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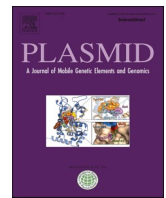
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Novel class 1 integron harboring antibiotic resistance genes in wastewater-derived bacteria as revealed by functional metagenomics

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ABSTRACT

Combating antibiotic resistance is critical to our ability to treat infectious diseases. Here, we identified and characterized diverse antimicrobial resistance genes, including potentially mobile elements, from synthetic wastewater treatment microcosms exposed to the antibacterial agent triclosan. After seven weeks of exposure, the microcosms were subjected to functional metagenomic selection across 13 antimicrobials. This was achieved by cloning the combined genetic material from the microcosms, introducing this genetic library into *E. coli*, and selecting for clones that grew on media supplemented with one of the 13 antimicrobials. We recovered resistant clones capable of growth on media supplemented with a single antimicrobial, yielding 13 clones conferring resistance to at least one antimicrobial agent. Antibiotic susceptibility analysis revealed resistance ranging from 4 to >50 fold more resistant, while one clone showed resistance to multiple antibiotics. Using both Sanger and SMRT sequencing, we identified the predicted active gene(s) on each clone. One clone that conferred resistance to tetracycline contained a gene encoding a novel tetA-type efflux pump that was named TetA(62). Three clones contained predicted active genes on class 1 integrons. One integron had a previously unreported genetic arrangement and was named In1875. This study demonstrated the diversity and potential for spread of resistance genes present in human-impacted environments.

1. Introduction

Antibiotic resistance continues to be an ongoing challenge with our ability to prevent and to treat infectious diseases. Complications arising from bacteria harboring resistance genes are routinely observed in clinical, agricultural, and industrial settings. While antibiotic resistance genes are ancient traits that evolved millions of years ago, our use and sometimes overuse of antibiotics provides selective pressure for their dissemination (Allen et al. 2009; Bhullar et al. 2012; Popowska et al. 2012; Ready et al. 2004; Tian et al. 2012). Continued exposure to these agents exerts ongoing selective pressure for antimicrobial resistant microbes. The issue is concerning for two reasons: first, microbes harboring resistance genes can share those traits with other bacteria through horizontal gene transfer, and second, in many cases multiple resistance genes can be transferred between bacteria by transferring a single DNA segment (Zhang et al. 2017).

While sequence-based metagenomic interrogations of natural

microbial communities can significantly inform on the potential for antibiotic resistance, annotation is biased to antibiotic resistance genes (ARGs) previously entered into public repositories. Furthermore, metagenomics paired to either metatranscriptomics or metaproteomics is needed to link sequence to activity, but even this cannot nail down an active gene without experimental validation. Here, we bypass both these issues by employing functional metagenomic selections. The advantages and limitations of employing functional metagenomics in the search for novel ARG has been previously reviewed (Mullany 2014). In this approach, metagenomic DNA is heterologously expressed in *E. coli* under particular conditions—in this case, antibiotic selection—to uncover clones with metabolic activities of interest (Böhm et al. 2020; Handelsman 2004; Handelsman et al. 1998; Sabree et al. 2009; Xu et al. 2017). Functional metagenomics has been applied to various microbiomes with various metabolic targets, for example cellulases, proteases, and other biocatalysts (Daniel 2004; Delmont et al. 2011; Gill et al. 2006; Leis et al. 2015; Moore et al. 2011; Yaung et al. 2015).

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Importantly, though, functional metagenomic interrogations of soil and gut microbiomes have unearthed novel antibiotic resistance determinants (Allen et al. 2009; Donato et al. 2010; Im et al. 2016; Riesenfeld et al. 2004; Sommer et al. 2009). Metagenomic techniques have also been successfully applied to activated sludge communities to identify novel antibiotic resistance genes (Parsley et al. 2010).

The goal of this study was to identify antibiotic resistance genes, particularly those found on mobile genetic elements from a municipal wastewater treatment-derived microbial community in order to advance understanding of the role that wastewater treatment plays in selecting for resistant bacteria. Wastewater communities impact and are impacted by human activities in multiple ways. They are responsible for many of the degradative processes that remove organic material and other nutrients from wastewater. These microbial communities are continually exposed to a variety of organic compounds, some toxic, that can affect various traits of the overall community (Carey et al. 2016). A growing number of studies have cataloged the antibiotic resistance genes present in wastewater communities and have reported the possibility that exposure to antimicrobial agents in the wastewater exerts a selective pressure on these communities to harbor resistance genes (LaPara et al. 2015; Li et al. 2016; Zhang et al. 2016).

Here, we used functional metagenomics to interrogate the types of ARGs in synthetic wastewater treatment reactors exposed to the antibacterial agent triclosan. After seven weeks, these microcosms were combined and used to construct a metagenomic library for antibiotic selection. In total, 13 clones were recovered that conferred resistance to at least one of the antibiotics. Five of the resistant clones contain ARG that are associated with mobile genetic elements. The set of resistant clones included one novel tetracycline resistance gene, and one integron with a previously unreported genetic arrangement. This study highlighted the need for continued efforts to identify and to characterize new genetic determinants contributing to antibiotic resistance.

2. Methods

2.1. Enriching wastewater-derived bacteria

Activated sludge was collected from a municipal wastewater treatment facility located in central Minnesota and consisted of a nitrifying conventional activated sludge process. The sample was collected and stored at -80°C in 10% glycerol. One aliquot was thawed and 70 μL of the previously frozen sample was added to each of eleven media flasks with 200 mL of synthetic wastewater medium made by dissolving 80 mg each of peptone, tryptone, and yeast extract with water to make one liter of medium. Cultures were also supplemented with triclosan to a final concentration of either 0, 1, 5, or 15 $\mu\text{g}/\text{mL}$. The cultures were grown aerobically at 27°C with shaking at 200 rpm for seven weeks in the dark. During the growth period, media was exchanged every 48 h. The exchange was achieved by removing the flasks from the shaker and allowing the bacteria to settle at room temperature. Spent supernatant was replaced with fresh synthetic wastewater medium supplemented with the appropriate amount of triclosan to maintain concentrations. After seven weeks of growth, cells were pelleted and stored at -20°C until they were subjected to metagenomic DNA extraction.

2.2. Metagenomic DNA isolation

Frozen cell pellets were thawed and suspended in Nanopure™ water, and the cell suspension was filtered through a 0.45 μm filter membrane with a filtration apparatus (Millipore, Darmstadt, Ger.). Metagenomic DNA was isolated from the cells according to the protocol of the Meta-G-Nome DNA isolation kit (Epicentre, Madison, WI).

2.3. End Repair of isolated metagenomic DNA and DNA precipitation

The ends of the metagenomic DNA were prepared for cloning

according to the protocol of the CopyControl Fosmid Library Production Kit (Epicentre). All of the isolated DNA was used in the 80 μL end-repair reaction that was conducted according to the manufacturer's instructions. After incubation, the reaction volume was increased to 100 μL with water, and then 80 μL isopropanol and 10 μL of 3 M sodium acetate were added to precipitate the DNA. The reaction was then incubated at -20°C for 10 min, and centrifuged at $21,130 \times g$ for 25 min at 4°C . The supernatant was discarded, and then the DNA pellet was washed with 500 μL of 70% ethanol and spun at $21,130 \times g$ for 3 min. The ethanol was discarded, and the pellet was air dried for 8 min. The pellet was resuspended in 8 μL of water, and the DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

2.4. Ligation of metagenomic DNA and pCC1FOS vector

The end-repaired DNA was ligated with pCC1FOS vector according to the CopyControl Fosmid Library Production Kit protocol. A target of 0.3 μg of metagenomic DNA was used in each reaction.

2.5. Phage packaging

25 μL of MaxPlax Lambda packaging extracts were incubated with the 10 μL ligation at 30°C for 2 h, at which point an additional 25 μL of MaxPlax Lambda packaging extracts were added to the reaction. After incubation, 940 μL of Phage Dilution Buffer (10 mM Tris-HCl [pH 8.3], 100 mM NaCl, and 10 mM MgCl_2) and 25 μL of chloroform were added.

2.6. Phage infection

EPI300-T1^R cells were grown to an OD_{600} of 0.8–1.0 in LB + 0.2% maltose + 10 mM MgSO_4 . The cells were infected in reactions consisting of 200 μL cells and 20 μL packaged phage, and incubated at 37°C for 1 h. The reactions were plated on LB+ 20 $\mu\text{g}/\text{mL}$ chloramphenicol (Cm) to select for the marker on the vector backbone, and incubated overnight at 37°C . After incubation, the number of colonies was counted across all plates to calculate the size of the library, and the plates were stored at 4°C for three days.

2.7. Library storage

Cells were scraped from the infection plates and combined into subpools. The cells on each plate were suspended in two washes of 600 μL LB + Cm and combined in microcentrifuge tubes. The cells were pelleted at $2500 \times g$ for 5 min. The majority of the supernatant was discarded to give a 1:1 ratio of pellet and supernatant. The pellets were resuspended, and then combined with other pellets to a volume of 800 μL . Sterile 30% glycerol in LB was added to the cell suspensions to a final concentration of 10% glycerol. The resulting cell suspensions were then stored at -80°C .

2.8. Selection for resistant clones

Samples of each library subpool were grown in LB + Cm for 4 h, and then diluted 10^{-2} and 10^{-3} -fold, and plated on LB supplemented with spectinomycin (spec, 60 $\mu\text{g}/\text{mL}$), tetracycline (tet, 10 $\mu\text{g}/\text{mL}$), triclosan (2 $\mu\text{g}/\text{mL}$), colistin (8 $\mu\text{g}/\text{mL}$), D-cycloserine (32 $\mu\text{g}/\text{mL}$), rifampicin (rif, 100 $\mu\text{g}/\text{mL}$), carbenicillin (carb, 50 $\mu\text{g}/\text{mL}$), ampicillin (amp, 100 $\mu\text{g}/\text{mL}$), ceftazidime (2.5 $\mu\text{g}/\text{mL}$), piperacillin (16 $\mu\text{g}/\text{mL}$), meropenem (16 $\mu\text{g}/\text{mL}$), cefotaxime (8 $\mu\text{g}/\text{mL}$), or kanamycin (20 $\mu\text{g}/\text{mL}$). Cultures diluted 10^{-5} and 10^{-6} were grown on LB + Cm to estimate total library size. The plates were incubated overnight at 37°C . Resultant colonies were streaked onto LB plates with the respective antibiotic, and a plate supplemented with Cm, and incubated overnight at 37°C . Clones that grew on both plates were further analyzed.

2.9. Fosmid DNA isolation

Fosmid DNA was isolated from resistant clones after cells were grown overnight at 37 °C with shaking in LB + Cm + arabinose to increase the fosmid copy number. Fosmids were extracted as follows: 3 mL of cells were pelleted by centrifugation for 1 min at 9400 ×g. The pellet was suspended in buffer P1 (50 mM Tris-HCl, pH 8; 10 mM EDTA, 100 µg/mL RNase A), and buffer P2 (200 mM NaOH, 1% SDS) was added to lyse the cells. Buffer P3 (3.0 M Potassium Acetate, pH 5.5) was added to neutralize the pH and precipitate chromosomal DNA and cell debris. The suspension was centrifuged at 21,130 ×g for 10 min, and the supernatant was isolated. To the supernatant, 3 M sodium acetate and isopropanol were added. The solution was then centrifuged for 25 min at 21,130 ×g to pellet the DNA. The supernatant was discarded, and the pellet was washed twice with 70% ethanol and centrifuged for 3 min at 21,130 ×g. The ethanol was discarded, and the pellet was resuspended in 20 µL water. DNA yield was quantified using the Nanodrop 2000 spectrophotometer.

2.10. Transposon mutagenesis

Transposon mutagenesis of the Tet 62 clone was performed using the EZ-Tn5 < KAN-2 > Insertion Kit (Epicentre Biotechnologies) according to the manufacturer's protocol. Sites of insertion were mapped by sequencing the flanking DNA using the primers provided by the manufacturer.

2.11. Antibiotic susceptibility testing

The susceptibility of each clone to the various antibiotics were determined using a serial dilution method as previously described (Donato et al. 2010).

2.12. Sequence Data

Active clones were sequenced by the University of Minnesota Genomics Center using a combination of SMRT and Sanger sequencing technologies. SMRT sequencing data was processed through SMRTLink to produce circular consensus sequence (ccs) reads (Pacific Biosciences). The ccs-processed data was assembled with the Sanger sequence data using SPAdes (Nurk et al. 2013). Assembled contigs containing full or partial fosmid inserts were initially annotated with prokka and refined with individual BLAST searches (Seemann 2014). Sequences of the clones from this project have been deposited in the NCBI database under the accession numbers: MN340011-MN340023 (Lehwark and Greiner 2019).

3. Results

The goal of this project was to capture the diversity of ARG in an activated sludge community. Sub-lethal doses of antibacterial agents have been reported to affect microbial communities (Carey et al. 2016; Kaplan et al. 2018; Ma et al. 2019; Zhang et al. 2016), so we reasoned that a complete library of genes would contain those that are enriched by low-dose antibacterials as well as those that are not enriched in those conditions. In order to capture the diversity of ARGs, an activated sludge microbial community was inoculated into synthetic wastewater microcosms, and pre-enriched by growing them for 7 weeks in a medium supplemented with various, low concentrations of the antibacterial agent, triclosan. Triclosan was a common antimicrobial in many household products, so it was reasoned that the activated sludge might contain bacteria harboring triclosan resistance genes due to repeated exposure to triclosan. At the conclusion of the pre-enrichment process, biomass was harvested and used as the starting material for construction of a fosmid metagenomic library housed in *E. coli* according to the scheme depicted in Fig. 1. The combined size of the library in this study

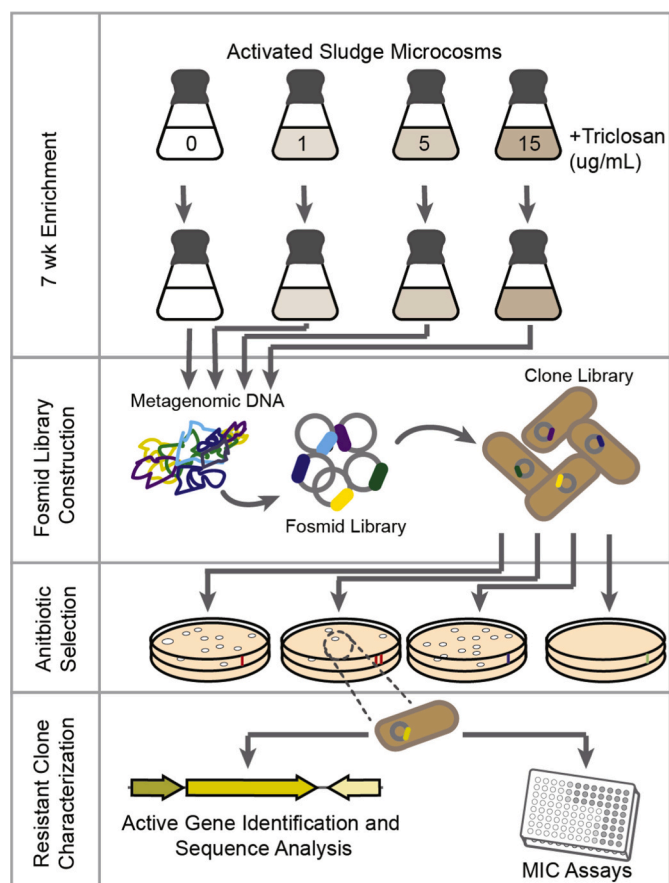


Fig. 1. Experimental scheme depicting the workflow for this study. Activated sludge microcosms were constructed and amended with either 0, 1, 5, or 15 µg/mL triclosan. These microcosms were harvested after 7 weeks, and the metagenomic DNA was used to construct a fosmid library. The resulting clone library was subjected to antibiotic selection. Resistant clones were then characterized using MIC assays, and the encoded putative active gene identified and the sequence analyzed.

was approximately 550,000 clones representing 20 Gb of DNA, or ~4000 microbial genome equivalents. The average insert size, estimated from a combination of agarose gel electrophoresis and bioinformatic analysis of fully sequenced clones, was 37 kb.

The library was selected for growth on medium supplemented with one of 13 antibiotics. Colonies of candidate resistant clones were further screened by re-introduction into fresh *E. coli* cells and repeated antibiotic resistance selection to eliminate false positives. A total of 13 unique clones were identified in this manner (Table 1). The set of resistant clones contains 4 clones with genes encoding integrons or transposases and one clone whose sequence suggested it contains DNA derived from an IncU plasmid.

Lab strain *E. coli* EPI300 was used as the host for heterologous expression of the metagenomic libraries. Therefore, we conducted antibiotic susceptibility testing and determined the resistance level of each clone relative to the isogenic *E. coli* EPI300 expressing an empty fosmid vector (Table 2). In each case, the decreased sensitivity to antibiotics ranged from 4-fold to >50-fold over the background levels exhibited by isogenic *E. coli* bearing an empty fosmid vector. Additionally, we determined antibiotic susceptibility across a panel of antibiotics in order to characterize multi-resistant clones. We found a single clone that we determined to be resistant to more than one antibiotic: Carb/Rif was found to be resistant to both carbenicillin and rifampicin.

The Carb/Rif, SpecC, and KanA clones each harbored their respective resistance genes on class 1 integrons. In general, a class 1 integron contains the gene for the *intI1* integrase, conserved promoter and *attC*

Table 1

The mechanism of resistance was determined through sequence-based analyses of clones conferring resistance. BLASTp was used to compare obtained sequences to known sequences within the GenBank database version available in April 2019. Clone names were assigned based on the antibiotic used for their selection. The plasmid sequence in the Carb/Rif clone was detected using PlasmidFinder (Carattoli et al. 2014). * indicates closest match in GenBank; however, the closest match to a known tetracycline resistance protein was 68% identical at the amino acid level.

Clone Name	Insights from sequence	Percent Identity Amino Acid level (Genus of closest match)	Predicted Resistance Genes (Gene name)
CarbB		100 (<i>Elizabethkingia</i>)	Metallo beta-lactamase (<i>blaB1</i>)
CarbD		100 (Uncultured)	Class A beta-lactamase (<i>carB6</i>)
CarbH		100 (<i>Elizabethkingia</i>)	Metallo beta-lactamase (<i>blaB1</i>)
Carb/Rif	Class 1 integron structure, multiple resistances, IncU plasmid sequence	100 (Multiple)	Class D beta-lactamase (<i>blaOXA</i>)
KanA	Pre-clinical class 1 integron	100 (<i>Pasteurella</i>)	aminoglycoside (2'') nucleotidyltransferase (<i>aadB</i>)
SpecB		57 (<i>Neorhizobium</i>)	aminoglycoside nucleotidyltransferase (<i>ant1</i>)
SpecC	Novel class 1 integron structure	100 (Multiple)	streptomycin/spectinomycin adenyltransferase (<i>aadA1b</i>)
Tet 62	Novel tet resistance gene	86* (<i>Pseudochrobactrum</i>)	Multi-drug efflux pump (<i>tetR</i>)
TetE	Multiple transposases	100 (Multiple)	Tet efflux pump (<i>tetR</i>)
TetF		100 (Multiple)	Tet efflux pump (<i>tetR</i>)
TetI		98 (<i>Achromobacter</i>)	Tet efflux pump (<i>tetR</i>)
TricAE		80 (<i>Chlamydiales</i>)	enoyl-[acyl-carrier-protein] reductase (<i>fabI</i>)
TricAF		100 (Multiple)	trans 2-enoyl-CoA reductase (<i>fabV</i>)

insertion sites, and an adjacent *sulI* gene. In between the integration sites, there are variable numbers of other genes that typically encode antibiotic resistance genes. The structures of the Carb/Rif and SpecC integrons fit this general structure (Fig. 2A). The variable region of the Carb/Rif clone contained putative resistance genes for aminoglycoside antibiotics, beta-lactam antibiotics, chloramphenicol, and rifampin. The variable region of the SpecC clone contained putative resistance genes for aminoglycoside antibiotics, lincosamide, and beta-lactam

Table 2

The susceptibility of *E. coli* bearing each clone to selected antibiotics was determined. The control is EPI300 *E. coli*. Numbers indicate the MIC in µg/mL. N.D. indicates value not determined.

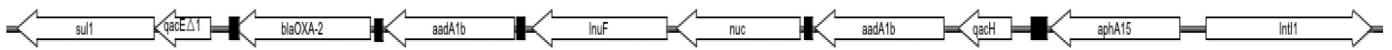
Clone Name	Carbenicillin	Kanamycin	Rifampicin	Spectinomycin	Tetracycline	Triclosan
Control	16	8	16	32	4	0.3
CarbB	256	8	N.D.	32	4	0.3
CarbD	256	8	N.D.	32	4	0.3
CarbH	256	8	N.D.	32	4	0.3
Carb/Rif	128	16	128	64	4	0.3
KanA	16	>64	N.D.	32	4	0.3
SpecB	16	8	N.D.	160	4	0.3
SpecC	16	8	N.D.	160	4	0.3
Tet 62	16	8	N.D.	32	16	0.3
TetE	16	8	N.D.	32	20	0.3
TetF	16	8	N.D.	32	20	0.3
TetI	16	8	N.D.	32	16	0.3
TricAE	16	8	N.D.	32	4	14
TricAF	16	8	N.D.	32	4	>14

antibiotics. All of the resistance genes on both of these clones were identical to known resistance genes. Querying the INTEGRALL database revealed the Carb/Rif integron is In37 (Moura et al. 2009). However, the arrangements of the specific resistance genes in the variable regions of each of the SpecC integron had not yet been reported and was deposited into INTEGRALL with the systematic name In1875. The cassette array on In1875 included a novel cassette that contained two genes without an intervening *attC* site, implying they may be transferred as a single cassette. One of these two genes was predicted to encode the *lnuF* lincosamide resistance gene. Although the ability to confer lincosamide resistance was not functionally assayed here, all of the amino acid residues responsible for both drug and nucleotide binding were identical to those reported for the *lnuF* gene from *E. coli* (Morar et al. 2009). The integron on the KanA clone appeared to be truncated. The *intI1* gene was present as well as the gene for an aminoglycoside nucleotidyl transferase; however, the 3'-conserved sequence was missing. This type of arrangement has been previously reported and indicates this integron arose before inclusion of the 3' conserved sequence (Ghaly et al. 2017). This type of integron was labeled as pre-clinical to indicate that evolutionary history. In keeping with the previous report, the integron on KanA also contained a transposon-associated DNA resolvase directly downstream of the 3' *attC* site. This integron is present in INTEGRALL and is known as In7.

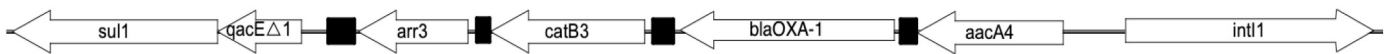
The integron on the SpecC clone contained an identical copy of the gene that confers spectinomycin resistance. The long read lengths associated with the SMRT sequencing technology were used to ensure this duplication was not an artifact of the sequence assembly process. This clone was fully sequenced using PacBio technology, and the resulting data was subjected to ccs analysis. Resulting ccs-processed reads that span this region were mapped onto the assembled clone (Fig. 2B). There was >10× sequence coverage across the region that contains the duplication. Additionally, there were seven unique sequences that contributed to the assembly and span all or part of both copies of the resistance genes.

The architecture of the active portion of the Tet 62 clone contained a predicted *tetR*, *tetA* gene pair. The TetA protein encoded by this gene exhibited 68% amino acid identity to the most closely related tetracycline resistance protein. This is below the 79% identity threshold that is used to determine novelty, so the corresponding gene was given the systematic name *tetA(62)*. The associated R gene was thus named *tetR(62)* as well (Fig. 3). Transposon insertions into this clone that integrated within the *tetA(62)* gene resulted in sensitivity to tetracycline, confirming that the functional *tetA(62)* gene was required to confer resistance to tetracycline. TetR proteins bind to the two palindromic *tetR* operator sites between the *tetR* and *tetA* genes. The *tetR* operator sites are 15-bp nearly perfect palindromes and are spaced 15 bp apart. This is consistent with the expectations for a TetR regulated system, suggesting that the *tetA(62)* gene expression was likely controlled by the TetR(62)

SpecC



Carb/Rif



KanA

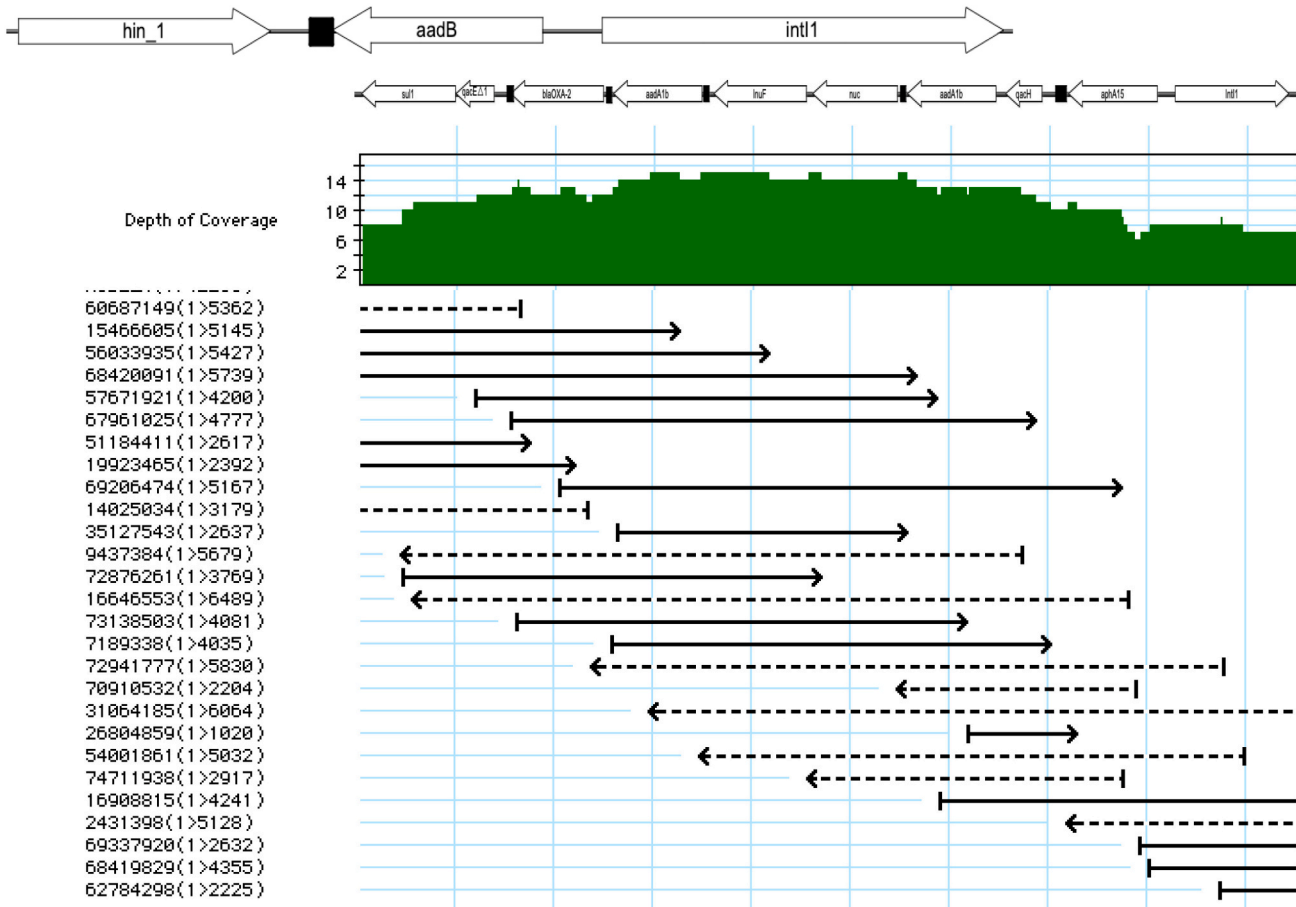


Fig. 2. (A) The genetic feature of each class 1 integron are listed. Black boxes denote *attC* sites. KanA contained a class 1 integron that does not include the *sul1* gene at the 3' end. Abbreviations are as follows: *aacA4*, aminoglycoside N(6') acetyltransferase; *aadB*, 2'' aminoglycoside nucleotidyltransferase; *aadA1b*, streptomycin/spectinomycin adenyllyltransferase; *aphA15*, aminoglycoside phosphotransferase; *arr3*, rifampin ADP-ribosyltransferase; *blaOXA-1*, class D beta-lactamase; *blaOXA-2*, class D beta-lactamase; *catB3*, chloramphenicol O-acetyltransferase; *int1*, class 1 integron integrase; *lnuF*, lincosamide resistance gene; *nuc*, purine nucleoside phosphorylase; *qacEΔ1* multidrug exporter; *qacH*, quaternary ammonium compound exporter; *sul1*, dihydropteroate synthase. Fig. 2 (B) The genetic architecture of the class 1 integron from the SpecC clone was shown in detail. Solid and dotted arrows indicated individual ccs-processed SMRT sequencing reads. Small rectangles between the genes indicated *attC* insertion sites.

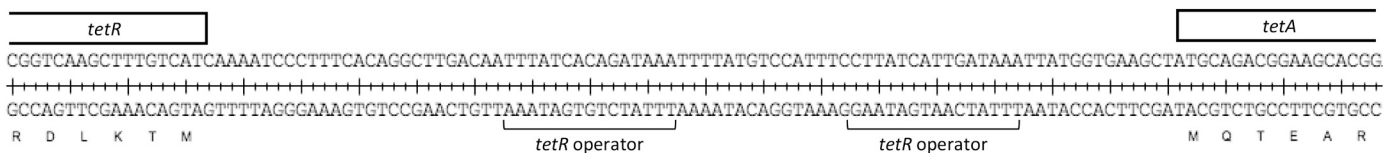


Fig. 3. The intergenic region between the *tetR*(62) and *tetA*(62) genes were shown. The two *tetR* palindromic operator sequences were indicated with square brackets.

transcriptional regulator (Ramos et al. 2005).

4. Discussion

The clones identified in this work included two types of novel features. The first type, found on the SpecC clone, contained an array of predicted antibiotic resistance genes harbored on a class 1 integron. To our knowledge, this specific arrangement of resistance genes on a class 1 integron has never been reported. By expanding the catalog of potential integron arrangements in this way, we can further understand the potential for co-selection of specific resistance elements. SpecC also contained a duplication of the gene that confers spectinomycin resistance. Although it is unclear what advantage this duplication offers in the case of SpecC, identical copies of a given antibiotic resistance gene embedded in a single class 1 integron have been reported to increase the MIC of specific antibiotics (Xu et al. 2018). These structural differences also shed light on the origins of the integrons. The integron from the KanA clone is considered to be a pre-clinical form of the class 1 integron as it does not include the 3' conserved sequences that includes the *sul1* gene (Ghaly et al. 2017). The presence of the *tniR* gene directly downstream of the 3' end of the *attC* site of the integron is also consistent with the previous report. *tniR* is a transposon-associated gene and its presence indicates that KanA class 1 may be embedded into a transposon suggesting a mechanism for the integron to become mobilized (Ghaly et al. 2017).

Comparison of the inferred amino acid sequences of the proteins encoded by the resistance genes reported here to those deposited in GenBank revealed the diversity of resistance determinants identified in this work ranged from 58% identical to 100% identical to known proteins. This level of diversity is consistent with previous reports of selection for antibiotic resistance determinants in *E. coli* (Allen et al. 2009; 2010; Forsberg et al. 2012). The diversity of genes discovered was likely a function of multiple factors, including the relatedness of the original bacterial host to the surrogate host, and the degree to which the original microbial community has been studied and its genes documented. The design of this study, while potentially increasing diversity among the genes identified, also likely played a role in limiting the number of genes recovered. Specifically, requiring that bacteria harboring active genes first grew in the enrichment culture(s), then requiring that the active genes conferred resistance to a surrogate bacterial host, likely excluded other potentially interesting genes. While acknowledging those limitations, one of the strengths of our approach was to potentially facilitate access to rare members of the wastewater community to enable characterization of their resistomes. This study further added to our growing knowledgebase by linking these specific resistance genes to potential mobile genetic elements, including plasmids, integrons, and transposons.

There was a large range of data in the antibiotic susceptibility test results. In this assay, clones of interest yielded values that represent a 4- to >50-fold decrease in susceptibility to each antibiotic when compared to the background strain. The range of these values is consistent with similar studies that have been previously reported for heterologous expression of metagenomic clones encoding antibiotic resistance genes (Allen et al. 2009; Donato et al. 2010; Amos et al. 2014; Xu et al. 2017). It is important to note that these are values that were determined through heterologous expression. The resistance level conferred by each of these genes may be higher in the original host bacterium where expression of the gene(s) is potentially higher.

These susceptibility tests led us to determine that the majority of clones identified in this work actively conferred resistance to a single antibiotic. Only one clone (Carb/Rif) actively conferred resistance to two antibiotics representing two distinct classes of antibiotics. Notably, bioinformatic analysis of the SpecC clone revealed a predicted beta-lactamase; however, no resistance to beta-lactam antibiotics was observed. This was likely a result of the putative beta-lactamase gene not being expressed in the surrogate host and is consistent with previous

reports of decreased gene expression from integrons in surrogate hosts as well as decreased translation of genes encoded towards the 3' end of class 1 integrons (Couvé-Deacon et al. 2019; Jacquier et al. 2009). The design of this study lent itself to the possibility of discovery of ARG that might be co-selected in the presence of sub-lethal doses of triclosan. However, none of the clones conferring active resistance to triclosan encoded active resistance genes to other antimicrobials within the ~37 kb insert. While there may not have been co-localization of active triclosan resistance genes with other active antimicrobial resistance genes, it is possible that co-resistance may have been propagated and not captured by the methods in this experiment because the alternative gene was located outside of the cloned portion. Conversely, at least one of the identified active resistance genes was linked to a putative triclosan resistance gene. The tet(62) genes were approximately 25 kb from a putative triclosan resistance gene. Although the Tet(62) clone did not confer active resistance to triclosan in *E. coli*, this triclosan resistance gene may have been active in its original host bacterium in the pre-enrichment. Therefore, it is still possible that in the original host bacterium the exposure to triclosan exerted a selective pressure that co-selected for the tet(62) tetracycline resistance genes.

The second novel clone was the tet(62) clone. Bioinformatic analysis of the tet(62)A gene revealed the gene encoded a putative efflux pump. The closest matches in the NCBI nr database are annotated as multi-drug efflux pumps. However, functional analysis of the resistance profile conferred by the tet(62)A determinant indicated that this determinant was specific to tetracycline when expressed in *E. coli*. This work highlighted the importance and need for coupling functional characterization with bioinformatic analyses.

5. Conclusion

This work highlighted the diversity of ARG harbored by wastewater bacteria. Many of these ARG have the potential for dissemination via their presence on mobile genetic elements. Future research will identify the role these mobile genetic elements play in the spread of ARG.

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