EVOLUTIONARY PROCESSES IN AQUATIC BACTERIA: THE TRANSFER AND TRANSPORT OF ANTIBIOTIC RESISTANCE IN INDUSTRIALLY CONTAMINATED SYSTEMS

by

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(Under the direction of J Vaun McArthur)

ABSTRACT

The presence of antibiotic resistant bacteria in non-clinical environments is the result of multiple selective pressures and evolutionary processes. To test the hypothesis that metal exposure is one selective pressure maintaining antibiotic resistance in aquatic bacteria, both bacterial community tolerance to metals and antibiotics and the relative abundance of antibiotic and metal resistance determinants were quantified along a gradient of metal contamination in freshwater microbial microhabitats on the Savannah River Site (SRS), SC, and in experimental freshwater microcosms. Phenotypic resistance to metals and antibiotics, and resistance determinants were greatest in metal-exposed bacterial communities supporting the hypothesis. The relative abundance of mobile genetic elements called class 1 integrons were then compared in contaminated and reference freshwater and estuarine microhabitats, and in experimental microcosms, to test the hypothesis that elements involved in the acquisition of exogenous sources of DNA are more abundant in bacterial communities exposed to metal contaminants . The structure and predicted function of the integron-associated gene cassette pool was then

compared using fragment analysis to assess how metal contamination shapes the gene cassette pool. Results indicate that class 1 integrons are abundant in environmental bacteria, and gene cassettes are a diverse genetic resource that can contribute to bacterial evolution. The complete nucleotide sequence of a 130 kb plasmid from a multidrug resistant *Escherichia coli* strain isolated from a contaminated estuary was determined to assess potential antibiotic resistance gene flow between clinical and environmental populations and the co-occurrence of metal and antibiotic resistance elements on the same plasmid. The plasmid contained several genes conferring resistance to multiple classes of antibiotics in a region with numerous insertion sequences, transposons, and integrons, yet no known metal resistance genes were identified. The sequence reveals that antibiotic resistance genes prevalent in clinically-derived isolates are prevalent in an environmental strain as well. The results of these studies indicate that antibiotic resistance persists in the environment, and that metal exposure selects for resistance phenotypes, genotypes, and genes involved in horizontal transfer.

INDEX WORDS: antibiotic resistance, metal resistance, bacteria, evolution, horizontal gene transfer, integron

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Bacterial adaptation to antibiotic selective pressures has generated much of what is known about evolutionary processes in bacteria. The dissemination of antibiotic resistance genes among clinical bacteria soon after the first usage of penicillin indicated that resistance genes were being exchanged among bacteria and the basic mechanisms involved in gene transfer relating to conjugation, transduction, and transformation were subsequently identified. For several decades, however, the dominant paradigm remained that bacteria evolve primarily through genetic mutation inherited vertically (27, 30). Through comparative analysis of gene content in microorganisms, it is now understood that horizontal gene transfer is also a driving force in microbial evolution (11). Furthermore, population-based studies indicate that bacterial genomes are highly dynamic with gene acquisition and loss, duplications, and rearrangements via transposase activity leading to heritable phenotypic variability within populations (3, 9).

Comparative genomic approaches also indicate that horizontal gene transfer has occurred throughout bacterial history (27), while an analysis of mobile genetic elements involved in antibiotic resistance transfer reveals that the genetic machinery involved is similar to those existing in bacteria prior to the antibiotic age. For example, metal-resistance plasmids predate the anthropogenic use of antibiotics. Mindlin et al. (33) suggested that the emergence of integron-carrying transposons that contain both antibiotic-resistance and mercury-resistance determinants is a relatively recent phenomenon because permafrost-derived *Pseudomonas* (~15 000–40 000 years old) contained closely related transposons found in many present-day bacteria, albeit devoid of antibiotic-resistance cassettes (34). In addition, the early characterization of R-plasmids from pre-antibiotic era culture collections of Enterobacteriaceae provided no evidence of transferable antibiotic resistance (23). Thus, the recent acquisition of antibiotic-resistance determinants on pre-existing R-plasmids must be evaluated within this context. Of interest is the

precise timescale at which preexisting metal-resistance elements acquired antibiotic resistance genes and whether this predated the widespread usage of antibiotics. The wide variety of elements involved in gene mobilization including multiple types of transposases, integrases, resolvases, and recombinases, also indicates mobile genetic elements are evolutionarily old.

Widespread antibiotic usage in clinical and agricultural settings has resulted in a strong evolutionary pressure favoring the acquisition of antibiotic resistance traits in bacteria in these settings. This is reflected by the current ineffectiveness of multiple types of antibiotics against major pathogens. What is also increasing is the number of multridrug resistant bacteria and extreme drug resistant bacteria. Furthermore, selective pressure from exposure to one antibiotic can lead to the selection or maintenance of other antibiotic resistance genes.

What is also apparent is the prevalence of antibiotic resistance in bacteria outside of these settings in habitats with varying degrees of anthropogenic antibiotic exposure (4). Multiple co-occurring mechanisms can be involved in the detection of antibiotic resistant bacteria outside of clinical or agricultural settings. First, as bacteria and other microbes are the original producers of antibiotics, these microbes have resistance mechanisms for self-immunity, and other bacteria have acquired or evolved resistance to these compounds (15, 30). Second, clinical, sewage, and agricultural settings act as sources of antibiotic resistance bacteria creating a conduit for their dispersal into environmental settings, carrying their genes with them (43). Third, antibiotics released from these same settings, though often at subinhibitory concentrations, also increase the selective pressure favoring antibiotic resistance genes (37). Fourth, co-selection mechanisms including co-resistance, cross-resistance, and co-regulatory responses may favor the maintenance of antibiotic resistance genes through bacterial exposure to other stressors as detailed below.

Neither the hypothesis that metal selective pressures co-select for antibiotic resistance has yet to be rigorously tested in environmental bacterial assemblages, nor has there been a mechanistic examination of potential processes involved in bacteria of environmental origin (but see (46, 53). The majority of studies that demonstrate co-selection at the population and community level are observational and have relied heavily on culture-dependent phenotypic analysis of isolates, making quantitative analyses of co-selection in microbial communities difficult. Mechanisms include co-resistance (the physical linkage of phenotypically unrelated genes on the same genetic element), cross-resistance (the same mechanism results in resistance to multiple stressors), or co-regulatory responses (stress-induced pathways regulate the expression of multiple genes).

Co-selection mechanisms

Co-resistance occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron (13). This physical linkage results in the co-selection for other genes located on the same element. Genetic resistance determinants can be contained within larger elements (e.g. integrons that exist within transposons) so that the transfer of the larger element also leads to the transfer of integral elements. The best-documented case with regard to co-resistance involves Tn21 and Tn21-like transposons, in which a mercury-resistance operon and an integron that contains multiple antibiotic resistance genes are co-located (6, 29, 53). Recent evidence into the evolutionary history of Tn21 identified the acquisition of the integron In2 as essential for attaining antibiotic-resistance genes (6, 19, 25, 29). Importantly, numerous studies have documented the presence of

Tn21 and Tn21-like transposons in both pathogenic and non-pathogenic Gram-positive and Gram-negative bacteria (6, 29, 33).

Plasmid sequencing in conjunction with bioinformatics and phenotypic analysis offers additional support for co-resistance, with numerous reports in the literature of plasmids that contain both antibiotic- and metal-resistance determinants. For example, plasmids that originate from sewage treatment plants contain genes that encode resistance to mercury (42, 47), chromate (48)and tellurite (49), along with multiple antibiotic-resistance genes. Sequence analyses indicate that many of these elements are flanked with insertion sequences and transposase sequences. Presumably, the plasmids have accrued these resistance determinants through multiple recombination events (17, 52). Additionally, genomic sequencing of the pathogen *Salmonella enterica* serovar Typhi CT18 revealed the presence of a conjugative plasmid (pHCM1) that confers resistance to trimethoprim (dhfr1b), sulfonamide (sulII), chloramphenicol (catI), ampicillin (bla) and streptomycin (strAB) and contains a mercury-resistance operon (35). These studies demonstrate that mobile genetic elements are mosaics of both metal and antibiotic resistance genes, and that large conjugative plasmids can play a role in the dissemination of metal and antibiotic resistance genes within bacteria of public health concern.

Cross-resistance can manifest through efflux of structurally dissimilar compounds using the same mechanism. To maintain homeostasis and import and export molecules bacteria have several classes of efflux pumps and porins used to transport these substrates across membranes. While expression of many of these transporters is tightly regulated, some transporters can pump multiple substrates. For example, the multiple-drug resistance (MDR) pump in *Listeria monocytogenes* can export metals in addition to antibiotics (31). Recent characterization of the MexGHI–OpmD efflux pump of *Pseudomonas aeruginosa* showed that the presence of the entire

pump operon in trans resulted in increased resistance to vanadium, ticarcillin and clavulanic acid compared with mutants that lack MexGHI–OpmD (1). Mutational analysis of a membrane-bound DsbA–DsbB disulfide bond formation system in *Burkholderia cepacia* followed by phenotypic analysis suggested that the DsbA–DsbB system is involved in the formation of a metal-efflux system and a multi-drug resistance system (21). Mutants without a functional DsbA–DsbBsystem were less resistant to a range of antibiotics and metals, including β-lactams, kanamycin, erythromycin, novobiocin, ofloxacin, sodium dodecyl sulfate, cadmium and zinc. Hernandez et al. (22)screened Enterobacteriaceae from contaminated oil refinery soils for isolates that could accumulate metals and found that two strains, *Escherichia hermannii* and *Enterobacter cloacae*, were markedly more antibiotic resistant when these isolates were grown in the presence of vanadate (compared with controls). Although the precise mechanisms underlying this co-resistance were not elucidated, the authors suggested that the MDR phenotype stimulated by vanadate addition was facilitated by membrane-bound efflux systems in these bacteria (22).

Bacteria have also evolved regulatory pathways that are activated under times of stress that can result in multple phenotypic changes in the cell. Known stress inducers include metals, UV, nutrient, temperature, and antibiotics and known stress response genes include SOS and mar induction. Perron et al.(38)addressed the issue of metal and antibiotic co-regulatory resistance in the Gram-negative bacterium *P. aeruginosa*. Isolates exposed to zinc were also found to be resistant to other heavy metals (cadmium and cobalt) and the carbapenem-class antibiotic imipenem. Analysis of the mechanisms that underlie cross-resistance to both zinc and imipenem revealed co-regulation of imipenem influx with heavy metal efflux (38). The *P. aeruginosa* two component sensor protein CzcS was subsequently found to be responsible for resistance to both

zinc and imipenem. Conejo et al. (14) found that zinc eluted from silicone latex urinary catheters exerted a negative effect on the expression of OprD2, a membrane porin responsible for carbapenem resistance in *P. aeruginosa*, which subsequently increased the overall resistance to this class of antibiotic.

Population and community level evidence for co-selection

Evidence for co-selection of antibiotic and metal resistance in the environment originates from diverse habitats contaminated with a variety of metals, which indicates that co-selection is not limited to a subset of metals, environments or microbial taxonomic groups. The strength of evidence presented by these studies ranges considerably between anecdotal reports of co-resistances to experimental studies that unambiguously implicate metals in antibiotic resistance co-selection.

Co-resistance of antibiotic- and metal-resistance traits

Reports of co-resistance to antibiotics and metals exist for clinical and environmental isolates and for bacterial populations and communities (2, 5, 7, 10, 16, 18, 39). In an example of co-resistance in clinical isolates, *Staphylococcus* species were resistant to multiple metals and antibiotics where the most common co-resistance involved chromium, lead and penicillin G (51). Potential public health concerns for the co-resistance of metal and antibiotic resistances were raised by Pettibone et al. (39) and Pathak and Gopal (36), who observed that bacterial isolates obtained from fish tissue commonly consumed by humans exhibited resistance to multiple metals and antibiotics. Although these studies do not directly address the hypothesis that metal exposure

co-selects for antibiotic resistance, they highlight the fact that metal and antibiotic resistances are commonly found within the same bacteria.

Resistance profiles from contaminated and reference settings

Corroborative support for co-selection stems from several studies that directly compare resistance profiles of bacteria collected from contaminated and reference sites. Although a direct assessment of the role of metals in co-selection is often hampered by the presence of other anthropogenic contaminants, these studies do link contaminant exposure with elevated antibiotic resistance. For example, the association of antibiotic- and metal-resistant phenotypes in environmental bacteria was hypothesized as early as 1974, when multiple antibiotic- and metal-resistant *E. coli* were more prevalent in sludge-contaminated estuarine sediment sites relative to reference locations (26). Additional studies conducted in marine systems observed elevated metal and antibiotic resistance co-occurring in isolates from contaminated sediments compared with reference sites [**57** (41, 50). Rasmussen and Sorensen (41) demonstrated an increased occurrence of conjugative plasmids in a contaminated site and showed that tetracycline- and mercury-resistance genes were located on plasmids, thereby providing indirect evidence of co-resistance. In freshwater systems, McArthur and Tuckfield (32) examined spatial patterns of antibiotic and metal resistance in contaminated and reference stream sediments and observed that isolates from industrially contaminated sediments were more resistant to kanamycin and streptomycin than those from a reference site. Furthermore, resistance to streptomycin was positively correlated with sediment mercury concentration.

Bacteria found within drinking water systems are exposed to a variety of metals, which is a potential selective force for metal resistance. To test this hypothesis and to ascertain whether

antibiotic-resistant bacteria were co-selected, Calomiris et al. (12) compared resistance profiles for isolates from a raw water intake site with isolates from multiple points along the drinking water system. In addition to the finding that isolates from within the drinking water system were more resistant to zinc, copper and lead than isolates from the raw water system, the authors demonstrated that metal-resistant isolates from within the drinking water system exhibited multiple antibiotic resistances whereas metal-sensitive isolates did not. Interestingly, this pattern did not hold true for all tested metals as cadmium resistance did not correlate with multiple-antibiotic resistance. The authors also noted that a gradient of metal and antibiotic resistances existed within the water system with isolates further from the source being more resistant than those at a site closer to the water intake.

Co-selection has also been observed in several agricultural-based studies. Huysman et al. (24) observed higher frequencies of resistance to a range of metals and antibiotics (including zinc, cadmium, nickel, cobalt, ampicillin, streptomycin, olaquindox and spiramycin) in copper-resistant isolates when compared with copper sensitive bacteria isolated from agricultural fields in which copper-contaminated pig manure had been applied (24). *Enterococcus faecium* isolates obtained from pigs, broiler chickens, calves and sheep were also tested for resistance to copper and erythromycin and vancomycin (20). The correlation between copper resistance and antibiotics was significant only for isolates obtained from pigs, which may in part be a result of the higher copper exposure in pigs through feed additives compared with other livestock in Denmark.

Recent developments in culture-independent flow cytometry have provided a more quantitative, high-throughput insight into co-selection in the environment. Using this technique, co-selection for resistant bacteria from freshwater aquatic systems that receive metal-laden coal

ash effluent has been detailed (45). Stepanauskas et al. (45)observed elevated tolerances to a range of metals and antibiotics in bacterioplankton collected from ash-settling basin water relative to bacterioplankton in intake water at three coal-fired power plants. Bacterial exposure to metals within the ash-settling basin was most probably the selective agent driving co-resistance (45).

Experimentally induced co-selection

Experimental studies in which metal exposure is directly manipulated to test for co-selection in bacterial communities are rare. Berg et al. (8) found that soil microbes isolated from a copper-amended field were more resistant to copper and antibiotics than strains isolated from control plots 21 months after copper amendment. Additionally, copper resistant strains were significantly more resistant to ampicillin and sulfonamide than copper-sensitive isolates, which strengthened the argument that the traits are co-selected. Experimental evidence for co-selection has been demonstrated using microcosms amended with a variety of metals or antibiotics, which resulted in an increased frequency of multiple resistance phenotypes (44). For example, addition of cadmium to microcosms that contained freshwater bacterioplankton resulted in increased resistance not only to this metal but also to nickel, ampicillin and gentamycin (44). Mercury contained within dental amalgams has been linked to co-selection of antibiotic-resistant bacteria by studies that examined isolates from intestinal and oral bacterial communities (46, 53). Ampicillin resistance profiles in isolates obtained after dental amalgam installation indicated that resistance to ampicillin increased relative to pre-installation levels (46). In a subsequent study, resistance to multiple antibiotics was more common in mercury-resistant isolates compared with those that were sensitive to mercury. Furthermore, the number of antibiotics to which isolates

were resistant correlated to the harbored *mer* locus, which is probably a result of the location of the *mer* operon on an integron containing transposon such as Tn21 (53). Yet, other studies have found no clear association between dental amalgam presence and antibiotic resistance (28, 40) illustrating the potential complexities of mechanisms and organisms involved in co-selection processes.

Summary

Because antibiotic resistance genes confer an observable phenotypic change in bacteria, they are well suited for examining evolutionary processes in bacteria. By examining antibiotic resistance patterns and dissemination we have a better understanding about interactions both within and among bacterial populations. We now realize that a complex set of ecological and evolutionary processes are interacting to increase the spread of resistance drugs among environmental and clinical bacteria. This has serious implications for our ability to combat bacterial infections.

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CHAPTER 2

BACTERIAL TOLERANCES TO METALS AND ANTIBIOTICS IN METAL-CONTAMINATED AND REFERENCE STREAMS[1]

[1]Wright, M.S., R. Stepanauskas, and JV McArthur. To be submitted to *Microbiology Ecology*.

Abstract

Anthropogenic-derived sources of selection are typically implicated as mechanisms for maintaining antibiotic resistance in the environment. Here we report an additional mechanism for maintaining antibiotic resistance in the environment through bacterial exposure to metals. Using a culture-independent approach involving flow cytometry and assays for bacterial membrane permeability, bacteria sampled along a gradient of metal contamination were more tolerant of antibiotics and metals compared to bacteria from a reference site. This evidence supports the hypothesis that metal contamination directly selects for metal tolerant bacteria while co-selecting for antibiotic tolerant bacteria. Additionally, to assess how antibiotic and metal tolerance may be transported through a stream network, we studied antibiotic and metal tolerance patterns over three months in bacteria collected from multiple stream microhabitats including the water column, biofilm, sediment, and *Corbicula fluminea* (Asiatic clam) digestive tracts. Sediment bacteria were the most tolerant to antibiotics and metals, while bacteria from *Corbicula* were the least tolerant. These differences between microhabitats may be important for identifying reservoirs of resistance genes and for predicting how these genes are transferred and transported in metal- contaminated streams. Temporal dynamics were not directly correlated to suite of physicochemical parameters such as precipitation or fluctuations in metal concentrations, suggesting that tolerance patterns within microhabitats are linked to a complex interaction of physicochemical characteristics of the stream.

Introduction

One aspect challenging public health efforts to minimize the spread of antibiotic resistance is the prevalence of resistant bacteria in the environment [1, 2]. Anthropogenic-derived sources of selection are typically implicated as mechanisms for maintaining antibiotic resistance in the environment whether it is through the release of antibiotics or resistant bacteria from confined animal feeding operations, hospital waste, or sewage treatment facilities [3-6]. An increasing number of studies have documented an additional mechanism for maintaining antibiotic resistant bacteria in the environment through co- or cross-resistance to metals or co-regulation of resistance pathways [7-13]. Therefore, it appears likely that metal exposure can directly select for metal resistant bacteria while co-selecting for antibiotic resistant bacteria.

Two scales must be considered when addressing the topic of antibiotic resistance in the environment. The first is the molecular scale of resistance genes and gene transfer. Resistance genes are frequently located on mobile genetic elements such as transposons and plasmids which can then be transferred to other bacteria [5, 14]. The second relevant scale is the landscape through which bacteria and their genes are transported through the stream network. Potential transport routes for bacteria in streams landscapes include aerial dispersal, groundwater connectivity, surface flow, or transport via other organisms such as invertebrates or fish. Factors influencing gene transport through streams are predicted to be those contributing to bacterial dislodgement from microhabitats (e.g., physical scouring due to stream flow, disruption of habitat due to invertebrate activities) and the degree of selective pressure favoring the establishment of the bacteria or gene (e.g., metal concentrations). Mechanisms for gene transfer through the stream likely include gene exchange in stream microhabitats where bacteria reach high densities and cells remain in close contact [15]. In order to identify critical locations at the

stream scale for antibiotic resistance gene transfer and transport, the prevalence of antibiotic and metal resistant bacteria in multiple stream microhabitats needs to be established. Thus locations with higher levels of resistance are likely to serve as sources for resistant bacteria and genes in these systems.

Four microhabitats common to most streams are biofilm on submerged woody debris, sediments, surfaces associated with stream organisms, and the water column. Each microhabitat represents a different degree of metal exposure and bacterial density. For example, biofilm microhabitats are areas in which bacteria reach high densities and exist in close contact or association with one another [16, 17]. Further, due to the metal binding properties of biofilms, bacteria within biofilms may be exposed to higher concentrations of metals relative to those in other microhabitats [18, 19]. These conditions may be conducive to gene transfer. Sediment microhabitats may also be conducive to the selection of resistance genes, as sediments are also a major site of metal deposition in streams. Bacteria residing here can reach high densities relative to other microhabitats due to organic matter availability. *Corbicula fluminea* (Asiatic clam), a representative stream organism common to many systems, reside in sediments where metals are typically deposited, and filter a large volume of water suggesting that many bacteria come in contact with *Corbicula* surfaces [20]. Finally, water column bacteria represent planktonic bacteria and those associated with particles in transport. Typically bacteria and metals are less abundant in the water column compared to other microhabitats. Investigating antibiotic and metal resistance dynamics at the stream scale will allow for an assessment of which microhabitats are likely to be important sources of resistance.

Trace metals are significant contaminants in many aquatic systems in part due to anthropogenic processes such as industrial and mining inputs. More specifically, coal fired

power plants generate large quantities of ash through the combustion process, and this waste contains multiple trace metal residues such as arsenic and selenium. Ash residues are then commonly mixed with plant intake water before being deposited in settling basins, and this flow through water is then typically discharged into aquatic systems [21]. Multiple studies have documented deleterious effects of coal ash on ecological systems [21, 22], but less is known concerning coal ash effects on microbial communities (but see 8, 23, 24).

This study addresses whether sites in streams receiving metal contamination from coal-fired power plants have elevated antibiotic and metal tolerances relative to a reference site. Bacterial tolerance is defined as the ability of bacteria to persist after exposure to a toxin, and differs from a definition of resistance in that there is no assessment of bacterial growth ability. Stepanauskas et al. [8] recently found elevated metal and antibiotic tolerances in bacteria from an ash settling basin relative to plant intake waters at the Savannah River Site coal-fired power plant, demonstrating that metal inputs into the system are likely the selection mechanism acting to elevate metal and antibiotic tolerances. This study extends the spatial scale of Stepanauskas et al. [8] by examining whether metal and antibiotic tolerances are elevated in a stream receiving coal ash input and whether this pattern persists downstream. This information is of particular interest in light of the common practice of discharging effluent from coal-fired power plants into lotic systems. This study also identifies locations within streams that are likely to serve as reservoirs for tolerant bacteria.

We tested the hypothesis that metal exposure results in the co-selection of metal tolerant and antibiotic tolerant bacteria along a gradient of metal contamination. Additionally, we tested the hypothesis that microhabitats vary in regards to bacterial tolerance to metals and antibiotics by comparing antibiotic and metal tolerance values among multiple microhabitats, thereby

assessing which locations within streams are likely to serve as sources for tolerant bacteria. The final objective was to examine temporal dynamics in metal and antibiotic tolerances in bacteria, and how these dynamics relate to physicochemical attributes of the sites. As we predicted, both antibiotic and metal tolerances were highest at our most contaminated site and lowest at our reference site. Further, as we predicted, both tolerances would were generally highest within sediment and biofilm microhabitats due to higher metal bioavailability in these locations relative to other microhabitats within the sites. We also predicted that antibiotic and metal tolerances would be linked to changes in physicochemical attributes of the sites such as precipitation and fluxes in metal concentrations, yet these relationships were difficult to elucidate with our sampling regime.

Methods:

Study Site

We studied two streams located on the United States Department of Energy's Savannah River Site (SRS) near Aiken, South Carolina. Beaver Dam Creek drains approximately 2,159 ha and flows into the Savannah River. Bottom and fly ash residues generated by the D-400 coal-fired power plant located on the SRS travel through two settling basins and a wetland before entering Beaver Dam Creek near its headwaters. Combined residence time for water in the two settling basins is approximately two months [25]. Two sites on Beaver Dam Creek were used. Site A is near the wetland outfall as it enters into Beaver Dam Creek. Sediment at Site A consists mainly of cobble, *Corbicula* shell remnants, sand, and ash particles. Site D is 3.2 km downstream of Site A where the sediment is composed predominantly of sand. This site is located just upstream of where Beaver Dam Creek enters the Savannah River floodplain swamp. The reference site on

Meyers Branch is also predominantly sandy-bottomed and is located near its confluence with Steel Creek. Meyers Branch is a historically-unimpacted blackwater stream that drains approximately 5,491 ha.

Sample collection

Bacteria were collected from the following four stream microhabitats: water, sediment, biofilm, and *Corbicula fluminea* (Asiatic clam) hind guts. In order to investigate the bacterial community response to metal exposure, a transplant study was conducted involving the transfer of *Corbicula* and biofilm from the Meyers Branch reference site to the Beaver Dam Creek sites. This ensured that any observed differences in bacterial tolerances between sites were the result of site characteristics, and not artifacts from differences in clam populations or woody debris differences. *Corbicula* were collected from Meyers Branch and deployed in containment cages at Beaver Dam Site A and Site D, as well as back-transplanted into the Meyers Branch reference site. Four plastic containment cages (31 cm x 18 cm x 11 cm) containing *in situ* sediment and 20 clams were submerged and tethered to aluminum poles in the stream at each site. Clams were secured by attaching netting (1 cm mesh size) over the top of the cages. On each sampling date, clams were harvested from the cages at each site and returned to the laboratory on ice for processing. For the biofilm collection, *Acer rubrum* (red maple) branches were cut into 20 cm by 5 cm diameter pieces that were bound together to form woody debris bundles. Bundles were then tethered to aluminum poles and submerged at the reference site one week prior to the initiation of the experiment to allow for biofilm growth. These were then transplanted to the same three locations as the *Corbicula* transplants. Woody debris bundles were harvested from each site and returned to the laboratory on ice for processing. Samples for Day 0 were collected

from *Corbicula* and biofilm indigenous to each site instead of from the transplanted samples to assess *in situ* tolerance values. Water column and sediment microhabitats were sampled by collecting grab samples in sterile whirl packs. Only sediment less than 2 mm in diameter was used in samples to reduce potential variation associated with particle size differences. To examine temporal patterns in antibiotic and metal tolerance, samples were collected in quadruplicate from each microhabitat once per month for three months.

Sample processing

Bacteria from *Corbicula* hind guts were obtained by aseptically removing the hind gut and manually grinding this material in sterile phosphate buffered saline (PBS) solution. To dislodge bacteria, this solution was then vortexed, and shaken in an ultrasonicator bath (Fisher Scientific) for 10 minutes. Bacteria were separated from other particles using low speed centrifugation (350 x g for 3 minutes). The supernatant was used in antibiotic and metal tolerance assays. Biofilm bacteria were collected by aseptically scraping a known surface area (4 cm x 4 cm) of the woody debris bundle. Biofilm was enzymatically digested using β-galactosidase in a sodium pyrophosphate solution as previously described [26]. Modifications included omitting lipase and α-glucosidase from the enzymatic treatment, eliminating the addition of sodium dodecyl sulfate (SDS), shaking samples at a lower speed (300 rpm instead of 1000 rpm), and using an ultrasonicator bath instead of a ultrasonicator probe. These modifications were used to minimize damage to cell structures. Bacteria were then pelleted and resuspended in sterile PBS. To remove biofilm particle debris, bacteria in the PBS solution were then centrifuged at 350 x g for 3 minutes and the supernatant was used for the tolerance assays. Sediment bacteria were detached from particles through vortexing and sonicating 0.5 g of sediment in 4 ml of sterile PBS

for 10 minutes in a ultrasonicator bath. Bacteria were separated from other particles by centrifugation at 200 x g for 3 minutes and the supernatant was used in tolerance assays. Water column samples did not require further processing to separate bacteria from other particles. All detachment and separation methods were validated using plating, direct cell counts, and flow cytometry. For development of these methods, various protocols were compared to optimize detachment and separation of bacteria from other particles, while minimizing cell disruption and loss.

Antibiotic and metal tolerance assays

Each processed sample was subdivided into six aliquots (500 µl in 15 ml sterile culture tubes). Each aliquot was either exposed to one of four contaminants or served as a positive or negative control. To assess antibiotic and metal tolerance, bacteria were incubated under slow rotation for 18 hours at 25 °C in PBS with one of the following treatments: 100 mg L^{-1} ampicillin (β-lactams), 300 mg L^{-1} tetracycline (tetracyclines), 1 mM $CdCl_2$ (class B metal), or 1 mM $NiCl_2(OH_6)$ (transition metal). Concentrations were chosen to be similar to Stepanauskas et al. [8]. Positive controls (no contaminant added) and negative controls (5% formalin added) were also used. Bacterial tolerance was then operationally defined to be the ratio of the number of live bacteria in treatment aliquots compared to the number of live bacteria in control treatments at the end of the 18 hour incubation (e.g., the number of live bacteria in a cadmium aliquot compared to the number of live bacteria in the positive control from the same original sample).

Flow cytometric analysis

Live bacteria were quantified through fluorescent viability staining based on membrane permeability, where live bacteria were defined as those with intact membranes. Flow cytometry analysis was

conducted as in Stepanauskas et al. [8]. Briefly, samples were incubated for 10 minutes with 30 µM propidium iodide (non-membrane permeable, red-fluorescing) and 5 µM SYTO 13 (membrane permeable, green-fluorescing) nucleic acid stains (Molecular Probes, Inc., Eugene, OR, USA); 1mM Na-EDTA, 0.01% Tween-20; and a known quantity of 2.15 µm fluorescent SkyBlue particles as an internal calibration standard (Spherotech, Inc., Libertyville, IL, USA). Live bacteria were classified as events with intense green fluorescence on green versus red fluorescence bi-plots. Total bacteria (live plus dead) were enumerated using the above procedure without the addition of propidium iodide. Bacteria were then classified as events with intense green fluorescent particles on green fluorescence and light side scatter bi-plots. Flow cytometry samples were run on a standard configuration FACSCalibur instrument containing a 488 nm argon laser using CellQuest software (BD, Franklin Lakes, NJ, USA). Previous studies have shown the efficacy of this method for distinguishing between live and dead bacteria and determining bacteria tolerance to antibiotics and metals in ash settling basin studies [8, 27-29]. This method allows for a culture-independent analysis of the bacterial community. The fluorescent stains as used in this study demonstrated highly similar results to assaying esterase activity using carboxyfluorescein diacetate (CFDA) [8].

Metal analysis

Samples for trace metal analysis were collected on each sampling date at each location. Water samples for dissolved metal analysis were collected in acid-washed high-density polypropylene bottles. Dissolved samples (100 ml) were filtered through GHP Acrodisc GF 25 mm syringe filters with GF/0.45 µm GHP membranes (Pall Life Sciences, East Hills, NY, USA). Water samples were acidified with 5 ml of nitric acid and were stored at -40 ºC until analysis. All samples were assayed in the analytical facility at the Savannah River Ecology Laboratory. Samples were subjected to nitric acid/hydrogen peroxide microwave digestion (CEM, Matthews,

NC, USA) followed by inductively coupled plasma-mass spectrometry analysis (ICP-MS, Perkin-Elmer, Norwalk, CT, USA). Appropriate calibration standards and blanks were used in both the digestion and analysis processes.

Physical and Chemical Attributes

On each sampling date a suite of physical and chemical measurements were obtained at each site. Water temperature, pH, dissolved oxygen, and conductivity were measured in the field (Hydrolab, YSI, Inc., Yellow Springs, OH, USA). Water samples for turbidity measurements were analyzed upon returning to the laboratory. Sediment analysis included organic matter content and sediment grain size measures. Organic matter content was determined by quantifying the percent ash free dry mass (AFDM) of the sediment by ashing dried samples at 460 °C for 8 hours. To estimate sediment particle size distributions, sediments were sorted into >2mm, sand, silt, and clay fractions based on the hydrometer method [30]. Daily precipitation data for the SRS was obtained from SRS records.

Statistical analyses

The response variable used to quantify bacterial tolerances was the ratio of the number of live bacteria in a treatment aliquot relative to the abundance of live bacteria in the positive control aliquot. Data were log-transformed before conducting principal component analysis (PCA) using each of the four metal or antibiotic treatments to form the four principal component axes. The first principal component explained 84.9% of the variation and each metal or antibiotic contributed equally to the loading of the principal component (range of 0.48 for ampicillin to 0.51 to tetracycline). Thus, subsequent analysis of variance (ANOVA) to compare the main effects of site, microhabitat, and date and the interaction terms were performed on values from this first principal component. These values represent the overall tolerance of bacteria in each

sample to the toxicants and are reported as a tolerance index. Sites and microhabitats were compared with *a priori* contrasts.

Results

Metal analysis

Dissolved metal concentrations from water samples were highest at Site A and lowest at the reference site on Meyers Branch, resulting in a gradient of metal contamination across the three sites (Table 1). Dissolved metal concentrations at Site A were elevated for V, Ni, Cu, As, Se, Sr, and Cd relative to Site D and Meyers Branch. Meyers Branch and Site D had slightly higher dissolved concentrations of Zn and Pb than Site A. Concentrations at Site D were generally intermediate of those at Site A and Meyers Branch. There was no consistent temporal pattern for dissolved metal concentrations at any site (data not shown).

Physical and chemical attributes

The days prior to sampling on Day 28 experienced more rainfall than the other three sampling dates (Figure 1). Turbidity was highest on Day 0 and Day 84 for Site D ranging between 11 and four nephelometric turbidity units (NTUs), but highest on Day 28 for Meyers Branch, ranging between six and two NTUs. Turbidity did not fluctuate greatly over time at Site A, remaining around 2 NTUs. Site A had the highest mean temperature (30.9 °C) followed by Site D (27.1 °C) and Meyers Branch (22.5 °C). pH was circumneutral at the three sites ranging from 7.28 at Meyers Branch to 7.43 at Site A. Site A also had the highest mean conductivity with 0.293 µS/cm, while mean conductivity at Site D was 0.140 µS/cm and 0.060 µS/cm at Meyers Branch. Dissolved oxygen varied little between sites or sampling dates, with mean concentrations of 7.29, 7.00, and 7.48 mg/L at Site A, Site D, and Meyers Branch respectively.

Sediment organic matter was highest at Site D (2.32% of dry mass) followed by 1.76% at Meyers Branch and 0.36% at Site A. Sand was the predominant class of sediment particles less than 2 mm in size at all three sites, ranging from 94.5% of particles at Site A to 98.6% at Meyers Branch.

Bacterial Abundance

Total bacterial abundance in each sample increased over the 18 hour incubation (Figure 2). Initial bacterial abundance typically ranged from 10^6 cells per ml of water and per cm^2 of biofilm, 10^6 to 10^7 cells per *Corbicula*, and 10^7 to 10^8 cells per gram of sediment. At the end of the incubation, bacterial abundance in control samples typically ranged between 10^7 per ml of water,10^7 and 10^8 cells per cm^2 of biofilm and per *Corbicula*, and 10^8 and 10^9 per gram of sediment. Temporal patterns indicate that abundance was lowest on Day 28 for biofilm, *Corbicula*, and sediment samples across most sites, whereas bacterial abundance did not fluctuate greatly in water samples (data not shown).

Antibiotic and metal tolerance

Tolerance values to the tested metals and antibiotics were highly correlated, with correlation coefficients ranging from 0.70 between ampicillin and cadmium tolerance values and 0.85 between tetracycline and nickel tolerance values. Each site was significantly different with regards to antibiotic and metal tolerance across all microhabitats (Figure 3, Table 2, $p<0.001$). Bacteria at Site A had the highest tolerance followed by Site D, and Meyers Branch generally had the lowest tolerance. Within a site, microhabitats were also significantly different (Figure 3, Table 2, $p<0.001$). Sediment bacteria had the highest tolerance values followed by biofilm,

water, and *Corbicula*. However, bacteria in water and biofilm microhabitats at Site A exhibited comparable tolerance values for each toxicant (Figure 4). Water and biofilm tolerance values at Meyers Branch were comparable for the two antibiotics tested but not for the metals. Temporal patterns indicate that tolerance dynamics were more variable at Meyers Branch and Site D, whereas Site A tolerances were more consistent through time (Figure 5). Antibiotic and metal tolerances were highest on Day 28 and lowest on Day 84 for Site D and Meyers Branch. Among microhabitats, *Corbicula* tolerance values fluctuated the least over time (Figure 6). Biofilm and sediment patterns showed the same trend with increasing tolerance on Day 28 followed by decreases on Days 56 and 84. Bacterial tolerance in water microhabitats showed a contrasting pattern as tolerance increased on both Day 28 and Day 56 before declining again on Day 84.

Discussion

That bacterial tolerances to metals and antibiotics were highest at the most contaminated site supports the central hypothesis that metal exposure directly selects for metal tolerant bacteria while co-selecting for antibiotic tolerant bacteria. These findings are consistent with culture-based results that suggest an association between the two traits obtained from multiple study systems including marine and freshwater sediments, agricultural soils, drinking water systems, oral and fecal bacteria isolated from mercury-exposed primates [31]. However, this study is novel in that it directly tests the hypothesis that bacterial exposure to metal co-selects for antibiotic tolerant bacteria in a culture-independent assessment of the microbial community in multiple microhabitats. The operational definition of tolerance as used in this study is influenced by two processes: cell mortality and reductions in bacterial growth (cell division) rates in the treatment aliquots compared to reference aliquots. Other potential measures of tolerance include

a comparison of the ratio of the number of live to dead bacteria between treatment and control aliquots. The limitation of this method, however, is that it does not detect the effect of a toxicant on growth rates. Thus the response variable used in this study, though it may be potentially affected by differential growth rates between control samples, offers a more sensitive assay of tolerance.

Recent experimental studies support the conclusions obtained in this study. Stepanauskas et al. [32] recently reported experimental evidence using culture-independent techniques that metal exposure directly selects for metal tolerant bacteria while co-selecting for antibiotic tolerant bacteria in a microcosm-based study. This pattern was found after bacterial exposure to multiple metals and multiple antibiotics, which suggests that co-selection is not limited to a subset of metals or antibiotics. Berg et al. [7] documented elevated antibiotic resistance in an experimental field study in a copper-amended field using culture-based assays. Copper resistant isolates had a higher incidence of antibiotic resistance as compared to copper sensitive isolates, indicating that these metal and antibiotic traits are associated. These studies all support the hypothesis that metal exposure results in increased frequency of antibiotic tolerance in bacteria.

Potential mechanisms for co-selection fall into three broad categories: co-resistance (resistance determinants physically linked within a genetic element), cross-resistance (the same resistance determinant confers resistance to multiple toxins), and co-regulation (expression of resistance determinants under control of same regulatory pathway). Evidence from previous studies exists for all three mechanisms (e.g., 9, 33, 34). Discerning which mechanism is involved in a bacterial community is made difficult by the multitude of antibiotic and metal resistance pathways utilized by bacteria [35, 36]. Thus, further investigations are needed to

identify specific resistance determinants involved in conferring resistance to both antibiotics and metals.

One objective of this study was to assess how antibiotic and metal tolerance values varied along a gradient of metal contamination. Site D had intermediate levels of contamination and intermediate antibiotic and metal tolerance values as compared to Site A and Meyers Branch. This suggests that the selective pressure for antibiotic and metal tolerance had diminished by Site D which is approximately 3.2 km downstream of Site A. Bacteria collected from Site D microhabitats not only reflect *in situ* contaminant exposure, but are also comprised of those bacteria that have been transported downstream from more contaminated areas. A companion study examining metal uptake in *Corbicula* found no difference in *Corbicula* tissue concentrations at Site D as compared to Meyers Branch, although concentrations in clams from Site A were significantly higher (Gretchen Loeffler Peltier, unpublished data). This implies that the effect of metal contamination on microbial communities may persist further downstream as compared to other stream organisms. Overall, Meyers Branch had the lowest tolerance values, but antibiotic and metal tolerant bacteria were still present, and in some cases tolerance values were higher than the other two sites (Figure 4). McArthur and Tuckfield [10] also documented resistance to kanamycin and streptomycin in sediment bacteria from Meyers Branch, although the resistance frequency was lower compared to a nearby contaminated site. Interestingly, the entire Meyers Branch watershed is contained within the borders of the SRS; thus there are no anthropogenic sources of antibiotics or resistant bacteria in this watershed.

A second objective of this study was to assess which microhabitats within the stream are likely to serve as sources of antibiotic and metal tolerance. With a few exceptions, bacterial tolerance to metals and antibiotics was higher in sediment and biofilm compared to the water

column and *Corbicula* digestive tracts. It is plausible that this elevated tolerance is a result of higher selective pressure imposed upon microbial communities by elevated metal concentrations in sediments and biofilm. For example, Cd concentrations at Site A were 0.03-0.05 ppb in the water column and 40-160 ppb in the sediment (data not shown). Ni concentrations were 1.7-3.6 ppb and 1,200-3700 ppb in the water column and sediment respectively. Contrary to this trend, bacterial tolerance values at Site A were comparable for water and biofilm microhabitats. This may reflect the proximity of Site A to the ash settling basins, as bacteria in the water column at Site A are likely those that have been transported from the ash settling basins where they were exposed to high concentrations of metals in the basins. Elevated tolerance values and high bacterial abundance in sediments and biofilm suggest that these microhabitats are likely to be sources of tolerant bacteria and tolerance genes.

We predicted that metal and antibiotic tolerance values of bacteria isolated from *Corbicula* would be intermediate between water and sediment values due to the fact that the clams reside in sediments, and filter- and deposit-feed on particles both from the water column and sediment [20]. However, tolerance values for bacteria from *Corbicula* digestive tracts were consistently lower than water column bacteria. This may be due to selective feeding by *Corbicula* on a specific size class of bacteria and other particulate matter [37]. Therefore all particulate matter suspended in the water column may not be ingested by *Corbicula*. More likely, *Corbicula* digestive tracts may harbor a resident bacterial community distinct from that of the water column upon which they feed. Evidence for this includes the lower temporal variability in *Corbicula* tolerance values compared to other microhabitats and more homogenous (less scattered) flow cytometry plots for *Corbicula* as compared to water column samples (data not shown).

Microhabitats differ in their susceptibility to disturbance which likely influences bacterial transport potential. Sandy substrates are easily disturbed by flow events and bioturbation. Bacteria attached to or residing on these particles are then also susceptible to disturbance and transport. Transport from biofilm microhabitats is a function of processes contributing to biofilm disturbance such as stream discharge and biotic activity (e.g., invertebrate feeding), and dynamics within the biofilm such as sloughing of cells. Transport from *Corbicula* digestive tract habitats occurs upon gut content elimination or upon *Corbicula* consumption by predators. Bacteria from these microhabitats are then transported downstream in the water column being consumed by stream organisms, attaching to surfaces such as on submerged woody debris or rocks, or adhering to particles and settling. The distance that bacteria are transported from metal-contaminated areas will therefore influence the extent of area in which the co-selection for antibiotic resistance is observed.

A third objective was to examine temporal patterns in bacterial tolerances Microbial tolerance to metals and antibiotics exhibited pronounced temporal variation, although tolerance values at Site A were consistently higher than Site D and Meyers Branch. We examined possible attributes that we hypothesized may be predictive of these patterns such as precipitation (i.e., microhabitat disturbance) and fluxes in metal concentrations (i.e., selective pressure). We observed an increase in tolerance values on Day 28, the sampling point preceded by multiple days of rainfall, relative to the other sampling dates in sediment and biofilm microhabitats. Elevated discharge from rainfall events would disturb sediment and biofilm microhabitats, re-suspending particulate matter and associated metals and bacteria. Evidence supporting this increase in particle transport would be seen in turbidity data. However, turbidity values were variable and did not show any consistent temporal trend. Additionally, tolerance values for

bacteria collected from the water column were highest on Day 56, not Day 28 as would be predicted from precipitation patterns. Dissolved metal concentrations also showed no consistent patterns over time either within a site for the various metals or across sites for one metal. Thus, neither precipitation, turbidity, nor metal concentrations correlated with observed tolerance dynamics. Other measures such as bacterial abundance, organic matter content, and pH also did not correlate with observed temporal patterns. It is plausible that temporal variation in the observed variables was not synchronic, e.g., bacterial response to an increased metal concentration may be delayed. Thus, the temporal resolution of our sampling (once per month) may be too coarse to resolve the linkages between tolerance patterns and physicochemical data.

Temporal patterns for the biofilm and *Corbicula* microhabitats reveal no significant transplant effect as evidenced by a comparison of tolerance values for Day 28 at the three sites to Day 0 values for Meyers Branch (data not shown). A comparison of microhabitats may potentially be hampered by the fact that two microhabitats (biofilm and *Corbicula*) were transplanted, so that the microbial community was experiencing a transition thereby rendering comparisons to sediment and water column bacteria invalid. The lack of a transplant effect, however, suggests that the transition period was shorter than one month and suggests that comparisons of microhabitats can be made. Day 0 tolerance values at Meyers Branch represent the tolerance values of the bacterial communities that were transplanted, whereas Day 0 tolerance values at Site A and Site D represent those for the *in situ* biofilm and *Corbicula* bacterial communities at each respective site. For both *Corbicula* and biofilm microhabitats tolerance values on Day 28 increased at Site A and Site D as compared to those on Day 0 at Meyers Branch. However, tolerance values also increased at Meyers Branch on Day 28. Thus the distinction between an increase in tolerance values at Site A and Site D on Day 28 being the

result of a shift in the bacterial community (i.e., a transplant effect) can not be made from the overall effect of increasing tolerance on Day 28 potentially due to fluctuations in physicochemical properties.

Antibiotic resistance in the environment involves processes at multiple scales. This study identifies sediment and biofilm microhabitats as locations at the stream scale that are likely to serve as sources for antibiotic and metal tolerant bacteria and genes. Future work at the molecular scale is needed to address specific mechanisms involved in the association between metal exposure and antibiotic resistance. Also, a quantification of genetic elements that are involved in these mechanisms would yield pertinent information regarding the frequency of antibiotic and metal tolerance genotypes in the environment under varying degrees of selective pressures.

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Table 2.1. Mean and range of dissolved metal concentrations (μg/L) from water samples over the four sampling dates. ND: not detected.

	V	Ni	Cu	Zn	As	Se	Sr	Cd	Pb
Meyers Branch	0.35 (0.3 - 0.4)	0.05 (ND - 0.1)	0.4 (0.1 - 0.6)	27.0 (21.4 - 30.8)	0.2 (0.2 – 0.3)	0.3 (0.2 - 0.4)	22.5 (20.9 - 23.7)	0.02 (ND - 0.08)	0.07 (0.02 - 0.09)
Site D	1.2 (1.0 - 1.4)	0.6 (0.2 - 1.0)	1.5 (1.3 - 1.8)	26.0 (22.9 - 28.6)	1.7 (1.0 – 2.7)	0.5 (0.3 - 0.8)	42.2 (35.7 - 53.4)	0.03 (ND - 0.1)	0.06 (0.05 - 0.09)
Site A	8.3 (7.2 - 9.6)	2.7 (1.7 - 3.6)	2.5 (1.8 - 3.6)	18.7 (12.7 - 29.4)	18.3 (12.5 - 27.2)	2.8 (2.4 - 3.1)	186.8 (107.2 - 291.0)	0.13 (0.06 - 0.18)	0.02 (ND - 0.08)

Table 2.2 ANOVA table using principal component 1 values

Source	DF	Sum of Squares	Mean Square	F Ratio	p-value
Model	29	491.97017	16.9645	26.2308	<0.0001
Error	143	92.48358	0.6467		
Total	172	584.45375			
R^2	0.842				
Adjusted R^2	0.81				

Source	DF	Sum of Squares	F Ratio	p-value
Site	2	24.09977	18.6318	<0.0001
Time	3	40.04908	20.6416	<0.0001
Time*Site	6	12.44919	3.2082	0.0055
Habitat	3	325.61632	167.8249	<0.0001
Site*Habitat	6	7.55648	1.9473	0.0771
Time*Habitat	9	69.89154	12.0075	<0.0001

Figure 2.1. Precipitation record for study period. Sampling time points are indicated with enlarged circles where May 19, 2004 corresponds to Day 0.

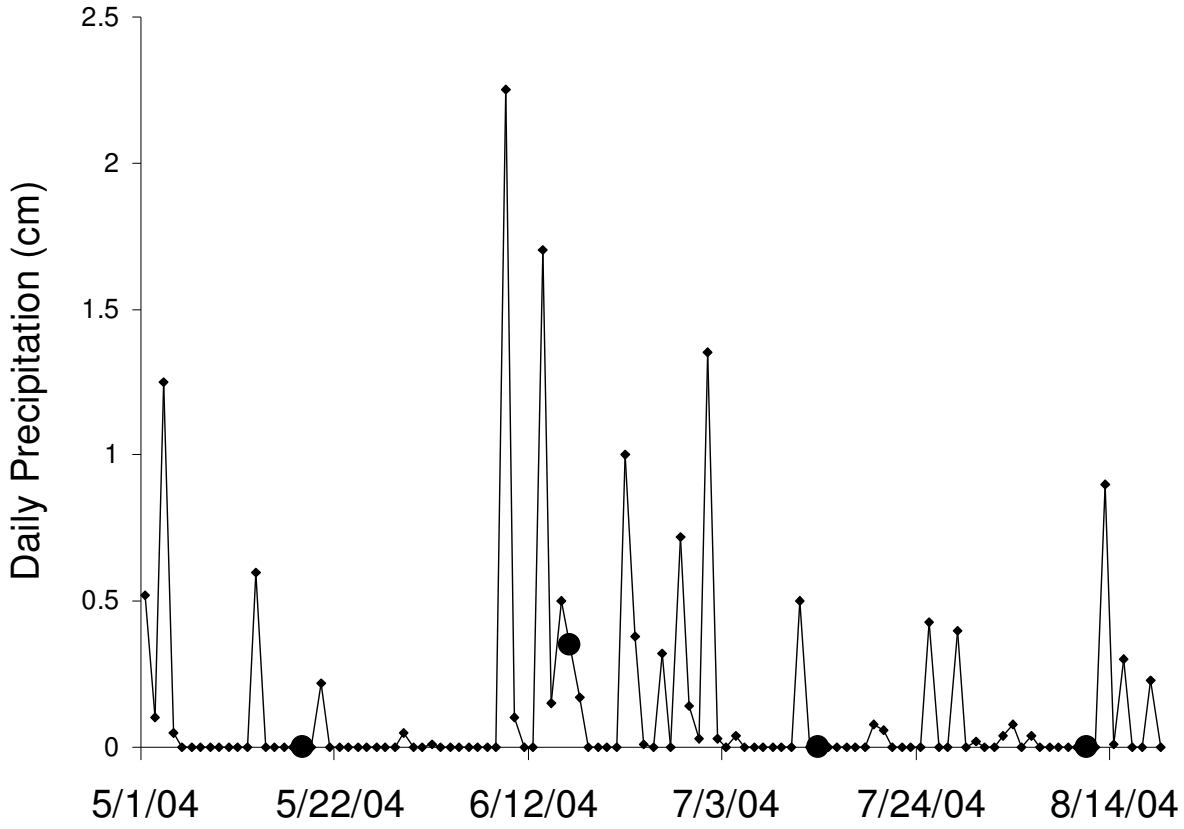


Figure 2.2. Initial and final total bacterial abundance after the 18-hr incubation averaged over the four sampling dates. Error bars are 1 standard error (n=16).

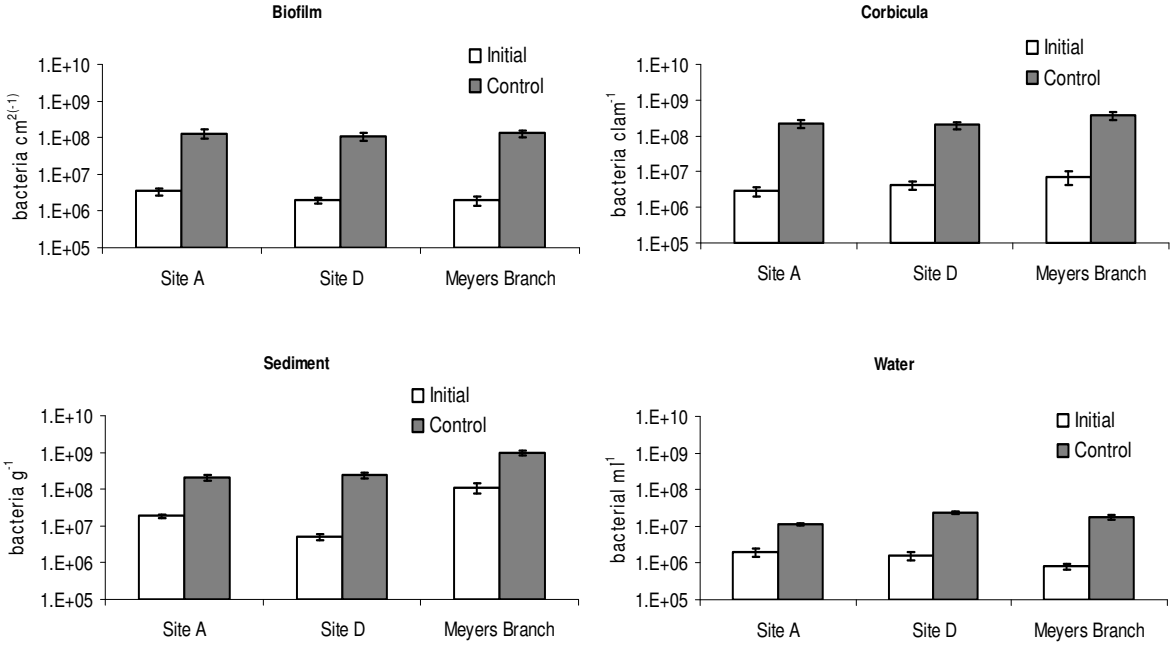


Figure 3. Mean tolerance index values generated from principal component analysis. The tolerance index value is the ratio of the number of live bacteria in a treatment aliquot compared to the number of live bacteria in a control aliquot. Data were log-transformed for subsequent principal component analysis. See text for further details. Error bars are 1 standard error (n=16).

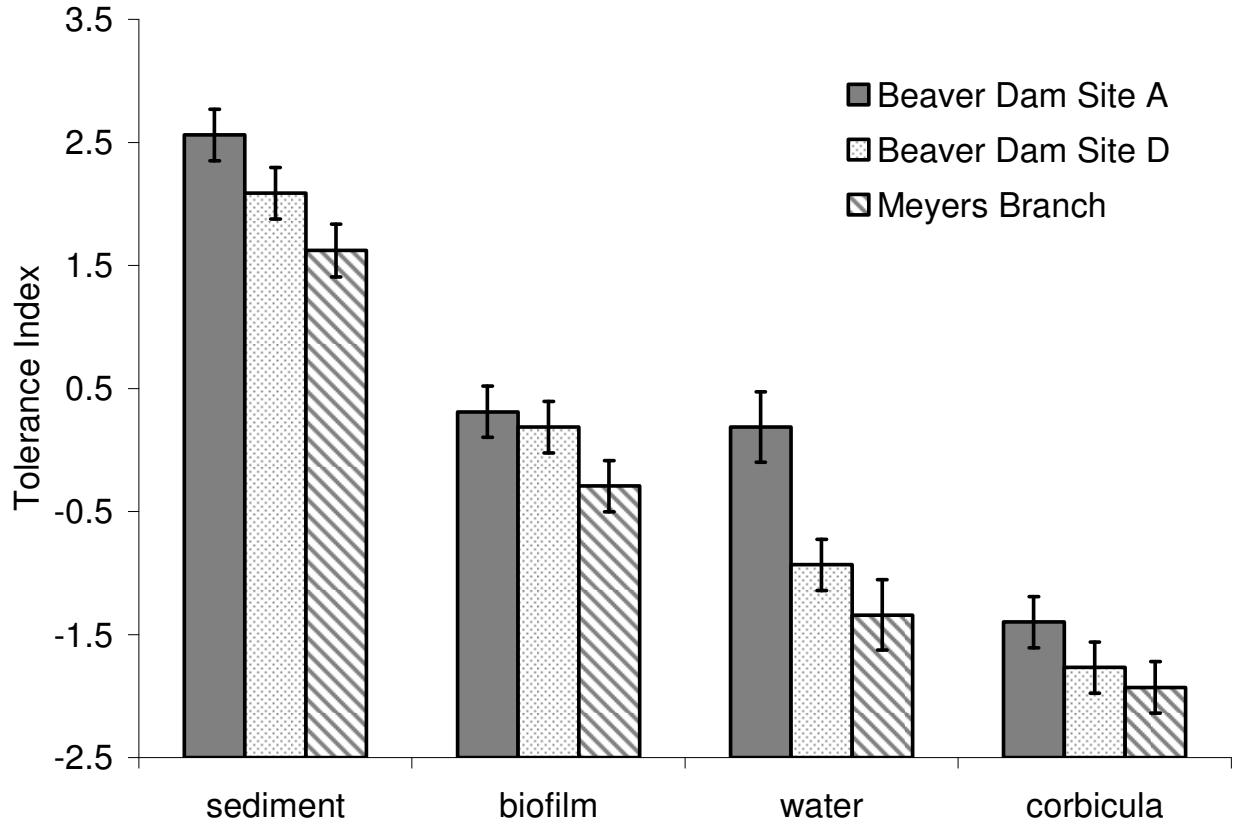


Figure 4. Bacterial tolerance, or the ratio of live bacterial abundance in treatment aliquots compared to the live bacterial abundance in control aliquots, for each toxicant. Each bar represents the mean ratio over the four sampling dates and error bars are one standard error (n=16).

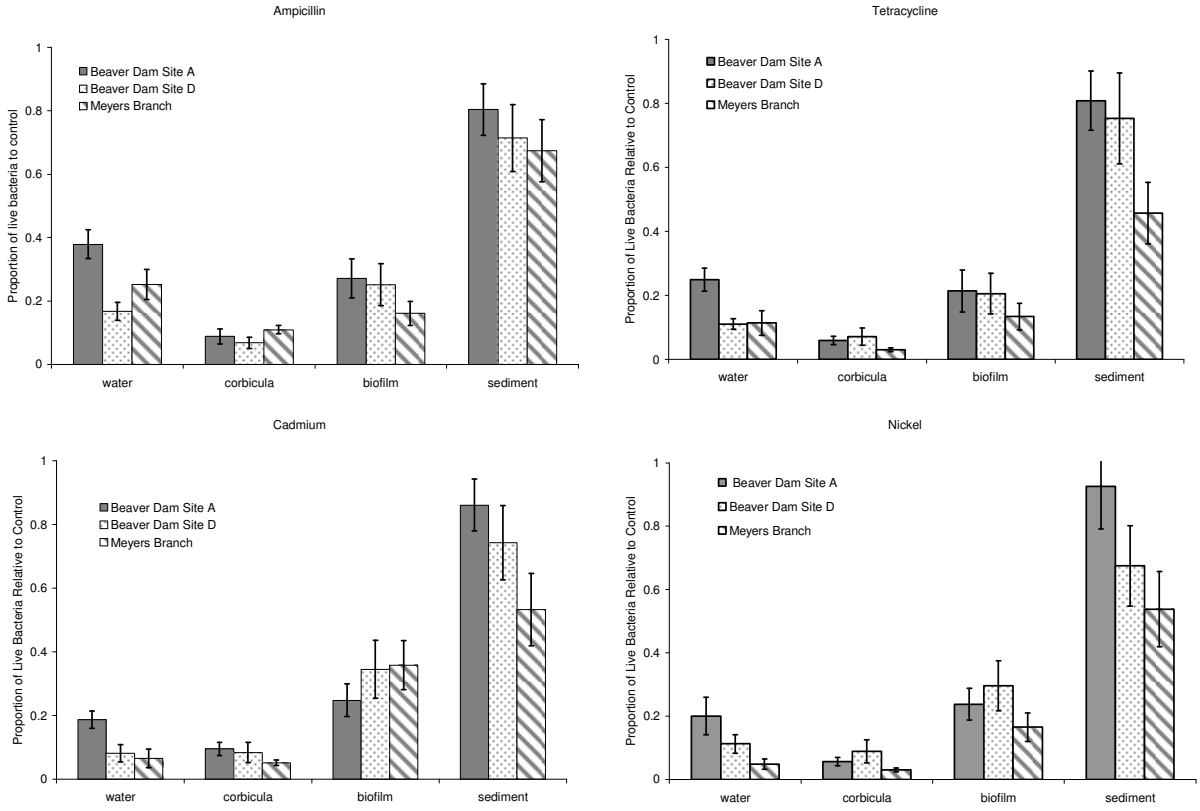


Figure 5. Mean tolerance index values over time for each site averaging over microhabitats generated from principal component analysis Errors are 1 standard error (n=16).

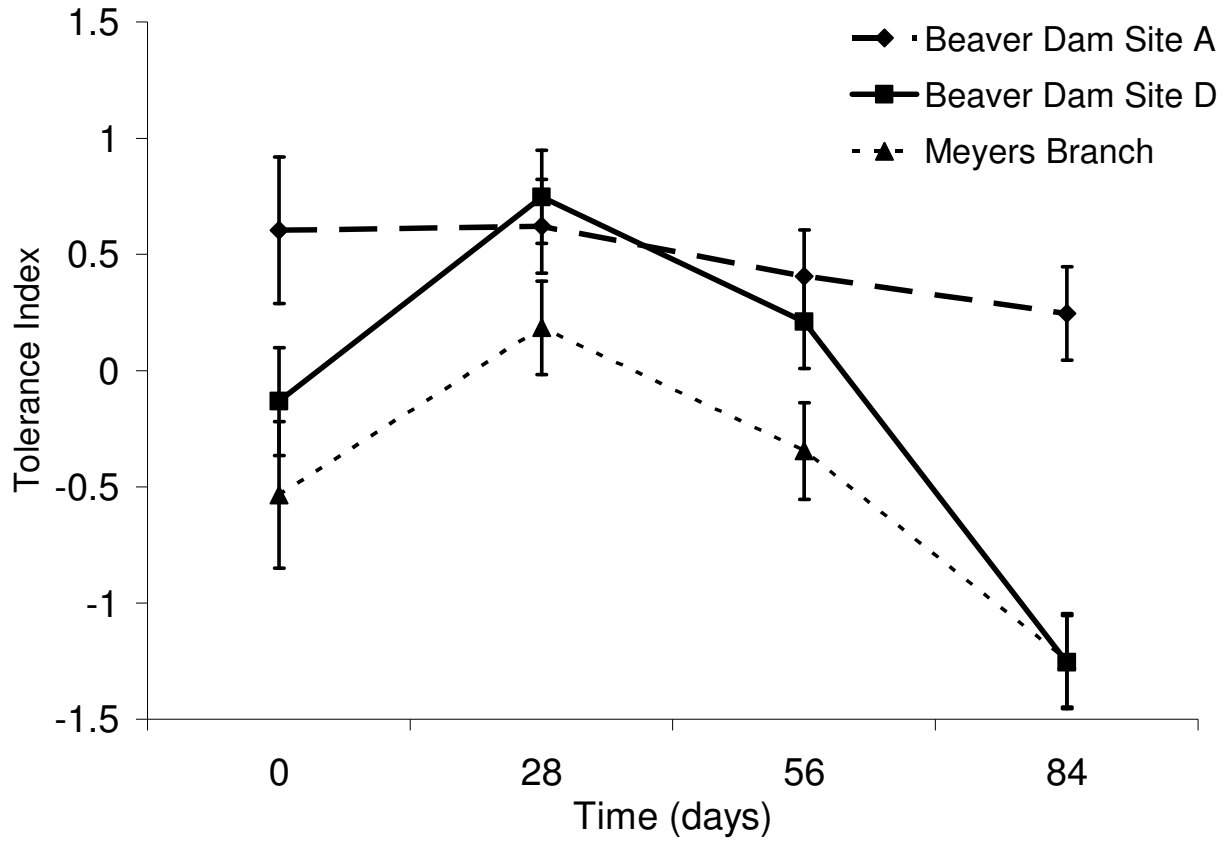
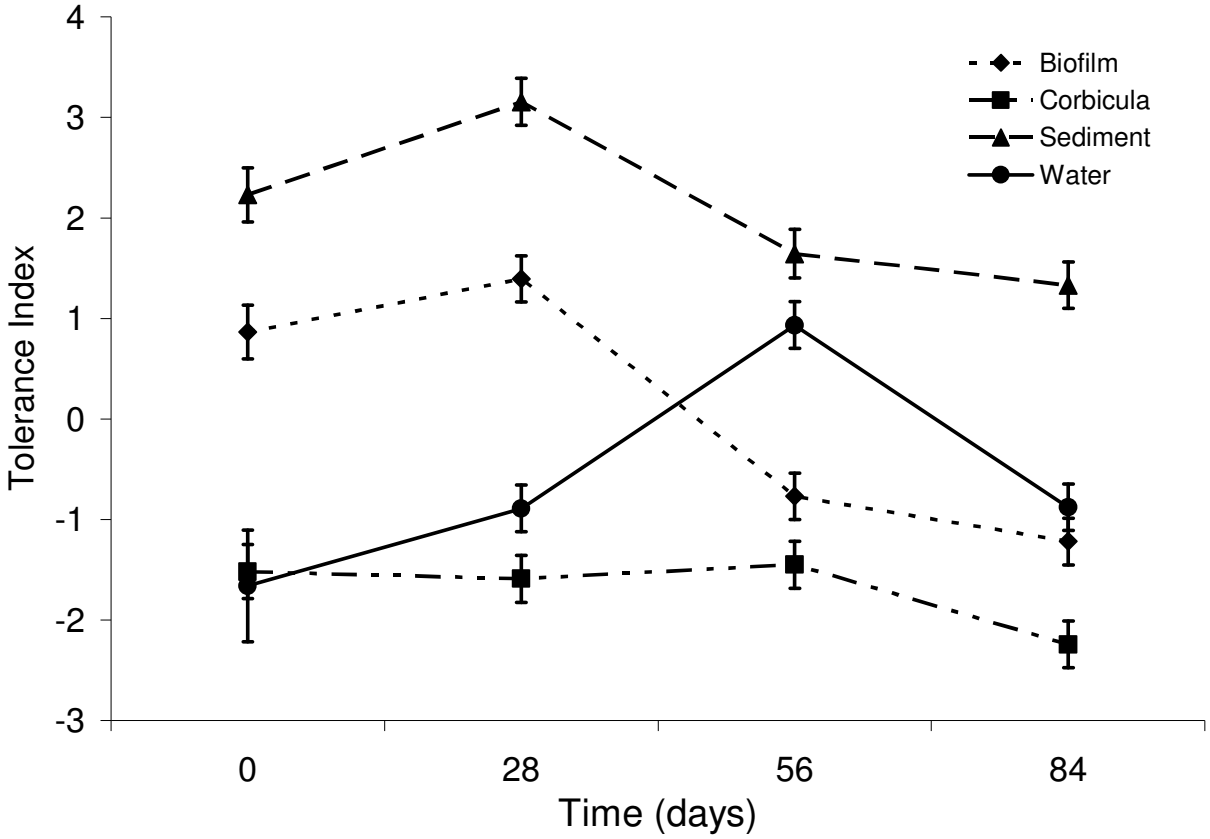


Figure 6. Mean tolerance index values over time for each microhabitat averaging over sites generated from principal component analysis. Error bars are 1 standard error (n=12).



CHAPTER 3

RELATIVE ABUNDANCE OF TETRACYCLINE (*tetA*) AND ARSENIC (*arsC*) RESISTANCE DETERMINANTS IN METAL EXPOSED BACTERIAL COMMUNITIES: EVIDENCE FOR CO-SELECTION? [1]

[1]Wright, M.S., A.H. Lindell, R. Stepanauskas, JV McArthur. To be submitted to *Applied and Environmental Microbiology*.

Abstract

Anthropogenic sources of antibiotics may not be the primary selective pressure maintaining antibiotic resistant bacteria and resistance genes upon their release into the environment. An additional selective pressure may be bacterial exposure to metals. To test the hypothesis that metal contamination maintains a reservoir of both metal and antibiotic resistance genes, we quantified *tetA* genes encoding a tetracycline efflux pump and *arsC* genes encoding an arsenate reductase from multiple bacterial microhabitats in metal-contaminated and reference riverine systems in South Carolina, USA using quantitative PCR. Additionally, we quantified *tetA* and *arsC* from experimental microcosms where bacterial exposure to antibiotics and metals was directly manipulated. *tetA* relative abundance was elevated in epiphytic biofilm, *Corbicula fluminea* (Asiatic clam) digestive tracts, sediment, and surface water microhabitats from multiple sites along a stream receiving coal ash effluent compared to a nearby reference stream. Copies of *arsC* were only detected in sediment microhabitats, but were significantly more abundant in the contaminated stream. *tetA* was most abundant in tetracycline-amended microcosms, but there was no significant difference in its abundance between cadmium-amended microcosms and control microcosms. *arsC* was not detected in any microcosms. Microcosm isolates possessing *tetA* genes were also resistant to several other structural classes of antibiotics and metals indicating that exposure to one bacterial stressor can result in the selection of additional resistance phenotypes. Results support the hypothesis that metal exposure can maintain a reservoir of antibiotic resistance genes in the environment, but which specific resistance gene is investigated is likely to influence the probability of detecting this occurrence.

Introduction

The persistence of antibiotic resistance genes in the environment potentially represents a challenge for public health efforts to minimize the transmission of antibiotic resistance. Phenotypic studies routinely identify environmental bacteria with multiple antibiotic resistances isolated both from habitats in which antibiotic exposure is likely (e.g., (1, 4, 13, 26), and from those environments where no significant anthropogenic source of antibiotics is apparent (10, 23, 31, 35). PCR-based studies also demonstrate that specific antibiotic resistance genetic determinants can be detected and quantified in environments with varying degrees of antibiotic exposure (3, 8, 20, 21, 27, 29, 34, 36). Thus it is evident that bacteria in the environment represent a reservoir for maintaining antibiotic resistance determinants that can potentially be mobilized to bacteria of human and agricultural importance.

Most of these studies investigate direct selection via bacterial exposure to antibiotics of anthropogenic origin (i.e., exogenous sources) as a mechanism for the persistence of antibiotic resistance genes in the environment. While settings such as sewage treatment plants and confined animal feeding operations, these settings may constitute a substantial source of antibiotic resistance genes for receiving systems, but they may not be the primary selective pressure acting to maintain resistance elements once released into the environment (28). For example, other sources of selective pressures to which bacteria adapt may also act to select for additional phenotypes through co-selection mechanisms (2, 6). One such potential selective pressure is bacterial exposure to metals resulting in the co-selection of metal and antibiotic resistance traits (6).

In previous studies, experimental and observational evidence documented elevated metal and antibiotic resistance phenotypic frequencies in metal-exposed bacteria relative to unexposed bacteria using culture-based and culture-independent methods (30, 35). In the current study, we examined the abundance of specific antibiotic and metal resistance genes in these environments where bacteria are not under direct antibiotic selection pressure, but are exposed to varying degrees of metal contamination. We tested whether specific antibiotic and metal resistance genetic determinants are selected for in metal-exposed bacterial communities by quantifying tetracycline (*tetA*) and arsenic (*arsC*) resistance genes from metal-contaminated and reference riverine systems and experimental microcosms. These specific resistance genes were chosen in part because of their location on mobile genetic elements, the high concentrations of arsenic in the study system, and because the tetracycline resistance phenotype was previously shown to be elevated in these systems (35). *tetA* encodes a tetracycline efflux pump of the major facilitator superfamily (MFS) class that can be transposon or plasmid-borne. *arsC* is part of an arsenic reduction operon encoding for an arsenate reductase that is present on the chromosome, transposons, or plasmids of a phylogenetically broad range of microbes (15, 18). *asrC* itself does not confer resistance but other genes within this operon do. Additionally, we examined whether gene abundance patterns were consistent across multiple bacterial microhabitats to identify potential reservoirs of resistance genes. Isolates possessing *tetA* genes were further characterized for their metal and antibiotic resistances to identify what additional phenotypes can be associated with *tetA*. We predicted that both *tetA* and *arsC* relative abundance would be greater in the contaminated sites and treatment microcosms.

Methods

Sampling

Savannah River Site

Two streams located on the United States Department of Energy's Savannah River Site (SRS) in South Carolina were sampled to test the effect of metal contamination on metal and antibiotic resistance gene abundance. Beaver Dam Creek receives effluent from ash settling basins associated with the SRS D-400 coal-fired power plant. Site A has elevated concentrations of a variety of metals associated with coal ash effluent including arsenic, selenium, and vanadium (35) Loeffler, unpublished data). Sediment at this site consists of cobble, sand, and coal ash particles. Site D is located 3.2 km downstream from Site A where sediment is primarily sandy. Meyers Branch is a historically unimpacted blackwater stream with sandy sediment which has previously been used as a reference stream on the SRS (35).

We collected samples from four bacterial microhabitats common to most streams including epiphytic biofilm, surface sediments, digestive tracts from *Corbicula fluminea* (Asiatic clam), and surface waters at each site on November 9, 2005 as in (35). To collect bacteria from epiphytic biofilm, oak wooden dowels (diameter: 2 cm x length: 45 cm) were tethered in the stream to mimic coarse woody debris on June 3, 2005 and periodically checked to ensure submersion. On the sampling date, three wooden dowels were collected and transported in sterile whirlpacks. To obtain sediment bacteria, grab samples from surface sediments (~ 50 g) were collected in sterile whirlpacks. Nine *Corbicula fluminea* (Asiatic clam) were collected from each site to represent bacteria

harbored in invertebrate digestive tracts. In autoclaved and UV-sterilized polycarbonate bottles, 1 L of stream water was collected in triplicate to sample bacteria suspended in the water column. All samples were returned on ice to the laboratory for further processing.

Savannah River Microcosm Series

To experimentally test the effect of bacterial exposure to metals and antibiotics on resistance gene abundance, a series of microcosms were constructed as detailed in Stepanauskas et al. (30). Briefly, water samples were collected from the Savannah River 36 km downstream of Augusta, GA, USA near the intake for the SRS D-400 coal-fired power plant on September 29, 2003. Water was aliquoted (1 L) into control, tetracycline treatment (30 mg/L), and cadmium treatment (0.1 mM) microcosms. In Stepanauskas et al. (30), multiple concentrations of tetracycline and cadmium were used in the experimental microcosms. Thus the concentrations used in the current study were chosen because phenotypic assays demonstrated that these concentrations generated the most significant effect on metal and antibiotic resistance phenotypes. After 7 days of incubation (23 °C on orbital shaker set at 100rpm), 250 mL of microcosm water was collected for further processing.

Sample processing and DNA extraction

Samples were processed within four hours of collection. Bacteria from epiphytic biofilm were detached from the wooden dowel by scraping the surface area with a sterile razor blade. DNA from this material was then extracted using the PowerSoil Extraction kit (MoBio) per the manufacturer's protocol. To obtain bacterial DNA from *Corbicula*

digestive tracts, individuals were dissected using aseptic techniques. Digestive tracts from three individuals were combined and weighed to obtain enough material from which to extract DNA using UltraClean Soil Extraction kit (MoBio). For each sediment replicate, DNA was extracted from 0.4 g of sediment using the PowerSoil Extraction kit. To collect bacteria for DNA extraction, 1 L of stream water was filtered onto 0.2 μm type filters. Multiple filters had to be used for each water sample which were combined in 10mL of autoclaved and UV-sterilized nanopure water in 50 mL sterile conical tubes. Tubes were vortexed until all visible particles were detached from filters and then centrifuged at 7000 rpm for 15 min to pellet particles and associated bacteria. Pellets were resuspended using the bead-beating solution from the PowerSoil extraction kit which was then used to extract DNA. This protocol was followed for microcosm water as well. DNA was quantified using AlphaEase FC Software version 3.1.2 (Alpha Innotech Corp.)

Quantitative PCR protocol

Primers

Primers previously designed and validated for use in qCPR assays were used to amplify genes encoding a tetracycline efflux pump (*tetA*) and arsenate reductase (*arsC*). Universal bacterial primers were used to quantify 16 S rDNA genes to account for potential PCR biases and differences in bacterial abundance between samples. Primers used and their annealing temperatures are listed in Table 1.

Standards

Calibration standards for use in qPCR assays were generated from conventional PCR products using the appropriate primer pair with DNA extracted from each microhabitat site as the template. After each conventional PCR reaction, products were run on a 1% agarose gel at 80 mV for 30 min to ensure amplicons were of the predicted size. PCR products were then purified using the Qiagen PCR purification product and quantified with standards using software AlphaEaseFC software. Six ten-fold dilutions were prepared with each purified PCR product to use as standards in qPCR assays.

qPCR conditions

Quantitative PCR assays were performed using a BioRad iCycleriQ Real-Time Detection System in BioRad iCycler 96-well plates. Reactions consisted of 1 μL template DNA, 400 nM primers, 12.5 μL of SuperArray SYBR Green master mix (SuperArray) and 9.5 μL of molecular grade PCR water (Sigma). For *arsC*, two primer sets were used to increase target range as in (32) resulting in 200 nM primer concentration for each forward and reverse primer. Six ten-fold dilutions were run for each qPCR assay to generate the standard curve while triplicate qPCR reactions were performed for each sample. Triplicate negative control blanks were also run for each assay. The thermocycler protocol for 16S rDNA and *arsC* reactions consisted of 95C for 15 min, followed by 40 cycles of 95°C for 30 sec, 30 sec at 53°C (60°C for *arsC*), 72°C for 30 sec, and a final 10 min at 72°C for extension. For *tetA*, the protocol was as follows: 95°C for 15 min, 5 touchdown cycles of 95°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec, with a 1 degree decrement of the annealing temperature for each cycle,

followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, 86°C for 18 sec. Fluorescence was recorded at 72°C and 86°C for *tetA* (a temperature above which potential primer-dimers melt).

To ensure specificity for each reaction, multiple analyses were conducted including a melt curve analysis after each qPCR run where fluorescence was measured for 55 cycles starting at 70°C with an increase of 0.5°C. The plot of -dFlour/dt was examined for the presence of a single peak to confirm reaction specificity. Additionally, qPCR product from randomly selected samples were run a 1% agarose gel in 10x TBE solution for 25 minutes at 80V to check for the presence of a single band of the appropriate size. A random subset of qPCR products were also sequenced.

Copy number of each target gene was calculated using the average molecular mass of 660 g mol^{-1} for a double-stranded DNA molecule. Estimates of 16S rDNA copy number were corrected for amplification in negative control reactions.

Microcosm Isolate Screening

Microcosm isolates characterized in (30) for their antibiotic and metal resistance phenotypes were screened for the presence of *tetA*. DNA was extracted from isolates obtained from control, tetracycline-amended, and cadmium-amended microcosms using the Bio101 FastPrep DNA Kit (Qbiogene) per the manufacturer's instructions kit (n = 30 isolates for cadmium, tetracycline, and control microcosm for a total n = 90 isolates). Primers and PCR protocols were used as above in duplicate conventional PCR reactions, and PCR products were then sequenced. Isolates positive for *tetA* amplicons were then subjected to further antibiotic and metal resistance assays to assess what additional

phenotypes can be associated with *tetA*. For antibiotic resistance screening, isolates were screened against a panel of 26 antibiotics using commercial dehydrated 96-well MicroScan® panels (Dade Behring, Sacramento, USA) according to the manufacturer's instructions. Antibiotics in this test (concentration ranges in mg/L) comprised nine drug classes, including aminoglycosides: amikacin (8–64), gentamicin (2-16), streptomycin (16–128), apramycin (8-32); β-lactams: ampicillin (4–32), amoxicillin (4–32), penicillin (16-128), imipenem (2-16), meropenem (2–16), ceftriaxone (8-64), cefoxitin (8-32), cephalothin (16-128), cephalexin (16-128); Folate synthesis inhibitors: trimethoprim (2–16), and trimethoprim-sulfamethoxazole (2/38-4/76); Sulfa agents: sulfathiazole (250-500); Nitrofurantoins: nitrofurantoin (16-128); Tetracyclines: oxytetraycline (4-32), tetracycline (4-32); Quinolones: ciprofloxacin (1-4), moxifloxacin (0.25–4), nalidixic acid (4-32), ofloxacin (1-8); Macrolides: azithromycin (2-8), erythromycin (16-128); and Chloramphenicols: chloramphenicol (8-32). For metal resistance assays, isolates were grown in triplicate in half-strength nutrient broth amended with either (change to μM) 1 g/L or 0.1 g/L cadmium, copper, nickel; or 1 μM or 10 μM mercury. Sensitivity to antibiotics and metals was recorded after two days growth in comparison to controls and blanks.

Sequencing and analysis of PCR products

qPCR and conventional PCR products were transformed into sequencing vectors using TOPO TA Cloning Kit (Invitrogen) and sequenced using an Applied Biosystems 3130xl sequencer. Sequences were edited with Sequencher software (Genecodes). Sequences were analyzed using BLAST searches to identify the most similar nucleotide

sequences and aligned using ClustalW (9). Sequences were deposited in GenBank under the following accession numbers.

Metal Analysis

Sediment samples were collected from each site to confirm that a gradient of metal concentrations existed among sites. Sediment samples were frozen until processing as in (35) at the Savannah River Ecology Laboratory analytical facility and analyzed using ICP-MS with the appropriate calibration standards and blanks during digestion and analysis.

Precipitation and Discharge Data

Precipitation data for the period leading up to the sampling date was obtained from the SRS Climate database. Discharge data for a gauging station midway between Site A and Site D on Beaver Dam Creek was obtained from the US Geological Survey Water Resources website (www.water.usgs.gov).

Statistical Analyses

qPCR

The relative abundance of each gene was calculated as the ratio of the target gene copy number to 16S rDNA copy number per ng DNA per sample unit for each sample (g for biofilm, *Corbicula*, and sediment; L for water) to account for potential PCR biases associated with PCR inhibition and differences in bacterial abundance. Site and microhabitat variables and their interaction term were used to test for statistical

differences between sites using an ANOVA and *a priori* LSMEANS statement in SAS where the response variable was the log-transformed ratio of target gene copy number to 16S rDNA copy number. Data were log-transformed to meet the assumptions of normality and because data ranged over multiple orders of magnitude

Results

Metals

Metal concentrations at both sites along Beaver Dam Creek were elevated above those at Meyers Branch. A variety of metals associated with coal ash residue including vanadium, selenium, and chromium were elevated in Site D sediments relative to those at Site A and Meyers Branch, yet arsenic, cadmium, and nickel were highest at Site A (Table 3).

Precipitation and Discharge

Prior to the sampling date, there was no precipitation at any site for over 28 days. Discharge data from a gauging station located between Site A and Site D indicate that discharge was elevated to approximately 100 ft^3s^{-1} for a period of seven days preceding the sampling date (Figure 1). Baseflow discharge for this site is approximately 55 ft^3s^{-1}.

16S rDNA abundance

16s rDNA qPCR reaction efficiencies for SRS and microcosms were 100.2% and 105.3% respectively, with correlation coefficients of 0.90 and 0.92 for the calibration curve. Melt curve analysis demonstrated one broad peak for each sample. For biofilm

samples, there was no significant difference in 16S rDNA gene abundance between sites, with mean values ranging from 9.55 x 10^7 to 1.3 x 10^8 copies g^{-1} biofilm material (Figure). There were significantly fewer 16S rDNA copies in samples from *Corbicula*, sediment, and water column microhabitats at Site D compared to the other two sites. 16S rDNA copies were significantly more abundant in sediment and water column samples from Meyers Branch than at Site A, but this pattern was reversed for *Corbicula* samples. 16S rDNA copy number declined over the 7 days of microcosm incubation, as the initial mean abundance of 4.5 x 10^7 copies ng DNA^{-1} L^{-1} was significantly greater than final abundances in control and treatment microcosms.

tetA abundance

The ratio of *tetA*:16S copy number was significantly greater at Site D in comparison with Site A and Meyers Branch across all microhabitats (Figure). *tetA* relative abundance was significantly greater at Site A than Meyers Branch in biofilm and sediment microhabitats, but there was no statistical difference in bacteria from *Corbicula* digestive tracts. However, this pattern was reversed for water column bacteria where the *tetA*:16S ratio was significantly greater at Meyers Branch than at Site A. In the microcosm series, the ratio of *tetA*:16S copy number increased over the initial sample value in all microcosms, but was significantly higher in the tetracycline-amended microcosms than control and cadmium microcosms. qPCR efficiencies for *tetA* reactions were 102.3% and108.4% for SRS and microcosm samples, where correlation coefficients for the calibration curve were 0.91 and 0.96. Melt curve analyses revealed a single peak at approximately 94°C.

arsC abundance

qPCR efficiencies ranged between 109.1% and 114.6% for SRS samples with correlation coefficients of 0.992 and 0.993. Melt curve analysis showed one sharp peak at 90°C. *arsC* copies were above detection only in sediment samples where the detection limit was estimated as 4.56 copies/ng DNA. The proportion of *arsC*:16S copy number was significantly greater in samples at both sites along Beaver Dam Creek than in Meyers Branch samples (Figure 4). No *arsC* copies were detected in any microcosm sample.

Microcosm Isolate Screening

Four isolates from the tetracycline microcosms were identified as possessing *tetA* copies, whereas no isolates were identified from control or cadmium microcosms (Table 3). Isolates were all β-Proteobacteria, comprising three unique 16S rDNA ribotypes all in the Comamonadaceae family. All isolates exhibited high levels of resistance, often exceeding the maximum tested concentration, to nearly all antibiotics (Table 3). Though isolates *Comamonas* 6-6 and *Comamonas* 24-1 had identical 16S rDNA sequences, they differed in antibiotic sensitivity, with resistances to 23 and 16 antibiotics, respectively. In general, minimum inhibitory concentrations (MICs) were lower for *Comamonas* 24-1 (data not shown). All isolates were resistant to the lower tested Hg and Cu concentrations, but only *Comamonas* 19-3 and *Delftia* 19-4 were able to grow in Cd and Ni (Table). *Comamonas* 19-3 was the only isolate able to grow in 1 g/L Cd while only *Comamonas* 6-6 was able to grow in 1 g/L Cu.

Sequencing results

Sequencing results confirmed the correct amplification for each of the targeted genes. All seven retrieved *arsC* sequences from the three SRS sites were identical and were highly similar to an *arsC* on the R478 IncH12 plasmid from *Serratia marcescens* (E- value = 0.0) varying in just two positions over 353 nucleotides, while the next most similar nucleotide matches were to an *arsC* from *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* genomes (E-value = $6e^{-43}$). *tetA* sequences from the four microcosm isolates were also similar to one another and only varied in two positions out of 567. All *tetA* sequences were highly similar to several *tetA* genes retrieved from the BLAST search including plasmids from *Escherichia coli*, *Salmonella* sp., *Shigella sonnei*, and plasmids from uncultured bacteria obtained from activated sludge.

Discussion

Metal contamination can select for metal and drug resistance genes in environmental bacterial communities. Relative *tetA* abundance was greater in a metal-contaminated stream in comparison to a nearby reference stream across multiple bacterial microhabitats supporting the hypothesis that metal contamination maintains tetracycline resistance genes in multiple types of microbial communities. That relative *tetA* abundances were consistently more abundant in microhabitats from Beaver Dam Creek signifies that each bacterial community constitutes a reservoir of *tetA* resistance genes in metal-contaminated systems. The exception to this is lower *tetA* abundance in Site A surface waters which may be related to increased plant discharge during the sampling period. Relative *arsC* abundance was also higher in sediment microhabitats from Beaver

Dam Creek where arsenic concentrations were substantially greater than at Meyers Branch indicating that arsenic contamination can directly select for arsenic resistance genes. There are no anthropogenic point sources of antibiotics at either Beaver Dam Creek or Meyers Branch minimizing the role direct selection pressures play in the maintenance of tetracycline resistance genes in these systems.

Within Beaver Dam Creek, *tetA* relative abundance was higher in microhabitats at Site D, the downstream site. While this is counter to the prediction that resistance genes would be most abundant in Site A, the most contaminated site, multiple metals associated with coal ash residues were elevated in Site D sediments on the sampling date relative to concentrations at Site A. The stream discharge record for Beaver Dam Creek indicates elevated discharge for several days prior to the sampling date though there was no measurable precipitation. We suggest that elevated discharge due to plant operations disturbed upstream sediments, bacteria, and associated metal residues resulting in higher abundances of resistance genes and sediment metal concentrations at Site D. Also, gene abundance and metal concentrations at Site D are additionally a function of cumulative downstream transport from more contaminated upstream sites. Sediment metal patterns were not consistent for all tested metals including arsenic, cadmium, and nickel perhaps due to differential transport of metals related to coal properties or metal chemistry and particle binding properties. Regardless of variations in gene abundances and metal concentrations within Beaver Dam Creek, metal concentrations and *tetA* and *arsC* relative abundances were consistently greater than those observed in Meyers Branch. It should be noted that multiple classes of genes confer resistance to tetracycline (3, 17, 27, 29, 36), and therefore the abundance of *tetA* likely underestimates the abundance of

tetracycline resistance genes in these systems. Furthermore, a phenotypic assessment of tetracycline resistance in Beaver Dam Creek and Meyers Branch microhabitats also demonstrated elevated tetracycline resistance in metal-exposed bacterial communities (35).

There was no evidence supporting the hypothesis that metal exposure maintains a pool of antibiotic resistance genes in the experimental microcosm series based on the relative abundance of the selected resistance genes. Similarly, in a previous phenotypic assessment of culturable bacteria from this experiment, there was an observed increase in tetracycline resistance occurrence in isolates from the tetracycline microcosms, but not in isolates from the cadmium microcosms (30). Despite no increase in a tetracycline resistance phenotype, multiple isolates from cadmium microcosms were resistant to ampicillin (β-lactams) and gentamycin (aminoglycosides) suggesting that these resistance phenotypes were co-selected with the selected metal resistances. It remains unclear whether this co-selection was the result of isolates possessing ampicillin or gentamycin resistance genes linked to metal resistance genes, or alternatively was mediated by co-regulatory or cross-resistance mechanisms (6). No *arsC* copies were detected in any of the microcosms and neither were copies of *cadA*, a gene encoding for a cadmium efflux pump, in preliminary testing (unpublished data), thereby limiting assessment of direct selection for metal resistance genes in the experimental microcosms.

Further screening of *tetA*-positive microcosm isolates indicates that additional resistance phenotypes were associated with *tetA* including resistances to Cd, Ni, Cu, and Hg and a remarkable number of antibiotics encompassing all major structural classes of antibiotics. Two isolates, *Comamonas* 6-6 and *Comamonas* 24-1 had identical 16S

rDNA sequences, yet exhibited contrasting susceptibility to the tested antibiotics highlighting the mosaic nature of antibiotic resistances. Furthermore, *Delftia* 19-4 had identical 16S rDNA sequence to two other isolates from the same tetracycline microcosm, *Delftia* 19-1 and *Delftia* 19-8, yet neither of these isolates possessed *tetA*. All isolates originated from the Savannah River where the nearest municipal sewage input is in Augusta, GA, approximately 26 km upstream, though there are several other potential point and nonpoint sources of antibiotics and resistant bacteria in the watershed.

The inability to detect *arsC* in a majority of the sampled bacterial microhabitats or in any of the microcosms may be a function of the narrow target range of the primer sets, and not an actual absence of bacteria possessing *arsC* in these systems. Because of low sequence similarity across the various clades of organisms possessing *arsC*, primers used in this study were designed by (32) to target a conserved region of *arsC* in the Enterobacteriaceae bacterial group. (32) was also unable to detect *arsC* in a majority of their soil samples, citing the narrow hybridization range of the primers and low sequence conservation for this gene as a likely explanation. From molecular surveys and culture-based assessments, it is hypothesized that a large proportion (~20-50%) of bacteria representing a phylogenetically broad group of organisms possess the ability to detoxify arsenic (15, 16, 18, 24, 33). Thus the primer sets used in this study likely underestimate true *arsC* abundance in the sampled systems. A thorough analysis of *arsC* abundance across a taxonomically representative group of isolates in response to metal and antibiotic exposure is required to validate the applicability of this approach.

Elevated tetracycline resistance in metal-exposed bacteria may be due to the occurrence of *tetA* on mobile genetic elements also containing metal resistance

determinants. Multiple types of metal and drug resistance genes can be located on the same genetic element resulting in a situation in which the selection for one resistance gene can result in the co-selection of other genes located on that element. The 50-kb IncN plasmid R46 exemplifies this linkage, containing an arsenic resistance operon, *tetA* and *tetR* genes, and a class 1 integron conferring resistance to beta-lactams, sulphonamides, and aminoglycosides (7, 14). Additionally, the 274-kb IncH12 megaplasmid R478 has acquired multiple resistance determinants including multiple drug resistances (tetracycline, kanamycin, and chloramphenicol) and multiple metal resistances (arsenic, tellurite, copper, mercury, and silver) (12). Comparative sequence analysis to other IncH plasmids suggests that R478 has undergone modular evolutionary events, acquiring its resistance determinants from bacterial chromosomes, other plasmids and mobile genetic elements such as class 1 integrons (12). Interestingly, all retrieved *arsC* sequences in this study were nearly identical to the *arsC* on R478. In addition to plasmids, both metal and antibiotic resistance genes are disseminated on transposons. For example, the widespread occurrence of *tetA* is in part because of its location on numerous transposons including Tn10, Tn1721 and Tn1721-like transposons. These transposons can be found in conjugative plasmids including broad host range plasmids of the IncH, IncU, and IncP groups (12, 19, 22, 25) increasing their mobility and likelihood to be disseminated among a taxonomically broad range of bacteria. These examples serve to highlight the potential role that broad host range plasmids and transposons can play in the simultaneous dissemination of drug and metal resistance genes.

Conclusions

Field data suggest that antibiotic resistance genes are associated with elevated metal concentrations and transport of these elements may be influenced by changes in environmental conditions such as elevated stream discharge and sediment transport. Experimental manipulation of bacterial exposure to metals did not in this case result in the co-selection of selected antibiotic and metal resistance traits perhaps due to a lack of association between cadmium and tetracycline resistance genes on the same genetic element in the initial bacterial community. However, *tetA*-positive isolates were highly resistant to a number of additional antibiotics and tested metals suggesting that this gene can be co-selected with several additional resistance phenotypes. The potential to detect co-selection for antibiotic resistance via co-resistance is likely a function of what antibiotic resistance gene is examined. For example, genes captured by mobile genetic elements like class 1 integrons and transposons including *tetA* (tetracyclines), *aad* (aminoglycosides), *dfr* (trimethoprim), *sul* (sulphonamide) and *bla* (β-lactams) drug resistance gene families may be more likely to be found on larger genetic elements also containing genes for additional phenotypes. Comprehensive sequence analysis of plasmids from environments with varying degrees of metal exposure are needed to clarify the potential association between antibiotic resistance and metal resistance genes and their role in maintaining an environmental reservoir of antibiotic resistance genes.

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Table 1. Targets and primers used for quantitative PCR and conventional PCR assays.

Target	Primer	Sequence (5'→ 3')	Amplicon size (bp)	Annealing Temperature (°C)	Reference
tetA	tetAC-150F	GCTRTATGCGTTGRTGCAAT	568	58	(36)
	tetAC-716R	TCCTCGCCGAAAATGACC			
arsC	srmc-42F	TCACGCAATACCCTTGAAATGATC	334	60	(32)
	srmc-376R	ACCTTTTCACCGTCCTCTTTCGT			
arsC	amlt-42F	TCGCGTAATACGCTGGAGAT	334	60	(32)
	amlt-376R	ACTTTCTCGCCGTCTTCCTT			
16S rDNA	Eub338	ACTCCTACGGGAGGCAGCAG	~200	63	(11)
	Eub518	ATTACCGCGGCTGCTGG			

Table 2. Sediment (mg/g) metal concentrations from Savannah River Site samples. BD: Below detection limits.

	Be	V	Cr	Ni	Cu	As	Se	Sr	Cd	Sb	Hg
Site A	0.09	3.03	BD	4.72	3.87	2.59	1.75	0.53	0.22	0.02	0.07
Site D	0.25	8.33	3.85	2.88	3.16	1.02	3.34	BD	0.04	BD	BD
Meyers Branch	BD	1.36	BD	1.47	0.74	BD	1.43	BD	0.02	BD	BD

Table 3. Phenotypes of microcosm isolates screened positive for *tetA*. Adapted from Stepanauskas et al. (31).

Isolate	Drug Resistances[a]	Metal Resistances[b]	Closest type strain in RDP	Closest isolate in GenBank
Comamonas 6-6	Ak AmAmx Apr Azi Cax Cex Cf Cfx Cp Fd Gm Imp Mer Mox NA Ofl Otet P St T Te	Cu, Hg	Comamonas aquatica (AJ430344) 96%	LMG 5 (AJ430346), 100%
Comamonas 19-3	Ak AmAmx Apr Azi C Cax Cex Cf Cfx Cp E Fd Gm Imp Mox NA Ofl Otet P St Sz T Te	Cd, Cu, Ni, Hg	Comamonas aquatica (AJ430344) 92%	BM-9_6 (AY635871), 98%
Comamonas 24-1	Am Amx Apr C Cex Cf Cfx Cp Fd Imp Mer Ofl Otet P T Te	Cu, Hg	Comamonas aquatica (AJ430344) 96%	LMG 5 (AJ430346), 100%
Delftia 19-4	Ak AmAmx Apr Azi C Cax Cex Cf E Fd Gm Mer Otet P St T Te	Cd, Cu, Ni, Hg	Delftia acidovorans (AF078774), 95%	As3-4 (AY367028), 99%

[a]Abbreviations used: Ak, amikacin; Am, ampicillin; Amx, amoxicillin; Apr, ampramycin; Azi, azithromycin; C, chloramphenicol; Cax, ceftriaxone; Cex, cephalexin; Cf, cephalothin; Cfx, cefoxitin; E, erythromycin, Fd, nitrofurantoin; Gm, gentamicin; Mer, meropenem; Mox, moxifloxacin; NA, nalidixic acid; Otet, oxytetracycline; P, penicillin; St, streptomycin; Sz, sulfathiazole, T, trimethoprim; Te, tetracycline.
[b]Abbreviations used: Cu: copper; Cd: cadmium; Ni: nickel; Hg: mercury

Figure Legends.

Figure 1. Discharge data for gauging station midway between Beaver Dam Creek Site A and Site D. The sampling date is indicated with the arrow.

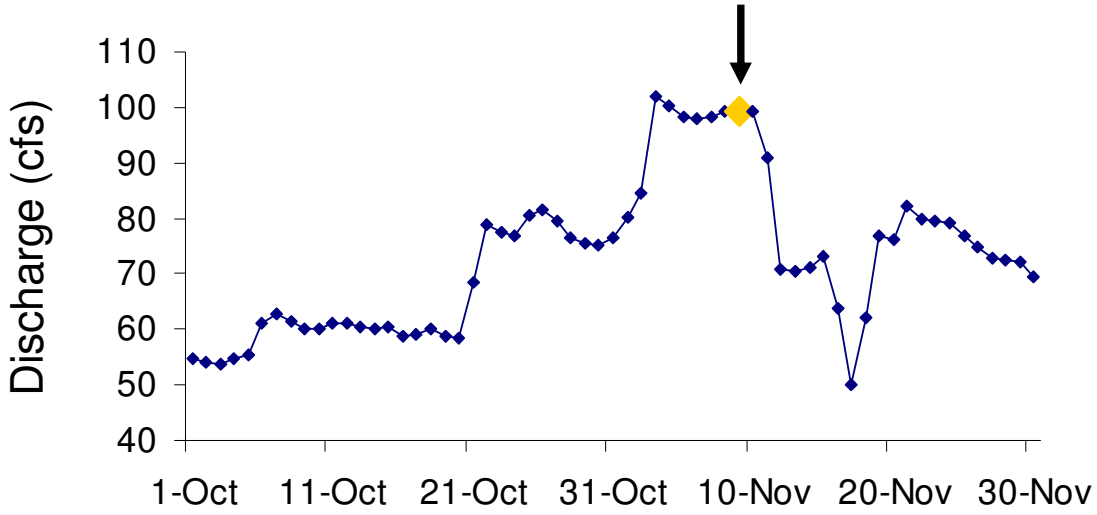
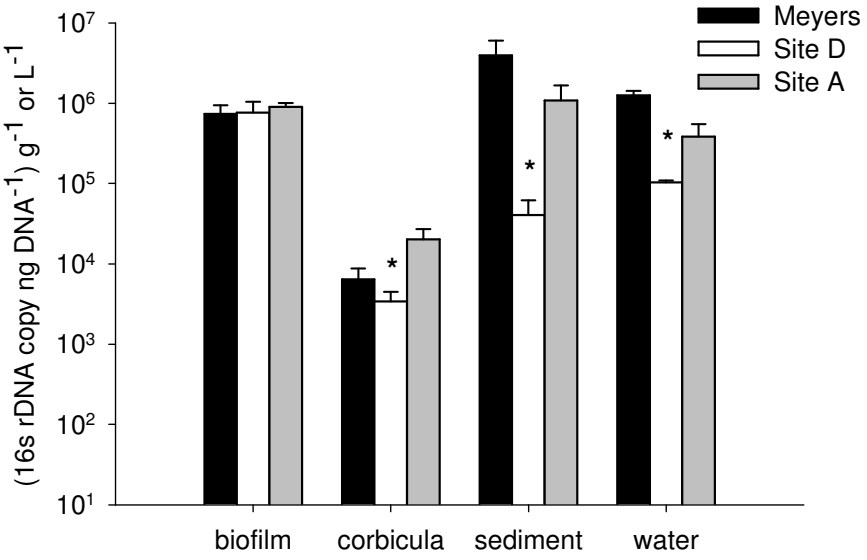


Figure 2a and b. Abundance of 16S rDNA genes in (a) biofilm, *Corbicula*, sediment and water microhabitats from Meyers Branch, Beaver Dam Creek Site D and Site A on the Savannah River Site, SC; and (b) from the initial bacterial community, and from control, cadmium-exposed, and tetracycline-exposed microcosms seven days later. Error bars represent ±1 SE (n = 9).



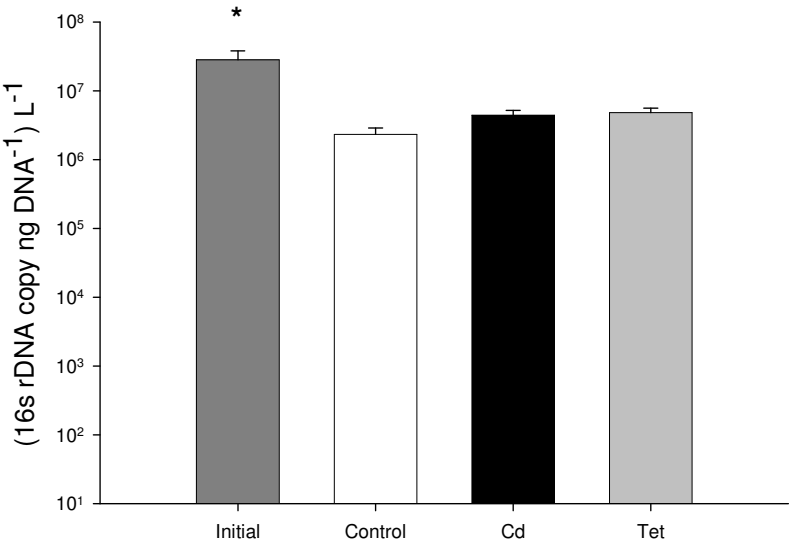
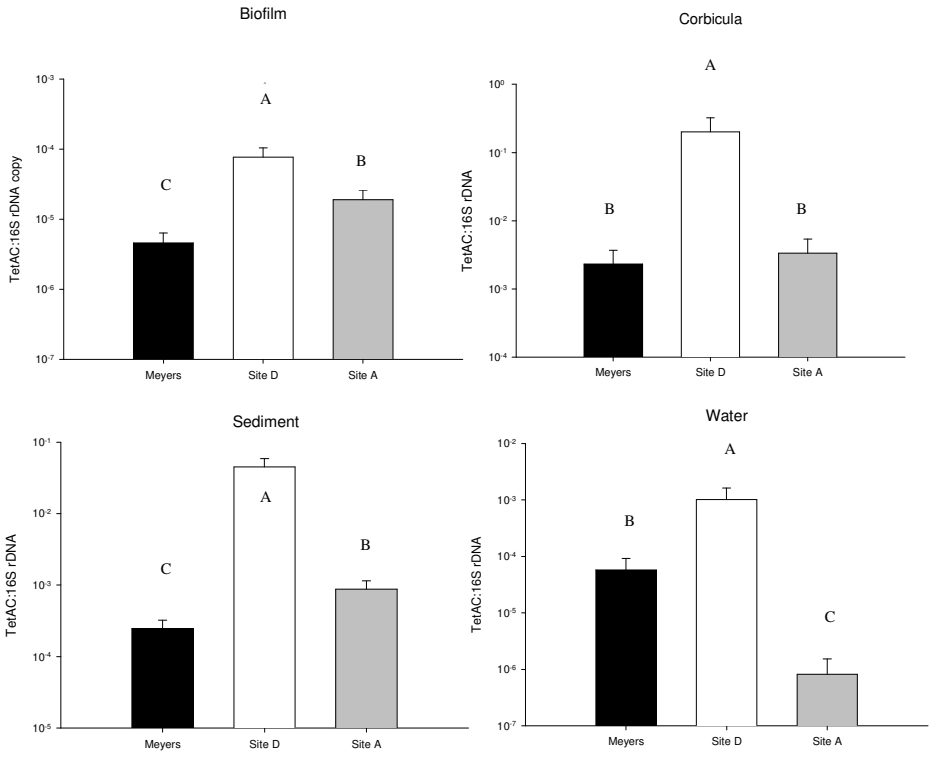


Figure 3a and b. Relative abundance of *tetA* (open bars) in bacterial microhabitats from Meyers Branch, Beaver Dam Creek Site D and Site A (a) and experimental microcosms (b). Response variable used for analysis is the log-transformed ratio of *tetA*:16S rDNA abundance. Error bars represent ±1 SE (n=9).



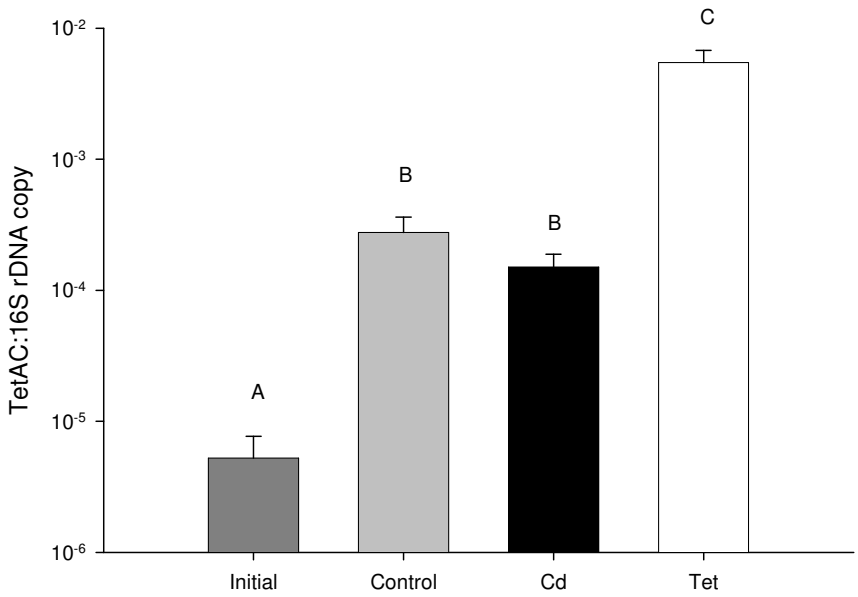
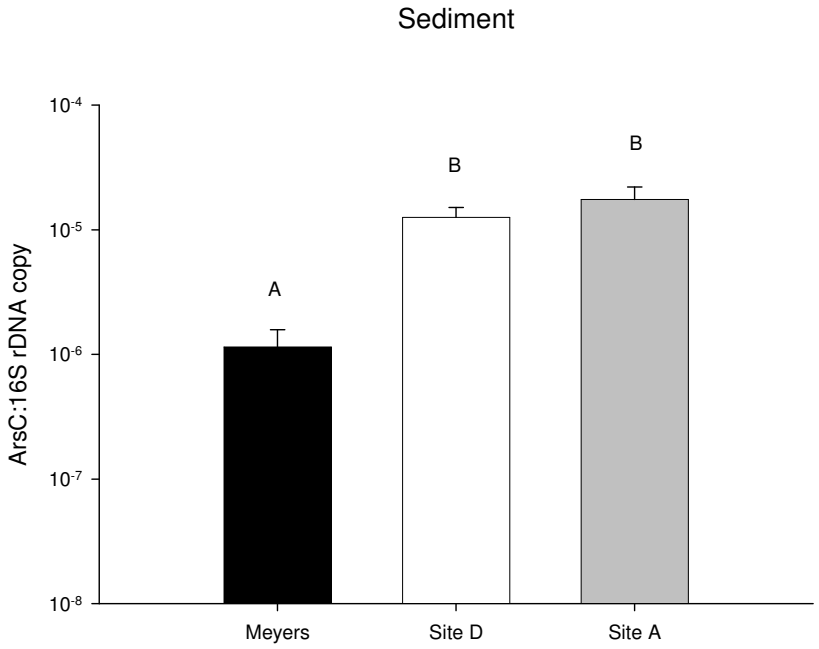


Figure 4. Relative abundance of *arsC* (open bars) from sediment microhabitats. Response variable used for analysis is the log-transformed ratio of *arsC*:16S rDNA abundance. Error bars are ±1 SE (n=9).



CHAPTER 4

THE ABUNDANCE AND DIVERSITY OF INTEGRONS AS A FUNCTION OF INDUSTRIAL CONTAMINATION[1]

[1]Wright, M.S., C. Baker-Austine, A.H. Lindell, R. Stepanauskas, and JV McArthur. To be submitted to *Applied and Environmental Microbiology*.

Abstract

The acquisition of new genetic material via horizontal gene transfer allows bacteria to rapidly evolve, but the ubiquity of this process is in environmental bacteria is poorly understood. One key to estimating the role of horizontal gene transfer in the evolution of environmental bacteria is to quantify the abundance of mobile genetic elements (MGEs) in bacterial communities under varying selective pressures. In this study, we estimated the relative abundance of class 1 integrases (*IntI1*) in multiple contaminated and reference riverine and estuarine microhabitats, and in experimental microcosms where bacterial exposure to metals and antibiotics was directly manipulated. *IntI1* was more abundant in contaminated sites for all tested habitats, indicating that gene transfer potential is higher in bacterial communities exposed to industrial contamination. A second key to assessing the role of integrons in bacterial evolution is to examine the gene pool associated with these elements. We compared the richness, similarity, and predicted function of gene among sites, finding that gene cassette pool is diverse, but contamination has no clear effect on cassette richness. However, gene cassettes were more similar within sites than among sites, although bacterial community composition was not more similar within sites as assessed by tRFLP analysis. This suggests that selective pressures shape the structure of the gene cassette pool available for bacterial acquisition. Of 46 sequenced gene cassette products, the vast majority were novel sequences, while the nine gene cassettes that were similar to GenBank sequences were primarily hypothetical proteins. That class 1 integrons are ubiquitous and abundant in environmental bacterial communities regardless of contaminant exposure indicates that they can play a substantial role in the acquisition of a diverse array of gene cassettes.

Introduction:

Because bacteria do not undergo sexual reproduction in the eukaryotic sense, changes in their genetic composition must come from processes involving mutation-based alterations or the acquisition of exogenous DNA via horizontal gene transfer. Mobile genetic elements (MGEs) enable bacteria to rapidly evolve as is best evidenced by the rapid and global dissemination of antibiotic resistance genes (30, 31). Approaches to examining the role of horizontal gene transfer in microbial evolution typically revolve around the use of a comparative approach to reconstruct historical gene transfer events (6, 10, 14, 21), the use of *in vivo* or *in vitro* gene transfer experiments where gene transfer rates are typically estimated under controlled conditions (12, 52), or molecular investigations of cellular regulation and machinery involved in the process (22, 46). While these methods have merit for revealing evolutionary relationships among organisms and regulations of gene transfer processes, they rarely reflect *in situ* or real-time horizontal gene transfer potential in environmental microbial communities (but see (27).

One key to estimating gene transfer potential and its role in bacterial evolution is to first estimate how abundant MGEs are in bacterial communities under varying degrees of selective pressure. Several studies have demonstrated the ubiquity of MGEs such as plasmids, transposons, and integrons in essentially all tested habitats (e.g., (17, 37, 38, 52), but rarely in a quantitative manner due to previous methodological constraints. However, mercury exposure was recently demonstrated to increase the abundance of IncP plasmids as assessed with PCR-based techniques (36). Also, Dahlberg et al. (11) quantified multiple classes of transposons using primers targeting the *tnpA* genes to

assess their occurrence in microbial communities, estimating that from 1 in 1,000 to 10,000 bacteria possess these elements. How the proportion of bacteria possessing MGEs is affected by environmental conditions and selective pressures remains to be tested fully.

Increased competency and acquisition of exogenous genetic material has been shown to be one bacterial response to stressors like antibiotic exposure, or nutrient limitation (4, 8, 18, 51, 53). Thus, mobile genetic elements involved in the acquisition and recombination of new genes such as integrons may be more abundant in stressed bacterial communities. Additionally, MGEs may be more abundant in stressed communities because they can confer a selective advantage to bacteria that possess them by containing genes that allow bacteria to either tolerate or utilize the stressor (49, 52).

According to evolutionary theory, gene content in a given bacterial community should reflect selective pressures present in that environment, and this should apply to MGEs as well (50). For example, antibiotic resistance genes are often more abundant in bacterial communities exposed to antibiotic contamination (19, 32) and mercury resistance genes are more abundant in mercury-contaminated sediments (36). However, previous studies have documented that additional genotypes and phenotypes can be co-selected for along with traits under direct selection including antibiotic resistance in metal-contaminated systems (Wright et al. Chapter 3) (1, 3, 43, 54). Furthermore, genotypes may persist in a bacterial community even after a given selective pressure no longer exists (15). Turner et al. (50) predict that the more intense the selective pressure, the narrower the range of genotypes that can persist, thereby resulting in a more homogenous (less diverse) pool of MGEs. Thus how a selective pressure influences the

MGE gene pool in a bacterial community remains poorly understood, and is of interest from a basic science perspective of understanding evolutionary processes in bacteria, and from a more applied perspective for understanding the role of these elements in the maintenance of an antibiotic resistance reservoir and dissemination of genes involved in pollution degradation.

In this study, we examined the potential role of mobile genetic elements called integrons in bacterial evolution. Integrons are genetic elements that incorporate exogenous sources of DNA called gene cassettes into the recipient genome via site-specific recombination. Various classes of integrons have been identified (28, 29), but they share a common structure including the integrase gene (*IntI*), an enzyme that catalyzes the recombination of a gene cassette at the *attI* site, and a downstream promoter (Figure). Class 1 integrons were first identified due to their role in the dissemination of antibiotic resistance gene cassettes in clinical bacteria (40), but have since been found in a phylogenetically broad range of clinical and environmental bacteria including Gram-negative and Gram-positive bacteria (27, 42). Environmental surveys of gene cassettes indicate that the majority of cassettes are novel sequences unrelated to known antibiotic resistance genes (20, 41), with one conservative estimate of cassette richness being at least 2,343 different gene cassettes in a 50-m^2 sediment plot (26). Integrons therefore represent a potential genetic resource by which bacteria in a variety of environments can adapt through the acquisition of new genetic material.

To estimate the potential role of integrons in the evolution of aquatic bacteria, we compared the relative abundance of class 1 integrons and quantitatively examined the structure and function of gene cassettes under varying degrees of bacterial stress. We

compared the abundance of *IntI1* in bacterial communities from industrially contaminated and reference riverine and estuarine systems, and in freshwater microcosms where bacterial exposure to metals was directly manipulated. To further investigate how environmental conditions influence the structure and function of genes contained within mobile genetic elements, we quantified gene cassette diversity and similarity metrics and assessed their predicted function. We predicted that integrons would be more abundant in the industrially impacted sites, while the diversity and structure of the cassette gene pool would reflect site-specific selective pressures and encode for functions other than that of antibiotic resistance.

Materials and Methods

Sampling

Savannah River Site

We collected samples from two streams located on the United States Department of Energy's Savannah River Site (SRS) near Aiken, SC, on November 9, 2005. Effluent from ash settling basins associated with the SRS D-400 coal-fired power plant enters into Beaver Dam Creek at Site A, the first sampling point. Sediment here consists of cobble, sand, and coal ash particles with elevated concentrations of a variety of metals associated with coal ash effluent including selenium, arsenic, and strontium (55). Beaver Dam Creek Site D is located 3.2 km downstream from Site A where sediment is primarily sandy, and dissolved metal concentrations are typically lower than at Site A. Meyers Branch is a historically unimpacted blackwater stream with sandy sediment which has

previously been used as a reference stream on the SRS (25, 55). Neither stream has a point source of anthropogenic antibiotics.

We collected bacteria from four common stream microhabitats to examine the abundance of mobile genetic elements in a variety of microbial communities including epiphytic biofilm, *Corbicula fluminea* (Asiatic clam) digestive tracts, surface sediments, and surface water. Epiphytic biofilm was collected from oak wooden dowels deployed at each site on June 3, 2005. On the sampling date, we transported three dowels from each site back to the laboratory in sterile whirlpacks on ice for further processing. To represent microbial communities harbored in the digestive tract of invertebrates, we collected nine *Corbicula fluminea* (Asiatic clam). Grab samples of surface sediments were collected in triplicate in sterile whirlpacks to analyze sediment bacterial communities, and we collected 1 L of stream water in triplicate in autoclaved and UV-sterilized polycarbonate bottles to sample bacteria communities in the water column.

Estuarine sites

We sampled three estuaries located along the Georgia and South Carolina coasts that vary in degree and type of industrial contamination in October 2005. Both Shipyard Creek (Charleston, SC) and the LCP Site (Brunswick, GA) are designated as US EPA Superfund sites due to their history of industrial contamination. Sediments contain elevated concentrations of chromium and cadmium (Shipyard Creek), and mercury (LCP) among others (Baker-Austin,. unpublished data). The Ashepoo-Combahee-Edisto (ACE) Basin estuary (Beaufort, SC), a historically unimpacted US National Wildlife Refuge,

served as a reference site. We collected samples in triplicate as above for surface sediments, and water samples during both ebb and flood tides.

Savannah River Microcosms

Bacterial exposure to metals and antibiotics was experimentally manipulated using a series of microcosms as described in (39). We collected surface water from the Savannah River near the intake for the SRS D-400 coal-fired power plant on September 29, 2003 which we then aliquoted into triplicate tetracycline (30 mg/L), cadmium (0.1mM), or control microcosms. Selected concentrations represent treatment microcosms from (39) that generated an increase in metal and antibiotic resistance phenotypes relative to controls in the microcosm series. Microcosms were incubated for seven days at 23°C with slow rotation (100 rpm on an orbital shaker) after which 250 mL of water were collected for DNA extraction.

Sample processing

Upon return to the laboratory, all samples were processed immediately. For biofilm samples, we aseptically scraped biofilm material from the wooden dowels using a sterilized razor blade and extracted DNA from 0.25 g of this material using the MoBio PowerSoil DNA Extraction kit per the manufacturer's instructions. To extract DNA from *Corbicula* digestive tracts, we aseptically removed and combined the hind guts of three individuals per replicate to obtain enough material to process using the MoBio UltraClean DNA Extraction kit. We extracted sediment DNA from 0.4 g of sediment using the Mo Bio PowerSoil DNA Extraction kit. For water samples, we filtered 1 L of

water (or 250 mL for the microcosms), onto 0.2 μm filters. Because of suspended particles, multiple filters had to be used which were combined in 50 mL Eppendorf tubes containing10 mL of autoclaved, UV-sterilized nanopure water. Tubes were votexed until all visible particles were detached and centrifuged at 7000 rpm for 15 min to pellet bacteria. We extracted DNA from the pellets using MoBio UltraClean DNA Extraction kit. DNA was quantified using gel standards with AlphaEase FC Software version 3.1.2 (Alpha Innotech Corp.)

Quantitative PCR (qPCR) protocol

Primers

Primers targeting the class 1 integrase (*IntI1*) gene were used to estimate the abundance of class 1 integrons (Diana Nemergut, personal communication) (Figure 1). To account for potential differences in bacterial abundance and PCR inhibitors, universal primers were used to quantify 16S rDNA genes. Primers used and their annealing temperatures are detailed in Table 1.

Calibration standards

Calibration standards for use in qPCR assays were generated from conventional PCR products using the appropriate primer pair with DNA extracted from each sample as the template. We then ran PCR products on a 1% agarose gel at 80mV for 30 min to ensure amplicons were of the predicted size. We purified PCR products using the Qiagen PCR Purification kit and quantified with standards using software AlphaEaseFC

software. Six ten-fold dilutions were prepared with each purified PCR product to use as standards in qPCR assays.

qPCR conditions

qPCR reactions for *IntI1* and 16S rDNA genes consisted of 1 μL template, 2 μL of 1 μM *IntI1* primers (10 μM 16S rDNA primers), 12.5 μL of SuperArray SYBR Green Master Mix (SuperArray), and PCR-grade water to a reaction volume of 25 μL. Triplicate reactions for each sample were performed using the BioRad iQCycler Real-Time Detection System in BioRad iCycler 96-well plates where triplicate no-template controls were also used.

The thermocycler protocol for *IntI1* and 16S rDNA reactions consisted of 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec, 30 sec at 62°C (53°C for 16S rDNA), 72°C for 30 sec, and a final 10 min at 72°C for extension. To ensure specificity for each reaction, multiple analyses were conducted including a melt curve analysis after each qPCR run where fluorescence was measured for 55 cycles starting at 70°C with an increase of 0.5°C. The plot of -dFlour/dt was examined for the presence of a single peak to confirm reaction specificity. Additionally, qPCR product from randomly selected samples were run on a 1% agarose gel in 10x TBE solution for 30 minutes at 80V to check for the presence of a single band of the appropriate size with an additional subset of *IntI1* PCR products selected for sequencing.

Copy number of each target gene was calculated using the average molecular mass of 660 g mol^{-1} for a double-stranded DNA molecule. Estimates of 16S rDNA copy number were corrected for amplification in negative control reactions.

Gene Cassette Profiling

To assess the structure of the gene cassette community, fluorescently labeled primers targeting the *attC* site (HS286 and HS287) were used to amplify gene cassettes from freshwater and estuarine sediment samples and microcosms as in Michael et al. (26). PCR reactions were run in triplicate for each sample consisting of 400 nM of each primer, 1 X PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.02 U JumpStart Taq (Sigma), and 5 ng of template DNA in a 25 μL reaction volume. PCR conditions were as follows: 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 2 min 30 sec, and a final extension at 72°C for 10 min. We then analyzed the PCR amplicons using fragment analysis on an ABI 3130xl sequencer to separate gene cassettes by size, combining 1 μL of PCR product with 1.5 μL internal NAUROX size standard (to 1002 bp) (13), and 7.5 μL of HiDi:formamide. We limited analysis of cassette sizes to those within 250-999 bp, as the false-positive rate for PCR fragments smaller than 250 bp is too high (as determined from sequencing results and as in (26) and size class calling accuracy decreases with increasing amplicon size. To account for variability in PCR reactions, three triplicate PCR reactions were analyzed separately for each sample using GeneMapper (Applied Biosystems) with the peak detection algorithm set to detect peaks greater than 20 fluorescence units above the threshold. We compiled the data from the three runs by retaining peaks that were present in at least two out of the three PCR runs and calculated mean peak area as a measure of relative cassette abundance.

tRFLP

We assessed bacterial community structure by amplifying 16s rDNA fragments for tRFLP analysis. PCR reactions consisted of 400 nM the universal primers, 5'-FAM labeled E334F and unlabeled E939R, 1 X PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.02 U JumpStart Taq (Sigma), and 5 ng of DNA. The thermocycler program consisted of 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. Duplicate PCR reactions were run for each sample, combined, and purified using Qiagen PCR Purification kit. PCR product was then quantified using DNA standards and the AlphaImager system and software. To generate restriction fragments, we incubated 30 ng of PCR product with 1 μL of RsaI enzyme (New England Biolabs), 2 μL of NEBuffer 1, and PCR-grade to a reaction volume of 20 μL at 37 °C for one hour, followed by 20 minutes as 65 °C to inactivate the enzyme. 10 μL products were then purified, precipitated, and resuspended in TE following the methods of Sambrook and Russell (34). For fragment analysis on the ABI 3130xl sequencer, we denatured 1 μL of the purified digest product with 0.7 μL of NAUROX internal size standard, and 13 μL of 1:1 HiDi:formamide at 95 °C for five minutes. Peaks above 20 fluorescence units were detected and sized using a global Southern algorithm in GeneMapper software (Applied Biosystems).

Sequencing

We assessed potential gene cassette function by transforming randomly selected gene cassette PCR products from each sample type using TopoTA Cloning Kit (Invitrogen). Clones were screened for the presence of the insert using conventional PCR

with M13 primers. We ran PCR products on a 1% agarose gel for 30 min at 80 mV and selected clones representing a range of sizes. Sequencing reactions using M13 forward and reverse primers and BigDye 3.1 Terminators (Applied Biosystems) were resolved on an ABI 3130xl sequencer. We edited and contiged sequences using Sequencher software (GeneCodes) to ensure accuracy of the reads. After confirming the presence of both HS286 and HS287 primer sequences in the obtained sequences, we both manually identified predicted ORFs and used NCBI's ORF Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) to locate predicted proteins within gene cassettes. To assess potential function of predicted proteins, we conducted a BLASTX search to identify similar amino acid sequences.

IntI1 PCR products were also transformed with the TOPO TA Cloning Kit and sequenced using M13 primers as above.

Statistical Analyses and Gene Cassette Diversity and Similarity Metrics

Differences among sites in log-transformed gene cassette relative abundance (IntI1 copies $\cdot$16S rDNA copies^{-1} $\cdot$ ng DNA^{-1}) were analyzed by ANOVA in SAS (SAS Institute, Cary, NC) where site was the main effect. Site means were compared using the LSMEANS option with α=0.05. Each sample set (freshwater, esturaine, microcosms) and each microhabitat were analyzed separately because of potential differences in PCR inhibitors among bacterial communities.

Gene cassette richness (Sobs), diversity (Shannon), and community similarity (Chao's Abundance-based estimate of Jaccard similarity) values were calculated using EstimateS software version 7.5 (9). Richness and diversity estimates were calculated for

each site using compiled data from the three PCR reactions for each of the replicates at each site. Chao's Abundance-based estimate of Jaccard similarity was chosen because it corrects for undersampling of rich samples and incorporates a measure of cassette relative abundance (7). A distance matrix was constructed for each sample set based on similarity values and a multidimensional scaling plot was generated using these data in SAS. The same procedure was followed for analyzing tRFLP profiles for similarity among samples at each site.

Results

16S rDNA abundance

16S rDNA qPCR reaction efficiencies for ranged between 100.2% and 105.3% among the sample types, with correlation coefficients between0.90 and 0.96 for the standard calibration curves. Melt curve analysis displayed one broad peak confirming the specificity of the reactions. For freshwater samples, 16S rDNA did not differ between sites for biofilm samples, but there were significantly fewer 16S rDNA copies at Site D in *Corbicula*, sediment, and water column microhabitats (Figure 2a). 16S rDNA copies were significantly more abundant in Meyers Branch sediment and water column samples than at Site A, but this pattern was reversed for *Corbicula* samples. 16S rDNA copy number was greatest in the initial samples but declined over the seven days of incubation for all microcosms (Figure 2b). For estuarine sites, in both ebb and flood samples, there were significantly fewer copies of 16S rDNA genes at the SYC site, but there were no site differences among sediment samples (Figure 2c).

IntI1 relative abundance

The ratio of *IntI1*:16S rDNA copy number was significantly greater in bacteria from contaminated sites compared to reference bacterial communities across the tested microhabitats. *IntI1*:16S rDNA was significantly lower for biofilm, sediment, and water microhabitats at the reference riverine site, Meyers Branch, but there was no statistical difference in the ratio for *Corbicula* samples (Figure 3a). *IntI1* was significantly more abundant at Site D than Site A only for sediment samples, while there was no statistical difference between the sites for the other microhabitats. *IntI1* relative abundance was greatest in the cadmium treatment, followed by the tetracycline treatment, while there was no statistical difference between initial and control microcosms (Figure 3b). Compared to the two Superfund estuarine sites, significantly fewer copies of *IntI1* were detected in ACE Basin for ebb, flood, and sediment samples, while the ratio of *IntI1*:16S rDNA was significantly greater in SYC than LCP samples for ebb samples (Figure 3c). qPCR reaction efficiencies ranged from 85.5 to 86.8%, while standard curve correlation coefficients ranged from 0.910 to 0.982. All *IntI1* sequences were highly similar to previously characterized class 1 integrases and have been deposited in GenBank under the following accession numbers.

Gene Cassette Structure

Gene cassette richness and Shannon diversity varied significantly among sites, but with no consistent pattern between environmental DNA extracted from contaminant-exposed and reference communities (Table 2). Richness and diversity were significantly greater in sediment communities at Beaver Dam Creek sites A and D in comparison with

the reference site, Meyers Branch. However, cassette richness and diversity were significantly greater in ACE and LCP sites than in SYC samples. For the microcosm series, richness and diversity were significantly greater in the cadmium and tetracycline treatment microcosms than in initial and control samples. Gene cassette composition varied among sites and microcosms, as cassette profiles were more similar within rather than among each site or microcosm treatment (Table 2). Replicates from each sample clustered together in comparison to replicates from other sites or treatments in a multidimensional scaling plot (Figure 4).

Gene Cassette Function

Of the 46 sequenced gene cassettes, 43 had predicted open reading frames (ORFs) with start and stop codons in the typical gene cassette orientation, while the three with no obvious ORF were all less than 209 bp in length. Several ORFs had putative stop codons out of frame (n = 15) or stop codons in the middle of the predicted coding sequence (n = 8). The majority of gene cassette sequences contained ORFs that had no similar matches in GenBank at either the nucleotide or amino acid level. Only nine gene cassettes were similar to GenBank sequences at the amino acid level (Table 3): two were similar to previously detected gene cassettes, four cassettes were similar to hypothetical proteins. Of the other cassettes with similarity to GenBank sequences, no antibiotic or metal resistance genes were recovered, but similar proteins included partial matches to magnesium chelatases, N-acetyltransferases, and pantothenate synthetases. However, the Cadmium Microcosm_01 cassette likely underwent a frameshift as there was an inframe stop codon in the middle of the coding sequence, and no inframe stop at the end. The

gene cassette similar to pantothenate synthetases involved in vitamin B5 synthesis had an inframe stop codon as did the Initial Microcosm_06 cassette.

tRFLP Community Similarity

Bacterial community composition as assessed by the similarity of tRFLP profiles was not distinct between sites, as Chao's Abundance-based Jaccard similarity values indicate that similarity was not greater within a site than among sites (Table 2) as analyzed for each sample set. Additionally, replicates from each site did not cluster together in a multidimensional scaling plot (Figure 5).

Discussion

Integron abundance

Genetic elements involved in the acquisition of exogenous sources of DNA were more abundant in contaminant-exposed bacterial communities across a variety of microbial habitats and in experimental microcosms where metal and antibiotic exposure was manipulated. That this effect of industrial contamination on integron abundance was observed across multiple bacterial communities in multiple types of ecosystems indicates its generality. Similarly, class 1 integrons were also more abundant in metal-exposed bacteria from river sediments in Australia compared to upstream reference communities (Carly Tucker, personal communication). In this study, *IntI1* was relatively more abundant in Site D than Site A in the contaminated stream counter to the prediction that *IntI1* would be most abundant in the most contaminated site. However, this same pattern was observed in these sites for the relative abundance of *tetA*, a gene encoding for tetracycline resistance, where *tetA* was more abundant at Site D than Site A in these same

samples (Wright et al. Chapter 3). As discussed in Wright et al., metal concentrations were elevated at Site D on the sampling date likely due to a period of increased discharge associated with the coal-fired plant operations and the cumulative effects of transport from more contaminated sites upstream, resulting in similar selective pressures at Site D on the sampling date.

Culture-based and molecular estimates of class 1 integrase abundance indicate that between one and ten percent of bacteria possess these genes (Baker-Austin unpublished data, (27)), although Rijavec et al. (33) found that 26% of uropathogenic strains of *E. coli* isolates harbored class 1 integrases and Biyela et al. (5) found class 1 integrons in over 50% of isolates with multiple drug resistances from an urban South African river. Variation in the proportion of bacteria possessing class 1 integrons likely is the result of several factors including: 1) distance to a source of integrons and integron dispersal ability; 2) the taxonomic composition of the community; 3) horizontal gene transfer potential in a given habitat; and 4) intensity of selection.

To date, Class 1 integrons have primarily been characterized from bacteria of clinical origin, but have more recently been detected in environmental bacteria from diverse habitats. Based on a BLASTN search of 759 bacterial genomes (completed and whole genome shotgun projects) in the Microbial Genome Resources database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html), eight genomes (1.1%) yielded sequences similar to *IntI1* (e-value cutoff: e^{-50}). Seven of these genomes were from enteric γ-proteobacterial pathogens of clinical origin, while the exception to this was the Gram-positive human pathogen *Corynebacterium diphtheriae* (Actinobacteria) (Table 3). In a BLASTN search of all GenBank bacterial sequences,

more than 299 unique nucleotide sequences produced similar alignments to *IntI1* (e-value cutoff: e^{-50}). Again, the majority of these were from plasmids and transposons in clinical isolates of Gram-negative proteobacteria pathogens reflecting the bias in the database towards pathogenic bacteria and antibiotic resistance studies. However, a broader group of bacteria likely possess these elements as evidenced by Nandi et al. (27) detecting *IntI1* in Gram-positive bacteria and Stokes et al. (42) recent description of four nonpathogenic soil microorganisms (all β-proteobacteria) possessing class 1 integrons. Furthermore, three additional *IntI1* sequences were identified from two environmental metagenomic studies: one sequence from an enhanced biological phosphorous removal (EBPR) sludge community (24), and one sequence each from the Global Ocean Sampling project in Delaware Bay, NJ, and Sargasso Sea samples (35). A BLASTN search of archaeal sequences found no matches to *IntI1* indicating that to date no class 1 integrons have been detected in Archaea (date of analyses: March 27, 2007). Thus sequence database evidence to date suggest that class 1 integrons are concentrated in Gram-negative γ-proteobacteria, but this picture will likely change with the accumulation of more environmental sequences.

To assess what additional phenotypes were associated with *IntI1* in the sampled habitats, *Escherichia coli* isolated from the estuarine sites (n = 433), and isolates from the microcosm series (n = 90) were screened using conventional PCR with the above *IntI1* primers and PCR conditions. The nine *IntI1*-positive *E. coli* isolated from the three estuarine sites were resistant to more antibiotics than *E. coli* isolates without *IntI1* (8.6 vs. 3.0 drugs, respectively), and had a higher incidence of resistance to specific drug classes including aminoglycosides (streptomycin), sulphonamides (sulfathiazole), and folate

inhibitors (trimethoprim) (Baker-Austin in review?). These resistances are encoded by genes routinely found within class 1 integrons including *aad*, *sul* and *dfr* gene families, respectively, and a gene cassette within one isolate, *E. coli* SYC Sed 3-5 contains a *dfr* gene within a class 1 integron on its plasmid (add what type of aad). Additionally, two *IntI1*-positive isolates, *Delftia* 19-1 sp. and *Delftia* 19-4 sp. (β-proteobacteria) from the microcosm series (tetracycline treatments), were resistant to 19 and 18 out of 26 tested antibiotics respectively, including multiple cephalosporins, aminoglycosides, quinolones, tetracyclines, and penicillins often exceeding the maximum tested antibiotic concentration (Table). Additionally, the minimum inhibitory concentration (MIC) for the *Delftia* sp. isolates grown in half-strength nutrient broth was 0.1 g/L for cadmium, nickel, and copper, and 0.0001 g/L for mercury. Attempts to recover gene cassettes from isolates using HS286 and HS287 primers were not successful from the *E.coli*, but gene cassettes from the *Delftia* sp. isolates were or were not similar to this (add soon). In all isolates, *IntI1* was clearly associated with multiple drug resistance (MDR) phenotypes, and what could be considered extreme drug resistance (XDR).

Whether this association between *IntI1* and antibiotic resistance is the general case for environmental bacteria is still unclear, but evidence from Stokes et al. (42) suggest that it is not. Antibiotic resistance gene cassettes have been found primarily in clinical class 1 integrons in which the 5' conserved segment contains transposase features, whereas class 1 integrons from environmental bacteria do not always have these sequences. Stokes et al. (42) hypothesize that the acquisition of transposases by 'conventional' class 1 integrons has contributed to the dispersal ability of class 1 integrons via transposition into larger mobile genetic elements and is related to the

dissemination of antibiotic resistance cassettes within these elements. We screened the nine *IntI1*-positive *E. coli* isolates and two *Delftia* sp. isolates using primers that distinguish between the 'clinical' type of class 1 integrons and those lacking the transposes sequences, finding that eight of the *E.coli* possess integrons of the 'clinical' type of class 1 integrons, while neither of the *Delftia* sp. do (Wright, unpublished data). Thus whether class 1 integrons are contributing to the dissemination of antibiotic resistance gene cassettes in environmental bacteria may depend on prevalence of 'clinical' type 1 integrons in bacterial communities.

Elevated class 1 integron abundance can either be the result of selection for the integron itself (the ability to acquire and insert new genetic material in a site-specific location), gene cassettes contained within integrons (direct selection), or other genetic elements to which the integron is physically linked (co-selection). In support of the last hypothesis, class 1 integrons are frequently detected on larger mobile genetic elements including insertion sequence common regions (ISCRs), transposons, and plasmids (42, 44, 45, 47, 48, 53) that contain genes encoding for other functions. The classic example of this is Tn21, a transposon containing a mercury resistance transposon in which a class 1 integron is embedded (23). Selective pressures favoring the maintenance of genes within these genetic elements (e.g, metal contamination maintaining metal resistance genes) may therefore maintain other genes including integrons that are physically linked.

Evidence supporting the hypothesis that integrons were more abundant in the metal contaminated sites because of the integron itself (i.e., the ability to acquire new genetic elements) includes recent studies on the regulation of gene transfer systems including induction to competence via the SOS response (4). These studies demonstrate

the increased expression of genes involved in uptake of DNA with bacterial exposure to antibiotics, DNA damage, or nutritional stress. Integrons represent a relatively safe gene acquisition system in terms of potential evolutionary cost compared to those that insert into the host genome at random such as some transposons, because gene cassettes are inserted in a site-specific location, likely minimizing their impact on the rest of the genome. Thus, integrons may be more abundant in stressed bacteria. However, what regulates integron activity, gene cassette insertion and excision frequency, and the expression of the gene cassettes within is yet to be fully understood.

Gene Cassette Structure and Function:

The effects of industrial contamination on gene cassette pool richness and diversity are equivocal, as the riverine and microcosm data suggest that this selective pressure increases the richness and diversity of the gene cassette pool, whereas this pattern was not observed in the estuarine sites, where cassette richness and diversity was lower at SYC. We predicted that contamination would have a homogenizing effect on the gene cassette pool, as selective pressures would favor the propagation of cassettes conferring a selective advantage (50), but perhaps the sampled sites are too heterogeneous to favor the dominance of a few cassettes.

The analysis of cassette richness using this method likely underestimates gene cassette richness. This is in large part because the primers used do not recover all cassettes (26). Other potential errors relate to the use of gene cassette size as a proxy for distinguishing between different cassettes. Previously, Michael et al. (26) detailed this potential bias where the same cassette size class can contain multiple cassettes.

However, this problem is not unique to this method, as the same limitation is present in techniques that rely on fragment-based analysis of communities such as tRFLP and DGGE. These biases all result in richness values that underestimate the true number of gene cassettes in a bacterial community, but what is apparent is that gene cassettes are a diverse genetic resource for bacterial evolution in the environment.

Similarity patterns indicate that the gene cassette pool at each site or microcosm treatment were more similar in gene cassette size composition and relative abundance compared to the other sites for each sample set. This finding likely reflects differences in selective pressures at each site maintaining a different pool of cassettes, but alternatively, could reflect taxonomic or biogeographic effects. However, 16S rDNA bacterial community fingerprints as assessed by tRFLP analysis indicates that overall community structure was not distinct among sites, strengthening the conclusion that gene cassette pools were distinct at each site because of selective pressure differences. Biogeography may have an effect through dispersal limitation of gene cassettes between sites, or through variations in gene cassette composition entering each site due to watershed differences.

One key to assessing whether gene cassettes are selected for at the various sites is to identify the potential function of gene cassettes. An assessment of whether any of the most abundant gene cassettes is of potential adaptive significance is hampered by the lack of recovery of the most abundant cassette sizes, and by the fact that most sequenced cassettes were novel. Of interest was the fact that several gene cassettes likely encode for nonfunctional proteins due to frameshifts, and inframe stop codons in the middle, indicating that they are not encoding a protein under selection. Those cassettes that are

similar to GenBank sequences are of mostly unknown function, whereas three sequences were similar to proteins with identified function. Two cassettes (Initial Microcosm_02 and SRS Sed_01) had low level similarity at the amino acid level to gene cassettes previously recovered, indicating that cassettes themselves evolve. The only cassette recovered from IntI1-positive isolates were a *dfr* from *E. coli* March SYC Sed 3-5 encoding resistance to trimethoprim, and a gene cassette with no database match from both of the microcosm *Delftia* sp. isolates (same cassette from each isolate). The finding that the majority of gene cassettes are novel is consistent with previous surveys of environmental gene cassettes supporting the hypothesis that this pool is a diverse and distinct genetic resource from that found statically in bacterial chromosomes.

Summary

Integrons are abundant in bacterial communities regardless of whether industrial contamination is present, demonstrating that these MGEs play a substantial role in the acquisition of new genetic material in environmental bacteria. That they were more abundant in sites with contamination across a variety of habitats and ecosystems suggests that bacteria at these sites have a higher potential for gene exchange, whether it is for the acquisition of antibiotic resistance gene cassettes or otherwise. Whether this finding is generalizable to other MGEs besides class 1 integrons remains to be tested. The gene cassette pool is a diverse resource available for bacterial acquisition via horizontal gene transfer, whereas the composition of the pool reflects selective pressures at each site.

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Table 1. Primers used in this study, annealing temperature used in conventional (*attC* and 16S rDNA tRFLP) and quantitative (*IntI1* and 16S rDNA qPCR) PCR reactions, and expected amplicon size.

Target	Primer	Sequence (5'→ 3')	Amplicon size (bp)	Annealing Temperature (°C)	Reference
IntI1	qIntI1F	ACCAACCGAACAGGCTTATG	~286	62	Nemergut
	qIntI1R	GAGGATGCGAACCACTTCCAT			
attC	HS286	(6-FAM)-ATCCTCSGCTKGARCGAMTTGTTAGVC	Variable	55	(41)
	HS287	GGGATCCGCSGCTKANCTCVRRCGTTAGSC			
16s rDNA qPCR	Eub338	ACTCCTACGGGAGGCAGCAG	~200	63	(16)
	Eub518	ATTACCGCGGCTGCTGG			
16s rDNA tRFLP	E334F	CCAGACTCCTACGGGAGGCAGC	~605	55	(2)
	E939R	CTTGTGCGGGCCCCCGTCAATTC			

Table 2. Gene cassette richness (Sobs), diversity (Shannon), gene cassette and tRFLP similarity (abundance-based estimate of Jaccard similarity) both within a site and between a site for sediment samples as calculated in EstimateS (9). Values in parentheses represent 1 std. dev. (n = 3). n/a: Not applicable because there is only one initial sample.

Sample	**Cassette Richness**	**Cassette Diversity**	**Cassette Similarity within group**	**Cassette Similarity among groups**	**tRFLP Similarity within group**	**tRFLP Similarity among group**
Meyers	202 (4.2)	4.0	0.77 (0.05)	0.30 (0.02)	0.25 (0.04)	0.28 (0.08)
Site D	259 (5.9)	4.9	0.48 (0.12)	0.31 (0.03)	0.29 (0.01)	0.25 (0.07)
Site A	268 (5.5)	4.6	0.77 (0.02)	0.36 (0.02)	0.27 (0.04)	0.26 (0.09)
ACE	255 (6.6)	4.2	0.91 (0.03)	0.66 (0.05)	0.88 (0.03)	0.67 (0.17)
LCP	255 (6.8)	4.5	0.86 (0.04)	0.69 (0.06)	0.75 (0.07)	0.71 (0.17)
SYC	234 (5.0)	4.3	0.90 (0.01)	0.59 (0.07)	0.63 (0.30)	0.60 (0.19)
Initial	21 (n/a)	2.2	n/a	0.052 (0.02)	n/a	0.48 (0.16)
Control	30 (5.2)	3.1	0.07 (0.04)	0.027 (0.01)	0.48 (0.15)	0.45 (0.13)
Cadmium	91 (8.3)	4.1	0.04 (0.04)	0.041 (0.01)	0.77 (0.09)	0.52 (0.13)
Tetracycline	95 (7.6)	3.6	0.11 (0.09)	0.025 (0.01)	0.543 (0.31)	0.53 (0.14)

Table 3. The nine gene cassettes out of 46 sequenced gene cassettes with GenBank matches based on amino acid similarity (BLASTX).

Cassette Name	Cassette Size (bp)	E-Value	Predicted Protein	Accession Number	Stop Codon in Middle	No Inframe Stop Codon	Organism and Accession Number
Initial Microcosm_01	359	5.00E-22	panthothenate synthetase		Yes		Acidothermus cellulolyticus 11B (YP_871971)
Initial Microcosm_02	494	1.00E-10	gene cassette ORF-SIK7-1341		Yes		Vibrio cholerae (AAL68646)
Initial Microcosm_03	533	3.00E-35	GNAT family N-acetyltransferase				Pseudoalteromonas tunicata D2 (ZP_01131714)
Initial Microcosm_04	555	1.00E-30	hypothetical protein				Xylella fastidiosa Temecula 1 (NP_779100)
Initial Microcosm_05	596	2.00E-52	hypothetical protein				Saccharophagus degradans str 2-40 (YP_527802)
Initial Microcosm_06	621	5.00E-35	hypothetical protein		Yes		Delftia acidovorans (ZP_01577442)
Control Microcosm_01	620	7.00E-20	hypothetical protein				Hahella chejuensis KCTC 2396 (YP_436158)
Cadmium Microcosm_01	401	1.00E-24	Mg-chelatase ATPase subunit D		Yes	Yes	Rhodopseudomonas palustris (YP_487620)
SYC Sed_01	448	8.00E-16	gene cassette			Yes	Environment (AAL24528)

Table 4. Bacterial genomes containing sequences similar to *IntI1* (accession number) from Microbial Genome Resources (date of BLASTN analysis: March 27, 2007).

Organism	Accession Number	Score	E-value	Location
Vibrio cholerae B33	NZ_AAWE01000022.1	523	2e-146	WGS
Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67	NC_006856.1	523	2e-146	Plasmid
Yersinia pestis biovar Orientalis str. IP275 Ypesb_01_105	NZ_AAOS01000105.1	518	1e-144	WGS
Escherichia coli B171 EcolB_01_89	NZ_AAJX01000089.1	518	1e-144	WGS
Escherichia coli B7A EcolB7_01_12	NZ_AAJT01000012.1	518	1e-144	WGS
Corynebacterium diphtheriae NCTC 13129	NC_002935.2	518	1e-144	chromosome
Salmonella enterica subsp. enterica serovar Typhi str. CT18	NC_003384.1	518	1e-144	Plasmid
Pseudomonas aeruginosa PA7 PaerP_01_16	NZ_AAQE01000016.1	440	2e-121	WGS

Table 5. Antibiotic resistances of *IntI1*-positive *E. coli* from estuarine sites and microcosm isolates (SRMC) as assessed with commercial dehydrated 96-well MicroScan® panels (Dade Behring, Sacramento, USA) according to the manufacturer's instructions. Conventional class 1 integron structure assessed using primers detecting whether class 1 integrase gene has the transposase sequences. Adapted from Stepanauskas et al. (39), Baker-Austin, unpublished data.

Taxa	Isolate and Source	Conventional Class 1 integrons	Number of Resistances	Drug Resistances
E. coli	June SYC Flood 3-4	Yes	5	E, Otet, St, Sz, Te
E. coli	Oct ACE Flood 1-9	Yes	6	E, P, Otet, St, Sz, Te
E. coli	Oct LCP Flood 2-8	Yes	6	E, P, Am, Amx, Cf, T
E. coli	Oct ACE Flood 2-7	Yes	8	E, P, Azi, C, Otet, St, Sz, Te
E. coli	Oct ACE Flood 2-9	Yes	8	E, P, Azi, C, Otet, St, Sz, Te
E. coli	Oct SYC Sed 4-5	Yes	8	E, P, Am, Amx, Azi, Otet, T, Te
E. coli	March SYC Ebb 3-8	Yes	9	E, P, Azi, Fd, Gm, Otet, St, Az, Te
E. coli	March SYC Flood 1-8	No	11	E, P, Am, Amx, Azi, Otet, St, Sz, T, T/S, Te
E. coli	March SYC Sed 3-5	Yes	16	P, Am, Amx, C, Cex, Cf, Cp, Mox, NA, Ofl, Otet, St, Sz, T, T/S, Te
Delftia sp. (DQ104942)	SRMC Tet 19-4	No	18	E, P, Ak, Am, Amx, Apr, Azi, C, Cax, Cex, Cf, Fd, Gm, Mer, Otet, St, T, Te
Delftia sp. (DQ104942)	SRMC Tet 19-1	No	19	E, P, Ak, Am, Amx, Apr, Azi, C, Cax, Cex, Cf, Fd, Gm, Mox, Ofl, Otet, St, T, Te

[a]Abbreviations used: Ak, amikacin; Am, ampicillin; Amx, amoxicillin; Apr, ampramycin; Azi, azithromycin; C, chloramphenicol; Cax, ceftriaxone; Cex, cephalexin; Cf, cephalothin; Cp, ciprofloxacin; E, erythromycin, Fd, nitrofurantoin; Gm, gentamicin; Mer, meropenem; Mox, moxifloxacin; NA: nalidixic acid; Ofl, ofloxacin; Otet, oxytetracycline; P, penicillin; St, streptomycin; Sz, Sulfathiazole; T, trimethoprim; TE, tetracycline; T/S, trimethoprim/ sulfamethoxazole;

Figure 1. Structure and orientation of integrons. The integrase enzyme, *IntI*, catalyzes the site-specific recombination of the gene cassettes *attC* site at the *attI* location downstream of the promoter, *Pc*.

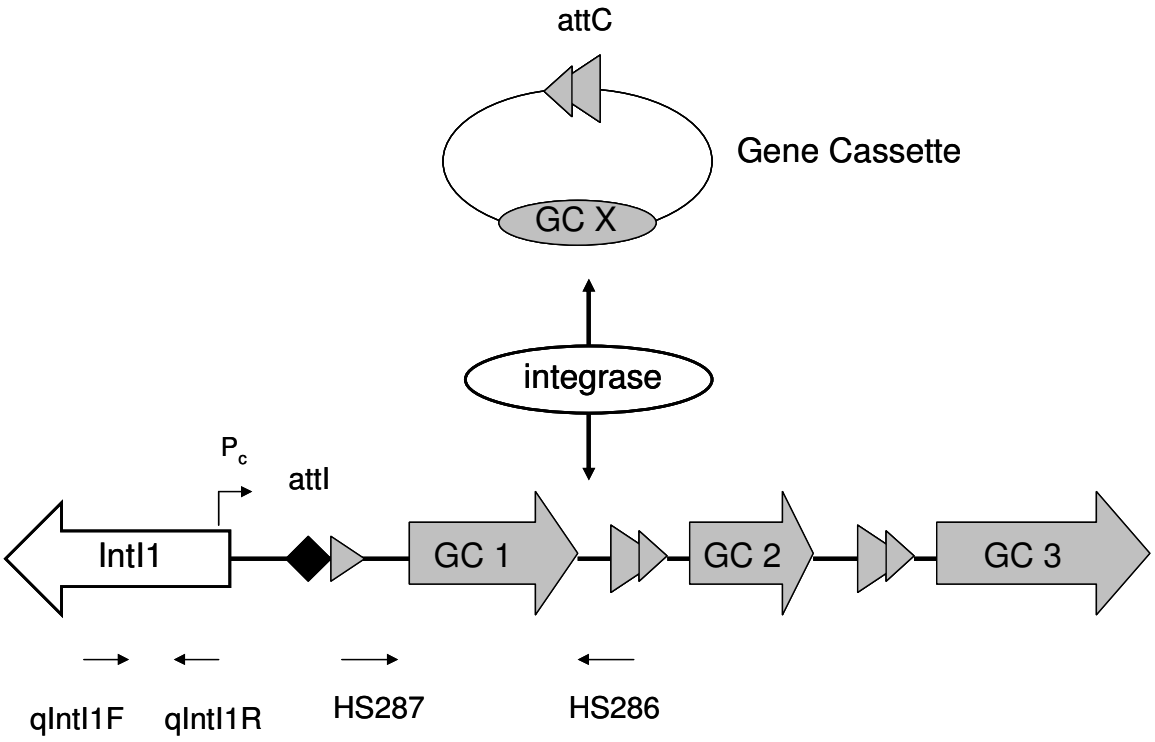


Figure 2. 16S rDNA copy number for various sites and microhabitats as assessed by quantitative PCR. A) Savannah River Site microhabitats; B) Estuarine sites; C) experimental microcosms. Error bars are 1 std err (n = 9).

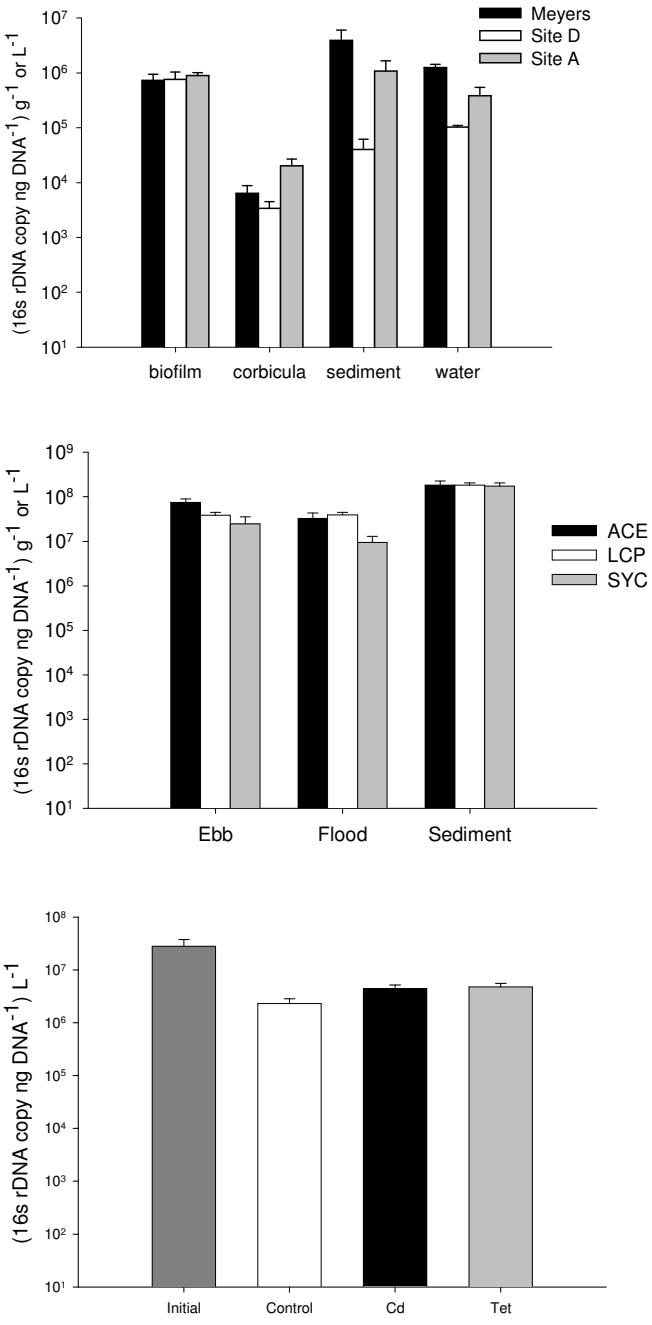


Figure 3. *IntI1*:16S rDNA copy number for A) Savannah River Site microhabitats; B) Estuarine sites; C) experimental microcosms. Means and significant differences are from *a priori* LSMEANS comparisons in ANOVAs for each sample set, with site as the main effect and α=0.05.

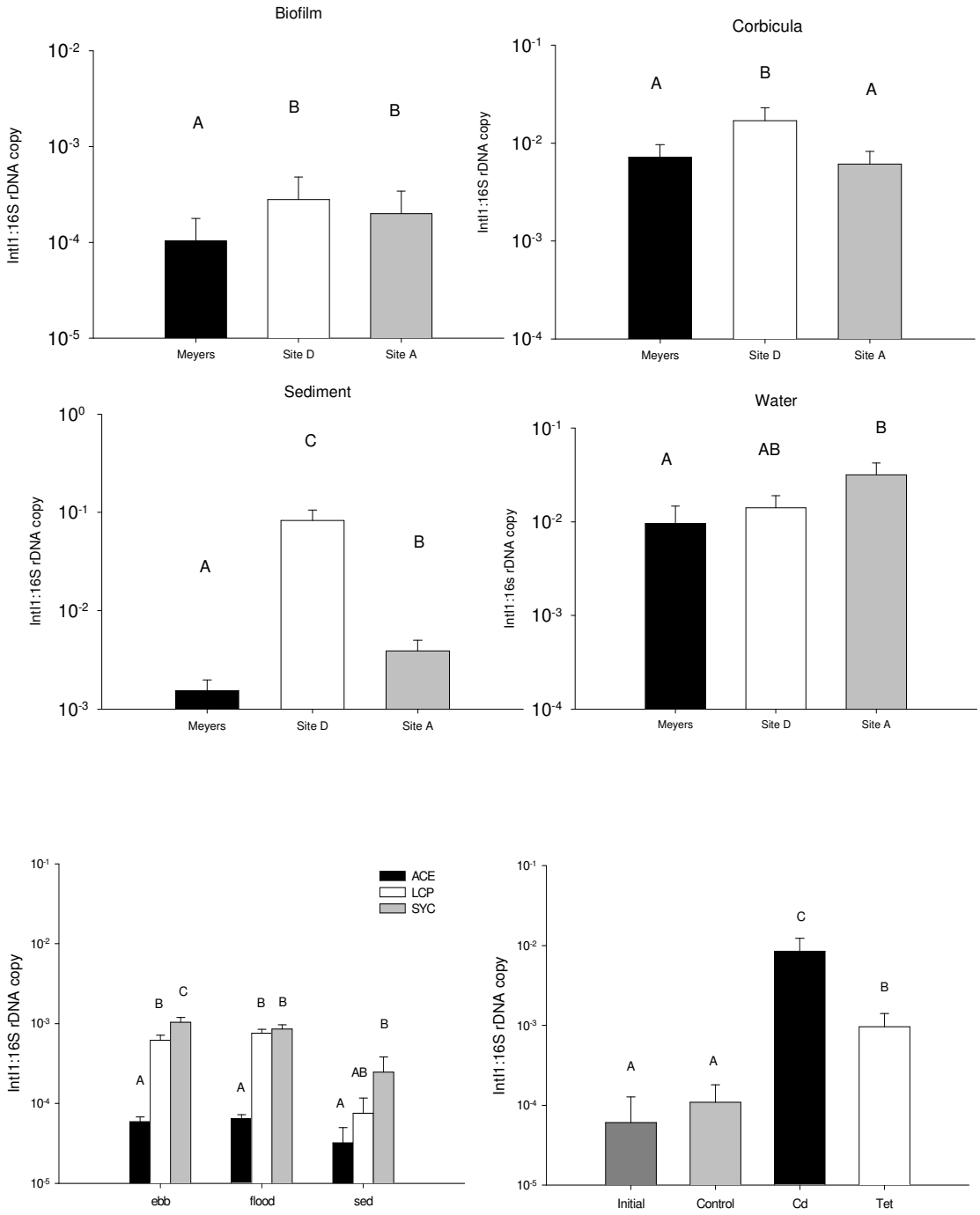
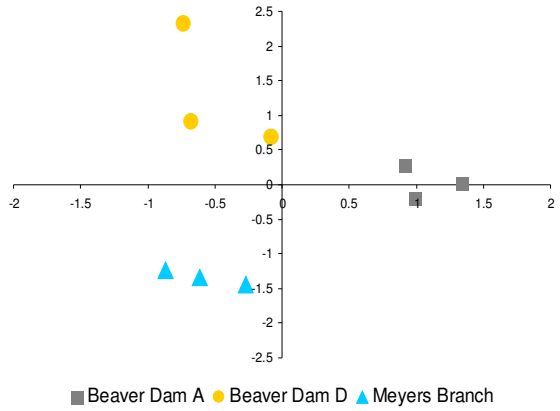
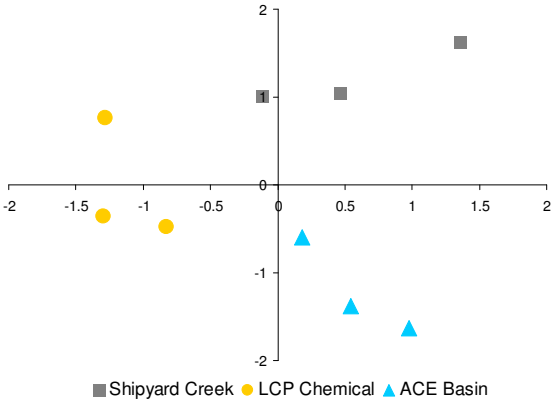


Figure 4. Multidimensional scaling plot of sediment gene cassette pool sampled by amplifying gene cassettes with HS286 and HS287 primers (Figure 1) based on a distance matrix calculated from abundance-based Chao estimators of Jaccard similarity (see Table 2 for similarity values).





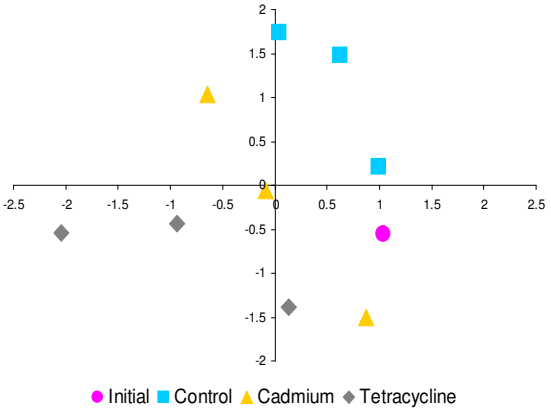
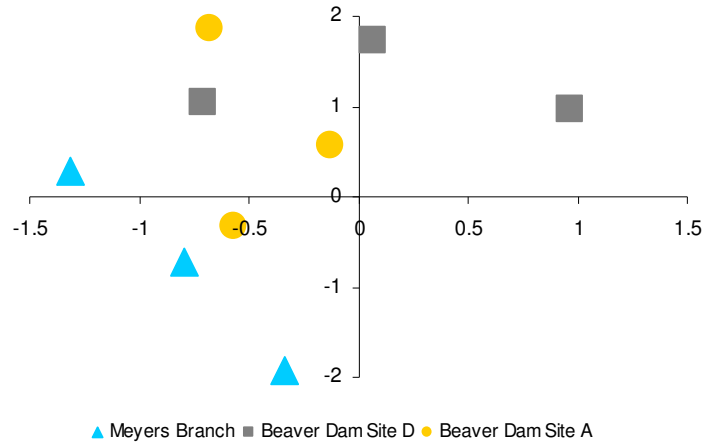
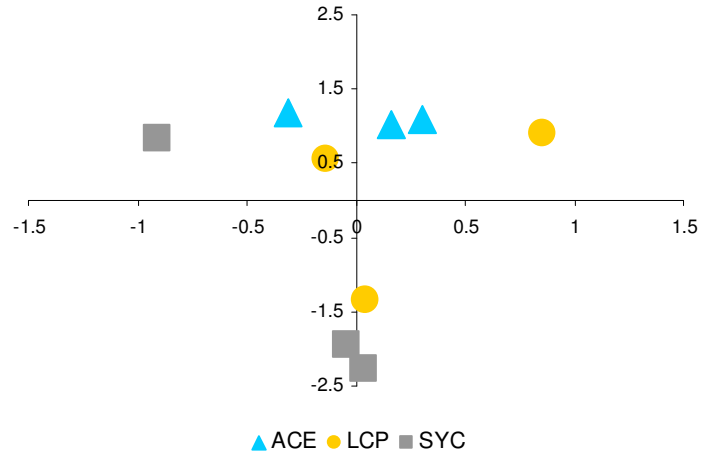
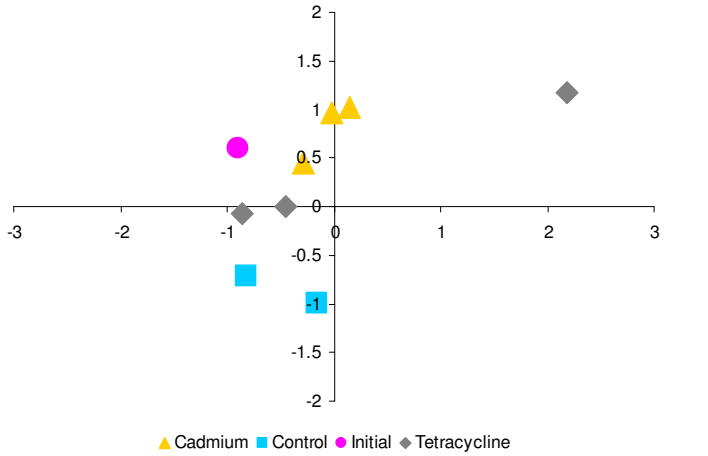


Figure 5. Multidimensional scaling plots of bacterial community structure as assessed by 16S rDNA tRFLP analysis, using abundance-based Chao estimators of Jaccard similarity.







CHAPTER 5

COMPLETE NUCLEOTIDE SEQUENCE OF A MULTIDRUG RESISTANT PLASMID ISOLATED FROM AN ENVIRONMENTALLY DERIVED STRAIN OF *ESCHERICHIA COLI* SECEC SMS3-5 REVEALS SHARED ANTIBIOTIC RESISTANCE REGIONS WITH CLINICAL BACTERIA[1]

[1]Wright, M.S., W.F. Fricke, C. Baker-Austin, J. Ravel, and R. Stepanauskas. To be submitted as part of a manuscript to *Journal of Bacteriology*.

Abstract

The complete nucleotide sequence of a pSMS3-5 from *Escherichia coli* SECEC SMS3-5 was determined to assess potential gene flow between clinical and environmental settings in this human and zoonotic pathogen. *E. coli* SECEC SMS3-5 was isolated from an industrially contaminated estuary off the coast of South Carolina, USA, and was demonstrated to be highly resistant to several classes of antibiotics. We assessed whether gene content on pSMS3-5 was similar to those in clinical bacteria and what mobile genetic elements may be involved in conferring multidrug resistance to *E. coli* SECEC SMS3-5. Additionally to examine whether the co-occurrence of antibiotic and metal resistance genes on pSMS3-5 is a potential mechanism for the co-selection of antibiotic resistance in metal contaminated systems, we assessed the metal resistance content of this plasmid. The 129,000 bp IncFIB plasmid contained 199 predicted genes that could be delineated into four distinct plasmid domains: 1) a conserved backbone region encoding for plasmid function genes; 2) a pathogenicity region containing genes involved in *E. coli* virulence; 3) an antibiotic resistance region; and 4) a region of unknown function. Overall, pSMS3-5 was most similar to pAPEC-01-ColBM, a plasmid from an avian pathogenic *E. coli*, and to p1658/97 from an enteric pathogenic *E. coli*, sharing a large syntetic 67.5 kb region with both. pSMS3-5 contained genes associated with virulence including colicin M production, hemolysin F, *vagC* and *vagD*, and the iron acquisition operon sitABCD. Antibiotic resistance genes included several resistance genes commonly observed on other *Enterobacteriales* mobile genetic elements including *tetA*, *cat2*, *aph*, *sul2*, and *aadA2*, *aadA4*, and *sat.* These were located in a region with numerous insertion sequences, transposons, integrons, and a putative *IS*CR. Surprisingly, no known metal resistance genes were detected on pSMS3-5, though a region of unknown function contained genes with conserved domains

potentially involved in metal homeostasis. The plasmid sequence indicates that gene flow between clinical and environmental settings via horizontal gene transfer has contributed to the global dissemination of resistance genes and the spread of multidrug resistant bacteria. This reservoir of resistance genes in environmental bacteria has serious implications for efforts to minimize the spread of antibiotic resistance genes in pathogenic bacteria, particularly for those with populations that persist in the environment.

Introduction:

The acquisition of large conjugative plasmids can rapidly alter the genetic composition of bacteria which can manifest phenotypically as increased catabolic or pathogenic capabilities or toxicant resistance (12). That plasmids contribute to the dissemination of antibiotic resistance and pathogenicity genes has resulted in substantial research efforts to understand the mechanisms governing gene flow between individuals. From these efforts, it is evident that plasmids are themselves mosaics of genes acquired through the insertion of mobile genetic elements (MGEs) including insertion sequences, integrons, transposons, and insertion sequence common regions (*IS*CRs) (33). This combinatorial process of accruing genes from varying sources contributes to the genetic diversity within a bacterial population, and the accessory genes encoded within these MGEs contribute to bacterial adaptation.

Evolutionary theory maintains that those mobile genetic elements (MGEs) that confer a selective advantage to the bacterial host will become more prevalent in bacterial assemblages. Thus antibiotic resistance genes accumulate in bacterial assemblages in response to antibiotic exposure as demonstrated by the rapid appearance of resistance genes with the advent of antibiotic usage (9, 14), and in environments contaminated with toxicants and xenobiotics, genes involved in tolerance to or the metabolism of these compounds accumulate (e.g, (21, 32).

The rate and extent to which genes located on MGEs disseminate across physical boundaries and among bacterial assemblages is likely to be a function of both MGE mobility and stability, and the selective advantage conferred to the host upon receipt of the element (evolutionary stable strategy). Horizontal gene transfer via conjugation can occur across phylogenetic boundaries, although the process occurs more readily between closely related individuals (18), and the biogeography of antibiotic resistance elements indicate that genes can

be transferred across wide geographical areas (20). For example, current surveillance projects monitoring drug resistance in clinical settings indicate that the rapid spread of resistance to extended spectrum beta lactamases (ESBL) is related to the location of $bla_{CTX\text{-}M}$ genes on *IS*CRs that can efficiently mobilize genes to a phylogenetically broad group of bacteria via a range of plasmid incompatibility groups across geographically distant areas (4, 31) and is likely related to an increased usage of cephalosporins.

To what extent MGE genes can cross boundaries such as those between clinical and environmental settings and what role environmental reservoirs of resistance genes play in the dissemination of antibiotic resistance in clinical settings remains poorly understood. That many genes conferring resistance to antibiotics detected in clinical settings have their origin in environmental bacteria suggests that gene flow is bidirectional (20). Bacterial taxa inhabiting both habitats such as the γ-proteobacterial pathogens *Escherichia coli*, *Salmonella enterica* typhimurium, and *Vibrio cholerae* thus potentially play a substantial role in this gene exchange. Furthermore, how MGEs persist in the environment when not under direct selective pressure is unclear and is as likely to depend on host characteristics as it is on plasmid characteristics (3, 10, 11, 13, 22). Additionally, other selective pressures like metal exposure may maintain a pool of antibiotic resistance genes due to the physical linkage of metal and antibiotic resistance traits on the same MGE and other co-selection mechanisms (2). However, evolutionary theory predicts that those genes not under direct selective pressure will accumulate mutations and eventually be lost from the gene pool. What time scale this process occurs at is not well understood, particularly if there is a continual source of antibiotic resistance genes entering the system.

To better understand the interplay between clinical and environmental populations in evolutionary processes and the maintenance of antibiotic resistance, we compared the gene

content and organization of a plasmid isolated from an environmental *E. coli* strain to previously characterized genetic elements. Because *E. coli* are habitat generalists in that they are ubiquitous commensals in avian and mammalian hosts, and can persist in aquatic and terrestrial habitats, it is critical to explore the genetic content of plasmids recovered from environmental strains of *E. coli* and their role in the maintenance and transmission of resistance genes. Additionally, this *E. coli* strain was isolated from an industrially contaminated estuary off the southeastern coast of the US as part of a larger project testing the hypothesis that antibiotic resistance traits are co-selected for in bacteria exposed to metal stress. *E. coli* SECEC SMS3-5 was thus also chosen for whole genome sequencing to elucidate whether the co-occurrence of antibiotic and metal resistance elements on the same MGE is one mechanism for the apparent co-selection of these elements, and whether metal exposure is one selective pressure favoring the maintenance of antibiotic resistance genes in the environment.

Methods:

Isolation and strain characterization

E. coli SECEC SMS-3-5, identified as serotype O19:H34, was isolated from Shipyard Creek, an industrially contaminated estuary near Charleston, SC, USA in March 2005 as part of a larger study investigating the co-selection of antibiotic resistance in metal-exposed bacterial communities. Shipyard Creek is contaminated with a variety of metals associated with industrial activities including cadmium and chromium, but lacks any proximal point source of sewage or agricultural discharges. *E. coli* SECEC SMS-3-5 was chosen for genomic sequencing because of its resistance to high concentrations of 16 of 26 tested antibiotics including aminoglycosides, penicillins, cephalosporins, fluoroquinolones, macrolides, phenicols, sulphonamides, and

tetracyclines as assessed with commercial dehydrated 96-well MicroScan® panels (Dade Behring, Sacramento, USA) (Table 1).

Genome sequencing and annotation

Plasmid assembly was conducted from a whole-genome shotgun sequencing project of *E. coli* SECEC SMS-3-5. Multiple sizes of insert libraries were sequenced on a 3730xl DNA analyzer (Applied Biosystems) including: 1) a 3- to 4-kb insert size; 2) an 8- to 10-kb insert size; and 3) a 30- to 40-kb insert size fosmid library, and assembled at the Institute for Genomic Research's Joint Technology Center. Plasmid closure was conducted as described in (23), auto annotated using Glimmer Gene Finder in MANATEE (www.tigr.org), and manually annotated. Careful manual annotation is particularly necessary in horizontally transferred regions due to variations in codon usage that algorithms using Hidden Markov Models fail to detect (12).

Comparative analyses

To identify plasmid regions homologous to previously characterized mobile genetic elements and those unique to pSMS3-5, we initially conducted BLASTP searches for each predicted coding sequence (CDS) using all available GenBank sequences. From this analysis we identified plasmids that shared high homology with large fractions of the plasmid genome and also included pMOL30 from *Ralstonia metalluridans* as an example of a plasmid obtained from a metal-contaminated site (Table 2). MEGABLAST analyses were also used to detect regions of

nucleotide similarity among the examined plasmids. We then assessed gene synteny using WebACT, an Internet-based Artemis Comparison Tool on selected regions to these plasmids (1).

Results and Discussion:

General plasmid features

pSMS3-5 is a 129,303 bp RepFIB plasmid encoding for 198 predicted proteins of which 29 were hypothetical proteins with no similar sequences in available databases. The backbone region of the plasmid encodes for core plasmid transfer and maintenance genes characteristic of F plasmids including the full suite of *tra* and *trb* genes (Table 3). Plasmid maintenance genes include plasmid stabilization and segregation proteins (*sopA* and *sopB*), plasmid SOS inhibition proteins (*psiA* and *psiB*), plasmid addiction toxin and antitoxin proteins (*pemI* and *pemK*), and the *sok* component of the *hok/sok* postsegregational killing plasmid maintenance system. Though no predicted *sok* gene was identified, just downstream of *sok* is a conserved hypothetical protein that has the conserved domain of a parB-like nuclease that may have a similar function as *hok*. pSMS3-5 additionally has components of RepFIIA replication (repA1 and repA2). The presence of these plasmid transfer and maintenance genes suggests that pSMS3-5 is able to self-transfer and is stably maintained in recipients, but needs to be verified experimentally.

Gene content and order as assessed by megablast scores and Artemis Comparison Tool, indicates that pSMS3-5 is most similar in the replication and transfer regions to pAPEC-01-ColBM (15), an avian pathogenic *E. coli* recovered from the lung tissue of an infected chicken, and to p1658/97 recovered from a pathogenic *E. coli* isolated during a clonal outbreak at a Polish hospital (34) (Table 2). pSMS3-5 shares approximately 45% synteny with pAPEC-01-ColBM in the RepFIIA and conjugative transfer region,. Both pAPEC-01-ColBM and p1658/97 were able

to transfer to recipient strains providing additional evidence that pSMS3-5 is capable of transfer as well. pSMS3-5 is also similar to pRSB107, a F-type plasmid recovered from wastewater treatment plant in Germany, and the prototypical plasmids F and R100 (Table 2).

Gene content in pSMS3-5 and pAPEC-01-ColBM and p1658/97 is similar from repA1 (ORF 39) to a break point at OmpT (ORF 151), although within these sequences is a region encoding for sitABCD located in both pSMS3-5 and pAPEC-01-ColBM that is lacking in p1658/97. pAPEC-01-ColBM has additional genes associated with virulence in this region that are not present in pSMS3-5 or pAPEC-01-ColBM. The common region shared between pSMS3-5 and p1658/97 extends upstream of this region to include additional genes associated with plasmid maintenance (*tir*, *pemI*, and *pemK*). Outside of these regions, pSMS3-5 contains genes more similar to *Salmonella enterica* plasmids (see below). Gene content in the backbone area thus suggests that homologous F-plasmids are able to transfer to multiple populations of *E. coli* including avian pathogens, enterotoxigenic pathogens, and an environmentally-derived strain.

Pathogenicity region

Multiple genes involved in *E. coli* virulence are found in pSMS3-5 in a region homologous to that of pAPEC-01-ColBM. This region contains the *sitABCD* operon encoding for chelated iron uptake mechanisms flanked by IS1 sequences as in pAPEC-01-ColBM, and is also homologous to a region on pUTI89 from an uropathogenic *E. coli* (5). Iron scavenging mechanisms are often associated with pathogenic bacterial strains due to iron sequestration responses by hosts (24), but also may be beneficial to bacteria living in low iron environments as well. Other genes associated with virulence include *vagC* and *vagD* that are thought to be involved in pathogenicity, but may also be involved in plasmid stability, and *hlyF*, a gene

encoding for a hemolysin protein. Additionally, the *cba* and *cma* activity genes which encode for the bacterial toxins colicin B and colicin M respectively were present in this region. While *cba* and the gene coding for immunity to it, *cbi*, are found on multiple plasmids, *cma* and *cmi*, its immunity gene, were only found on pAPEC-01-ColBM. However, in pSMS3-5, this region contains transposases associated with IS2 inserted between a truncated *cbi* and a complete *cbi* gene not seen in pAPEC-01-ColBM. The *cba* gene in both plasmids is truncated and a resolvase with 100% nucleotide identity precedes the *cma* genes in both plasmids. Colicins are secondarily related to *E. coli* virulence in animal hosts, as the primary function of colicin production is as a bacteriocide to lyse other bacteria. pSMS3-5 differs from pAPEC-01-ColBM in that pAPEC-01-ColBM has multiple inserts in this region encoding for several other pathogenicity genes homologous to other APEC plasmids involved in iron uptake. These pathogenicity genes are completely lacking in pSMS3-5 and also in p1658/97 suggesting that these genes were acquired by pCOL-01-BM after the divergence of the three plasmids.

Antibiotic Resistance Features

Genes encoding for resistance to chloramphenicol (*cat2*), sulfonamides (*sul2*), aminoglycosides (*sat*, *strA*, *strB*, *aadA2*, *aadA4*, *aph*), tetracyclines (*tetA*), and folate inhibitors (*dhfr*) are located in a region containing numerous insertion sequences, transposons, and integrons (Table 3). Phenotypically, *E. coli* SMS3-5 was resistant to the drugs to which these genes confer resistance including chloramphenicol, sulfathiazole, kanamycin, streptomycin, tetracycline and oxytetracyline, and trimethoprim. The antibiotic resistance region diverges from pAPEC-01-ColBM and p1658/97 in that pAPEC-01-ColBM did not possess any known antibiotic resistance genes, whereas p1658/97 had fewer resistance genes, and gene content and

order differed. p1658/97 contained a copy of bla_{SHV-5} encoding for an extended spectrum β-lactamase that was not present in pSMS3-5, yet p1658/97 lacked copies of *strA*, *strB*, *tetA*, *cat2*, and *aph*. Also, class 1 integron gene cassette composition varied between the two as p1658/97 contained *aacA4*, *aacC1*, *orfX*, *aadA1*, *qacΔ1*, and *su1l*. The only gene cassette within the class 1 integron in pSMS3-5 was *dfr* encoding for trimethoprim resistance.

The suite of antibiotic resistance genes detected on pSMS3-5 occur on several multidrug resistance plasmids of varying incompatability groups, but the particular order and combination seen in pSMS3-5 has not been described, reflecting the mosaic nature of horizontally transferred genes associated with multiple transfer and recombination events. Overall antibiotic resistance gene content is similar to that observed on pSC138 from the zoonotic pathogen *Salmonella enterica* serovar Choleraesuis pSC138 isolated in China (7)and *Klebsiella pneumonia* pK245 from Taiwan (6).

Tn1721 and Tn1721-like transposons in which *tetA* and its repressor *tetR* are located are common components of plasmids. In pSMS3-5, a relaxase gene, the regulatory protein *pecM*, and a conserved hypothetical protein are located within this transposon as well. This gene array is seen on pSC138, pAPEC-02-R from another avian pathogenic *E. coli* (16), the IncP-1β plasmids pB10 and pTB11 from a wastewater treatment plant in Germany (25, 30), pRAS1 from the fish pathogen *Aeromonas salmonicida* (28), and *Bordetella bronchiseptica* pKBB4037 (17). Thus the diversity of organisms and MGEs, and geographic extent of *tetA* and *tetR* as associated with Tn1721 indicates that this is an evolutionary successful combination of a MGE with a set of genes potentially conferring a selective advantage to carriers of it. Additionally, that *tetA* is abundant in several environmental systems regardless of the extent of antibiotic exposure (Wright et al.), but is more abundant in metal-exposed bacterial communities, suggests this

resistance gene can persist in the environment and is subject to co-selection pressures. Whether this is due to its dissemination by Tn1721 is yet to be investigated.

Another gene combination in pSMS3-5 that has been previously detected is that of *sul2, strA, strB* which is identical to a sequence recovered from pK245 in a clinical strain of *Klebsiella pneumonia* in Taiwan, and according to a BLASTN search, has been described in 21 other plasmids and chromosomal antibiotic resistance islands in enteric bacteria and *V. cholerae* with either 99 or 100% nucleotide identity over the length of the coding sequences (date of analysis: April 14, 2007). The prevalence and geographic distribution of this operon-like structure indicates that these elements can be transferred as a unit within clinical strains of bacteria and persist in the environment. It is unclear when this combination originated and whether it developed in multiple lineages. Additionally the location of a putative *repC* fragment adjacent to a transposase fragment upstream of *sul2* was detected in both pSMS3-5 and pK245. A similar pattern was found in the multidrug resistance island on the chromosome of *Salmonella enterica* serovar Typhimurium isolate DT193 from Ireland with an intact *repC* gene (8). This pattern was first identified on the IncQ broad host range plasmid RSF1010 from *E. coli* (26).

Recent studies have implicated a MGE with an IS91-like transposase termed IS common regions (*IS*CRs) capable of mobilizing adjacent genes through rolling-circle replication as a possible mechanism for the widespread dispersal of antibiotic resistance elements (31). The association of *IS*CRs with class 1 integrons is believed to be in part responsible for the role of *IS*CRs in antibiotic resistance gene dispersal. There were no apparent IS-91 like sequences in this region, though as detailed in Toleman et al. (31), these sequences can be truncated or deleted resulting in a cryptic *IS*CR. Toleman et al. (31) identified a cryptic *IS*CR of type 2 in RSF1010 downstream of *sul2* in the region homologous to pSMS3-5. This suggests that an ISCR may

have also contributed to the transfer of antibiotic resistance genes into pSMS3-5 such as the *sul2, strA, strB* combintation. Supporting this claim is the fact that antibiotic resistance genes in *IS*CRs typically have no apparent flanking 59-be sequences associated with the *attC* site of typical gene cassettes (31), and previous attempts to recover gene cassettes from pSMS3-5 using primers targeting the 59-be sequence only recovered the *dfr* gene cassette immediately downstream of *IntI1* (unpublished data).

Class 1 integrons are well known for their role in the acquisition of antibiotic resistance genes in clinical bacteria. Gene cassettes conferring resistance to antibiotics typically belong to a few resistance gene families including *dfr*, *aad*, *aac*, *qnr*, *sul*, and *qac*. In pSMS3-5, only *dfr* is associated with a class 1 integron, although *sat, aadA2* and *aadA4* were also found in the antibiotic resistance region. Class 1 integrons are found in both Gram negative and Gram positive bacteria and are associated with multiple plasmid types (20). Some class 1 integrons are flanked by transposition sequences that increase their mobility indicating that they can play a major role in the transmission of genes associated with them.

Insertion sequences can play a major role in shaping the gene content and organization of bacterial genomes (27) as evidenced in pSMS3-5. IS26 is prevalent throughout the antibiotic resistance region. BLASTP analysis in available GenBank sequences indicate that this MGE is present in multiple copies in several genomes primarily in γ-proteobacteria, but similar sequences are seen in *Corynebacterium* sp. (Actinobacteria), *Solibacter* sp. (Acidobacteria), and *Bacillus* sp. (Bacillus), with expect values ranging from $1e^{-84}$ to $9e^{-52}$, respectively for a sequence of 240 amino acids. IS26 is able to form compound transposons, as evidenced by both *cat2* and *aph* being bounded by two copies of IS26 in the same orientation, which indicates that these resistance genes can be mobilized by transposition. Both *cat2* and *aph* are found in this

configuration on pSC138 and in pK245. The combination of IS26 copies flanking the *aph* gene encoding for kanamycin resistance is encountered less frequently when compared to the other resistance genes on pSMS3-5, but has been found on the F-plasmid pRSB107 recovered from a wastewater treatment plant in Germany, on a plasmid in the α-proteobacteria *Oceanicaulis alexandrii* isolated from the Sargasso Sea, in another *K. pneumoniae* plasmid, pRMH760, from Australia, and in the actinomycete opportunistic pathogen, *Corynbacterium stiatum* M828 pTP10. These examples of the distribution of antibiotic resistance genes present on pSMS3-5 illustrate the extent to of antibiotic resistance gene flow between disparate geographic settings and across phylogenetic barriers.

The majority of antibiotic resistance genes that are present in pSMS3-5 encode for resistance to drugs in use for several decades and that are natural compounds produced by actinomycete bacteria, primarily by *Streptomycetes* (except for the synthetic sulfonamides and folate inhibitors). For example, streptomycin and sulfonamides were released in the 1940s, followed by tetracycline in 1955, and trimethoprim (folate inhibitor) in1961. Of the six aminoglycoside resistance genes on pSMS3-5, five relate to streptomycin or spectinomycin resistance, which is reflected in the antibiotic resistance profile, as *E. coli* SMS3-5 was not resistant to other tested aminoglycosides including amikacin, apramycin, and gentamicin.

The absence of certain classes of antibiotic resistance genes can shed light on how antibiotic resistance genes flow between environmental and clinical settings. In comparison to similar multidrug resistance plasmids from clinical enterobacteria such as p1658/97, there are notable genes lacking from pSMS3-5. One such class of genes is the group of genes encoding resistance to extended spectrum β-lactams (ESBLs) including bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$. The first detection of a plasmid-borne bla_{SHV} occurred in 1983, and the prevalence of these genes has

increased dramatically in subsequent years due to their presence of broad host range MGEs and are often associated with other antibiotic resistance drugs (4). Therefore, there may not have been sufficient time for these elements to disperse into environmental populations such as *E. coli* SECEC SMS3-5. Alternatively, the absence of these genes could be because there is no selective pressure favoring the establishment of genes encoding for resistance to semi-synthetic antibiotics.

Metal Resistance Features

Of significance is the lack of any known metal resistance genes on pSMS3-5 considering this isolate was obtained from a heavily contaminated site. pSMS3-5 shares very few genes with pMOL30 from *Ralstonia metalluridans,* an isolate from a heavily contaminated soil with numerous metal resistance genes (21).Other closely related F plasmids have metal resistance operons including resistance to arsenic, silver, and chromate (16, 25, 29). For example, pSC138 has a similar antibiotic resistance gene content to pSMS3-5, but also possesses a mercury resistance operon. Mercury resistance operons are common features of antibiotic resistance plasmids due to the association between Tn21-borne mercury resistance operons and class1 integrons (19). Interestingly, a region homologous to a region from R64 plasmid and *E. coli* p300 is inserted within the antibiotic resistance domain and contains *vagC* and *vagD*, genes putatively encoding for plasmid maintenance proteins or for virulence. The region also contains two conserved hypothetical proteins, each with conserved domains structurally similar to ABC-type transporters. ORF 8 contains conserved domains associated with an ATPase involved in DNA-repair (cdd: 30768) and an ABC-transporter motif associated with metal transport (cdd: 72999). Orf212 contains a conserved domain with an ABC-type transporter associated with

siderophore uptake (cdd: 72973). Downstream of this region is a proQ homolog (Orf 014) putatively an osmoprotectant transporter, followed by ORF015 which is structurally similar to a permease. An *arsR*-type transcriptional regulator completes this region. As *arsR* is known to regulate metal responses in bacteria, and the presence of genes involved in potential DNA repair and metal transport, suggests that this region may be involved in metal homeostasis and tolerance. This needs to be verified experimentally.

Conclusions

The antibiotic resistance gene content of pSMS3-5 is similar to several previously detected MGEs demonstrating the occurrence of gene flow between clinical and environmental settings across both geographic and species boundaries. It also demonstrates the persistence of antibiotic resistance genes outside of a direct selective pressure, although these resistance determinants may be involved in resistance to antibiotics produced by other members of the bacterial community. The detection of gene combinations observed in multiple organisms indicates that these gene combinations are disseminating in tandem. The extent of this process is likely a function of MGE mobility and selective pressure favoring the maintenance of genes contained within them. How the gene pool of MGE responds to changes in antibiotic consumption over time is less apparent, and whether MGEs characteristic of clinical isolates disseminate beyond γ-proteobacterial pathogens remains less clear. Sequence analysis of plasmids and other MGEs from a broader taxonomic range from a variety of habitats will yield pertinent information regarding gene flow between populations via horizontal gene transfer.

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Table 1. Antibiotic resistance profile for E. coli SMS3-5 as assessed using Dade-Behring commercial plates. Minimum inhibitory concentration (MIC, μg/mL) is given for each antibiotic. S: sensitive.

	E	P	Ak	Am	Amx	Apr	Azi	C	Cax	Cex	Cf	Cfx	Cp	Fd	Gm	K	Imp	Mer	Mox	NA	Ofl	Otet	St	Sz	T	T/S	Te
E. coli SMS3-5	S	>128	S	>32	>32	S	S	>32	S	32	64	S	>4	S	S	>128	S	S	>4	>32	>8	>32	>128	>500	>16	>4/76	>32

[a]Abbreviations used: E, erythromycin; P, penicillin; Ak, amikacin; Am, ampicillin; Amx, amoxicillin; Apr, ampramycin; Azi, azithromycin; C, chloramphenicol; Cax, ceftriaxone; Cex, cephalexin; Cf, cephalothin; Cfx, cefoxitin;, Cp, ciprofloxacin; Fd, nitrofurantoin; Gm, gentamicin; K, kanamycin; Mer, meropenem; Mox, moxifloxacin; NA, nalidixic acid; Otet, oxytetracycline; St, streptomycin; Sz, sulfathiazole, T, trimethoprim; T/S Trimethoprim/Sulfamethoxazole; TE, tetracycline. S: sensitive.

Table 2. Plasmid sequences used for comparative analysis to determine most similar gene order and gene content. Score, query coverage, and E-value are the result of a MEGABLAST search with default parameters using pSMS3-5 as the query sequence.

Plasmid	Organism	Plasmid Size	Accession Number	Score	Query Coverage	E value
p1658/97	*Escherichia coli*	125 kb	AF550679	$1.4e^{5}$	83%	0.0
pAPEC-01-ColBM	*Escherichia coli*	101 kb	DQ381420	$1.3e^{5}$	83%	0.0
R100	Plasmid R100	94 kb	AP000342	$7.9e^{4}$	61%	0.0
pRSB107	Uncultured bacterium	121 kb	AJ851089	$7.0e^{4}$	33%	0.0
pSC138	*Salmonella enterica* serovar Choleraesius str. SC-B67	139 kb	AY509004	$5.3e^{4}$	17%	0.0
R64	*Salmonella typhimurium*	121 kb	AP005147	9675	7%	0.0
pMOL30	*Ralstonia metalluridans* CH4	234 kb	X71400	244	0%	3e-64

Table 3. Gene content of pSMS3-5. Nearest match in GenBank based on predicted coding sequence and percent nucleotide identity.

ORF	gene	Start	Stop	BLASTx Match	Accession	% identity	% similarity	alignment length	bit score
1	tnpA	836	120	IS26	NP_872582.1	100	100	238	501
3	pifA	815	1603	phage T7 exclusion protein	AAP42498.1	99.58	100	238	446
4		2552	1653	conserved hypothetical protein	NP_061418.1	100	100	299	589
5		2832	2542	YebA	NP_863396.1	100	100	96	191
6	vagC	3057	3185	VagC	YP_001008278.1	90.48	95.24	21	42.4
7		3127	3357	conserved hypothetical protein	NP_863395.1	100	100	51	105
8	vagD	3354	3770	VagD	NP_863394.1	99.28	100	138	249
9		6070	3932	conserved hypothetical protein	NP_863393.1	99.86	99.86	712	1383
10		6308	6466	hypothetical protein					
11		6424	6681	conserved hypothetical protein	NP_863392.1	100	100	85	174
12		6681	7271	conserved hypothetical protein	NP_863391.1	100	100	196	350
13		7528	9090	conserved hypothetical protein	NP_863390.1	100	100	518	982
14		9109	9243	hypothetical protein					
15		9282	9899	conserved hypothetical protein	NP_863389.1	100	100	205	400
16		11271	10192	Permease	AAP42482.1	94.69	95.23	377	640
17	arsR	11376	11699	transcriptional regulator, ArsR family	NP_862992.1	100	100	107	212
18	tnpA	12949	12233	IS26	YP_001007636.1	73.68	84.21	57	87
19	tnpA	12928	13197	transposase 1721-like, truncated	NP_872582.1	100	100	238	501
20	tnpA	13278	13859	transposase for transposon 1721	ABF71538.1	98.44	100	64	135
21		13917	14159	Relaxase	ABF71538.1	100	100	193	386
22	tetR	14868	14191	tetracycline repressor	YP_190207.1	98.75	100	80	125
23	tetA	14872	16146	tetracycline resistance	YP_025723.1	100	100	225	416
24	pecM	17062	16178	regulator protein PecM	CAF31521.1	100	100	424	756
25		17607	17200	conserved hypothetical protein	NP_858027.1	100	100	294	484
26		18660	18409	hypothetical protein					
27	tnpA	19943	19227	IS26	ABF71538.1	100	100	84	178
28	tnpA	19922	19996	IS26 truncated	NP_872582.1	100	100	238	501
29	tnpA	19996	20281	IS26 truncated	ABG56822.1	92.45	93.4	106	193
30	aph	21232	20471	aminoglycoside 3'-phosphotransferase	NP_478145.1	100	100	253	530
31	tnpA	21425	22141	IS26	NP_872582.1	100	100	238	501

32	insB	23009	22749	IS1 InsB	NP_414798.1	98.68	98.68	76	167
33	tnpA	22189	22752	Tn3-like	ABN54710.1	100	100	177	350
34	pemK	23784	23452	stable plasmid inheritance protein K	NP_862963.1	100	100	110	192
35	pemI	24043	23786	protein PemI	NP_052993.1	100	100	85	172
36	tir	24789	24136	transfer inhibition protein	NP_052992.1	100	100	217	431
37		24908	24786	conserved hypothetical protein	ABD51697.1	80	95	20	38.9
38		25027	24887	conserved hypothetical protein	NP_858394.1	89.13	95.65	46	92
39	repA1	26598	25729	IncFII RepA protein family	YP_443947.1	98.96	99.65	289	476
40		25622	25759	conserved hypothetical protein	CAD87782.1	47.22	61.11	36	33.9
41		26735	26848	hypothetical protein					
42	repA2	27139	26879	replication regulatory protein repA2	S11883	100	100	63	125
43		27845	27378	conserved hypothetical protein	NP_862957.1	100	100	147	300
44		28198	27989	conserved hypothetical protein	NP_862956.1	100	100	69	142
45	finO	28765	28244	fertility inhibition protein	YP_190110.1	95.78	97.59	166	335
46		29160	28948	conserved hypothetical protein	NP_862954.1	100	100	69	147
47	finO	29909	29298	fertility inhibition protein	NP_085417.1	96.55	97.54	203	338
48	traX	30659	29913	pilin acetylase TraX	NP_862952.1	100	100	236	447
49	traI	35949	30679	relaxase protein TraI	ZP_00719298.1	97.89	98.63	1756	3224
50	traD	38120	35949	coupling protein TraD	AAC44181.2	98.2	98.76	723	1426
51	traT	39149	38373	TraT	YP_190117.1	99.61	100	258	418
52	traS	39651	39136	TraS	YP_443959.1	100	100	167	281
53	traG	42476	39654	protein TraG	YP_190118.1	99.79	99.89	940	1761
54	traG	43849	42473	pilus assembly protein	YP_190119.1	100	100	458	766
55	trbJ	44148	43846	conjugal transfer protein	AAR00493.1	100	100	100	172
56	trbB	44683	44138	pilin assembly thiol-disulfide isomerase TrbB	YP_190121.1	100	100	181	321
57	traQ	44954	44670	pilin chaperone TraQ	YP_190122.1	100	100	94	122
58	trbA	45420	45073	TrbA	AAR00492.1	96.52	97.39	115	225
59	traF	46179	45436	pilin assembly protein TraF	NP_052969.1	99.6	100	247	403
60	trbE	46432	46172	inner membrane protein TrbE	YP_538723.1	96.51	96.51	86	164
61	traN	48264	46456	mating-pair stabilization protein TraN	ZP_00719306.1	99.5	99.83	602	1153
62	trbC	48899	48261	F pilus assembly protein TrbC	YP_538721.1	97.64	98.58	212	414
63				hypothetical protein					
64		49870	49409	conserved hypothetical protein	NP_065355.1	43.14	60.78	153	106
65	traU	50888	49896	TraU	YP_788074.1	100	100	330	670
66	traW	51517	50885	TraW	NP_052962.1	99.52	100	210	423
67	trbI	51900	51514	TrbI	NP_052961.1	100	100	128	256
68	traC	54524	51897	TraC	YP_190131.1	99.43	99.77	874	1706

69	traR	54857	54684	conserved domain protein	YP_190135.1	100	100	14	35
70	traV	55555	55040	TraV	NP_061462.1	100	100	57	125
71	trbG	55803	55552	TrbG	YP_788070.1	93.75	100	16	33.1
72	trbD	56117	55800	TrbD	ZP_00715068.1	98.83	99.42	171	297
73	traP	56686	56114	TraP	YP_190137.1	100	100	83	174
74	traB	58103	56676	protein traB	YP_190138.1	100	100	105	209
75	traD	58831	58103	secretin TraK	YP_190139.1	98.95	100	190	361
76	traE	59384	58818	TraE	NP_061457.1	100	100	475	895
77	traL	59717	59406	TraL	NP_061456.1	100	100	242	476
78	traA	60091	59732	pilin TraA	NP_061455.1	100	100	188	350
79	traY	60352	60125	conserved domain protein	ZP_00714044.1	99.03	100	103	221
80	traJ	61192	60446	functional relaxosome complex initiator	YP_190144.1	100	100	119	198
81	traM	61706	61323	protein TraM	P05836	100	100	60	122
82		61983	62630	transglycosylase SLT domain protein	YP_538703.1	97.58	98.39	248	489
83		62790	62656	hypothetical protein					
84		62958	62809	hypothetical protein					
85		63748	62927	conserved hypothetical protein	YP_001096463.1	93.18	95.45	44	87
86		64158	63871	conserved hypothetical protein	NP_862914.1	100	100	273	558
87		64302	64183	conserved hypothetical protein	NP_862913.1	100	100	95	193
88		64277	64450	hypothetical protein					
89		64560	64793	conserved hypothetical protein	YP_190160.1	97.3	97.3	37	73.6
90		64955	64800	hypothetical protein					
91	sok	65518	65381	conserved hypothetical protein	ABD51591.1	100	100	20	48.5
92	psiA	66237	65518	PsiA	YP_053129.1	88.89	93.33	45	89.7
93	psiB	66671	66234	plasmid SOS inhibition protein B	NP_863050.1	100	100	239	452
94		68687	66723	transcriptional regulator, parB like	NP_863049.1	99.31	100	144	259
95		69000	68740	conserved hypothetical protein	ABD51586.1	99.85	99.85	654	1203
96	ssb	69556	69029	single-stranded DNA-binding protein	ABD51585.1	98.84	100	86	183
97		69722	69582	conserved hypothetical protein	P28044	98.86	99.43	175	256
98		69773	69904	hypothetical protein					
99		69959	70192	conserved hypothetical protein	ABD51583.1	100	100	39	88.2
100		70447	70199	hypothetical protein					
101		71010	70447	conserved hypothetical protein	YP_190163.1	79.59	81.63	49	67.8
102		72417	71056	conserved hypothetical protein	NP_863044.1	100	100	187	375
103		72699	72469	conserved hypothetical protein	NP_863043.1	100	100	453	880
104		72933	72796	hypothetical protein					
105		73063	72923	conserved hypothetical protein	YP_788041.1	97.06	100	34	80.5

106		73114	73539	conserved hypothetical protein	NP_863042.1	99.12	100	114	195
107		73875	73693	conserved hypothetical protein	NP_863041.1	100	100	60	122
108		74246	73875	conserved hypothetical protein	ABD51578.1	99.19	100	123	218
109	ard	74875	74336	antirestriction protein	ABD51577.1	100	100	170	333
110		74841	75011	conserved hypothetical protein	NP_863426.1	90.57	98.11	53	103
111	tnpA	75187	76134	Transposase	NP_863038.1	100	100	247	453
112	tnpA	76690	76187	IS InsB	NP_052905.1	100	100	167	352
113		76913	76701	conserved hypothetical protein	YP_053131.1	97.14	98.57	70	142
114		77482	77339	conserved hypothetical protein	NP_863035.1	100	100	47	100
115		77861	77448	conserved hypothetical protein	NP_863035.1	100	100	79	160
116	sopB	79460	78489	protein SopB	NP_863034.1	100	100	323	596
117	sopA	80635	79460	protein SopA	NP_863033.1	100	100	391	794
118	tnpA	81429	80926	IS1 InsB	NP_052905.1	100	100	167	352
119		81652	81440	conserved hypothetical protein	YP_053131.1	95.71	97.14	70	140
120		83281	81779	conserved hypothetical protein	NP_863030.1	100	100	500	1036
121		81665	81796	hypothetical protein					
122		84330	83881	conserved hypothetical protein	ABD51734.1	100	100	149	262
123		84448	84927	conserved hypothetical protein	NP_863028.1	100	100	159	273
124		85197	85003	hypothetical protein					
125		85213	85353	hypothetical protein					
126		85743	85561	putative enolase	NP_863027.1	100	100	60	127
127		86245	86099	hypothetical protein					
128	sitD	87068	86211	chelated iron transport system	YP_444051.1	99.65	99.65	285	480
129	sitC	87922	87065	chelated iron transport system	AAT11266.1	99.65	99.65	285	477
130	sitB	88743	87919	chelated iron transport system	YP_444049.1	100	100	274	534
131	sitA	89660	88746	chelated iron transport system	AAT11264.1	100	100	304	608
132		89974	89783	hypothetical protein					
133	tnpA	90210	90713	IS1 protein insB	NP_569348.1	100	100	167	351
134		90715	90891	plasmid stability protein	YP_308776.1	98.11	100	53	107
135	umuC	91240	90893	protein UmuC	NP_863024.1	100	100	115	240
136	tnpA	91896	91540	IS1 insA	NP_863023.1	100	100	110	225
137		91937	92113	conserved hypothetical protein	YP_194849.1	100	100	57	122
138	tnpA	92181	92492	IS911 truncated ORF1	AAW51749.1	100	100	103	203
139	tnpA	92520	92687	IS911 truncated ORF2	ZP_00713120.1	100	100	55	116
140	tnpA	92612	93358	IS911 truncated ORF2	ZP_00713119.1	100	100	225	481
141		93627	93379	hypothetical protein					
142		93740	93910	hypothetical protein					

143	repA	93903	94880	replication protein RepA	Q51651	99.38	99.69	325	547
144		95075	94956	hypothetical protein					
145	int	95905	95165	Int E2	NP_863020.1	100	100	246	497
146		96062	96187	hypothetical protein					
147	Mig-14	97496	96588	Mig-14, transcriptional regulator	ABD51759.1	100	100	302	639
148	HlyF	98668	97559	hemolysin F	NP_863018.1	100	100	336	681
149		98918	99073	hypothetical protein					
150	ompT	100054	99101	Protease	YP_444072.1	100	100	317	632
151		100130	100297	hypothetical protein					
152		100398	100547	conserved hypothetical protein	YP_444073.1	100	100	49	104
153		100499	100639	hypothetical protein					
154	tnpA	101485	101327	truncated S1400	YP_444075.1	98.08	100	52	107
155		101495	101617	hypothetical protein					
156	tnpA	101669	101995	IS629	YP_538619.1	98.77	98.77	81	124
157	tnpA	101995	102882	IS629	ABD51641.1	100	100	295	600
158		102848	102994	conserved hypothetical protein	NP_311519.1	80.77	84.62	26	51.2
159	tnpA	103271	103152	IS911	ABD51766.1	100	100	39	89
160		103326	103538	conserved domain protein	YP_053131.1	95.71	97.14	70	140
161	tnpA	103549	104052	IS1	NP_052905.1	100	100	167	352
162		104362	104637	hypothetical protein					
163		105225	104944	conserved hypothetical protein	ABD51689.1	100	100	93	190
164	cba	105425	105565	colicin B truncated	ABD51691.1	97.44	100	39	85.9
165	cbi	105729	105583	colicin-B immunity protein	P22426	100	100	37	76.3
166	tnpA	105789	106154	IS2	NP_058408.1	100	100	121	237
167	tnpA	106178	107017	IS2	ABD51690.1	98.59	99.3	142	253
168	cbi	107497	106988	colicin-B immunity protein	ZP_00736352.1	99.64	100	279	575
169		107594	107478	hypothetical protein					
170	cma	107690	108505	colicin-M	ABD51688.1	99.63	99.63	271	548
171	cmi	108980	108555	colicin-M immunity protein	ABD51687.1	100	100	141	263
172	int	109862	109086	site specific recombinase/integrase	ABD51686.1	99.61	100	258	507
173		110173	109874	conserved hypothetical protein	NP_862970.1	100	100	86	172
174	tnpA	110186	110902	IS26	NP_872582.1	100	100	238	501
175		111658	111422	hypothetical protein					
176	tnpA	111671	112387	IS26	NP_872582.1	100	100	238	501
177	cat2	113172	112531	chloramphenicol acetyltransferase	YP_209316.1	100	100	213	444
178		113822	113322	conserved hypothetical protein	ABG56838.1	100	100	166	335
179	tnpA	113890	114606	IS26	NP_872582.1	100	100	238	501

180	tnpM	114953	114801	TnpM truncated	ABE73753.1	67.24	74.14	58	72.8
181	intI1	116067	115054	integrase/recombinase	NP_052898.1	100	100	337	651
182	dhfr	116150	116320	dihydrofolate reductase	NP_569370.1	100	100	35	74.7
183	tnpA	116775	116662	truncated IS15	CAJ77077.1	87.1	90.32	31	60.5
184	tnpA	116754	117470	IS26	NP_872582.1	100	100	238	501
185	tnpR	117518	117838	resolvase interrupted	CAC14695.1	100	100	84	161
186		118200	117853	hypothetical protein					
187	tnpA	117892	120807	Tn3-like	YP_145631.1	100	100	871	1703
188	tnpA	120886	121890	IS4321R A	YP_001102014.1	100	100	334	610
189	int	122248	122072	phage integrase	YP_001102013.1	96.88	96.88	32	69.7
190	rep	122247	122390	repC-like truncated	CAH64749.1	100	100	34	73.9
191	sul2	122578	123393	dihydropteroate synthase	NP_065288.1	100	100	271	486
192	strA	123454	124257	streptomycin 3"-kinase 2.7.1.87	YP_001096379.1	99.63	100	267	547
193	strB	124257	125093	streptomycin resistance protein	AAN40998.1	100	100	278	562
194	tnpR	125613	125320	resolvase	AAA25052.1	100	100	40	79.7
195	aadA4	125639	125508	streptomycin-spectinomycin resistance protein	P21424	96.77	100	31	60.8
196	tnpA	125652	126368	IS26	NP_872582.1	100	100	238	501
197	sat	126737	127816	streptothricin acetyl transferase	YP_209360.1	100	100	359	697
198		128188	128520	conserved hypothetical protein	YP_209359.1	100	100	46	99.4
199	aadA2	128578	129369	streptomycin 3"-adenylyltransferase	YP_001102251.1	100	100	259	511

CHAPTER 6

CONCLUSION

Recent bacterial outbreaks in food sources highlight the toll bacteria can take on human health and the economic impact these organisms can have. For example, the recent 2007 *E. coli* outbreak in spinach sickened 205 people, three of whom died, and cost upwards of $74 million in losses to the California agricultural community alone. The toll bacteria can have on human health and economic factors is in part minimized by our ability to treat bacterial infections with antibiotics, which can facilitate the recovery of infected individuals and reduce potential economic impacts. However, this ability is severely hampered by bacterial resistance to these drugs.

Widespread usage of antibiotics began in the 1940s with the introduction of penicillin, while reports of bacteria resistant to antibiotics emerged soon after. In fact, the first β-lactamase gene was first identified even before β-lactam drugs were even in use. Important bacterial pathogens such as *Escherichia coli*, *Salmonella enterica*, *Vibrio cholerae*, and others that cause disease in humans and food animals are now resistant to many of the drugs in use today. Additionally, multidrug resistance in which a bacterium is resistant to more than five antibiotics is now common in these bacteria. The use and misuse of these drugs have been implicated in the increase in bacterial resistance, resulting in many bacteria of concern for human and agricultural health being resistant to the drugs used to treat infections.

Antibiotic resistant bacteria are found not only in hospital and agricultural settings where exposure to antibiotics is likely, but are also found in terrestrial, freshwater, and marine habitats. Because bacteria that cause disease in humans and food animals such as *E. coli*, *S. enterica*, and *V. cholera* are also found in such habitats, it is critical for our understanding of the dissemination of antibiotic resistance in bacteria of clinical concern to investigate antibiotic resistance in bacteria in environmental settings.

Antibiotic drugs are detected in rivers and streams below sewage treatment plants and confined animal feeding operations in settings. Additionally, these settings can serve as sources of antibiotic resistant bacteria and resistance genes into receiving environments. Multiple studies document the occurrence of resistance genes in agricultural and wastewater treatment plant settings demonstrating that these can be significant sources of resistance in the environment. However, there may be additional selective pressures maintaining these genes once released into the environment.

One such selective pressure is bacterial exposure to metals. Bacteria found in metal-contaminated streams are not only more tolerant to the metals cadmium and nickel, but are also more tolerant to ampicillin and tetracycline drugs compared to bacteria from a reference stream with no metal contamination. This observation is consistent across multiple types of bacterial communities including bacteria collected from river sediments, water column, invertebrate digestive tracts, and from biofilm growing on submerged woody debris in streams. Additionally, genes conferring resistance to the antibiotic tetracycline are more abundant in the metal-contaminated streams suggesting that metals maintain a pool of resistance genes in this system. The role of metals in maintaining a pool of antibiotic resistant bacteria and resistance genes may be due to multiple mechanisms. One such mechanism is the physical linkage of antibiotic and metal resistance genes on the same genetic element, so that when one gene is selected for, other genes to which it is attached are also selected.

Bacteria are able to acquire antibiotic resistance genes by a process called horizontal gene transfer in which foreign genes are inserted into their genome either by bacteria taking up free DNA from the environment (transformation), viral transfer of DNA from bacterium to another (transduction), or through transfer from one bacterium to another via cell-to-cell contact

(conjugation).. Multiple types of genetic elements are involved in these processes including elements called plasmids, transposons, and integrons. Not only are antibiotic resistance genes are more abundant in metal-exposed bacteria, but so are integron genes. Integrons are involved in bacterial acquisition of new DNA via horizontal gene transfer. Thus bacteria in metal-contaminated systems have a higher potential to acquire genes associated with integrons compared to bacteria from reference sites.

Bacteria in settings outside of clinical and agricultural settings are more likely to be exposed to metals than antibiotics at levels that would be toxic to them. The current extent of metal contamination on a global scale greatly exceeds that of antibiotic contamination from sewage treatment or agricultural inputs into aquatic systems. Furthermore, metals are commonly used in a variety of materials as antibacterial treatments including the recent addition of silver to athletic clothing to minimize bacterial growth, indicating that metal exposure is likely a driving force in bacterial evolution, and one effect of this exposure can also be increased bacterial resistance to antibiotics.

Bacteria have demonstrated a remarkable ability to evolve resistance mechanisms to toxicants including industrial pollutants, metals, and antibiotics. The fact that resistance to different classes of toxicants can be co-selected for in bacteria warrants more attention in implementing antibacterial treatments.