harboring AR genes. Non-marine taxa may be washed in with rain, people, or sewage spills. By using metagenomics, I was able to elucidate the AR gene reservoir in Newport Beach coastal water.

CHAPTER 1

Gene amplification uncovers large previously unrecognized cryptic antibiotic resistance potential in *E. coli*

Stacy A. Suarez and Adam C. Martiny

Introduction

The rapid spread and emergence of antibiotic resistance makes it one of the major threats to global public health (1, 2). Antibiotic resistant bacteria annually infect nearly three million people in the United States (1) and are projected to cause 10 million human deaths worldwide per year by 2050, more than the current rate for cancer (3). Therefore, it is critical to understand the evolution of antibiotic resistance to effectively tackle this worldwide crisis.

The emergence of antibiotic resistance is generally due to acquired, intrinsic, or adaptive resistance (4). Acquired resistance is the traditional pathway, which includes mutations in chromosomal genes and horizontal gene transfer. Intrinsic resistance refers to the inherent properties (such as efflux pumps) of the bacterial cell that can influence resistance. Intrinsic antibiotic resistance (AR) genes contribute to resistance at wild type expression level (5). Adaptive resistance, which includes cryptic resistance, does not have a universally accepted definition (6), but it has been defined as 'a temporary increase in the ability of a bacterium to survive antibiotic insult due to alterations in gene and/or protein expression as a result of exposure to an environmental trigger' (4). Contrary to acquired and intrinsic resistance, adaptive resistance is dependent on the antibiotic resulting in an unstable phenotype.

Latent resistance is a form of adaptive resistance, and latent AR genes have the

potential to contribute to resistance if their expression is changed from the wild type (5).
Latent antibiotic resistance may occur by the activation of unclassified (cryptic) AR
genes in the bacterial cell (5, 7–10). Cryptic genes can be any gene not commonly
known to confer antibiotic resistance. Only until recently have studies emerged
thoroughly investigating the link between antibiotic resistance and the amplification of
unrecognized AR genes (5, 7–10).

There may be a large potential for an unrecognized and diverse reservoir of latent
AR genes in pathogens as cryptic resistance can occur without major mutation and
horizontal transmission. Additionally, the vast biochemical diversity harbored within
bacterial genomes furthers the potential for the presence of cryptic genes that could
confer resistance when necessary. For example, the method Scalar Analysis of Library
Enrichments was used to identify genomic regions that, when upregulated, led to cryptic
aminoglycoside resistance in *Pseudomonas aeruginosa* (7). Genes that increased
aminoglycoside resistance encoded products related to DNA repair, O-antigen
synthesis, and transcriptional and translational processes. Gene expression variability
was measured in *E. coli* adapted to ampicillin, tetracycline, or n-butanol, showing that
the top three categories for overexpressed genes were metabolic and biosynthetic
processes, membrane components, and response to stimuli (9). A transposon tool,
GeneHunter, has also been used to identify cryptic/latent AR genes in *Salmonella
enterica* (10). Recently, intrinsic and latent resistance genes were identified in *E. coli* via
a disk diffusion assay (5). Understanding cryptic resistance is crucial to ultimately
reduce the evolution to current and new antibiotics. The molecular mechanisms and

types of antibiotics that lead to cryptic resistance are still unclear but delineating these will further elucidate the emergence of antibiotic resistance.

Adaptive (latent) resistance may provide a link to mutational resistance, which endures in the absence of the antibiotic (6). *E. coli* adapted to amoxicillin, tetracycline, and enrofloxacin exposure showed that an initial differential gene expression response led to mutations conferring higher antibiotic resistance (11). Adaptive resistance, which is not classified as tolerance or resistance but rather a connection between the two, leads to transient resistance to low antibiotic concentrations for long periods of time. Tolerance, which has shown to facilitate the development of mutational resistance to antibiotics in *E. coli* (12), allows cells to resist high antibiotic concentrations for short periods of time (6). Adaptive resistance could be an opportune time for bacterial cells to develop more efficient resistance mechanisms such as tolerance and permanent resistance to higher antibiotic concentrations. Additionally, overexpression of unrecognized AR genes imparts a minor to zero effect on fitness in the absence of the antibiotic (5, 8). In contrast, antibiotic resistance mutations can be costly, for example, fluoroquinolone resistance in pseudomonads can hinder motility (13). Our overarching hypothesis is that bacteria harbor an extensive array of diverse cryptic latent AR genes that will confer resistance when amplified. We predict that these genes will be associated with the antibiotic mechanism of action and that cryptic resistance will be less common in the presence of newer antibiotics due to their stronger activity.

Here, we developed a plasmid assay adapted from functional metagenomics, which incorporates a high throughput method to determine if a large increase in gene copy number can cause an AR phenotype in *E. coli* in the absence of chromosomal

mutations. We specifically asked the following: (i) what are the genes that confer an AR phenotype when amplified and (ii) which types of antibiotics will induce resistance in this manner? If we find cryptic genes conferring an AR phenotype when amplified, then this may demonstrate a prevalent resistance mechanism, allowing us to identify genes not known to be considered AR genes.

Methods

Strain, Media, and Culture Conditions

E. cloni 10G Supreme cells (Lucigen, Middleton, WI, USA), the wildtype strain, were grown in Luria-Bertani (LB) media and incubated overnight in 37°C unless otherwise stated.

Resistance Profile

To appropriately screen clones for cryptic antibiotic resistance, the minimum concentration of antibiotic needed to inhibit the growth of 10^6 E. cloni cells was determined for all antibiotics (Table 1) using LB agar plates. The listed antibiotics were tested to include a range of classes (mechanisms of action) and origins (natural, semisynthetic, or synthetic) if available. The range of concentrations tested for each antibiotic was 0.032 – 512 ug/mL. Growth was identified as more than 10 colonies. The lowest concentration that led to no growth on 2 out of 3 replicates was used to screen clones for cryptic antibiotic resistance.

Cloning and Screening

Genomic DNA was extracted from E. cloni cells using the Wizard Genomic DNA purification Kit (Promega Corporation, Madison, WI, USA). At least 10 micrograms of genomic DNA were sheared to a target size of 2 kb using a Covaris S220 Focus

Acoustic Shearer (Covaris Inc., Woburn, MA, USA). Fragments of 1 to 3 kb were extracted from a 1% agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA was treated with the NEBNext End Repair Module to create blunt ends on the fragmented DNA (New England Biolabs, Ipswich, MA, USA). The end-repaired DNA was purified using the DNA Clean and Concentrator-10 kit (Zymo Research). DNA was ligated into pSMART-HCKan vector (accession number AF532107) and then electroporated into *E. cloni* cells as per the CloneSmart Blunt Cloning Kit (Lucigen). This vector relies on endogenous promoters. A vector background control and a positive control insert DNA (HincII-digested lambda DNA) were processed as well to determine the ligation and transformation efficiencies. Transformed cells were recovered at 37 °C for 1 h. Cultures were then diluted 1:10 and 1:100, and 100 μL of each was plated on LB Lennox agar containing kanamycin (30 μg/mL) to determine the total colony-forming unit and the number of plasmids tested on each antibiotic. 50 μL of the vector background control and 5 μL of the positive control insert DNA were plated on LB Lennox kanamycin agar plates.

To test for cryptic antibiotic resistance, 150 μL of undiluted recovered transformants was plated on LB Lennox kanamycin agar containing one of one of eighteen antibiotics (Table 1). After overnight incubation, resistant transformants were pooled for each antibiotic using 1-2 mL of phosphate-buffered saline (PBS). Prior to pooling, 2 colonies from each plate were re-streaked onto agar containing the same antibiotics to confirm resistant clones. Pooled plasmid DNA was extracted from each PBS suspension (9 total samples, 1 from each resistance positive antibiotic) using the ZR Plasmid Miniprep kit (Zymo Research) and stored in -20°C. Plasmid inserts

containing the AR genes were amplified via polymerase chain reaction (PCR). This PCR used 25 µL reactions, including 12.5 µL of AccuStart II PCR SuperMix 2X (Quantabio), 3 µL (1.5 ng) of plasmid DNA, 4.5 µL of nuclease-free water, and 2.5 µL of SL1 and SR2 primers (Lucigen). The reaction cycle conditions follow those delineated for AccuStart II PCR SuperMix 2X (Quantabio). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen).

The cloning was repeated to obtain the AR clones' minimum inhibitory concentrations (MIC) and the range of resistance conferred by gene amplification. 150 µL of undiluted recovered transformants was plated on concentrations 2, 4, and 8 times above the MIC (Table 2). This was done for the 9 resistance positive antibiotics (Table 1). All clones grown above the MIC were re-streaked onto agar containing the same antibiotics. Then, these re-streaked clones were grown in LB broth containing the same antibiotics and incubated overnight. Plasmid DNA was individually extracted from each culture (1 from each AR clone) using the ZR Plasmid Miniprep kit (Zymo Research) and stored in -20°C.

Library Preparation, Sequencing, and Analysis

For pooled plasmids, library preparation was performed according to the PCR barcoding genomic DNA (SQK-LSK109) protocol for the MinION device (Oxford Nanopore Technologies). 200 fmol of each library was end-prepped for ligation with barcode adaptors using the NEBNext Ultra II End-Repair/dA-tailing module (New England Biolabs). DNA samples were purified using 1x volume AMPure XP beads (Beckman Coulter). Barcode adapters (Oxford Nanopore Technologies) were ligated onto the end-prepped DNA libraries using the Blunt/TA Ligase Master Mix (New

England Biolabs). After bead-cleaning DNA libraries, barcodes from PCR barcoding expansion 1-12 (Oxford Nanopore Technologies) were added onto the samples via PCR using LongAmp Taq 2x Master Mix (New England Biolabs). Barcoded libraries were bead-purified and equimolar-pooled. The pooled libraries were end-prepped for ligation of sequencing adaptors and subsequently purified using beads. Sequencing adaptors were ligated onto the end-prepped DNA using Ligation Buffer (Oxford Nanopore Technologies), NEBNext Quick T4 DNA Ligase (New England Biolabs), and Adapter Mix (Oxford Nanopore Technologies). The reaction mix was bead-purified and quantified using the Invitrogen Qubit fluorimeter (Thermo Fisher Scientific).

Sequencing was done on the MinION flow cell (FLO-Min106 R9.4.1 version; Oxford Nanopore Technologies) using the MinION device (Mk1B version). A platform QC and priming was done on the flow cell prior to sequencing according to the manufacturer's instructions. The final library, mixed with Sequencing Buffer and Loading Beads (Oxford Nanopore Technologies), was added to the flow cell via the SpotON sample port.

Base-calling was done in real-time using MinKNOW software (Oxford Nanopore Technologies) on a local computer. The sequencing run was carried out for 15 hours, and the barcoded base-called reads were subsequently demultiplexed and analyzed using the 'Barcoding' workflow on the EPI2ME Desktop Agent software. Demultiplexed reads were aligned and mapped to the *E. coli* reference genome (Lucigen) using Bowtie 2 (14). Mapped reads were assembled and processed with Anvi'o (15) which provided coverage, identity, and location within the reference strain for each aligned gene.

We chose genes that had coverage within the 99% confidence interval as

putative resistance genes, which totaled 174 individual genes (292 total, taking into account repetition) across all resistance positive antibiotics. Gene identities were confirmed with NCBI BLASTx, and gene names present within the Comprehensive AR Database (16) were identified as known AR genes. For each resistance positive antibiotic, we identified the gene with the highest coverage as the most probable resistance gene when multiple genes were located within close proximity respective to the reference strain. After taking this into account, we found a total of 78 AR genes for the analysis (Fig. 3). 81% of the 78 AR genes were associated with at least one other gene, demonstrating that the majority of plasmid inserts harbored more than one gene.

For individual plasmid extractions, Sanger sequencing was used to identify genes causing resistance at concentrations above the MIC. SL1 and SR2 primers (Lucigen) were used at 5 uM for sequencing. Gene identities were confirmed with NCBI BLASTx, and gene names present within the Comprehensive AR Database (16) were identified as known AR genes. Sequencing yielded 2 genes for each plasmid extraction, totaling 18 unique genes. If a beta-lactamase gene was either of the 2 genes, we identified the beta-lactamase as the most probable resistance gene. After taking this into account, we found 15 AR genes for the analysis (Fig. 6).

Fig. 1: Gene amplification assay we developed to test for latent and cryptic antibiotic resistance.

Results and Discussion

Through a gene amplification approach, we manipulated *E. coli* by randomly cloning genes into a high copy vector and then reintroducing the vector into the *E. coli* host (Fig. 1). Utilizing a high copy number plasmid increases the gene copy number and thereby increasing the template for expression. AR clones were then selected by plating on 18 antibiotics spanning 8 antibiotic classes (Table 1). First, we evaluated the minimum concentration of antibiotic needed to inhibit the growth of the wildtype strain (*E. cloni*, a laboratory *E. coli* strain). These concentrations were then used to screen clones for cryptic resistance. In this study, "resistance" is in reference to the wildtype strain and means that clones were able to grow at a concentration at which the wildtype was inhibited. Clones were subsequently tested for their MIC by plating transformants on antibiotic concentrations higher than the wildtype's MIC (Table 2). Plasmid inserts were sequenced from resistant clones and compared to the Comprehensive AR Database (16). We conducted a quantitative analysis of latent AR genes according to their functional categories (Fig. 2 and 3) and then qualitatively analyzed latent AR genes shared between antibiotic classes (Fig. 4 to 6). We analyzed unclassified (cryptic) AR genes and antibiotic characteristics that led to latent and cryptic antibiotic resistance. We then examined the relation between antibiotic origin (natural, semisynthetic, synthetic) and resistance (Fig. 7 and 8). Thus, we were able to systematically characterize genes that conferred an AR phenotype when amplified in *E. coli* via this gene amplification assay.

Table 1: Total antibiotics tested and their respective properties

Biochemical property	Site of action	Class and subclass	Origin	Antibiotic (Ab)	Ab concentration[a]	Resistance[b]	No. of clones[c]
Hydrophilic	Cell wall	Beta-lactams					
		Penicillins	Natural	Penicillin	64	+	16
		Cephalosporins	Semisynthetic	Ampicillin	8	+	50
				Cephalothin	32	+	15
				Cefoxitin	64	–	0
				Cefotaxime	0.25	+	67
				Cefepime	0.125	+	45
		Monobactams	Synthetic	Aztreonam	0.25	+	19
		D-cycloserine	Natural	D-cycloserine	32	+	24
Amphipathic	Cytoplasmic membrane	Polymyxins	Natural	Polymyxin B	0.5	+	>1,000
Hydrophobic	Protein synthesis	Chloramphenicol	Synthetic	Chloramphenicol	8	+	16
		Aminoglycosides	Natural	Gentamicin	4	–	0
			Semisynthetic	Amikacin	16	–	0
		Tetracyclines	Natural	Tetracycline	4	–	0
			Natural	Chlortetracycline	4	–	0
			Semisynthetic	Doxycycline	4	–	0
	DNA synthesis	Fluoroquinolones	Synthetic	Nalidixic Acid	4	–	0
			Synthetic	Norfloxacin	0.125	–	0
		Nitrofurans	Synthetic	Nitrofurantoin	1	–	0

[a]The minimum concentration of antibiotic (mg/ml) needed to inhibit the growth of *E. cloni* cells (Lucigen). This concentration (MIC) was used to screen clones for cryptic antibiotic resistance.

[b]If resistance occurred in our study, this is denoted as "+".

[c]The number of colonies that appeared if resistance occurred at the MIC.

Resistance occurred in response to 50% of the antibiotics tested. Antibiotics included chloramphenicol, D-cycloserine, polymyxin B, and six beta-lactams (Table 1). Known AR genes (i.e., CARD positive) comprised 13% of the total identified genes whereas the majority (87%) of the identified genes were unclassified AR genes (i.e., CARD negative). Genes related to stress response and/or DNA repair conferred resistance to all resistance positive antibiotics (Fig. 2) and was highly represented (17-75% of genes for each antibiotic). However, many uncharacterized or hypothetical proteins conferred resistance to each positive antibiotic except for chloramphenicol and polymyxin B. Genes from all functional categories (beta-lactamase, efflux pump/transporter, membrane structure, stress response/DNA repair, hypothetical/uncharacterized, and miscellaneous) conferred resistance to beta-lactam antibiotics (Fig. 2 and 3). On the contrary, genes from only 2-4 functional categories conferred resistance to chloramphenicol, D-cycloserine, and polymyxin B. Genes

affecting membrane structure comprised 32% and 40% of genes conferring resistance

to beta-lactams and D-cycloserine, respectively (Fig. 3). This is possibly to alleviate the

stress on cell wall biosynthesis of beta-lactams and D-cycloserine (17). We observed

that a wide diversity of genes conferred resistance to beta-lactams, and stress

response/DNA repair genes conferred resistance to all resistance positive antibiotics.

Table 2: Total number of clones when testing concentrations above the MIC

Antibiotic (Ab)	Ab concentration MIC[a]	No. clones MIC[b]	No. clones 2× MIC[b]	No. clones 4× MIC[b]	No. clones 8× MIC[b]
Penicillin	64	16	8	0	0
Ampicillin	8	50	9	5	0
Cephalothin	32	15	4	2	0
Cefoxitin	64	0			
Cefotaxime	0.25	67	1	0	0
Cefepime	0.125	45	0	0	0
Aztreonam	0.25	19	3	0	0
D-cycloserine	32	24	0	0	0
Polymyxin B	0.5	>1,000	0	0	0
Chloramphenicol	8	16	0	0	0
Gentamicin	4	0			
Amikacin	16	0			
Tetracycline	4	0			
Chlortetracycline	4	0			
Doxycycline	4	0			
Nalidixic acid	4	0			
Norfloxacin	0.125	0			
Nitrofurantoin	1	0			

[a]The minimum concentration of antibiotic (*m*g/ml) needed to inhibit the growth of *E. cloni* cells (Lucigen). This concentration (MIC) was used to screen clones for cryptic antibiotic resistance.

[b]The number of clones indicates the number of colonies that appeared if transformants showed resistance at the MIC, 2x the MIC, 4x the MIC, and 8x the MIC.

We next identified AR genes shared between antibiotic classes (Fig. 4). The

genes conferring resistance to more than one antibiotic class (multiple beta-lactams and

chloramphenicol) when amplified were *soxS* and those in the *marRAB* operon. These

are known AR genes that encode transcriptional regulators for general stress responses

(18). When overexpressed, they may activate the multidrug efflux pump AcrAB and

decrease expression of porin OmpF to decrease cell permeability. In contrast, there were no unclassified AR genes that conferred resistance to more than one antibiotic class, suggesting that cryptic antibiotic resistance may stem from a certain gene response specific to the antimicrobial mechanism of action.
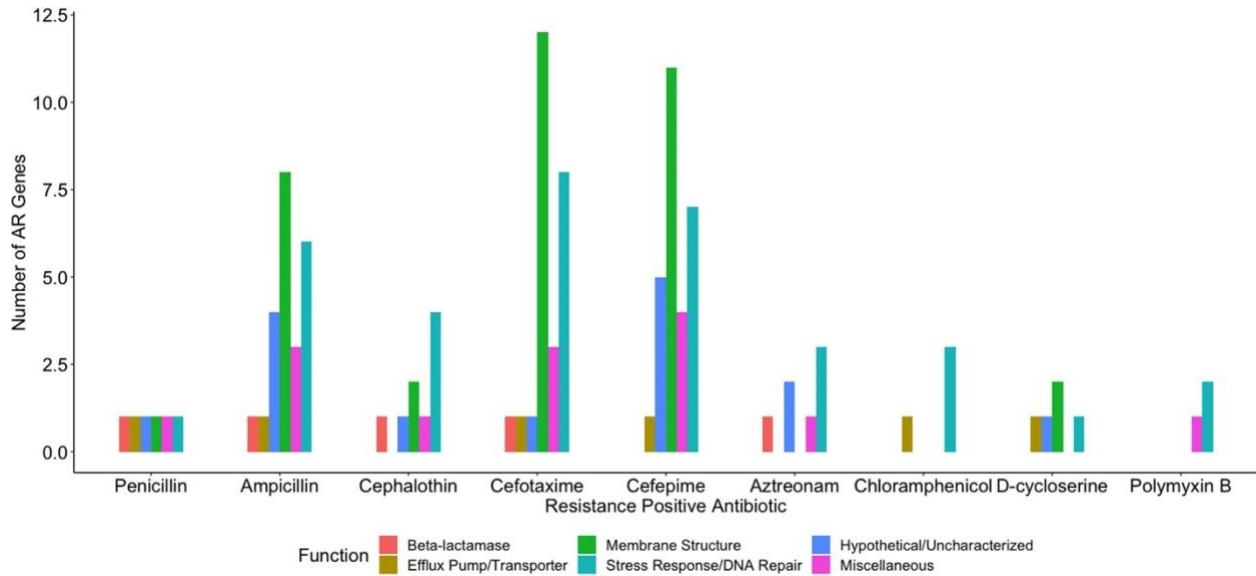
Fig. 2: Number of antibiotic resistance genes conferring latent resistance to antibiotics at the minimum inhibitory concentrations. Penicillin-aztreonam are beta-lactams.
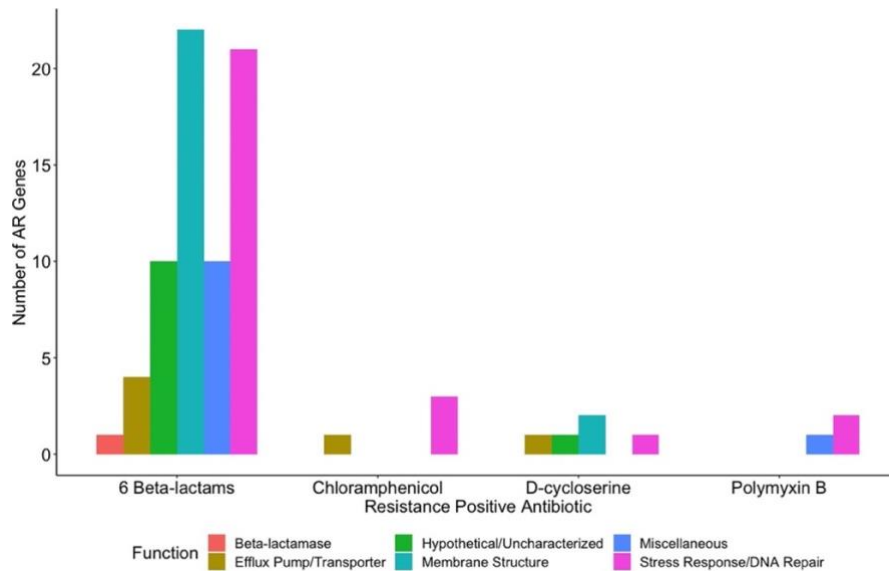
Fig. 3: Number of antibiotic resistance genes conferring latent resistance to antibiotics at the minimum inhibitory concentrations, separated by class.

We uncovered a diversity of previously unclassified AR genes (CARD negative) that conferred cryptic resistance to D-cycloserine or polymyxin B (Figure 4). The *alaA* and *ddlA* genes, which encode glutamate-pyruvate aminotransferase and D-alanine-D-alanine ligase A, respectively, conferred resistance to D-cycloserine when amplified in our study. Inhibiting the biosynthesis of amino acids integrated within the Gram negative peptidoglycan peptide stem has been investigated as a putative approach for novel antibiotics (19), and *alaA* and *ddlA* hold an important role for L- alanine and D-alanine synthesis, respectively, in *E. coli*. *ddlA* has long been known to be the target gene for D-cycloserine (20).
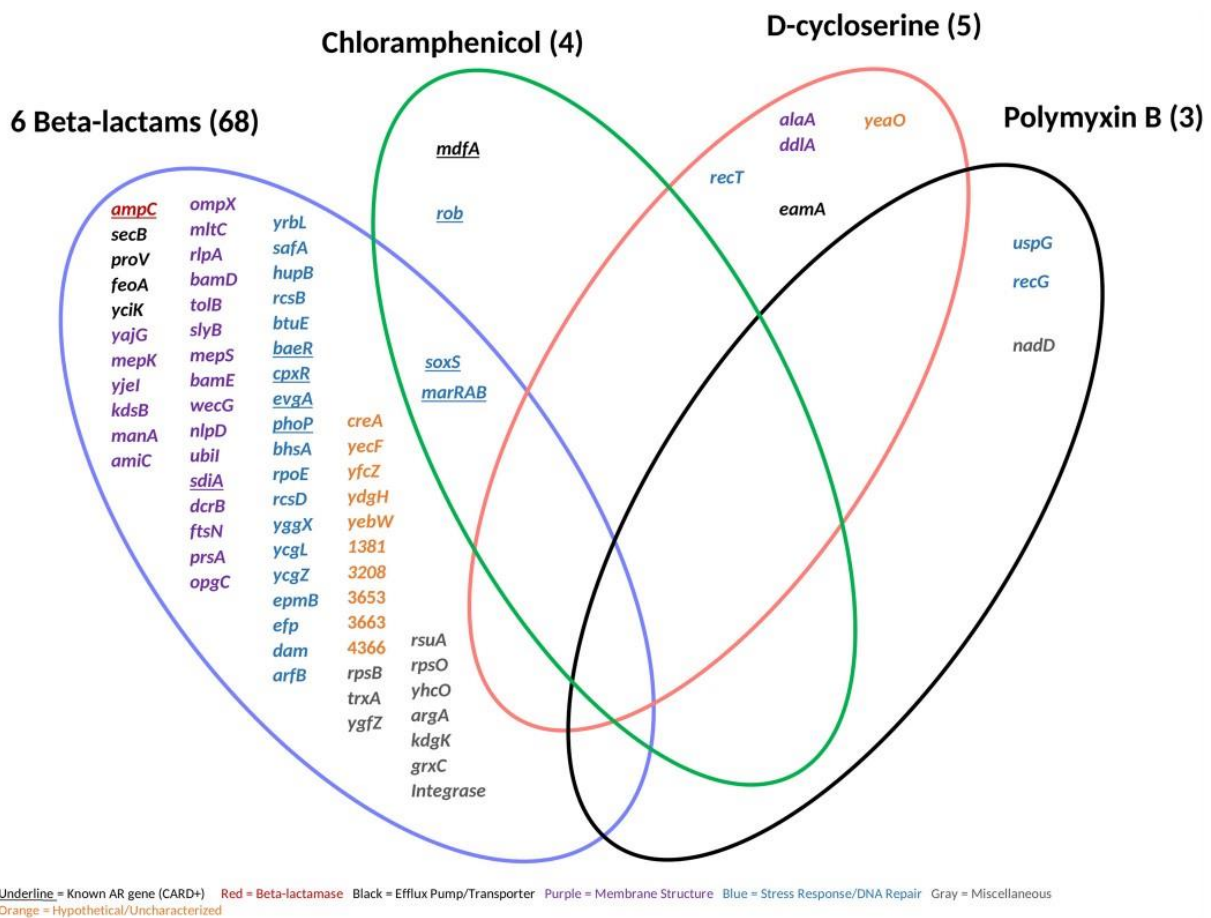
Fig. 4: Antibiotic resistance genes shared between all resistance positive antibiotics (9) separated by class. We identified 78 antibiotic resistance genes (shown) causing resistance at the minimum inhibitory concentrations. Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.

This was the only case in which gene amplification of the antibiotic target gene conferred latent resistance in our study. Similarly, this occurred to only 4 out of 31 antibiotics when observing for latent antibiotic resistance in a previous study (5). Overexpression of target genes occurs primarily to antimicrobial agents that act on a single target gene. This is less common for Gram negative antibiotics as they usually inhibit a family of related enzymes or act on non-protein targets such as the cytoplasmic membrane (21). For example, many beta-lactams bind multiple targets (penicillin binding proteins), which catalyze peptidoglycan cross-linking; polymyxins disrupt the integrity of the cytoplasmic membrane. Here, *eamA* (previously named *ydeD*) also conferred D-cycloserine resistance. Although this is an unclassified AR gene we have identified in this study, EamA is an exporter classified within the Drug/Metabolite Transporter Superfamily (22). A high copy number of DNA repair proteins RecT and RecG conferred resistance to D-cycloserine and polymyxin B, respectively in our study. RecT has not been previously linked to resistance to our knowledge, but RecG has been shown to decrease polymyxin B susceptibility when upregulated in *P. aeruginosa* (23). Here, universal stress protein G, UspG, also conferred resistance to polymyxin B, and it has been previously shown to be regulated during colistin (a polymyxin drug) treatment in *E. coli* (24). *nadD*, which encodes an essential enzyme involved in both the *de novo* biosynthesis and salvage of NAD+ and NADPH (25), also conferred resistance to polymyxin B in our assay. This gene has been shown to be a promising antimicrobial target with broad spectrum activity (25). No unknown AR genes conferred resistance to chloramphenicol in our assay. Genes within the stress response/DNA repair functional category have a broad AR potential as they conferred cryptic resistance to nearly all

14

positive antibiotics (Fig. 4 and Fig. 5). Even though some of the genes identified have been previously linked to antibiotic resistance, they have not been established as the culprit of resistance. This assay demonstrates that these unknown genes conferred an AR phenotype when present in high copy number.
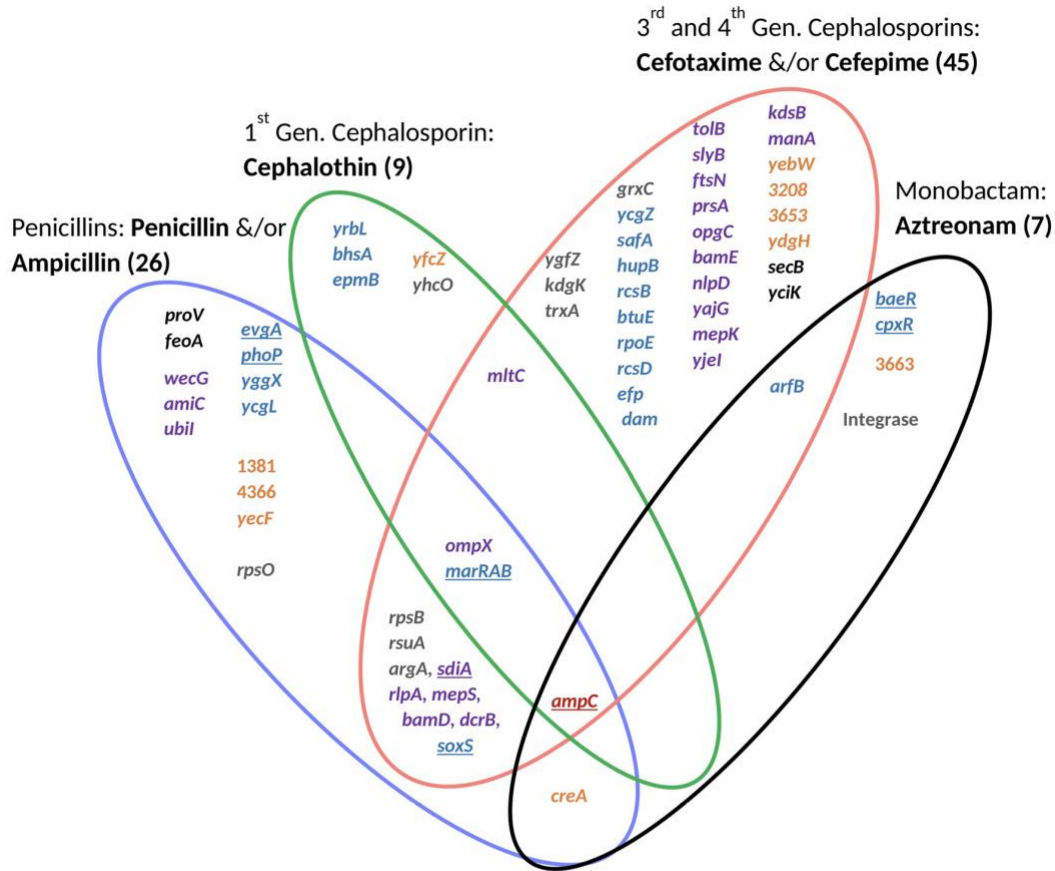
Underline = Known AR gene (CARD+)   Red = Beta-lactamase   Black = Efflux Pump/Transporter   Purple = Membrane Structure   Blue = Stress Response/DNA Repair   Gray = Miscellaneous
Orange = Hypothetical/Uncharacterized

Fig. 5: Antibiotic resistance genes shared between 6 resistance positive beta-lactam antibiotics separated by subclass and/or generation. We identified 68 antibiotic resistance genes (shown) conferring beta-lactam resistance at the MICs. Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.

We also found a diversity of unknown AR genes that conferred cryptic resistance to beta-lactam antibiotics (Fig. 4 and 5). The majority (6 out of 11) of the unclassified AR genes that conferred resistance to multiple generations of beta-lactams had functions

related to membrane structure. Three genes [*rlpA* (26), *mepS* (27), and *mltC* (26)] were related to cell wall/peptidoglycan recycling. Even though these genes are not antibiotic targets, *rlpA* is directly upstream *dacA,* which encodes penicillin binding protein 5 (Pbp5) (28). A Pbp5 associated protein has shown to increase cephalosporin resistance when overexpressed in *Enterococcus faecium* (29). Therefore, genes associated or in close proximity to Pbp5 may be capable of conferring cryptic resistance when amplified. Outer membrane protein X, encoded by *ompX*, also conferred resistance to multiple beta-lactams in our assay. Overexpression of *ompX* can repress expression of OmpC and OmpF porins and lead to a decreased susceptibility to beta-lactams (30). We also saw that genes related to maintaining cell membrane permeability and integrity conferred resistance to the penicillins and cephalosporins (Fig. 5). These proteins included WecG (31), UbiI (32), TolB (33), SlyB (34), PrsA (35), OpgC (36), KdsB (37), ManA (38), AmiC (39), NlpD (39), YajG (40), and MepK (41). The latter four proteins are involved in cell wall synthesis and recycling. We found that at least one stress response gene conferred resistance to each beta-lactam antibiotic, and many of these genes were associated with a global stress response and/or two-component regulatory systems. For example, *ycgL*, which conferred resistance to the penicillins in our assay is a gene that is potentially regulated by SOS, a global response to DNA damage (42, 43). Similarly, *yrbL*, which conferred resistance to cephalothin, is regulated by PhoP, a part of a two-component system that senses and responds to a variety of environmental changes (44, 45). The PhoP/PhoQ system is activated by *safA* (46), which conferred resistance to cefotaxime in our study. The PhoP/PhoQ system is connected to the EvgS/EvgA two-component system, and *safA*, the "connector," connects these two

systems (47). Here, a high copy number of YcgZ led to cefepime resistance, and this protein interacts with the Rcs two-component regulatory system while being regulated by *marA* (48). The Rcs system consists of the response regulator RcsB and phosphotransferase RcsD (49), and these proteins conferred resistance to cefotaxime and cefepime, respectively, in our assay. *creA,* which conferred resistance to multiple beta-lactams here, has an uncharacterized function, but it is adjacent to the CreBC two-component regulatory system (50). We saw that more hypothetical proteins conferred beta-lactam resistance than unclassified AR efflux pumps/transporters when amplified. The low number of efflux pumps/transporters causing latent resistance could be due to the antibiotic target site and cell structure (51, 52). Beta-lactams do not need to cross the cytoplasmic membrane to reach their target and thereby face the Gram negative cell wall as their primary barrier. We found that beta-lactam antibiotic resistance was dominated by genes related to the cell wall and general stress transcriptional regulators.

We observed more genes conferring latent resistance to semisynthetic antibiotics compared to natural or synthetic (Fig. 7 and Fig. 8). There were most semisynthetic resistant positive antibiotics [4], compared to natural [3] or synthetic [2] antibiotics. There is a significant difference between the antibiotic origin groups (P <0.05) driven by a difference between the semisynthetic and synthetic antibiotic groups (P <0.05). We had predicted that latent resistance would be less common in the presence of synthetic antibiotics due to their stronger activity, but this was not the case. Semisynthetic antimicrobials are generally made to act against bacteria that developed resistance to the prior generation (53), suggesting that semisynthetic antibiotics can be specifically optimized to prevent resistance. *E. coli* may be more capable of developing latent

resistance to semisynthetic antibiotics, specifically beta-lactams as the resistance positive semisynthetic antibiotics consisted of cephalosporins. 35% of genes conferring resistance to semisynthetic antibiotics were related to membrane structure (Fig. 7). This is likely to lessen the impact of cephalosporins on cell wall biosynthesis. Cell membrane related genes did not confer latent resistance to synthetic antibiotics in our study even though a beta-lactam was present (Fig. 7 and Fig. 8). This potentially indicates that synthetic antibiotics can overcome the effect of highly amplified cell membrane genes.

Underline = Known AR gene (CARD+) Red = Beta-lactamase Black = Efflux Pump/Transporter Purple = Membrane Structure Blue = Stress Response/DNA Repair Gray = Miscellaneous Orange = Hypothetical/Uncharacterized

Fig. 6: Antibiotic resistance genes conferring resistance above the MICs. Genes are shared between five resistance-positive beta-lactam antibiotics separated by subclass and/or generation. We identified 15 antibiotic resistance genes (shown). Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.

29-55% of AR genes for all three origins were related to stress response/DNA repair, highlighting the broad AR potential for this functional category. This study did not determine a link between antibiotic origin and latent resistance as most antibiotics from each origin were beta-lactams, showing a stronger link between antibiotic mechanism of action and latent resistance.
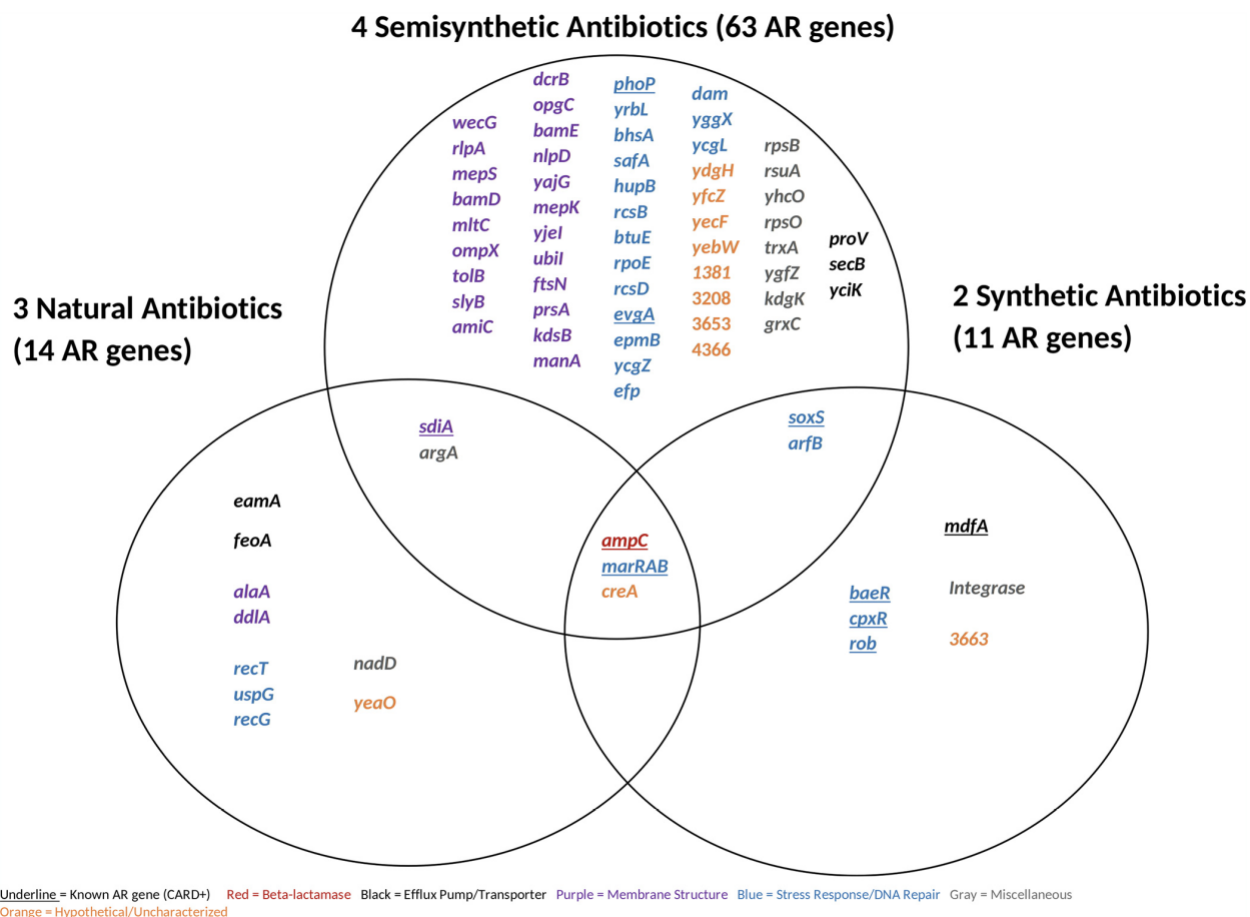
Fig. 7: Antibiotic resistance genes shared between resistance positive antibiotics classified by origin. We identified 78 antibiotic resistance genes causing resistance at the minimum inhibitory concentrations. Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.
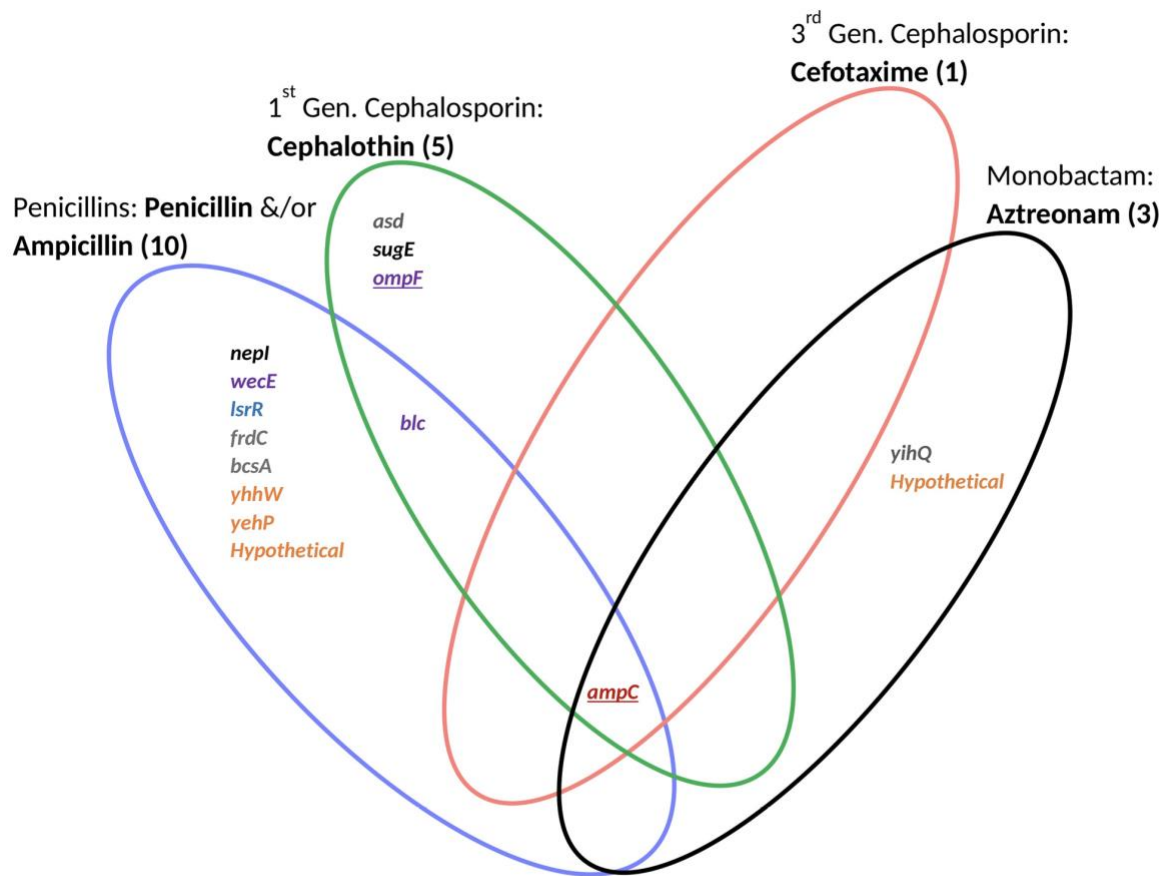
Certain antibiotic characteristics may contribute to cryptic and/or latent antibiotic resistance. Resistance occurred to nearly all beta-lactams tested, chloramphenicol, D-

cycloserine, and polymyxin B. Beta-lactams (51) and D-cycloserine (54, 55) are hydrophilic and inhibit cell wall biosynthesis. Polymyxin B inhibits the cytoplasmic membrane and is amphipathic (56). The Gram negative bacteria outer membrane acts a first defense mechanism against antibiotics due to the hydrophobic lipid bilayer and specifically sized aqueous pores (51, 52, 57). Antibiotics can penetrate the outer membrane by dissolving in the lipid bilayer or crossing through the pores, the hydrophobic or hydrophilic mechanism, respectively (51, 52). Antibiotics with targets on the outer surface of the cytoplasmic membrane (exposed) need to cross the lipid matrix, facing the outer membrane barrier (52). Beta-lactams and polymyxin B have exposed targets, while D-cycloserine, although hydrophilic, needs to permeate the outer and cytoplasmic membrane to reach its' target. Hydrophobic antibiotics usually need to penetrate the outer and cytoplasmic membrane since their target is generally involved with DNA or protein synthesis (52). Therefore, it may be biochemically simpler for *E. coli* to inhibit antibiotics with hydrophilic properties as opposed to hydrophobic antibiotics. Resistance did not occur in the presence of nitrofurans, fluoroquinolones, tetracyclines, and aminoglycosides which are all hydrophobic (Table 1) and need to cross the cytoplasmic membrane to reach their target (52, 58, 59). Resistance also occurred to chloramphenicol which is noteworthy because it is now synthetically made and hydrophobic (60), but only known AR genes conferred resistance to it in our study (Fig. 4). We found that beta-lactams, hydrophilic antibiotics, and those that inhibit the cell wall or cytoplasmic membrane were more likely to induce latent resistance in *E. coli*.

We captured many known AR genes (Fig. 4), suggesting this is a robust approach to examine the effect of gene amplification on latent resistance profiles. Gene

amplification of *ampC* conferred latent and higher ranges of resistance to all beta-lactams except for cefoxitin and cefepime (Fig. 5 and 6), second and fourth generation cephalosporins, respectively (61). There may have been no resistance to cefoxitin as it is stable against *ampC* activity and cefepime is a weak substrate for *ampC* (62). This gene is encoded on the chromosomes of many *Enterobacteriaceae*, but it is commonly weakly expressed (62). *sdiA,* which encodes a cell division regulator and activates AcrAB multidrug efflux pump (63), conferred resistance to multiple generations of beta-lactams in our assay (Fig. 5). Gene amplification of the two-component regulatory systems BaeSR, CpxAR, EvgAS, and PhoPQ also conferred resistance to multiple beta-lactams. Two-component systems, which activate responses to environmental stress, are known to increase antibiotic resistance via several mechanisms including upregulation of multidrug efflux pumps and changes in cell permeability (64). A multidrug efflux pump, MdfA, conferred resistance to chloramphenicol in our study (Fig. 4). MdfA was originally classified as the CmlA/Cmr chloramphenicol exporter (65), further validating the chloramphenicol resistance phenotype. We observed that the amplification of *soxS, rob*, and genes from the MarRAB operon conferred resistance to several beta-lactams and/or chloramphenicol. These genes encode transcriptional regulators for general stress signals such as oxidative stress, acidic pH, and antibiotics (18). When upregulated, they may activate the multidrug efflux pump AcrAB and repress expression of porin OmpF to decrease cell permeability (18). However, high copy numbers of *soxS, rob*, and genes from the MarRAB operon did not confer resistance above the MIC in this study (Fig. 6), showing that these genes are limited in their resistance potential. The identification of known AR genes validated this method as

an effective way to test for latent resistance genes in a high throughput manner.
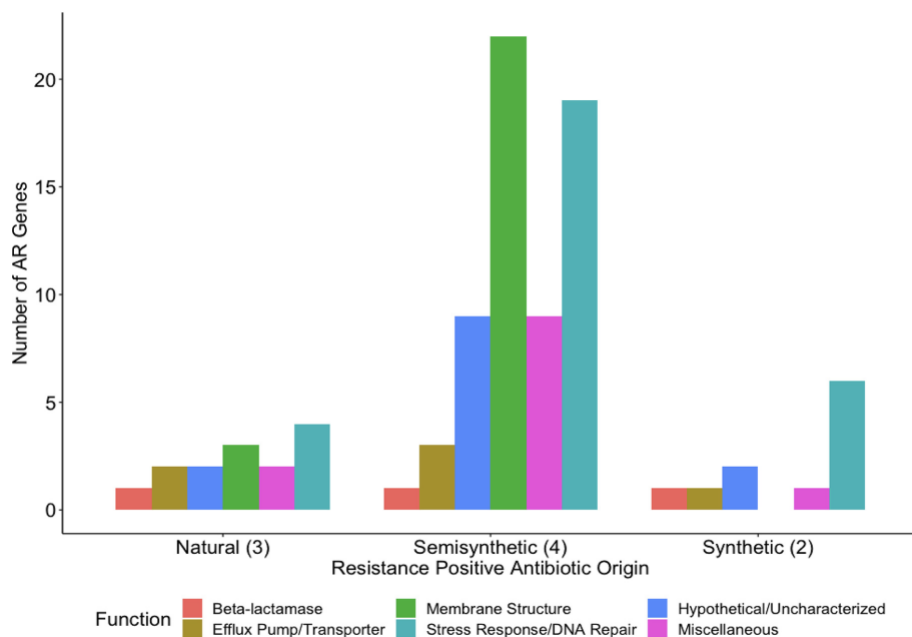
Fig. 8: Number of antibiotic resistance genes conferring latent resistance to antibiotics at the MICs, classified by origin. The Kruskal Wallis Rank Sum Test determined a significant difference between antibiotic origin groups (P <0.05). The Dunn test was used post-hoc to determine which pairs of groups are different. There is a significant difference in the number of antibiotic resistance genes between the semisynthetic and synthetic antibiotic groups (P <0.05).

A caveat of this study is that our assay cannot discern multiple or complex gene regulation. First, this assay is unable to capture mechanisms, whereby two genes are required for resistance but are not co-located. Second, this assay did not distinguish coregulation that occurred between genes present on the plasmid insert. In these cases, we took a probabilistic approach and called the gene with the highest coverage as the putative AR gene. Even though coverage varied across antibiotics for some genes, we chose the gene with the highest coverage to maintain consistency and accuracy. Opting for smaller insert sizes (1-2 kb) may cause less co-occurrence of genes. Due to the high frequency (81%), most gene calls were subject to co-occurrence, but choosing the gene with the highest coverage ensures that the most

probable AR gene was identified. Last, it is also a caveat that this study cannot yet be interpreted in a clinical sense as our MIC methodology is not clinically standard. We needed to replicate the approach (LB agar plate) that we used to screen clones for cryptic antibiotic resistance, as our main goal was to demonstrate the biological mechanism more than the clinical relevance. Our study demonstrates that *E. coli* is capable of increasing the concentration of antibiotic for which they can grow in. Translation for a clinical setting would require further examination of the inhibition concentrations using a clinical standard.

A diverse repertoire of latent AR genes may be a widespread phenomenon among bacteria. Microbiomes from humans (66), sea gulls (67), soil (68, 69), river (70), and ocean water (71) have shown to be reservoirs of diverse known and unknown AR genes. Even though these functional metagenomic assays were used to survey AR genes in a certain environment, this technique can also be used to identify silent resistance genes which are capable of conferring resistance when amplified in other hosts but not in their native context (72). Thus, the presence of cryptic genes activated by gene amplification may be a widespread phenomenon. However, the use of a surrogate host to identify resistance genes can confound results as phenotypic resistance in donor strains may not translate to resistance in the native genomic context. Therefore, we have developed an assay that circumvents this limitation and expresses genes in the organism of interest. The diversity of microbes, which appears to be the principle of latent resistance, suggests this could be important for the emergence of resistance to antibiotics. As this platform is used on other pathogens, a predictive model could be built to classify types of antibiotics and organisms that are

less likely to promote latent resistance while also identifying novel antibiotic resistance genes.

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CHAPTER 2

Intraspecific variation in antibiotic resistance potential

Stacy A. Suarez and Adam C. Martiny

Introduction

The antibiotic resistance crisis is fueled by the rapid evolution and dissemination of resistance genes (1). To effectively mitigate this threat to human health (2, 3), it is important to identify and characterize antibiotic resistance (AR) genes as well as bacterial resistance mechanisms. Antibiotic resistance typically emerges due to acquired, intrinsic, or adaptive resistance mechanisms (4). Acquired resistance pertains to chromosomal mutations or the assimilation of genetic elements, while intrinsic resistance characterizes the innate properties of a bacterium to circumvent the impact of antibiotics. Adaptive resistance, caused by changes in gene expression, occurs in response to environmental conditions, such as antibiotic exposure. Latent resistance is a form of adaptive resistance and can occur from the activation of unknown (cryptic) AR within the cell (5–10). Thus far, few studies have looked at the link between antibiotic resistance and the upregulation of cryptic AR genes among micro-diverse lineages (5– 10).

Intraspecific variation, which includes the genomic diversity found within populations, can correspond to variation in a wide range of functional traits including antibiotic resistance (11). Intraspecific genomic diversity furthers the potential for an unreported and diverse reservoir of latent AR genes in pathogens, because cryptic resistance can occur without major mutations or horizontal transmission. Large differences in genome content have been shown among closely related *E. coli* strains

(12). For example, one paper detailed that three distinct *E. coli* strains shared about 40% of genes, and two of the three were clinical, pathogenic strains. These two clinical strains were as different from each other as they were from the non-pathogenic strain. The acquisition of genomic islands encoding virulence factors led to pathogenicity in the clinical strains (12).

One approach to investigate intraspecific latent antibiotic resistance has been functional metagenomics. Microbiomes from humans (13, 14), sea gulls (15), soils (16, 17), rivers (18), and ocean water (19) have revealed reservoirs of diverse known and unknown latent AR genes. Functional metagenomics is an efficient and powerful technique for AR gene detection (20, 21), due to three key advantages: (i) no need to culture microorganisms apart from donor strain, (ii) no prior knowledge required about resistance gene sequence, and (iii) direct association between a genotype and a demonstrated resistance phenotype (22). Functional metagenomics uses a surrogate host to identify resistance genes, but this can confound results as phenotypic resistance in donor strains may not translate to resistance in the native genomic context.

To overcome this limitation, we have developed an assay that circumvents this limitation and expresses genes in the organism of interest (10). Delineating the intraspecific potential for cryptic antibiotic resistance is important to further elucidate the emergence of antibiotic resistance. We use our method to test the hypothesis that there is a highly diverse reservoir of cryptic latent AR genes between strains of the same species that confer an AR phenotype when upregulated. We therefore predict that strain origin will affect which genes and antibiotics induce resistance. For example, we expect that known resistance genes will primarily cause latent resistance within clinical strains.

Additionally, we predict that cryptic resistance will occur to hydrophilic antibiotics due to the highly hydrophobic outer membrane in *E. coli* (10). Here, we use a functional metagenomics assay that induces a large increase in gene copy number to assay intraspecific variation in AR potential. We specifically ask: (i) what are the groups of orthologous genes (orthogroups) among *E. coli* strains that confer an AR phenotype when upregulated and (ii) how do strain and antibiotic origin affect which orthogroups induce latent resistance in this manner?

Methods

Strains, Media, and Culture Conditions

E. cloni 10G Supreme cells (Lucigen, Middleton, WI, USA), *E. coli* 40B, and *E. coli* 72 were grown in Luria-Bertani (LB) media and incubated overnight in 37°C unless otherwise stated. *E. coli* 40B and *E. coli* 72 were isolated from the blood of babies with bacteremia at the Children's Hospital Orange County. In addition, genomic DNA from the following strains was obtained from the American Type Culture Collection (ATCC): *E. coli* FDA strain Seattle 1946, *E. coli* H10407, *E. coli* Crooks, *E. coli* RMID 0509952, *E. coli* AMC 198. We assessed latent antibiotic resistance in eight strains by transforming fractions of their DNA into *E. cloni*.

Resistance Profile

The minimum concentration of antibiotic needed to inhibit (MIC) the growth of 10^6 *E. cloni* cells was determined for all antibiotics (Table 1) as described in (10). The listed antibiotics were tested to include a range of classes (mechanisms of action) and origins (natural, semisynthetic, or synthetic) if available.

Cloning and Screening

       The following methods were completed separately for each strain (10). Genomic DNA was extracted from *E. cloni, E. coli* 40B*,* and *E. coli* 72 cells using the Wizard Genomic DNA purification Kit (Promega Corporation, Madison, WI, USA). At least 5 micrograms of genomic DNA from each strain (including the ATCC strains) were sheared to a target size of 2 kb using a Covaris S220 Focus Acoustic Shearer (Covaris Inc., Woburn, MA, USA). Fragments of 1 to 3 kb were extracted from a 1% agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA was treated with the NEBNext End Repair Module to create blunt ends on the fragmented DNA (New England Biolabs, Ipswich, MA, USA). The end-repaired DNA was purified using the DNA Clean and Concentrator-10 kit (Zymo Research). DNA was ligated into pSMART-HCKan vector (accession number AF532107) and then electroporated into *E. cloni* cells as per the CloneSmart Blunt Cloning Kit (Lucigen). Transformed cells were recovered at 37 °C for 1 h.

       To test for cryptic antibiotic resistance, 150 µL of undiluted recovered transformants was plated on LB Lennox kanamycin agar containing one of eighteen antibiotics (Table 1). After overnight incubation, resistant transformants were pooled for each antibiotic using 1-2 mL of phosphate-buffered saline (PBS). Pooled plasmid DNA was extracted from each PBS suspension (1 from each resistance positive antibiotic) using the ZR Plasmid Miniprep kit (Zymo Research) and stored in -20°C. Plasmid inserts containing latent AR genes were amplified via polymerase chain reaction (PCR). This PCR used 25 µL reactions, including 12.5 µL of AccuStart II PCR SuperMix 2X (Quantabio), 3 µL (1.5 ng) of plasmid DNA, 4.5 µL of nuclease-free water, and 2.5 µL of

SL1 and SR2 primers (Lucigen). The reaction cycle conditions follow those delineated for AccuStart II PCR SuperMix 2X (Quantabio). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified using the Invitrogen Qubit fluorimeter (Thermo Fisher Scientific). A low quantity of DNA was generated from cephalothin and polymyxin B resistant clones, suggesting that these were not plasmid containing colonies. Therefore, these clones were excluded from subsequent analysis and sequencing for all strains.

Library Preparation, Sequencing, and Analysis

For pooled plasmids (separated by strain), library preparation and sequencing were performed (10). Sequencing was done on the MinION flow cell (FLO-Min106 R9.4.1 version; Oxford Nanopore Technologies) using the MinION device (Mk1B version). Base-calling was done in real-time using MinKNOW software (Oxford Nanopore Technologies) on a local computer. Each sequencing run was carried out for about 15 hours, and the barcoded base-called reads were demultiplexed by MinKNOW during the sequencing run. Demultiplexed reads were trimmed post-sequencing using MinKNOW to remove barcodes. Trimmed reads were aligned and mapped to their respective *E. coli* reference genome using Bowtie 2 (23). Mapped reads were assembled and processed with Anvi'o (24) which provided coverage, identity, and location within the reference strain for each aligned gene.

We fit a gamma distribution to gene coverage values and selected genes that had coverage within the 95% confidence interval as putative resistance genes. Gene coverage values were normalized by total coverage values for each resistance positive antibiotic prior to obtaining the confidence interval. Gene identities were confirmed with

38

NCBI BLASTx, and gene names present within the Comprehensive AR Database, CARD (25), were identified as known AR genes. Gene names not present within CARD were designated as cryptic/unknown AR genes. Latent AR genes include known and cryptic AR genes. For each resistance positive antibiotic, we identified the gene with the highest coverage as the most probable resistance gene when multiple genes were located within close proximity in the respective reference strain (i.e., the eight *E. coli* strains). After taking this into account, we found a total of 66 individual AR genes across all resistance positive antibiotics from all strains.

OrthoFinder 2.0 (26) was used to find groups of orthologous genes (orthogroups), and the Interactive Tree of Life v5 (27) was used to build the phylogenetic tree showing the genetic relatedness among all strains (Fig. 1). For heatmap hierarchical clustering of orthogroups and strains (based on positive antibiotic resistance genes), R's "ggdendro" package was used. The "ggplot2" package was used for displaying the heatmap dendrogram clustering. To determine the correlation between the dendrograms generated based on phylogeny (Fig. 1) and resistance profile (Fig. 5), R's "vegan" package was used to perform the Mantel test.

Table 1: Total antibiotics tested and their respective properties

Biochemical Property	Site of Action	Class and Subclass	Origin	Antibiotic	Antibiotic Concentration[a]
Hydrophilic	Cell Wall	Beta-lactam			
		Penicillins	Natural	Penicillin	64
		Cephalosporins	Semisynthetic	Ampicillin	8
				Cephalothin	32
				Cefoxitin	64
				Cefotaxime	0.25
				Cefepime	0.125
		Monobactams	Synthetic	Aztreonam	0.25
		D-cycloserine	Natural	D-cycloserine	32
Amphipathic	Cytoplasmic Membrane	Polymyxins	Natural	Polymyxin B	0.5
Hydrophobic	Protein Synthesis	Chloramphenicol	Synthetic	Chloramphenicol	8
		Aminoglycosides	Natural	Gentamicin	4
			Semisynthetic	Amikacin	16
		Tetracyclines	Natural	Tetracycline	4
			Natural	Chlortetracycline	4
			Semisynthetic	Doxycycline	4
	DNA Synthesis	Fluoroquinolones	Synthetic	Nalidixic Acid	4
			Synthetic	Norfloxacin	0.125
		Nitrofurans	Synthetic	Nitrofurantoin	1

[a]The minimum concentration of antibiotic (mg/ml) needed to inhibit the growth of *E. cloni* cells (Lucigen). This concentration (MIC) was used to screen clones from all *E. coli* strains for cryptic antibiotic resistance.

Results and Discussion

Through a modified functional metagenomics approach, we tested for the intraspecific potential of cryptic antibiotic resistance in eight *E. coli* strains (Fig. 1). The strains were chosen to represent clinical and laboratory origins. In this study, "resistance" meant that clones were able to grow at the MICs determined from the original host strain (*E. cloni*). We conducted a quantitative analysis of latent AR genes according to their functional categories and between strains. We examined the relation between cryptic/latent resistance and strain origin and antibiotic type. Thus, we characterized the intraspecific variation of the cryptic/latent AR potential by this gene amplification assay.
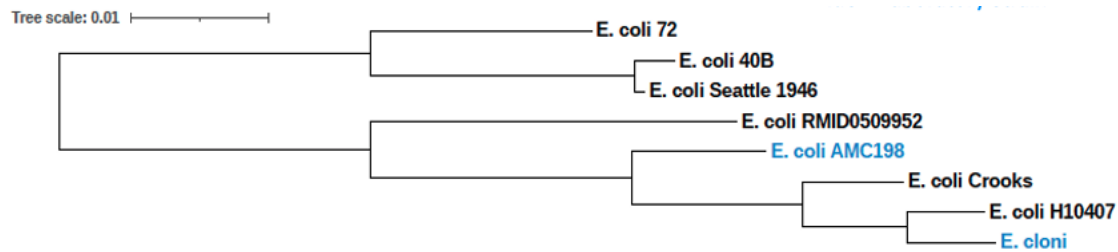
Fig. 1: Genetic relatedness between all strains tested for cryptic antibiotic resistance. The strains in blue are laboratory strains, and those in black are clinical strains.
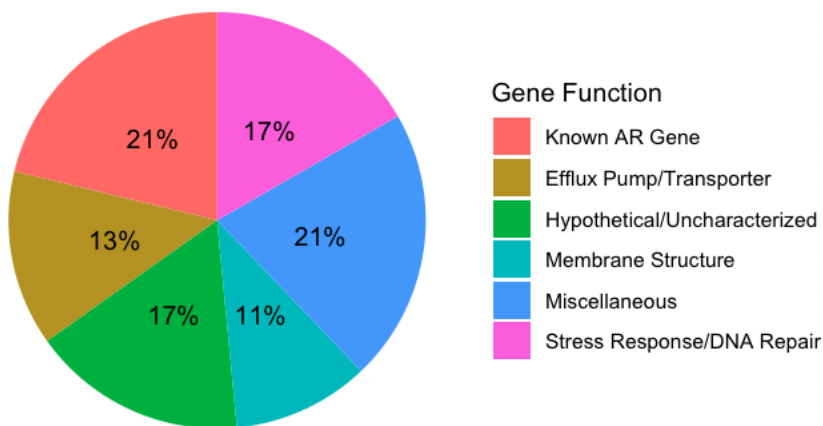
Fig. 2: Distribution of antibiotic resistance gene functional categories conferring latent resistance at the MICs. We identified a total of 66 individual resistance orthologous genes across all resistance positive antibiotics from all strains.

We observed a wide diversity of latent AR genes. We found a total of 66 individual genes conferring latent resistance to 11 out of 16 tested antibiotics. Known resistance types (CARD positive) comprised 21% of identified AR genes, whereas the majority (79%) of the identified AR genes were unclassified (CARD negative) (Fig. 2). Resistance positive antibiotics included chloramphenicol, D-cycloserine, nitrofurantoin, norfloxacin, tetracycline, and six beta-lactams (Fig. 3). Latent AR gene functions vary for each antibiotic, but genes related to stress response/DNA repair, known AR genes, or miscellaneous genes conferred resistance to the highest number of antibiotics.

Genes from all functional categories (known AR genes, efflux pump/transporter, hypothetical/uncharacterized, membrane structure, miscellaneous, and stress response/DNA repair) conferred resistance to the beta-lactam antibiotic class (Fig. 4). However, genes from at least three functional categories conferred resistance to each of the remaining resistance positive antibiotics, demonstrating the diversity of resistance mechanisms within a given antibiotic. Hypothetical/uncharacterized,
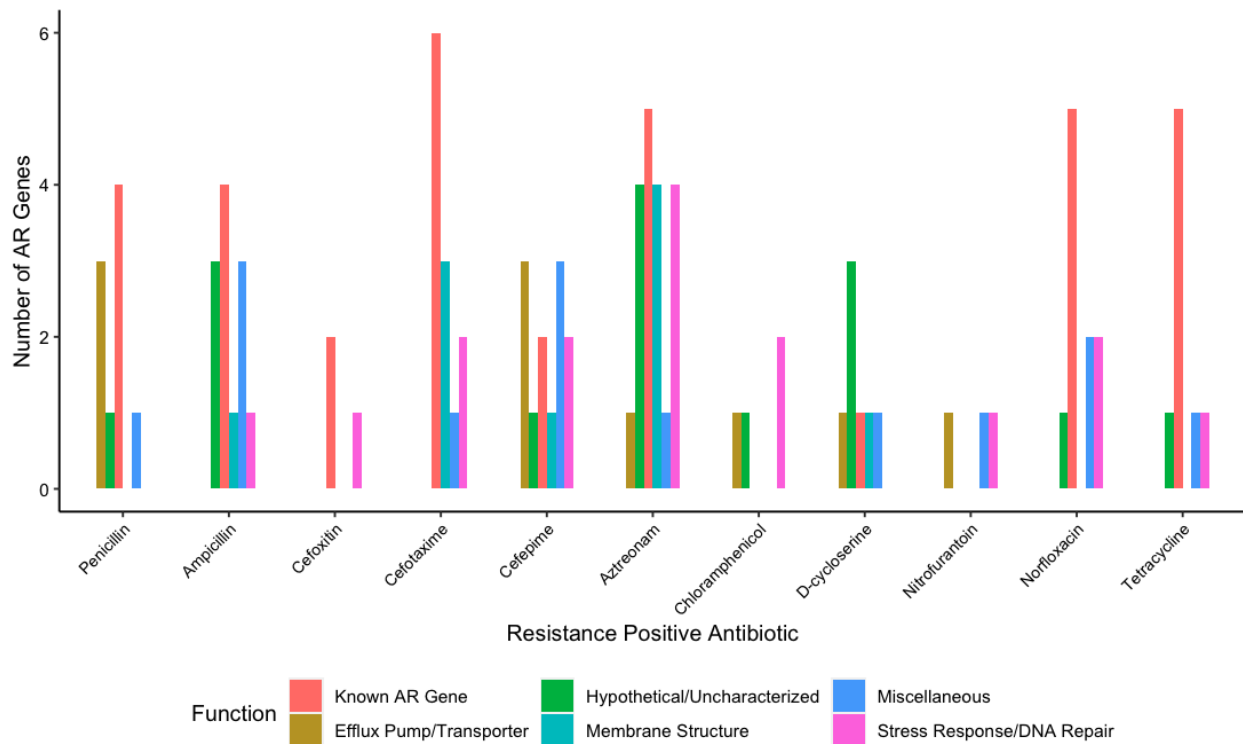
Fig. 3: Number of antibiotic resistance genes conferring latent resistance to antibiotics at the MICs. Penicillin-Aztreonam are beta-lactams.

stress response/DNA repair, and miscellaneous genes conferred resistance to all antibiotic classes except for nitrofurantoin, D-cycloserine, and chloramphenicol, respectively. Genes related to membrane structure conferred resistance to beta-lactams and D-cycloserine (Fig. 4). This result was expected as beta-lactams and D-cycloserine are the only resistance positive antibiotic classes that inhibit cell wall synthesis. Stress

response/DNA repair genes were highly represented (13-50%) of genes for each class (Fig. 4), demonstrating a wide resistance potential.
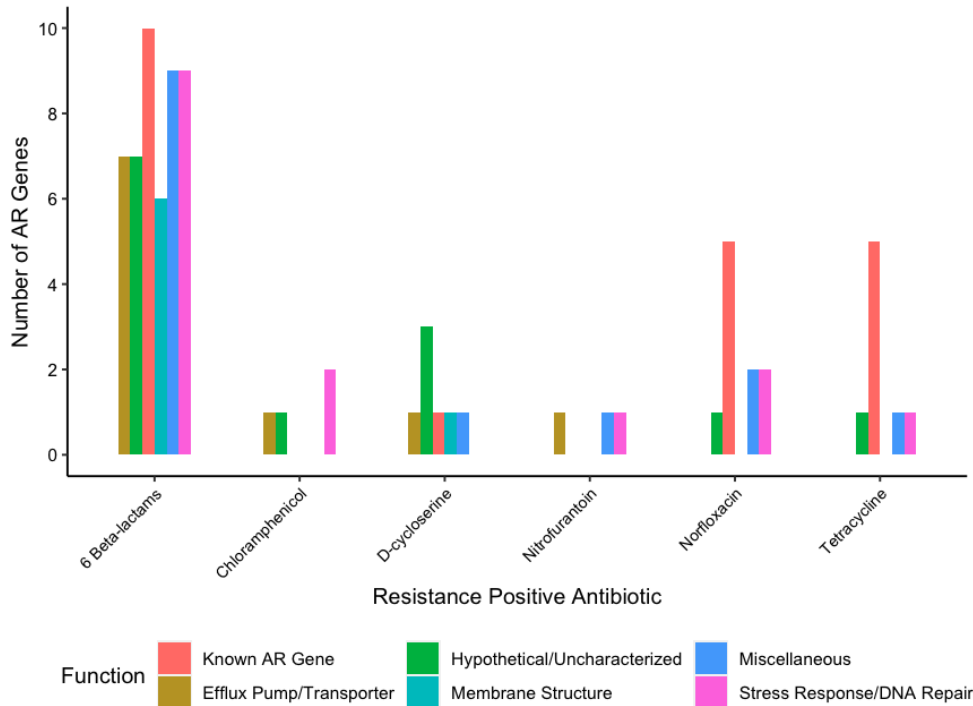
Fig. 4: Number of antibiotic resistance genes conferring latent resistance to antibiotics at the MICs, separated by class.

We next analyzed AR orthogroups shared between *E. coli* strains and the antibiotics resisted by each orthogroup (Fig. 5). Between the eight strains, a total of 35,823 genes were classified into 5551 orthologous groups, including single gene groups. The proportion of positive orthologues (conferred resistance in at least one strain) was 1.2% or 66 genes. Eighty-six percent of AR genes (57 genes) were shared between the eight strains, and nine AR genes were not shared within all eight strains (Fig. 5). Sixty-four percent of positive orthologues conferred resistance to only one strain, demonstrating high intraspecific variability of latent AR genes. This result is noteworthy because the majority of AR genes were shared within all strains.
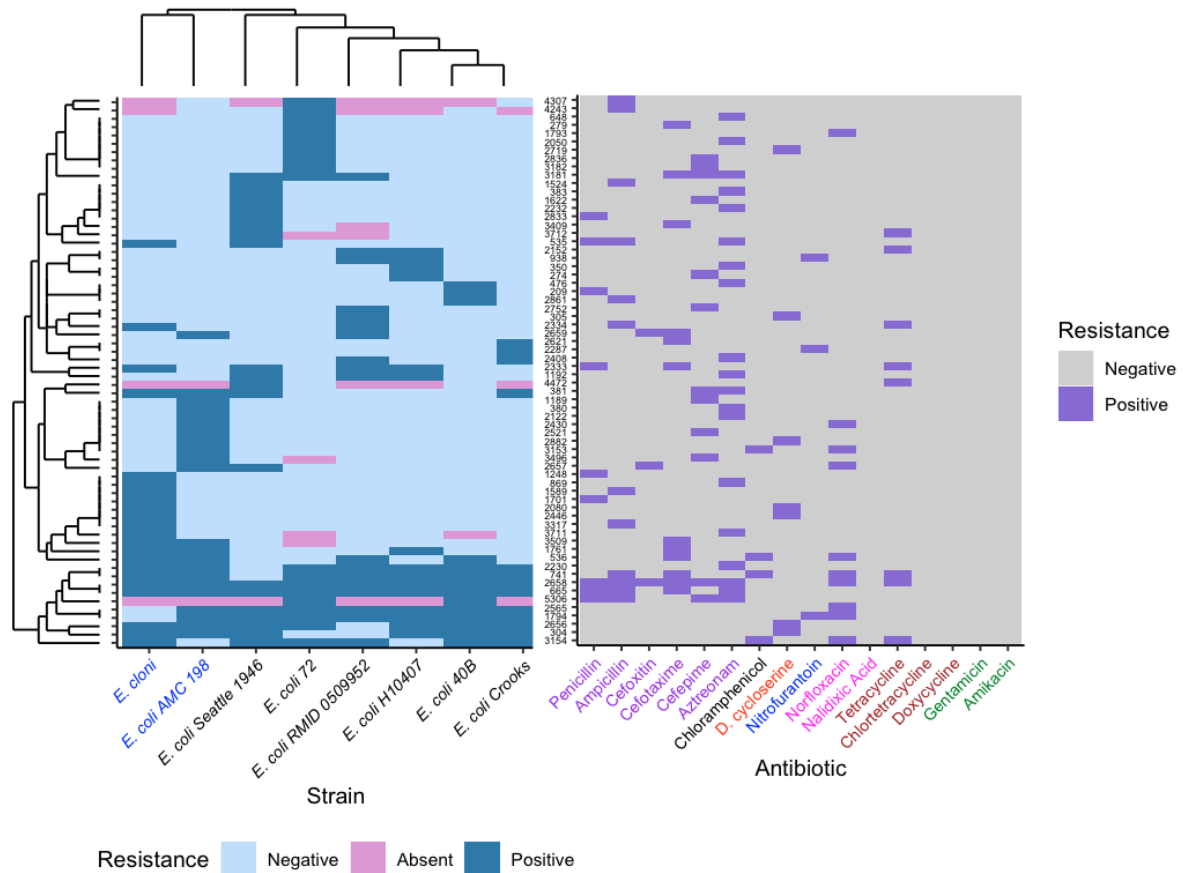
Fig. 5: Resistance positive shared groups of orthologous genes (left) conferring latent resistance to antibiotics (right) at the MICs. The proportion of resistance-positive orthogroups (shown) is 1.2%. The strains in blue are laboratory strains, and those in black are clinical strains. Dendrograms were built based on positive antibiotic resistance genes. Antibiotics are color-coded by class.

Multiple reasons could be the cause for high intraspecific variability, including mutation or movement of the gene within each strain. The genomic background of the gene may vary across strains. Bacterial species have shown considerable variations in genetic diversity and have displayed historical rates of recombination, horizontal gene transfer, and recurrent mutation (28–30). For example, TEM-1 beta-lactamase (orthogroup 5306), may have been horizontally transmitted in *E. coli* 72 and *E. coli* 40B as this gene conferred latent resistance while being absent in the remaining strains (Fig. 5). TEM beta-lactamases are normally transferred by plasmids (31). Also, sampling error could be the culprit resulting in an incomplete screen of the genome for latent AR

genes. However, with an average of 75,000 clones being tested on each antibiotic, the

probability of missing a gene is very low. Eighteen percent (12 genes) of positive

orthogroups conferred resistance in at least half of all strains (0.22% of all orthologues).

Three of the 12 genes are unclassified AR genes: *arfB*, *recA*, and *eamA*. Alternative

Rescue Factor A (ArfB) encodes a ribosome rescue system commonly present in

bacteria (32). Although ArfB has not been directly linked to antibiotic resistance,

ribosome rescue inhibitors have been suggested as potential antibiotic mechanisms

(32, 33). ArfB also contributes to heat stress resistance in *Azotobacter vinelandii* (34),

demonstrating how ribosome rescue mechanisms can play a role in tolerance to

stressors. Even though *recA* is not a classified AR gene, it has been well known to

induce antibiotic resistance via the SOS response (35–37). EamA is an exporter

classified within the Drug/Metabolite Transporter Superfamily (38). Three percent of

positive orthologues conferred resistance in all eight strains. These include two known

AR genes: *ampC* and *marA*. *ampC* is encoded on the chromosomes of many

Enterobacteriaceae but is normally expressed at low levels (39). Mutation and plasmid

mediation of *ampC* can lead to overexpression, resulting in beta-lactam resistance (39).

We uncovered a highly variable intraspecific reservoir of latent AR genes which

uncommonly develop cross cryptic resistance within multiple strains.

We found that cryptic AR genes present a low potential of developing cross

cryptic resistance to multiple antibiotics as compared to known AR genes (Fig 5).

Positive orthologues did not confer latent resistance to nalidixic acid, chlortetracycline,

doxycycline, gentamicin, or amikacin. Hence, *E. coli* strains may not have the potential

to develop latent or cryptic resistance to aminoglycosides. One orthogroup (*marA*,

known AR gene) conferred resistance to at least half of all antibiotics. Four orthogroups

of known AR genes conferred resistance to at least half of all antibiotic classes. These

genes are *marA*, *soxS*, *robA*, and *mdfA*. Thus, *E. coli* strains may not be as capable of

developing cross cryptic resistance to multiple antibiotics as known AR genes conferred

resistance to at least half of all antibiotics and antibiotic classes. Seventy-seven percent

of positive orthogroups conferred resistance to only one antibiotic, highlighting variability

of latent AR genes and suggesting that these genes may stem from a certain gene

response specific to the antibiotic. The dendrograms in Fig. 5 are generated based on

the resistance profile, and the dendrogram in Fig. 1 is generated according to genetic

relatedness between the strains. It is noteworthy that the two laboratory strains are

clustered within the same clade when based on the resistance potential. Although the

two dendrograms differ, there is phylogenetic conservatism to the antibiotic resistance

potential as there is a significant relationship between the resistance profile and

phylogeny (P <0.05).

　　We observed most latent AR genes (59%) conferring cross resistance in strains

from laboratory and clinical origins to be known AR genes (Fig. 6). However, 68% and

73% of genes conferring latent resistance in laboratory and clinical strains were

unclassified AR genes, respectively. This showed the vast reservoir of cryptic AR genes

across diverse strains. Hence, known AR genes may not always be the culprit of latent

resistance in clinical strains as we predicted. The cryptic AR genes conferring

resistance in strains from both origins are *arfB*, *recA*, *marB*, *creA*, *yecF*, *nlpD*, and

eamA. MarB is part of the multiple antibiotic resistance operon, marRAB, in which *marA*

and *marR* are classified AR genes (40). MarB has an unknown function, but it has

shown to increase the level of MarA. CreA has an uncharacterized function, but it is adjacent to the CreBC two-component regulatory system and *robA*, a known AR gene (41). *creA* was also shown to confer cryptic resistance to multiple beta-lactam antibiotics from varying origins (10). YecF has an uncharacterized function, but it has been shown to be upregulated in response to antibiotic exposure (10, 42). Additionally, YecF is adjacent to *sdiA*, a known AR gene (43). NlpD is involved in maintaining cell membrane permeability and integrity (44). Since *nlpD* conferred resistance to cefotaxime, this could
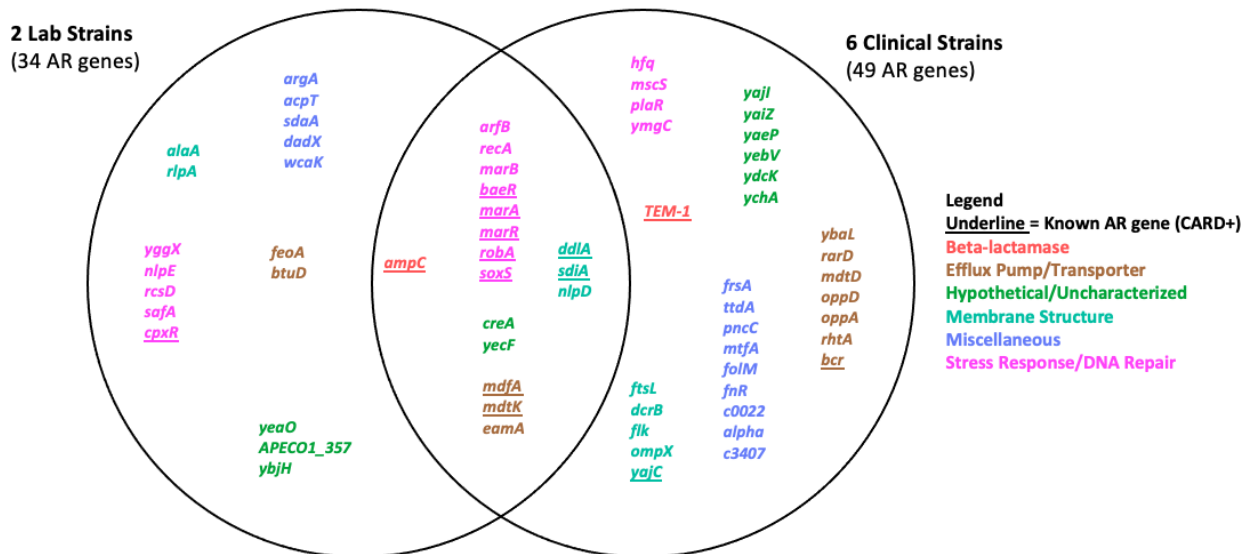
Fig. 6: Antibiotic resistance genes shared between all strains, separated by strain origin. We identified 66 antibiotic resistance genes (shown) causing resistance at the MICs. Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.

be to alleviate the stress on cell wall biosynthesis caused by the beta-lactam cefotaxime (45). Here, *eamA* conferred resistance to D-cycloserine within seven *E. coli* strains, and it has also shown to confer cryptic resistance to D-cycloserine in a laboratory *E. coli* strain (10). For both origins (individually and combined), stress response genes comprised the highest number of AR genes compared to other gene functions, demonstrating the broad intraspecific latent AR potential for this gene function. Bacterial

stress response mechanisms such as the general (46, 47), SOS (35–37), oxidative (48, 49), and envelope stress responses (50, 51) have been commonly shown to reduce antibiotic susceptibility. Even though known AR genes contributed to cross resistance within distinct strains, a diversity of cryptic AR genes led to cryptic resistance among *E. coli* strains.

We found that cross latent resistance to multiple origins of antibiotics is driven by known AR genes (Fig. 7). Specifically, 80% of genes conferring latent resistance to all antibiotic origins (natural, semisynthetic, and synthetic) are known AR genes. The only cryptic AR gene conferring resistance to all antibiotic origins is *creA.* Even though known AR genes primarily conferred cross latent resistance to multiple antibiotic origins, cryptic AR genes comprised the majority for natural [64%], semisynthetic [74%], and synthetic [70%] antibiotics. There were the fewest natural resistant positive antibiotics [3], compared to semisynthetic [4] or synthetic [4] antibiotics. We had predicted that latent resistance would be most common in the presence of natural antibiotics, but this was not the case as presented in this study and previously (10). Additionally, latent resistance occurred to all hydrophilic antibiotics (Table 1) as we predicted. This occurred possibly due to the highly hydrophobic outer membrane present in Gram negative bacteria being a barrier for hydrophilic (water-soluble) antibiotics (10). 64% of resistant positive antibiotics were hydrophilic and inhibited cell wall synthesis (Table 1), potentially showing a link between antibiotic mechanism of action and latent resistance. Antibiotics that inhibited the cell wall or cytoplasmic membrane also comprised most resistant positive antibiotics when testing for latent resistance in a laboratory strain of *E. coli* (10). For semisynthetic and synthetic antibiotics, stress response/DNA repair genes
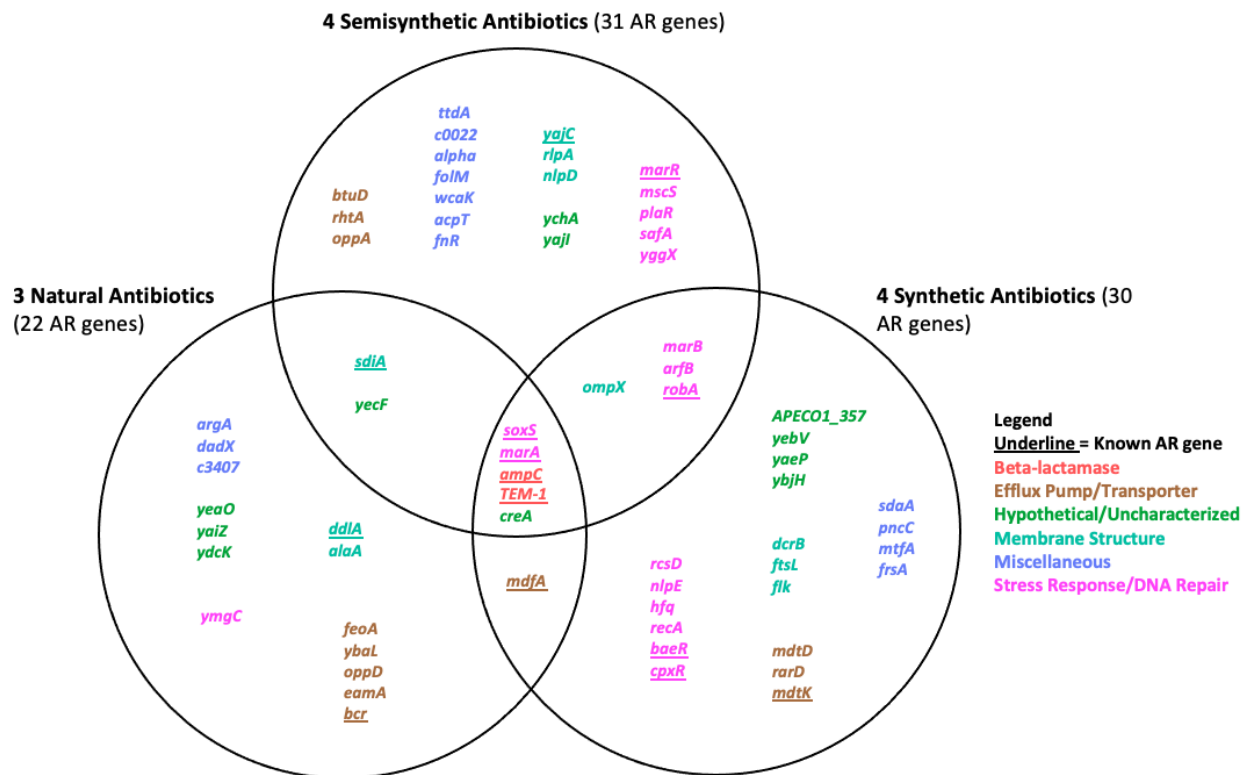
Fig. 7: Antibiotic resistance genes shared between all strains, separated by resistance-positive antibiotic origin. We identified 66 antibiotic resistance genes (shown) causing resistance at the MICs. Known antibiotic resistance genes were classified using the Comprehensive Antibiotic Resistance Database by gene name.

comprised the highest number of AR genes compared to other gene functions, highlighting the significant role of this gene functional category in latent resistance. While known AR genes were the main contributors to cross latent resistance, cryptic AR genes comprised the majority for natural, semisynthetic, and synthetic antibiotics.

This study encounters the same caveats discussed previously (10). These include the inability to capture complex gene regulation and/or coregulation that occurs between genes present on the plasmid insert used to amplify the gene copy number. This study cannot be interpreted in a clinical sense since our MIC methodology is not clinically standard. However, our study demonstrates the intraspecific potential of latent antibiotic resistance in *E. coli.*

Functional metagenomic studies have shown that latent AR genes are a common occurrence among bacteria. However, due to the small insert size harboring the resistance gene, functional metagenomic studies have limited information about the phylogeny of the original host organism (20). This holds true even if used in conjunction with sequenced based metagenomics. Functional metagenomic studies have been used to identify resistance genes from certain environments but rarely from strains with distinct origins against a comprehensive panel of antibiotics. Additionally, functional metagenomic studies utilize a surrogate host. We have addressed these limitations to better comprehend the intraspecific potential for latent and cryptic antibiotic resistance. Intraspecific genomic diversity may be a driving force in the emergence of antibiotic resistance. By utilizing this platform, we aim to gain an improved understanding of the antibiotic characteristics that promote latent resistance in multiple strains, while considering intraspecific diversity. This platform offers the potential to uncover genes and functional gene categories that could become responsible for inducing cross latent resistance to varying antibiotics within diverse strains. Thus, this study may prove valuable in the identification of novel antibiotic targets and mechanisms.

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CHAPTER 3

Metagenomics reveals dynamic coastal ocean reservoir of antibiotic resistance genes

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Introduction

Coastal waters may pose a risk to human health through exposure to antibiotic resistant bacteria. The combination of marine microbiome genome diversity and the influx of antibiotics and antibiotic resistant bacteria into the ocean forms the basis for a reservoir of resistance genes (1). During recreational activities at the beach, people may ingest bacteria harboring antibiotic resistance (AR) genes which can lead to infection and/or transfer of genes to commensal and opportunistic pathogens resident in the human microbiome (1, 2). Additionally, climate change can increase the frequency of AR genes (3–5). Climate change is driving an increase in high precipitation events. Such events increase flooding and stormwater runoff, transporting bacteria and substances that may promote the development of antibiotic resistance in seawater (3). An increase in temperature was shown to increase antibiotic resistance among common pathogens (4, 5) and can lead to antibiotic resistance in the lab, even in the absence of antibiotics (6). However, thus far, few studies have thoroughly studied the prevalence of AR genes in coastal water.

Seawater has shown to be a reservoir or AR genes (7–22). Specifically, coral reef regions and deep and coastal ocean water have been shown to harbor AR genes. Significantly higher abundances of AR genes have been found in coastal water

compared to deep ocean and Antarctic seawater samples, highlighting the pressure exerted from human activity on the biogeography of AR genes (7). However, antibiotic resistant and virulent *Staphylococcus aureus* have been found in Hawaiian beaches with limited human activity (22). Many beta-lactamases, including those uncommonly found outside of clinical environments, have been found at Australian beaches (18). Multi-resistant *Escherichia coli* were found at a touristic island in Portugal, and this reservoir of AR genes was mainly modulated by seagulls-derived fecal pollution (13). Among Southern California beaches, about 45% of total coliforms isolated were resistant to ampicillin and remained consistent during dry and wet seasons (10). Through functional metagenomics, Newport Beach, California was shown to harbor a diversity of known and unclassified AR genes carried by marine and non-marine taxa (15). Newport Beach, CA is an ideal location to study, because it is surrounded by one of the most densely populated communities in the nation and is one of the largest harbors for recreational vessels in the United States (23). However, there is a lack of studies investigating AR genes in coastal water over an extended period. Many studies analyzed samples collected within only one year using culture-based techniques (10, 11, 13–15, 17, 22). A ten-year time series would provide a comprehensive understanding of the prevalence of AR genes in coastal water.

Metagenomics is a powerful tool for assessing AR genes as it captures the diversity of all resistance mechanisms and the taxa carrying these genes (7, 8, 16, 24). Through metagenomics, one is not restricted to studying culturable bacteria and limited antibiotic resistance mechanisms as compared to culture-based techniques. Metagenomics is also less labor intensive and can generate large volumes of genomic

data relative quickly (16). The MICRO time-series station at Newport Pier, California has demonstrated how ocean warming such as an El Niño event can lead to shifts in marine microbial populations (25). We hypothesize that the seasons will greatly impact AR genes in coastal water. For example, we predict that antibiotic resistance frequencies will increase during the winter (due to increased rainfall) and the summer (due to a higher number of visitors). We also predict that terrestrial and enteric taxa that wash in during rain events or with visitors during the summer are the main carriers of AR genes in coastal water.

Here, we used a ten-year time series to determine the prevalence of AR genes in Newport Beach, CA coastal water using metagenomics. We specifically asked the following: (i) what are the role of seasons, number of visitors present on the beach, and *Enterococcus* levels on the frequency of AR genes and (ii) which taxa are harboring AR genes? If we find an increase in AR genes during certain times, then this suggests potential causes for the changes in frequencies. Therefore, this would allow us to delineate the AR genes that are present in coastal water and possibly pinpoint when and why their frequencies increase. Identifying the taxa will provide a better understanding of AR genes in seawater and the impacts of the factors studied.

Methods

Sample Collection and DNA extraction

Between 2011 and 2020, 255 surface seawater samples were collected weekly or monthly from the "Microbes in the Coastal Region of Orange County" (MICRO) time series station at Newport Pier in Newport Beach, California, USA (33.608˚N and 117.928˚W). Samples were collected and processed as previously described (25–27).

Bacterial DNA was extracted from Sterivex syringe filters (Millipore) as described previously (25). Briefly, the filters were incubated at 37°C for 30 minutes with lysozyme prior to adding Proteinase K and 10% SDS buffer then incubated at 55°C overnight. DNA was precipitated using ice-cold isopropanol and sodium acetate. DNA was then centrifuged and resuspended in TE buffer in a 37°C water bath for 30 minutes. DNA was purified using a genomic DNA Clean and Concentrator kit (Zymo Research Corp) and then quantified using a Qubit fluorometer (ThermoFisher).

Library Preparation, Sequencing, and Analysis

The U.S. Department of Energy Joint Genome Institute (JGI) performed the library preparation, sequencing of metagenomes, processing of reads, and functional and taxonomic annotations according to their metagenome workflow (28). In brief, sequencing was performed on the Illumina NovaSeq 6000 platform, and raw reads were processed using BBDuk version 38.79 from the BBTools package (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/). Filtered reads were assembled with metaSPAdes version 3.13.0. Assembled contigs were then passed onto the annotation module of the workflow, which first predicts noncoding RNA genes, followed by the identification of clustered regularly interspaced short palindromic repeats (CRISPR) and protein-coding genes (CDSs). Protein-coding genes were predicted using Prodigal 2.6.3 (29) and GeneMarkS-2 1.07 (30). The last step of the feature prediction module combines the results from all tools and attempts to resolve overlaps between features of different types.

Functional annotation for metagenomes was done by associating protein-coding genes with KO (KEGG Orthology) terms, Enzyme Commission (EC) numbers, COG

(Cluster of Orthologous Genes) assignments, as well as other annotations. Genes are associated with KO terms and EC numbers based on the results of a sequence similarity search of metagenome proteins against a reference database of isolate proteomes using the large-scale alignment tool, LAST (31). The best LAST hits of CDSs were also used for the taxonomic annotation of metagenomes. The taxonomy of best hit was assigned to each metagenome protein.

Antibiotic Resistance Analysis

We obtained a total of 102 AR KOs from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (32–34). These AR KOs included beta-lactamase, aminoglycoside, multidrug, trimethoprim, macrolide, and tetracycline resistance mechanisms (S1 Table). Seventy-eight of 102 selected KOs were present within less than 5% of samples or were completely absent. These KOs were removed from further analysis. The remaining 24 KOs were present in at least 5% of samples. We referred to AR KOs by the gene(s) they correspond to (Table 1). We determined the frequencies of these 24 AR genes using their respective raw coverage values (Fig. 1). Coverage was defined as the average coverage of the gene within the contig that holds it. For example, a coverage of one is an average of one read per base pair in the contig. To quantify both seasonal (monthly reoccurrences across years) and interannual trends in the AR genes, we fit the following ANOVA model to our data (25):

$$Y_{ijk} = \mu + \alpha_{Year}X_{Year,j} + \beta_{Month}X_{Month,k} + \varepsilon_{ijk}$$

We conducted Type II ANOVAs on categorical linear regressions of AR genes (Y_{ijk}) as a function of year (X_j) and month (X_k) with corresponding regression coefficients α_{Year} and β_{Month}. R's "car" and "stats" packages were used to perform this analysis. Raw gene

coverage values were normalized by total sequences passed for each sample. Normalized coverages were then normalized zero to one to conduct the regression analysis. Linear model regression coefficients were scaled to center on zero prior to plotting Fig. 2.

Precipitation records at Newport Pier in Newport Beach, CA from 2011-2020 were obtained from the National Oceanic and Atmospheric Administration. Data documenting the number of visitors at Newport Beach, CA from 2015-2020 were obtained as a public records request from the City of Newport Beach. The recorded levels of *Enterococcus* at the 15S station located at 15th/16th St. in Newport Beach, CA from 2011-2020 were obtained from the State of California Water Boards.

The correlations between AR gene coverages and *Enterococcus* levels, number of people, and precipitation levels at Newport Beach, CA were quantified through the Pearson correlation coefficient (Fig. 6). The linear model regression coefficients (unscaled) as a function of month and year for AR gene coverages, *Enterococcus* levels, number of people, and precipitation levels were used to obtain the Pearson correlation coefficients.

Results and Discussion

Through a ten-year time series, we delineated the prevalence of AR genes in Newport Beach, CA coastal water (n=250, Fig. 1). We identified the seasonal (monthly reoccurrences across years) and interannual trends of AR genes (Fig. 2) as well as the taxa harboring these genes (Table 1). We then examined the impact of rainfall, number of visitors on the beach, and *Enterococcus* levels (Fig. 3-6) on the trends of AR genes.

Therefore, we were able to characterize the presence of AR genes in coastal water and the variables potentially impacting them.

We detected a total of 24 out of 102 possible AR gene types that were present in at least 5% of samples. One out of 24 AR genes did not consist of any genes present within the Comprehensive Antibiotic Resistance Database, which documents classified AR genes (35). This gene is *penP* (Table 1), but *penP* is known to confer beta-lactam resistance in *Bacillus* (36). We determined the coverage values of the 24 AR genes (Fig. 1). We identified four beta-lactamase, five aminoglycoside, seven multidrug, three chloramphenicol, one macrolide, one trimethoprim, and three vancomycin resistance gene types. There was variability in coverages within each antibiotic category, but beta-lactamase and vancomycin resistance genes were the most prevalent (Fig. 1). *penP* (class A beta-lactamase) and *vanY* (vancomycin resistance gene) had the highest coverages (32–34).
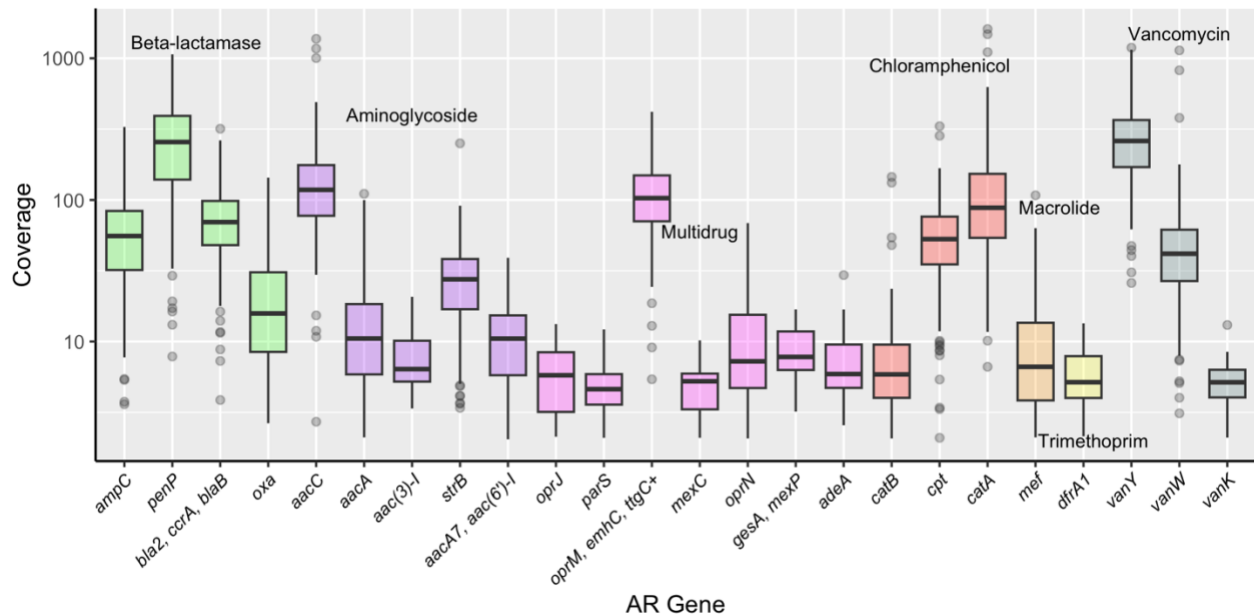
Fig. 1: Raw coverage of antibiotic resistance genes present in at least 5% of samples. Coverage is defined as the average coverage within the contig that contains the gene. For example, a coverage of one is an average of one read per base pair in the contig. Genes are color-coded by resistance mechanism.

Table 1: Antibiotic Resistance Genes and the Taxa Harboring them

| ARG Type	KO [a]	Gene [a]	Genus 1 [b]	Genus 2 [c]
Beta-lactamase	K01467	*ampC*	*Pseudohongiella*	*Thalassomonas*
	K17836	*penP*	*Synechococcus*	*Chryseolinea*
	K17837	*bla2, ccrA, blaB*	*Woeseia*	*Pseudohongiella*
	K17838	*oxa*	*Pseudohongiella*	Unclassified *Verrucomicrobiae*
Aminoglycoside	K00662	*aacC*	*Campylobacter*	*Lishizhenia*
	K00663	*aacA*	*Cognatiyoonia*	*Candidatus Planktophila*
	K03395	*aac(3)-I*	*Sphingopyxis*	*Alcanivorax*
	K04343	*strB*	*Devosia*	*Drancourtella*
	K18816	*aacA7, aac(6')-I*	*Rhizobium*	*Salmonella*
Multidrug	K08721	*oprJ*	*Pseudomonas*	*Marinobacter*
	K18139	*oprM, emhC, ttgC, cusC, adeK, smeF, mtrE, cmeC, gesC*	*Pseudomonas*	*Psychrobacter*
	K18295	*mexC*	*Pseudomonas*	*Halioglobus*
	K18072	*parS*	*Pseudomonas*	*Bradyrhizobium*
	K18300	*oprN*	*Salinisphaera*	*Pseudomonas*
	K19595	*gesA, mexP*	*Unclassified Halieaceae*	*Limibacillus*
	K18145	*adeA*	SAR116	*Halomonas*
Chloramphenicol	K00638	*catB*	*Halomonas*	*Acinetobacter*
	K18554	*cpt*	*Henriciella*	*Streptomyces*
	K19271	*catA*	*Formosa*	*Mucilaginibacter*
Macrolide	K08217	*mef*	*Gordonibacter*	*Rhodoluna*
Trimethoprim	K18589	*dfrA1*	*Vibrio*	
Vancomycin	K07260	*vanY*	*Synechococcus*	*Prochlorococcus*
	K18346	*vanW*	*Flavobacterium*	*Synechococcus*
	K18354	*vanK*	*Catenulispora*	*Nocardioides*

[a]Antibiotic resistance KOs and their respective gene names were obtained from KEGG.
[b]The taxa hosting the highest percentage of coverage for the corresponding gene.
[c]The taxa hosting the second highest percentage of coverage for the corresponding gene.

Specific groups of AR genes fluctuated in coverage during certain months and specific years throughout the time series (Fig. 2). Generally, genes presented a seasonality trend, remained constant, or displayed an inconsistent trend. For example, *oxa, aacA, catA, aacC, vanW, aacA7, aac(6')-I*, and *ampC* displayed a potential

seasonality trend as they had an increasing trend in January. Total AR genes also increased in January. *gesA* and *mexP* showed a seasonality trend since they experienced a prominent increase in July. Genes that remained constant throughout the time series were *bla2, ccrA, blaB, adeA, mef, catB, aac(3)-I,* and *vanK*. Genes that displayed an inconsistent trend were *oprN, oprM, emhC, ttgC*+ (K18139), and *oprJ*. The subsets of genes that displayed an increasing trend in May and June consisted primarily of multidrug resistance mechanisms, which may demonstrate a common driver of AR genes during these months. In 2015 and 2016, total AR genes increased. This may have occurred due to El Niño which peaked in this period (25) and is characterized by an increase in sea surface temperatures. Certain subsets of genes demonstrated trends in coverage levels occasionally related to AR mechanisms.
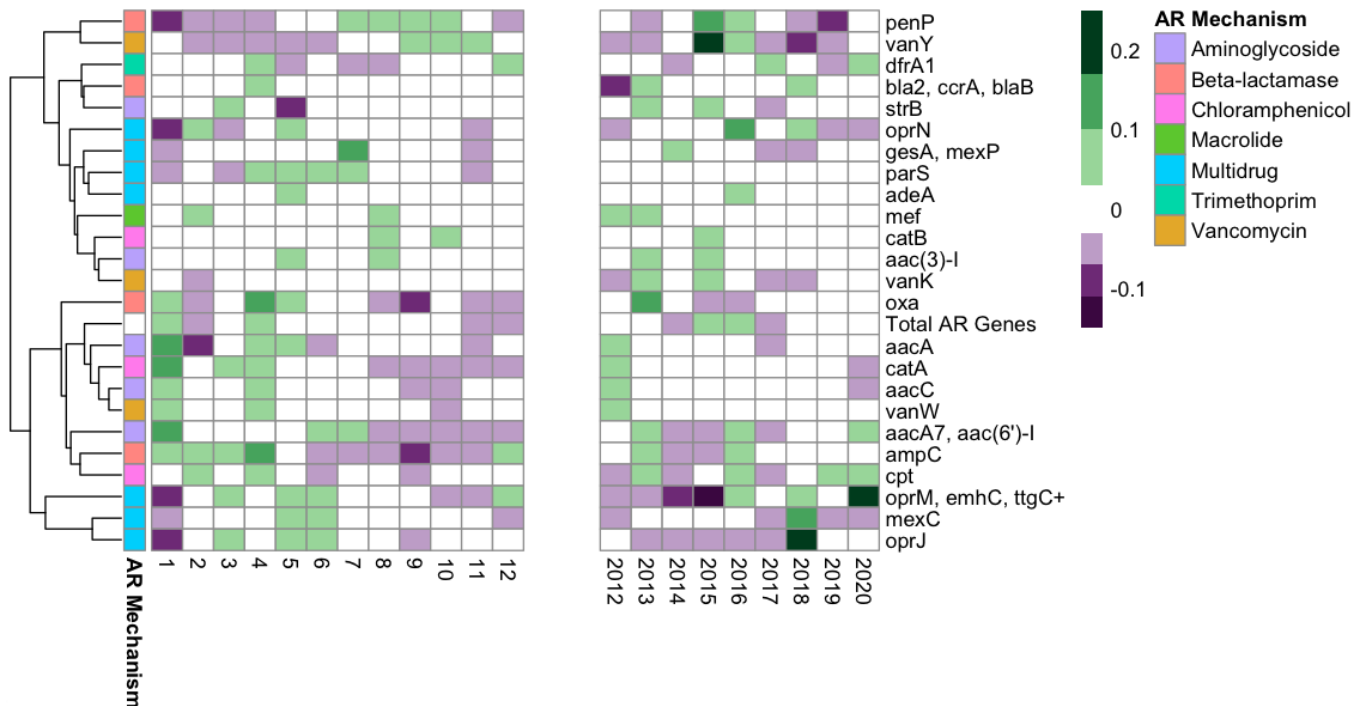

Fig. 2: Seasonal and interannual trends in antibiotic resistance genes present in at least 5% of samples. Linear model regression coefficients for normalized gene coverages as a function of month (1-12) and year (2012-2020) (see Methods). Each month is aggregated across years. Coefficients were scaled to center on zero. The regression model uses January and the first year of the time series (2011) as the reference category for month and year coefficients, respectively. A higher coefficient means an increasing trend at that month or year. Genes are color-coded by resistance mechanism.

Taxa harboring AR genes consisted predominantly of native/marine taxa, but there were also potentially pathogenic bacteria carrying AR genes (Table 1). Fourteen out of 24 AR genes were represented highest within marine genera which included *Pseudohongiella* (37)*, Woeseia* (38)*, Halomonas* (39)*, Henriciella* (40)*, Formosa* (41)*, Vibrio* (42)*, Synechococcus* (43), and *Flavobacterium* (44). These genes have beta-lactamase, chloramphenicol, and trimethoprim resistance mechanisms. Three out of the five top genera carrying aminoglycoside resistance genes (*aac(3)-I, strB, aacA7*, and *aac(6')-I*) were primarily found in the environment (excluding water sources). These genera are *Sphingopyxis* (45), *Devosia* (46), and *Rhizobium* (47). *Sphingopyxis* and *Devosia* have been commonly found in oil and pesticide contaminated soil. *strB*, *aacA7*, and *aac(6')-I* were also found within *Drancourtella* and *Salmonella*, respectively. This is noteworthy as *Drancourtella* and *Salmonella* reside within the intestines of humans and animals (48, 49). The top genus harboring *aacC* was *Campylobacter*. *Campylobacter* species are known pathogens in humans and animals (50). However, only 5.6% of coverage pertaining to *aacC* were within *Campylobacter* (S2 Table). Seventy seven percent of coverage for *aacC* was found within reads not assigned to a genus (S2 Table). The top genus hosting macrolide resistance gene *mef* was *Gordonibacter* (43.8% of coverage). *Gordonibacter* resides in the human gut and has been isolated from the stool of patients that are healthy (58) and suffering from acute Crohn's disease (59). Four of the seven multidrug resistance genes were found primarily (top genus) within *Pseudomonas* (Table 1). *Pseudomonas* species are commonly found in soil and water associated habitats and polluted environments, but they can also be opportunistic pathogens (51–54). For example, *Pseudomonas* species such as *Pseudomonas putida*

(53), *Pseudomonas pseudoalcaligenes* (51), and *Pseudomonas aeruginosa* (55) have caused infections in humans. Ninety-two percent of coverage for *oprJ* was found within *Pseudomonas* (S2 Table). OprJ is the outer membrane channel component of the MexCD-OprJ multidrug efflux complex. *P. aeruginosa* harbors this clinically relevant efflux complex making it innately resistant to several antibiotics (56). We found that the *Pseudomonas* species carrying these genes were primarily *P. pseudoalcaligenes*, *P. alcaliphila*, *P. putida*, and *P. balearica*. *P. pseudoalcaligenes* is a soil organism, but it is a rare opportunistic human pathogen (51). *P. alcaliphila* typically resides in seawater (52) but has been isolated from patients with cystic fibrosis (57). *P. putida* resides in soil and water-associated environments, but it also rarely causes infection in humans (53). *P. balearica* is usually found within aquatic and petroleum-polluted environments (54). Thus, *Pseudomonas* carrying AR genes in Newport Beach coastal water are likely marine lineages but have the potential to cause infection in humans.

Our data suggest that seasonal and interannual trends of AR genes vary by gene and the taxa carrying them, but rainfall and *Enterococcus* levels may be an accurate indicator for total AR gene levels. We hypothesized an increase of most resistance genes during the winter and summer months. Storm-water events increased AR genes at multiple Australian beaches, and the corresponding genes were linked to pathogenic genera associated with wastewater (9, 18). Throughout the time series, Newport Beach had the highest rainfall during December and January (Fig. 3). There was a significant positive correlation between precipitation at Newport Beach and *aacC, catA, vanW, vanK*, and total AR genes (Fig. 6). Thus, precipitation may be an indicator for total AR gene levels in Newport Beach coastal water. Newport Beach was visited more

frequently during June, July, and August (Fig. 4). The highest number of visitors

occurred in July throughout the time series. We found a significant positive correlation

between the number of people at Newport Beach and *gesA, mexP, parS*, and *catB* but

not between total AR genes and number of people (Fig. 6). Therefore, the number of

visitors is not a good indicator of total AR gene levels in Newport Beach coastal water.

On the contrary, a higher diversity of AR genes were found in an Antarctica freshwater

zone with greater human activity compared to freshwater zones with little human

intervention (60). An increase in tourism at the Galapagos Islands led to increases in

antibiotic resistant *E. coli* and *Enterococcus* levels (61). *Enterococci* are used as

indicators of human fecal pollution in recreational waters as high concentrations of this

genus are found in human feces (62) which has been linked to antibiotic resistance in

aquatic environments (63). Newport Beach may show differing trends as *Enterococcus*

concentrations were highest in the winter (Fig. 5) when tourism was at its lowest (Fig.

4). However, high *Enterococcus* levels occurred when precipitation was highest at
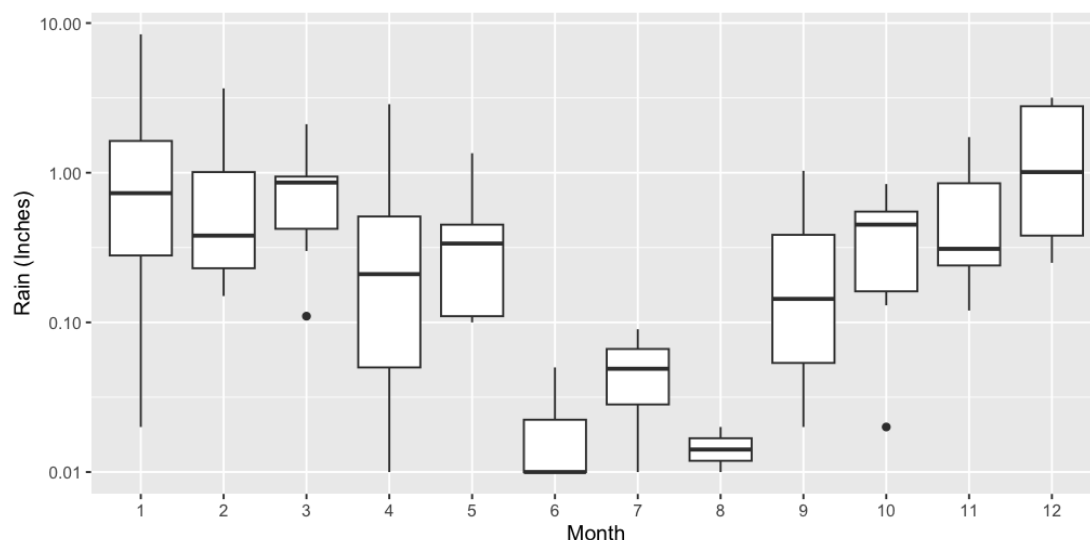
Fig. 3: Monthly precipitation at the Newport Beach Harbor, CA National Oceanic & Atmospheric Administration station from 2011-2020. Data for January 2011 and May 2012 is not available. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. The black line within the box marks the median. Points indicate outliers.
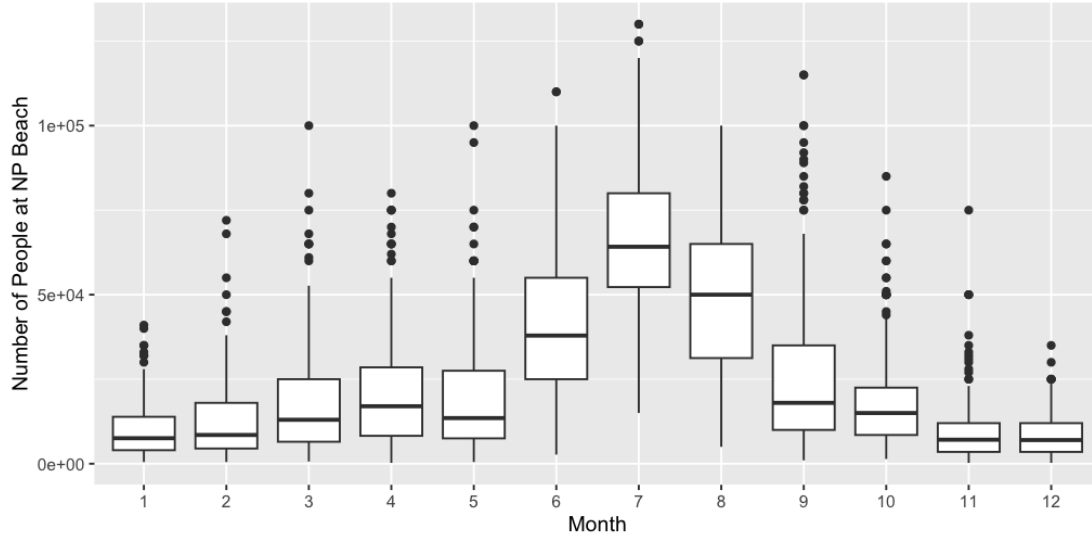
Fig. 4: The number of monthly visitors at Newport Beach, CA from 2015-2020. Data was obtained as a public records request from the City of Newport Beach. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. The black line within the box marks the median. Points indicate outliers.

Newport Beach (Fig. 3). Similarly, at an urbanized subtropical bay in Texas, rainfall was shown to be directly correlated with increased *Enterococcus* concentrations (63). Even though *Enterococcus* levels increased with rainfall, we did not observe AR genes carried by *Enterococcus* throughout the time series (Table 1). Similarly, increased
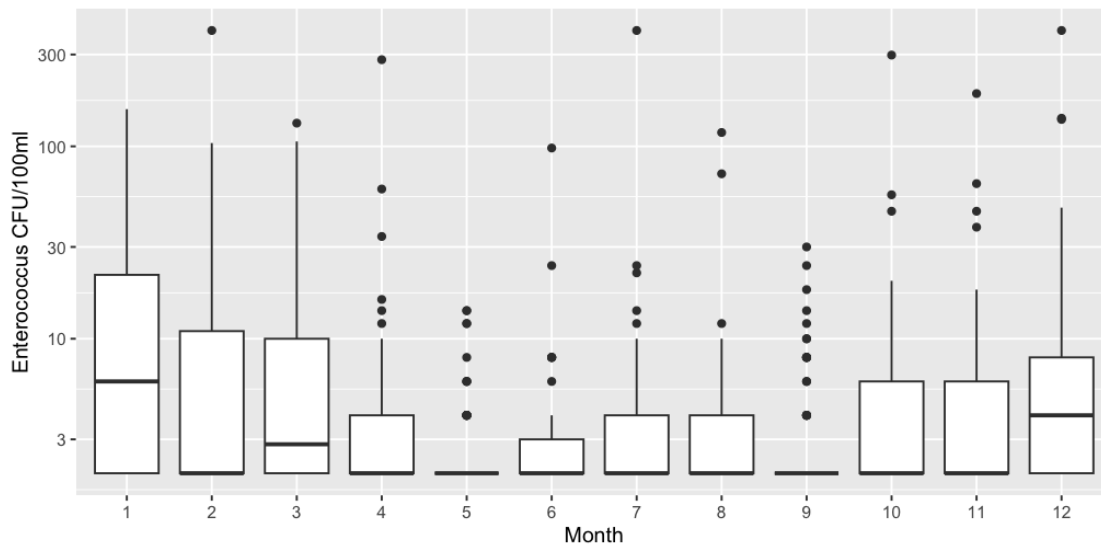
Fig. 5: Monthly *Enterococcus* levels at the State of California Water Boards 15S station located at 15th/16th St. in Newport Beach, CA from 2011-2020. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. The black line within the box marks the median. Points indicate outliers.

concentrations of *Enterococcus* were not correlated with an increase in antimicrobial-resistant *Enterococcus faecium* at an urbanized subtropical bay in Texas (63). However, we found a significant positive correlation between *Enterococcus* levels at Newport Beach and *aacC, catA, vanW, vanK*, and total AR genes (Fig. 6). Thus, *Enterococcus* levels may be an accurate indicator for total AR gene levels in Newport Beach coastal water even though *Enterococcus* was not a carrier of AR genes.
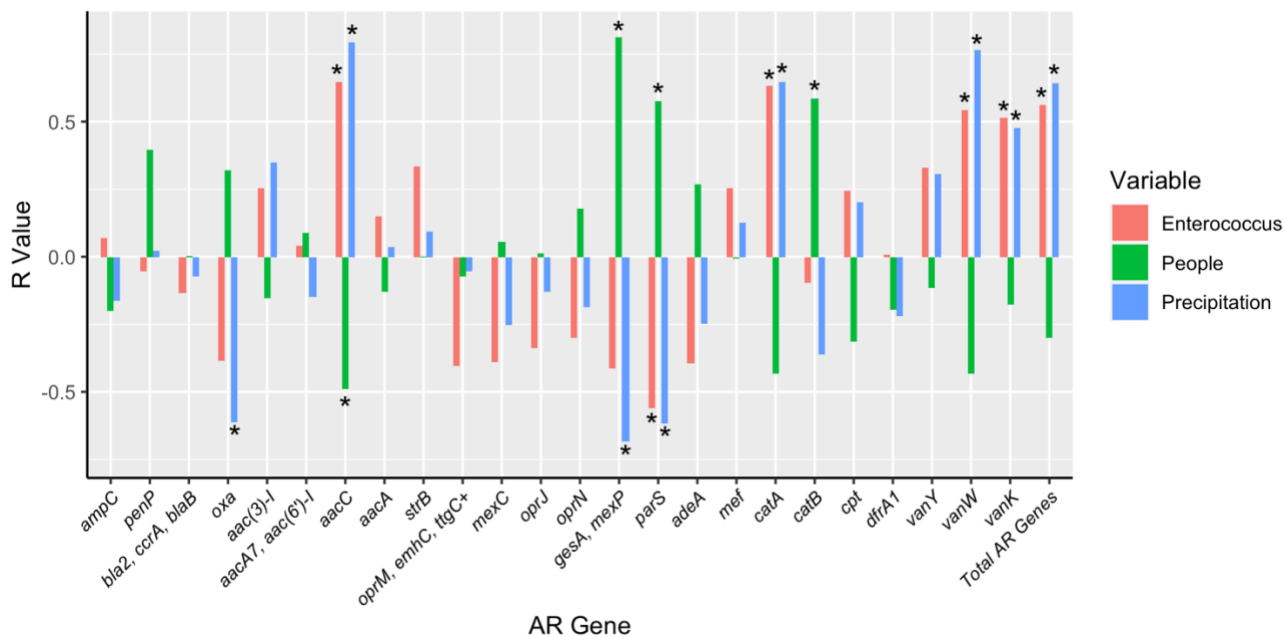
Fig. 6: Pearson correlation coefficients (R values) for the correlation between AR gene coverages and *Enterococcus* levels, number of people, and precipitation levels at Newport Beach, CA. The linear model regression coefficients as a function of month and year for AR gene coverages, *Enterococcus* levels, and precipitation levels were used to obtain the Pearson correlation coefficients. *, P ≤ 0.05.

Marine taxa are the primary sources of AR genes in Newport Beach coastal water. We had predicted that terrestrial and enteric taxa that wash in with people or during intense rain events were the primary carriers of AR genes. If the seasons were defined by enteric or terrestrial genera, then this indicates that factors such as rainfall or tourism are likely sources of AR genes. For example, colistin resistant genes within Croatian coastal water was found to come predominantly from pathogenic genera (21).

71

On the contrary, in Newport Beach we found that marine genera had the highest percentage of coverage for more than half of AR genes (Table 1). Similarly, the majority of AR genes found within Los Angeles Harbor and San Pedro Channel in CA came from marine taxa (15). This previous study also demonstrated an equal distribution of AR genes coming from marine and nonmarine taxa in Newport Bay, CA coastal water (15). We observed increasing trends of AR genes harbored within marine genera throughout the year. For example, *Synechococcus* harboring *penP* showed an increasing trend in July, August, September, and October (Fig. 2). *Synechococcus* reproduces more quickly as temperature increases (64–66), complementing the increasing trend during the summer months as observed in our study. *Synechococcus* hosting *penP* and *vanY* rose sharply in 2015 (Fig. 2). This may have occurred due to El Niño which peaked in 2015 (25) and is characterized by an increase in sea surface temperatures. *Vibrio* carrying *dfrA1* increased in April and December (Fig. 2). Conversely, *Vibrio* abundance is usually higher in the summer than in the winter (67). *Pseudohongiella* harboring *oxa* and *ampC* displayed increasing trends from January through May and December (Fig. 2). We found that AR genes may be driven by taxa that grow optimally during certain times.

A caveat of this study is that it is difficult to conclusively assign taxa to genus level. There are large percentages of unassigned (NA) taxa hosting AR genes at the genus level (S2 Table). However, it is common to have a large portion of unannotated reads from seawater samples as less than 10% of individual metagenomic reads from the ocean can be recruited on reference genomes (68). Using metagenomics allows us to identify diverse AR genes as well as the taxa that carry them, but we cannot always

fully identify them as this has been an ongoing problem in environmental microbial genomics (16, 68). Since we are using KO terms, a second caveat is that antibiotic resistance function from the selected KOs is only a prediction. Some AR genes may carry other functions within the cell. Additionally, genes may require changes in regulation before conferring resistance.

Studies have shown that AR genes are a common occurrence in coastal water. Since Newport Beach in California is a highly visited beach, up to 100,000 visitors each day in the summer (Fig. 4), it is important to understand the prevalence of AR genes from a public health perspective. Metagenomics can facilitate the scanning of AR genes from diverse environments as well as become a tool for monitoring water quality (69). Through a ten-year time series, we have provided a comprehensive understanding of the AR genes present within Newport Beach seawater, the temporal distribution of these genes, and the factors driving their frequencies.

S1 Table: Total Antibiotic Resistance-related KOs classified by Resistance Mechanism

Beta-lactamase		Aminoglycoside	Multidrug	Trimethoprim	Macrolide	Tetracycline
K18766	K19210	K17840	K18131	K19643	K00561	K18908
K18781	K19211	K18815	K18094	K19644	K06979	K18909
K18782	K19212	K17881	K19593	K19645		
K18795	K19213	K10673	K19594	K18590		
K18972	K19214	K12570	K18142			
K19095	K19216	K18845	K09476			
K19215	K19316	K19272	K18073			
K19217	K19317	K19274	K13632			
K02546	K19318	K19275	K18095			
K02547	K19319	K19276	K18129			
K18698	K19320	K19277	K18141			
K18699	K19321	K19278	K18898			
K18767	K19322	K19299	K18899			
K18768	K20319	K19301	K18906			
K18780	K20320	K19315	K18145			
K18790	K21266	K19543				
K18791	K21276	K19544				
K18792	K21277	K19883				
K18793	K22331					
K18794	K22332					
K18796	K22333					
K18797	K22334					
K18970	K22335					
K18971	K22346					
K18973	K22351					
K18976	K22352					
K19096	K24153					
K19098	K24159					
K19100	K24161					
K19101	K24162					
K19209						

S2 Table: Antibiotic Resistance Genes and the Taxa Harboring them

AR Mechanism	KO	Gene	Genus 1[a]	Genus 2[b]	Genus 3[c]
Beta-lactamase	K01467	ampC	Pseudohongiella (54%)	NA (25%)	Thalassomonas (5.45%)
	K17836	penP	Synechococcus (84.09%)	NA (9.04%)	Chryseolinea (1.47%)
	K17837	bla2, ccrA, blaB	NA (57.94%)	Woeseia (20.39%)	Pseudohongiella (4.92%)
	K17838	oxa	NA (42.14%)	Pseudohongiella (38.79%)	Unclassified Verrucomicrobiae (11.67%)
Aminoglycoside	K00662	aacC	NA (77.3%)	Campylobacter (5.59%)	Lishizhenia (3.85%)
	K00663	aacA	NA (46.53%)	Cognatiyoonia (23.54%)	Candidatus Planktophila (8.51%)
	K03395	aac(3)-I	Sphingopyxis (37%)	NA (20.25%)	Alcanivorax (19.15%)
	K04343	strB	NA (73.94%)	Devosia (6.09%)	Drancourtella (5.42%)
	K18816	aacA7, aac(6')-I	NA (60.66%)	Rhizobium (19.93%)	Salmonella (3.34%)
Multidrug	K08721	oprJ	Pseudomonas (91.56%)	NA (5.63%)	Marinobacter (2.81%)
	K18139	oprM, emhC, ttgC, cusC, adeK, smeF, mtrE, cmeC, gesC	NA (41.04%)	Pseudomonas (26.77%)	Psychrobacter (5.91%)
	K18295	mexC	Pseudomonas (57.37%)	Halioglobus (14.01%)	Tropicimonas (9.12%)
	K18072	parS	Pseudomonas (44.61%)	Bradyrhizobium (27.94%)	Halobellus (10.44%)
	K18300	oprN	Salinisphaera (31.27%)	Pseudomonas (13.44%)	NA (11.20%)
	K19595	gesA, mexP	Unclassified Halieaceae (88.04%)	Limibacillus (7.57%)	Enterovibrio (4.39%)
	K18145	adeA	SAR116 (73.91%)	Halomonas (18.63%)	NA (2.92%)
Chloramphenicol	K00638	catB	NA (40.1%)	Halomonas (33.78%)	Acinetobacter (7.16%)
	K18554	cpt	NA (56.68%)	Henriciella (6.91%)	Streptomyces (6.56%)
	K19271	catA	NA (82.31%)	Formosa (9.02%)	Mucilaginibacter (2.57%)
Macrolide	K08217	mef	Gordonibacter (43.8%)	Rhodoluna (20.53%)	Fusibacter (15.04%)
Trimethoprim	K18589	dfrA1	NA (59.06%)	Vibrio (40.94%)	
Vancomycin	K07260	vanY	Synechococcus (52.38%)	Prochlorococcus (20.6%)	NA (9.51%)
	K18346	vanW	NA (62.16%)	Flavobacterium (15.68%)	Synechococcus (8.89%)
	K18354	vanK	NA (68.97%)	Catenulispora (14.46%)	Nocardioides (9.65%)

[a]The taxa hosting the highest percentage (and the [b]2nd and [c]3rd highest percentage) of coverage for the corresponding gene. Unassigned genera are labeled as NA. Percentage of coverage harbored within the corresponding genus is indicated.

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