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Dissemination and Persistence of Plasmid Located, Integron Associated Antibiotic Resistant Genes in Wastewater Treatment Plant Effluent and Stream Water Bacteria

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Dissemination and Persistence of Plasmid Located, Integron Associated Antibiotic Resistant
Genes in Wastewater Treatment Plant Effluent and Stream Water Bacteria

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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Abstract

Transmissible plasmid-mediated integrons play important role in the persistence and dissemination of antibiotic resistance throughout the environment. Plasmids from 139 multi-antibiotic resistant *Escherichia coli* recovered from wastewater treatment plant effluent and upstream and downstream receiving stream water in Northwest Arkansas were extracted and profiled. Genes of class 1 and class 2 integrase (*intI*), mobilization (*mob*), sulfamethoxazole resistance (*sul*), and trimethoprim resistance (*dfr*) were detected using PCR and confirmed through DNA sequencing. Plasmids from almost half of the isolates (47%) were transmissible with *mob_{F12}* gene as the most frequently detected mobilization gene. Plasmid-borne class 1 with and without class 2 integrons were prevalent (46%). More isolates possessed plasmid DNA carrying single or coexistence of two or three *sul* genes (99.3%), and single or a combination up to five *dfr* genes (89.3%). The presence of integron, more than one *mob* gene, three *sul* genes, and three to five *dfr* genes all increased the proportion of *E. coli* possessing higher multiple antibiotic resistance numbers. Over an 11-day experiment, isolates persisted at concentrations greater than $\log 8.99 \text{ mL}^{-1}$ in the presence of low (sub-inhibitory) concentrations of sulfamethoxazole regardless of integron and mobilization gene designation. In the presence of trimethoprim, isolates harboring plasmids *mob⁺int⁺* were less persistent compared to isolates without either or with a gene from either group individually. Approximately 77% of *E. coli* possessing *mob_{F12}* disseminated plasmids into a recipient under controlled conditions at mean transfer rates of 2.14×10^{-3} (transconjugants/donors) or 7.90×10^{-3} (transconjugants/recipients). Isolates recovered from further downstream and possessing plasmid-mediated-resistance to three antibiotics with three *sul* genes had significantly higher transfer frequency than their counterparts. Effluent may be an important source of introduction MAR bacteria possessing

transmissible plasmids and integron serving as a mechanism for accumulating antibiotic resistance genes. *Escherichia coli* possessing three *sul* genes, regardless of integron or *mob* gene designation appear to be highly persistent and prone to dissemination of resistance genes, and thus may contribute to the spread and persistence of multiple antibiotic resistances among bacteria in the stream environment.

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Dedication

This doctoral dissertation is dedicated to my parents, teachers, wife, Wiwit Artika, and my precious daughter, Sofia Aleeya Suhartono, as well as my friends.

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List of Abbreviations

ARG	Antibiotic resistance gene
ARB	Antibiotic resistant bacteria
BHR	Broad host range
GLIMMIX	Generalized linear mixed models
GLM	Generalized linear models
LB	Luria bertani
MAR	Multiple antibiotic resistance
MGE	Mobile genetic elements
UTI	Urinary tract infections
WWTP	Waste water treatment plant

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

A. INTRODUCTION

Antibiotic resistant bacteria (ARB) are a major public health problem, with concern increasing about their dissemination throughout the environment. The bacteria that are no longer susceptible to current antibiotics generate significant costs, both in clinical and environmental settings (Smith and Coast, 2013). This reduced susceptibility may reduce the therapeutic options to combat infectious diseases as well as increase health risks and complications leading to the increase in patient mortality rates (Hanberger et al., 2014). Moreover, antibiotic resistance may inhibit drug development by disrupting drug discovery due to the surpassing bacterial innovation (resistance) over the human innovation to discover effective novel drugs (Georgopapadakou, 2014).

There are some mechanisms, resulting from external and internal factors to explain the increased number of ARB in the environment. Externally, ARB are promoted by the intensive and extensive applications of antimicrobial agents in clinical as well as agricultural settings (McManus et al., 2002). Furthermore, significant increases of organic and inorganic pollutants, including antibiotics, from hospitals, households, industries, urban areas, and agriculture entering the environment may be contributing to the proliferation of ARB populations within communities (Martinez, 2009). Internally, ARB arise because of the occurrences of considerable genetic variations resulting from recombination events, such as mutations or from genetic exchanges with other bacteria through horizontal gene transfer (HGT) (Baquero et al., 2008). Genetic exchange occurs when environmental bacteria mix with pathogenic organisms or their genes in the environment. The genes of the first bacterial population are functional but may not express antibiotic resistance phenotypically, termed as silent/cryptic resistance, but they might act as a resistome, or genes that are able to be restored

into antibiotic-resistance counterparts by either means of mutation or mobilization (D'Costa et al., 2006; Henriques et al., 2011; Forsberg et al., 2012).

Wastewater treatment plants (WWTPs) have an important role in environmental management. Wastewater treatment plants decompose fecal wastes and remove toxic organic as well as inorganic contaminants contained in wastes. Contaminants originate from various sources ranging from hospitals, homes, urban localities, farms, and industries (Rahube and Yost, 2010). Wastewater treatment plants have responsibilities to reduce chemically, physically and biologically the concentration of dissolved organic carbon, nitrogen and phosphorus and eliminate viable pathogens from the liquid effluents (Zhang et al., 2009). However, gram-negative ARB carrying antibiotic resistant genes (ARG) and broad-host-range (BHR) plasmids have been recovered in WWTP effluent that is discharged into receiving surface waters (Akiyama et al., 2010; Akiyama and Savin, 2010). Moreover a local investigation revealed that antibiotics, pharmaceuticals, and other organic compounds were detected in the stream water of northwestern and north-central Arkansas (Galloway et al., 2005; Haggard et al., 2006).

Wastewater treatment plants harbor a diverse microbial community, with the microbial community composition depending largely on the biochemical composition of influents received by the WWTP (Wagner and Loy, 2002). For example, more than 20 phyla of some environmental opportunistic and clinically significant bacterial pathogens, such as proteobacteria, chloroflexi, firmicutes, spirochaetes, and bacterioides have been successfully isolated and identified from WWTP samples (Wagner and Loy, 2002). Genetic exchange within these bacterial communities might be accomplished by means of HGT allowing ARG

to be disseminated among bacteria, not only in the same species or genus of bacteria but also among distantly related bacteria.

Antibiotic resistance genes are frequently associated with mobile genetic elements, such as plasmids and integrons, which can be horizontally exchanged among bacterial populations (Thomas and Nielsen, 2005). Many types of plasmids and other mobile genetic elements have been recovered from activated sludge of WWTPs conferring both resistance to a particular group of antibiotics such as aminoglycosides (Tennstedt et al., 2003), quinolones (Bönemann et al., 2006), or erythromycin (Schlüter et al., 2007), and resistance to multiple drugs (Szczepanowski et al., 2009). Furthermore, a recent investigation revealed that the genetic element of broad-host-range IncP-1 plasmids was found in wastewater treatment plants of Northwest Arkansas (NWA) (Asfahl and Savin, 2012). These self-transmissible plasmids are able to transfer and replicate in a wide range of hosts and are capable of encoding resistances to almost all types of clinically relevant antibiotics (Szczepanowski et al., 2009).

Previous investigations indicated that ARG might emerge in water and sediment of Northwest Arkansas surface waters. Akiyama and Savin (2010) reported that a number of *E. coli* possessing ARG and plasmids were recovered in treated effluent and receiving stream water in Northwest Arkansas. Despite disinfection protocols in WWTPs and reductions in BHR plasmid abundance, ARG and BHR plasmids were present in WWTP effluents (Asfahl, 2011), which lead to inputs of corresponding plasmids into receiving streams. Thus, further investigation is warranted to elucidate mechanisms for accumulation of ARG within bacteria, and relationships of mobile genetic elements to persistence and dissemination of ARG from bacteria isolated from WWTP effluent and receiving streams.

B. LITERATURE REVIEW

1. Antibiotic resistance

The prolonged use of antibiotics in both clinical and non-clinical settings has led to resistance in target organisms. In addition to compounds and conditions that occur in natural microbial communities, drugs promote selective pressures for the target organisms to evolve and spread ARG (Allen et al., 2010). Antibiotic or antimicrobial drugs inhibit susceptible organisms and select for genetic resistance determinants in surviving microorganisms (Levy and Marshall, 2004).

Among resistant microorganisms, there are various mechanisms used to counter antimicrobial drugs, including an impermeable membrane or lack of the antibiotic target (Allen et al., 2010). One mechanism is alteration of the protein target, through mutations, thereby reducing affinity towards the antibiotics. This mechanism, for example, applies to the alteration of penicillin binding proteins, reducing affinity towards β -lactam antibiotics (Waxman and Strominger, 1983). Some antibiotic resistant organisms produce β -lactamases, enzymes that inactivate the drugs by breaking the β -lactam ring, thereby avoiding their interaction with penicillin binding proteins (Beceiro and Bou, 2004). An alternative antibiotic resistance mechanism is modification of the expression of outer membrane proteins, namely porins, to reduce entry of the drugs into the cell and also activates efflux pumps which discharge the drugs (Nikaido and Vaara, 2003). A prevalent mechanism of antibiotic resistance is the expression of enzymes that are able to hydrolyze antibiotics.

The phenotypes of ARB are determined by the existence of ARGs in their genome. These ARG are genes responsible for eliciting antibiotic resistance determinants towards and compromising the effectiveness of major classes of antimicrobial drugs such as tetracycline,

aminoglycoside, chloramphenicol, sulfonamides and trimethoprim, and β -lactam. The protein/enzymes used for drug inactivation are expressed within genes that might be encoded both chromosomally and extra-chromosomally and inherited to the next generation. Those that are encoded extra-chromosomally within plasmids or other mobile genetic elements might be transferred to other organisms through HGT.

2. Sulfamethoxazole – trimethoprim and resistance

Since the 1990s, sulfamethoxazole, a sulfonamide bacteriostatic antibiotic, and trimethoprim, a synthetic derivative of trimethoxybenzyl-pyrimidine, have been first-line antibiotics of choice administered in combination to treat bacterial infections, particularly in urinary tract infections (UTIs) (Nicolle, 2003). Both antibiotics have synergistic effects in vitro (Bushby and Hitchings, 1968); however, the combination of sulfamethoxazole and trimethoprim had no clear evidence to support their synergisms in vivo from clinical applications (Brumfitt and Hamilton-Miller, 1982). Both sulfamethoxazole and trimethoprim disrupt folic acid synthesis in bacteria by means of inhibition of dihydropteroate synthetases (DHPS), i.e. enzymes catalyzing the transformation of dihydropteroic acid from dihydropteroate diphosphate and p-aminobenzenzoate (PABA), in the presence of sulfamethoxazole as well as inhibition of dihydrofolate reductase (DHFR), i.e. enzymes catalyzing the formation of tetrahydrofolate from dihydrofolate, in the presence of trimethoprim (Eliopoulos and Huovinen, 2001).

However, resistance of bacterial pathogens to both sulfamethoxazole and trimethoprim, particularly in the UTIs' treatment has been reported (Guneysel et al., 2009). This is mainly a consequence of the occurrence of insensitive DHPS and/or DHFR enzymes to

the drugs resulting from mutational or acquired changes of the enzymes (Eliopoulos and Huovinen, 2001). To date, there are three different genes associated with insensitive DHPS enzyme, namely *sul1*, *sul2*, and *sul3* (Gündoğdu et al., 2011), and more than 30 different genes associated with insensitive DHFR enzymes (Šeputienė et al., 2010). It is also important to note that all genes encoding insensitive DHPS and/or DHFR enzymes are transferable and highly associated with mobile genetic elements, such as integrons and insertion sequence common regions (ISCRs) (Shin et al., 2015).

3. Mobile genetic elements (MGE)

Mobile genetic elements (MGE) are defined as segments of DNA encoding enzymes and other proteins that mediate the movement of DNA intracellularly from one genetic location to another or intercellularly from one bacterial cell to another (Frost et al., 2005). The intracellular MGEs include integrons and gene cassettes, insertion sequences, that translocate DNA with or without involvement of replication, whereas the intercellular MGEs include conjugative plasmids, bacteriophages, and conjugative transposons, that involve replication during the DNA translocation (Bennett, 2008).

The MGE facilitate gene exchange between bacteria by means of horizontal gene transfer (HGT) that provide ecological benefits. Through HGT, bacteria possessing MGE may acquire particular genes from phylogenetically-related or distinct bacteria in the community, allowing them to adapt to dynamic ecological conditions and establish a niche (Wiedenbeck and Cohan, 2011). For example, MGEs mediate the transfer of genes to catabolize xenobiotic compounds, i.e. human-made compounds released and accumulated in the environment (Top and Springael, 2003). Additionally, MGEs may also assist bacteria to widely disseminate

pathogenicity as they often confer virulence genes to help bacterial pathogens penetrate and survive within hosts (Gyles and Boerlin, 2014). The two primary MGE associated with horizontal transfer of ARGs are plasmids and integrons; both will be further discussed in the following section.

4. Plasmids: Biology, properties, classification, and persistence

Plasmids are extra-chromosomal fragments of genetic units that replicate autonomously constituting 1% to greater than 10% of the bacterial species genome (Thomas, 2000). Plasmids are normally circular, although linear forms have been also described. They vary widely in copy number per bacterial cell, and vary widely in size from 1 kb to 200 kb to up to 1000 kb for the larger plasmids termed megaplasmids. Structurally, plasmids harbor two distinct regions, namely the backbone region and the accessory region. The backbone region is responsible for encoding functions involved in replication (*oriV* and *trfA*), transfer (*tra*), maintenance, and control of the plasmid (*ctI*); whereas the accessory regions confers genes encoding specific beneficial traits to the bacterial host (Sen et al., 2012). The genes, particularly in the accessory region, within a plasmid that might evolve over time through the acquisition of various MGEs, such as transposons, integrons, gene cassettes, genomic islands and insertion sequences, are located in one or two sites between essential DNA fragments i.e., between *oriV* (the origin of vegetative replication), and *trfA* (a gene for plasmid replication) and/or between the two transfer operons that encode mating-pair formation (*trfA*) and plasmid transfer (*trfB*) (Sota and Top, 2008).

There are three major characteristics of plasmids, 1) separateness (i.e. the potential to be physically distinct and to replicate autonomously) from the chromosome, 2)

transmissibility (i.e. the ability to transfer or be transferred as a discrete molecule), and 3) dispensability (i.e. lack of essential genes) (Slater et al., 2008). In their host population, plasmids tend to have discontinuous distribution meaning they can be physically lost at high frequency or acquired from other bacteria and can be horizontally exchanged among bacterial populations by conjugation as well as mobilization (Francia et al., 2004).

The presence of plasmids within bacterial cells could generate advantages and disadvantages. One of the advantages of plasmids is the increasing pathogenicity of host cells by encoding genes for bacteriocin production, adhesion, pathogenicity factors, resistance to antibacterial agents (antibiotic, ion, and radiation) and survival in stressful conditions, e.g. the limitation of substrate or presence of heavy metals (Schumann, 2001). Plasmid-encoded antibiotic resistance encompasses most of those classes of antibiotics that are at the forefront of antibiotic therapy, such as cephalosporin, fluoroquinolones and aminoglycosides (Bennett, 2008). Moreover, the presence of plasmids within bacterial cells is mainly associated with the acquisition of additional properties usually in the form of an enzyme that is able to modify antibiotics, circumvent the target, or result in the reduced accumulation of the antibiotic (Schuman, 2001).

One of the disadvantages of plasmids is the burden on bacterial cells to replicate and maintain a larger genetic burden because plasmids are not essential for host cell growth. Therefore, bacterial cells without a plasmid are expected to outcompete those with a plasmid just as bacterial cells with a smaller genome will outgrow those with larger ones (Schuman, 2001). Various measures may be taken by the plasmid to reduce the intrinsic burden it imposes on its host. Elimination of resistance genes themselves from a plasmid can lower the fitness burden of a plasmid, which can also lead to a selective sweep of a less versatile plasmid

(Bergstrom et al., 2000). Another mechanism is reduction in the number of plasmid copies maintained by the cell to decrease the energy consumption for DNA replication (Bahl et al., 2009).

In terms of plasmid classification, one of the most widely used is based on their incompatibility properties. A formal scheme to classify plasmids based on incompatibility was introduced by Datta and Hedges (1972). Basically, incompatibility is defined as the inability of two plasmids to stably coexist in the same cell line. Thereby, a cell containing two or more incompatible plasmids will give rise eventually to descendants containing either plasmid, but not both (Summers, 1996). The inability of coexistence in the same bacterial cell results from competition of both incompatible plasmids for common factors involved in plasmid replication or partitioning.

Furthermore, plasmids are also classified by their host range. A plasmid's host range is defined as the range of hosts in which the plasmids can replicate. Plasmids that replicate only in the limited number of closely related hosts are classified as narrow-host-range (NHR) plasmids; whereas those plasmids that can transfer to and be stably maintained in different host species are grouped as broad-host-range (BHR) plasmids. Both NHR and BHR plasmids have specific mechanisms allowing them to transfer, replicate, and persist in the limited and varied range of hosts, respectively (Sota and Top, 2008). Due to their transfer and replication in diverse bacteria at high frequencies as well as their host-beneficial genes, the BHR plasmids are important for bacterial adaptation to dynamic environments (Sen et al., 2011). Some BHR plasmids can partially integrate into the recipient chromosome, or mobilize non-conjugative vectors with a wider host range (Sota and Top, 2008) and retromobilize or retrotransfer, i.e.

capture genes or non-self-transmissible plasmids from a host in which they cannot replicate (Szpirer et al., 1999).

Another classification of plasmids is based on conjugative apparatus and mobility. In this classification, plasmids are grouped into transmissible plasmids (i.e. conjugative or self-transmissible plasmids; and mobilizable plasmids); and nonmobilizable plasmids (Smillie et al., 2010). The first possesses a complete set of conjugation genes comprising mobilization, *mob*, genes required for the processing of conjugative DNA, and mating pair formation (MPF) genes required for forming the mating channel; whereas the latter confers only *mob* genes allowing the plasmid to be mobilized by conjugation when it co-exists in the same donor cell with a conjugative plasmid (Smillie et al., 2010, Garcillan-Barcia et al., 2011).

5. Integrons: structures, classification, and their association with antibiotic resistance genes

Integrons are bacterial genetic elements containing a site-specific recombination system that is able to acquire, rearrange, and express of genes embedded within the gene cassette (GC) (Fluit and Schmitz, 1999). These DNA elements were first described, characterized and named as DNA integration elements or integrons by Stokes and Hall (1989) after they found novel DNA elements from several different locations that encode a variety of antibiotic resistance genes. Stokes and Hall (1989) discovered these novel mobile DNA elements have site-specific integration functions that are able to acquire and express various gene units by supplying the promoter for the inserted genes (gene cassette).

Structurally, an integron consists of three major components: the *intI* gene; the recombination site (*attI*); and the promoter (Pc). The *intI* gene encodes for an enzyme called

integrase that allows specific excision and integration of gene cassette adjacent to the recombination site (*attI*) followed by the transcription and expression of the gene cassette through the integron promoter (Pc) (Fluit and Schmitz, 1999, Stalder et al., 2012, Gillings, 2014). The gene cassettes are present in covalently closed circular forms of DNA (Collis and Hall, 1992) containing genes mainly encoding for resistance against a wide spectrum of antibiotics (Fluit and Schmitz, 1999). Despite the fact that integrons are not independently mobile, their association with other MGEs, such as plasmids and transposons, can allow ARGs encoded in the open reading frame (ORF) to be widely spread and exchanged among diverse bacteria (Fluit and Schmitz, 1999).

Integrons can be divided into two distinct groups: the mobile integrons (MIs), which are linked to mobile DNA elements and are primarily involved in the spread of antibiotic-resistance genes; and the chromosomal integrons (CIs) (Mazel, 2006, Cambray et al., 2010). Based on the sequence homology of the encoded integrases, MIs can be further divided into five classes, namely class 1 (C1), class 2 (C2), class 3 (C3), class 4 (C4), and class 5 (C5), integrons (Cambray et al., 2010). Of five classes of MIs, class 1 (C1) integrons and class 2 (C2) integrons are the most prevalent and clinically important, as they are extensively found in gram-negative clinical isolates (Xu et al., 2011), and occasionally identified in gram-positive bacteria (Nandi et al., 2004). They are associated with functional and nonfunctional transposons derived from Tn402, which can be further embedded in larger transposons such as Tn21 (Cambray et al., 2010).

Class 1 integrons contain two conserved segments, namely the 5'-conserved segment (5'-CS) and 3'-conserved segment (3'-CS). The 5'-CS contains the *intI* gene, the *attI* site and the promoter, while the 3'-CS codes for the *sulI* gene, conferring resistance to sulphonamides,

the *qacEΔ1* gene, conferring resistance to quaternary ammonium compounds, and *orf5* gene, conferring a protein of unknown function (Fluit and Schmitz, 1999). Antibiotic resistance gene cassettes are integrated between the 5'- and 3'-CS at the receptor *attI1* site. Class 1 integrons have been detected harboring up to seven gene cassettes, and in some cases multiple copies of the same cassette have been detected (Carattoli, 2001). Gene cassettes which confer resistance to β-lactams, aminoglycosides, erythromycin, fosfomicin phenicols, lincomycin, quaternary ammonium compound family antiseptics, rifampin, streptothricin and trimethoprim have been described from class 1 integrons (Mazel, 2006).

Class 2 (C2) integrons are the second most abundant group that are similar in structure to class 1 integrons in the 5'-CS region but are different in the 3'-CS region because of the absence of *sull* gene. The gene encoding the integrase in class 2 integrons contains a nonsense mutation in codon 179 (ochre 179), yielding a truncated and non-functional protein (Hansson et al., 2002). Gene cassettes in the class 2 integrons are mainly composed of the GC *dfrA1* (involved in the resistance to trimethoprim), *sat2* (involved in the resistance to streptomycin), *aadA1* (involved in the resistance to streptomycin and spectinomycin), and *orfX* (unknown function) (Hansson et al., 2002). Class 2 integrons are almost always associated with the Tn7 transposon and their derivatives, thus promoting their dissemination (Stalder et al., 2012).

The term 'super integron' was coined by Mazel et al. (1998) after the discovery of integron in the small chromosome of *Vibrio cholerae* with a very large array of gene cassettes incorporated within it. In terms of location and number of gene cassette arrays, super integrons have distinctive features compared to MIs. There are a large number of gene cassettes that are associated with the integron, and there is a high degree of identity (>80%) observed between the *attC* sites of these cassettes. Furthermore, the structure of super integron

is not likely to be mobile since it is located on the chromosome and is not associated with mobile DNA elements (Heidelberg et al., 2000).

It is noteworthy that all MIs are physically linked to mobile DNA elements, such as insertion sequences (ISs), transposons and conjugative plasmids, all of which can serve as vehicles for the intra-species and inter-species transmission of genetic material (Mazel, 2006). Some transposable elements are known to be associated with integrons to increase their mobility. Class 1 and 2 integrons are often carried by Tn21-transposable elements (Cambray et al., 2010) and Tn7-transposable elements (Ramírez et al., 2010) as well as have a relationship with insertion sequences (IS). Furthermore, integrons borne on transposons have been described as preferentially incorporated into conjugative plasmids, such as IncP and IncQ plasmids, which then guarantees a broad-host range horizontal transfer of the integrated gene cassettes such as those encoding antibiotic resistance (Poirel et al., 2010, Moura et al., 2012). It is evident that the association of a highly efficient gene-capture and expression system of integrons together with the capacity for vertical and horizontal mobility of plasmids may allow ARG to be potentially widely spread (Carattoli, 2001).

6. Horizontal gene transfers (HGT) in aquatic environments

There are two routes of evolutions for acquired resistance, vertically via mutation and selection (Darwinian evolution) or horizontally via exchange of genes between strains and different species (Jury et al., 2011). Vertical evolution is determined by natural selection whereby a spontaneous mutation in the bacterial genome leads to resistance in a bacterium and its progeny within the population. Horizontal evolution (or lateral gene transfer), on the

other hand, can occur by means of three mechanisms, conjugative transfer, transformation, and transduction (Andersson and Hughes, 2010).

Conjugative transfer or conjugation occurs between two different strains of bacteria assigned as donor and recipient. The donor will transfer its MGE (plasmid or transposon) to the recipient through a pilus, an appendage of the donor cell that allows physical contact between both cells. This process requires a viable and metabolically capable donor cell to transfer its genetic material to the recipient cell. There are two classes of plasmids according to their transmissibility by conjugation, namely conjugative plasmids and mobilizable plasmids. Conjugative plasmids contain a full set of conjugation genes comprising -MOB module, which carries genetic information essential for the processing of conjugative DNA and mating pair formation (MPF) module encoding a membrane-associated complex, which is a type 4 secretion system that forms the mating channel. An example of this plasmid is the enterobacterial AbR IncW plasmid R388 (Garcillan-Barcia et al., 2011). Mobilizable plasmids, on the other hand, contain only a minimal set of genes that allow them to be mobilized by conjugation when they coexist in the same donor cell with a conjugative plasmid. They are called mobilizable plasmids and the example is the IncQ1 plasmid RSF1010 (Garcillan-Barcia et al., 2011). These non-self-transmissible plasmids carry only the MOB module, and their transfer requires an MPF provided by another genetic element (Smillie et al., 2010).

Transformation, on the other hand, does not require physical contact between donor and recipient cells. In transformation, a 'competent' recipient cell incorporates extracellular plasmid or chromosomal DNA from the environment into its genome. This incorporated DNA

may be expressed by the transformed recipient cell and inherited by the next generation during replication in cell division.

Transduction has a quite different mechanism than the first two processes. In transduction, DNA from bacterial donor cells is indirectly transferred to recipient cells by bacteriophage particles. During the last phase of infection (packaging phage particles), rather than packaging the appropriate viral genome into the phage particle, a segment of donor cell DNA is erroneously packaged into the particles. Following lysis of the infected donor cell, the donor cell DNA carried by bacteriophage particles is released into the surrounding environment. Once the corresponding phage particles subsequently infect and propagate within a new recipient cell, the segment of DNA donor might be integrated into the latter recipient cell.

7. Possible dissemination of antibiotic resistant genes in WWTP effluent and receiving stream water

Wastewater treatment plants are facilities that manage and physically and biochemically decontaminate wastes originating from homes, hospitals, agriculture, and industries. It is not surprising that WWTPs also become natural reservoirs of biological contaminants including plasmid-borne antibiotic resistance genes. Various sources of sewage may provide habitats to facilitate interaction and communication among bacterial communities. Biofilm formation in the WWTPs may promote communication and interaction among bacteria (Schwartz et al., 2003). The presence of low concentrations of antibiotics in WWTPs may exert selective pressure for the acquisition or proliferation of antibiotic resistant organisms (Jury et al., 2011). Furthermore, through HGT, the ARGs spread among bacteria not

only from the same species, but also different taxonomic groups since the genes are encoded within mobile genetic elements. Inadequate and inappropriate treatment processes may not eliminate these pollutants leading to the escape into environment of ARGs with discharge of treated WWTPs effluent.

The detection of 140 plasmid-mediated ARGs of clinical isolates in the activated sludge and final effluents of WWTP signifies the plasmids' ARG reservoirs in WWTPs (Szczepanowski et al., 2009). Plasmids were recovered in the final effluent of the WWTP, implying the possibility of dissemination of these plasmids into surface water environments (Akiyama et al., 2010). Wastewater effluent carrying ARG is usually released into rivers and creeks and subsequently reaches geographically distant areas, such as lakes and coastal waters (McArthur et al., 2011). Dissemination of plasmid-borne antibiotic resistance from WWTP to environments depends on the persistence of isolates carrying these plasmids in the environments into which they are released. These antibiotic resistance plasmids do not always carry only ARGs. Other accessory genes, such as heavy metal resistance genes that are co- and cross-expressed, may be able to facilitate and sustain the antibiotic resistance plasmids in environments with or without antibiotic selective pressures (Baker-Austin et al., 2006).

C. GOALS AND OBJECTIVES

The overall goal of this research was to determine prevalence, dissemination and persistence potentials of antibiotic resistant *E. coli* possessing plasmid-mediated integron genes, mobilization genes, sulfamethoxazole and/or trimethoprim resistant genes that were isolated from wastewater treatment effluent and stream water.

The objectives of the research were to determine the occurrence of integrase, mobilization genes, and sulfamethoxazole and/or trimethoprim resistant genes encoded within plasmids, and the relationship with multiple antibiotic resistance (MAR) number in antibiotic resistant *E. coli* isolated from treated wastewater effluent and receiving stream water; to determine the influence of plasmid-mediated integron-mobilization gene combination towards the persistence of sulfamethoxazole-trimethoprim resistant *E. coli* isolates; and to determine the dissemination potential of MAR *E. coli* harboring transmissible plasmids.

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CHAPTER 2. TRANSMISSIBLE PLASMIDS AND CLASS 1 AND 2 INTEGRONS SHIFT
POPULATION OF ANTIBIOTIC RESISTANT *Escherichia coli* ISOLATED FROM
WASTEWATER TREATMENT PLANT EFFLUENT AND STREAM WATER TOWARDS
LARGER MULTIPLE DRUG RESISTANCE NUMBERS

Transmissible plasmids and class 1 and 2 integrons shift population of antibiotic resistant *Escherichia coli* isolated from wastewater treatment plant effluent and stream water towards larger multiple drug resistance numbers

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A. Abstract

Plasmid-mediated integron may be an important mechanism for accumulating antibiotic resistance genes in multiple antibiotic resistant bacteria, and thus contributing to the persistence and spread of antibiotic resistance throughout the environment. *Escherichia coli* recovered from wastewater treatment plant effluent and upstream and downstream receiving stream water in Northwest Arkansas exhibited resistance to up to six different antibiotics, including sulfamethoxazole and trimethoprim. Plasmids were extracted and profiled from 139 bacterial isolates grown on selective media. Genes of class 1 and class 2 integrase (*intI*), mobilization (*mob*), sulfamethoxazole resistance (*sul*), and trimethoprim resistance (*dfr*) were detected using PCR amplification and confirmed through DNA sequencing. Almost half of the total plasmids (47%) in the present study were transmissible with *mob_{F12}* gene as the most frequent mobilization gene. When two or three *mob* genes were detected in plasmids of isolates, there was a significant shift in the population towards a larger multiple antibiotic resistance (MAR) number. Plasmid-borne class 1 and/or 2 integrons were prevalent (46%). Integron presence also significantly shifted the isolate population towards larger MAR number. More isolates possessed plasmid DNA carrying single or coexistence of two or three *sul* genes (99.3%), and single or a combination up to five *dfr* genes (89.3%) than had exhibited phenotypic resistance to the respective antibiotics. These findings may support transmissible plasmids and integron as a mechanism for accumulating antibiotic resistance genes and thus contribute to the spread and persistence of MAR among bacteria in the stream environment.

B. Introduction

The emergence of antibiotic resistant bacteria (ARB), especially of bacteria that are no longer susceptible to most clinically relevant antibiotics, generates significant global health and management costs, both in clinical and non-clinical settings. Cost estimates for antibiotic resistance exceed \$55 billion, comprising \$20 billion in medical and \$35 billion in societal costs per year in the U.S. alone (Roberts et al., 2009, Smith and Coast, 2013). Moreover, ARB may limit the therapeutic options to counteract infectious diseases leading to increasing complications and mortality rates as well as inhibiting drug development in pharmaceuticals (Wright and Poinar, 2012, Georgopapadakou, 2014). The ARB may also generate significant impacts in wildlife and the environment. Antibiotic resistant bacteria harboring plasmids that exhibit multiple antibiotic -resistant phenotypes have been isolated from wild birds and mammals (Radhouani et al., 2014, Carroll et al., 2015). Thus, wildlife may serve as potential environmental reservoirs for the ARB leading to the widespread dissemination of zoonotic diseases as well as increasing potential problems of the medical treatment of wildlife (Radhouani et al., 2014, Carroll et al., 2015).

A combination of extrinsic and intrinsic causative factors may be linked to the increasing proliferation of ARB in the environment. Externally, ARB are promoted by the intensive and extensive application of antimicrobial agents in clinical and agricultural settings (Durso and Cook, 2014). Increasing organic and inorganic pollutants, including antibiotics, released into the environment from anthropogenic activities may be contributing to the proliferation of ARB populations within communities through selective pressure (Martinez, 2009, Andersson and Hughes, 2014, Martínez and Baquero, 2014). In terms of intrinsic factors, ARB may arise through genetic mutations or as a result of recombination events

increasing genetic variation or resulting in genetic acquisition from other bacteria within the population or community through horizontal gene transfer (HGT) (Baquero et al., 2008, Brochier-Armanet and Moreira, 2015). Genetic acquisition can occur when pathogenic bacteria or their genes mix with bacteria in the environment. The original host bacterial population harbors acquired and/or intrinsic resistance genes that encode antibiotic resistance determinants, while the latter bacterial population may not express antibiotic resistance phenotypes, but serve as a reservoir of resistance genes that could be activated through mobilization (Perry and Wright, 2014).

The presence of antibiotic resistance genes (ARG) in bacterial cells is strongly associated with mobile genetic elements, such as plasmids and integrons. Plasmids are extrachromosomal circular double-stranded DNA that autonomously replicate with widely varying copy number and size per bacterial cell (Carattoli, 2009). Plasmids can confer ARG allowing host cells to overcome the activity of antibiotics, even those in classes at the forefront of development, such as cephalosporin, fluoroquinolones and aminoglycosydes (Bennett, 2008). Plasmids, depending on their host range, conjugative apparatus, and mobility, play a major role in HGT, promoting dissemination of ARG among bacteria, not only in the same species or genus but also among distantly related bacteria (Thomas and Nielsen, 2005). In terms of conjugative apparatus and mobility, plasmids are classified into transmissible plasmids (i.e. conjugative or self-transmissible plasmids and mobilizable plasmids) and nonmobilizable plasmids (Smillie et al., 2010). For transmissible plasmids, the first possesses a complete set of conjugation genes comprising MOB genes required for the processing of conjugative DNA and mating pair formation (MPF) genes required for forming the mating channel; whereas, the latter confers only MOB genes allowing the plasmid to be mobilized by

conjugation when it co-exists in the same donor cell with a conjugative plasmid (Smillie et al., 2010, Garcillán-Barcia et al., 2011).

The mobility and rate of ARG dissemination of conjugative and mobilizable plasmids might increase when ARG are present within other mobile genetic elements, such as an integron. An integron is a bacterial genetic element containing a site-specific recombination system that is able to efficiently acquire, rearrange, and express genes embedded within a gene cassette (GC) (Fluit and Schmitz, 1999). Structurally, an integron consists of three major components: the *intI* gene; the recombination site (*attI*); and the promoter (Pc). The *intI* gene encodes for an enzyme called integrase that allows specific excision and integration of the gene cassette adjacent to the recombination site (*attI*) followed by the transcription and expression of the gene cassette through the integron promoter (Pc) (Fluit and Schmitz, 1999, Stalder et al., 2012, Gillings, 2014). Integrons have been highly associated with the occurrence of ARG, especially the genes related to the trimethoprim and sulfamethoxazole resistance (Chang et al., 2007, Ho et al., 2009). Within the integron structure, sulfamethoxazole resistance (*sul*) genes are commonly encoded in 3'- conserved segments, whereas trimethoprim resistance (*dfr*) genes are frequently found on the variable regions (gene cassette array) (Deng et al., 2015). It is not surprising that the occurrence of integrons might be used as an indicator the occurrence of *sul* and *dfr* genes (Šeputienė et al., 2010, Chen et al., 2015). Thus, the occurrence of integrons might be useful as an indicator the occurrence of *sul* and *dfr* genes.

Previous investigations indicate that ARG might emerge in water and sediment of Northwest Arkansas streams. It was reported that a number of *E. coli* phenotypically exhibiting multiple antibiotic resistance (MAR) to up to six different antibiotics, namely

ampicillin, gentamicin, ofloxacin, tetracycline, sulfamethoxazole and trimethoprim, plus plasmids were recovered in the effluent of WWTPs and stream water in Northwest Arkansas (Akiyama et al., 2010, Akiyama and Savin, 2010). Genotypically, these MAR determinants might be associated with mobile genetic elements of transmissible plasmids and integrons carrying sulfamethoxazole and trimethoprim resistance genes. Hence, the objectives of the research were to determine the presence of transmissible plasmids encoded by *mob* genes, integrons encoded by *intI* genes, sulfamethoxazole resistance genes encoded by *sul* genes, and trimethoprim resistance genes encoded by *dfp* genes, determine if transmissible plasmids and/or presence of integrons increases MAR number, and determine if sulfamethoxazole and trimethoprim resistance gene number increases with MAR number of *E. coli* isolated from treated wastewater effluent and receiving stream water.

C. Material and methods

A total of 139 *E. coli* isolates harboring plasmids and ARG were used as initial environmental sources. The isolates were recovered from four collection sites (WWTP effluent, 20 meters upstream, and 640 meters and 2000 meters downstream from effluent discharge) in an effluent-driven stream Mud Creek, a tributary of the Illinois River, located in Fayetteville, Arkansas (36.090° latitude, 94.111° longitude). Mud Creek tributary has a 293 ha of catchment area with 57.7%, 42.3%, and less than 1% of its land use for forests, urban, and pasture, correspondingly (Haggard et al., 2007). Initial water collection and data, and bacterial isolation and characterization procedures are provided in Akiyama and Savin (2010). Isolates were phenotypically resistant to up to six different antibiotics: 32 µg/mL ampicillin, 16 µg/mL gentamicin, 16 µg/mL tetracycline, 8 µg/mL ofloxacin, 4 µg/mL trimethoprim, and 80 µg/mL

sulfamethoxazole, as described in Akiyama and Savin (2010). Those isolates were stored in glycerol solution at -76 °C. Of 139 isolates, a total of 17 isolates are resistant to 5 and 6 antibiotics, whereas 30, 62, 11, and 19 isolates are identified to be resistant to 4, 3, 2, and 1 antibiotic, respectively.

E. coli strains were grown on selective Luria-Bertani (LB) agar plates containing antibiotic(s) which the isolates were originally determined to be resistant and incubated at 37 °C overnight. Single colonies were inoculated into 10 mL of selective LB broth (Mobio, Carlsbad, CA, USA) containing antibiotic(s) similar to LB agar plates which were incubated overnight at 37 °C with shaking at 150 rpm. Plasmid extraction was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instruction.

The isolated plasmids were profiled on 0.8% (w/v) agarose gels containing ethidium bromide at 50V for 200 min in 0.5X Tris-borate-EDTA (TBE) buffer and visualized using Kodak EDAS 290 gel documentation and analysis system (Eastman Kodak Co., Rochester, NY, USA). A lambda DNA *EcoRI* / *HindIII* molecular marker (Promega, Madison, WI, USA) was run in parallel with the plasmid samples to estimate the size.

The extracted plasmids of DNA were also used as templates to determine the occurrences of mobilization genes (*mob_{P11}*, *mob_{P14}*, *mob_{P51}*, *mob_{F11}*, *mob_{F12}*, *mob_{Q11}*, and *mob_{Qu}*); integrons (*intI1* and *intI2*); sulfamethoxazole (*sul1*, *sul2*, and *sul3*) and trimethoprim (*dhfrA1*, *dhfrA8*, *dhfrA12*, *dhfrA14*, *dhfrA17*, and *dhfrB3*) resistance genes using PCR amplification with primer sets outlined in Table 2.1. All PCR amplifications were performed in 20 µL reactions containing 1× PCR buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 400 ng/µL bovine serum albumin (Merck KGaA, Darmstadt, Germany), 0.5 µM of each primer, 1 µL of

template DNA, and 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). DEPC-treated water (EMD Millipore, Darmstadt, Germany) was used as no template control (NTC) run in parallel with samples. The PCR reactions were carried out using PTC-200 thermocycler (MJ Research, Waltham, MA) under conditions as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing (varied temperature, see Table 2.1) for 30 s, and 72°C for 60 s, with a final extension at 72°C for 8 min. The PCR products were analyzed on 1.5 % (w/v) agarose gels with ethidium bromide at 100V for 50 min in 0.5X TBE buffer and visualized using gel documentation to assess bands of the expected size. Additional confirmation of the PCR products was performed through DNA sequencing (Eurofin Genomics, Kansas City, Kansas, USA).

A statistical analysis was performed to evaluate the effects of occurrence of integron, mobilization genes, sulfamethoxazole resistance genes, and trimethoprim resistance genes towards the MAR number with 95% confidence intervals using GLIMMIX procedure on SAS 9.4 (Cary, North Carolina, USA). The data were analyzed based on a multinomial logit model with a cumulative logit link function and the results were back-transformed to the proportion scale for presentation of the results. Following the preliminary overall test for treatment effects contrasts were used to compare individual pairs of treatments ($P \leq 0.05$) on the cumulative logit scale.

D. Results

Plasmid profile analysis (Figure 2.1) showed the occurrence of multiple plasmids across MAR number. These plasmids conferred prevalent and diverse *mob*, *intl*, *sul*, and *dfr* genes that showed statistically unequal distributions across MAR number.

There was a statistically significant difference ($P = 0.0014$) in *mob* gene distribution among plasmids of isolates across MAR number (Figure 2.2). A total of 65 (46%) isolates conferred transmissible plasmids indicated by the presence of *mob* genes. A *mob* gene, *mob*_{F12}, was most prevalently detected on plasmids from 54 (39%) of total 139 isolates or 83% of the total transmissible plasmids. Interestingly, the *mob*_{F12} gene was not only detected singly (19% isolates) but also in coexistence with another *mob* gene (9% isolates) and with two other *mob* genes (4% isolates) across all MAR numbers. The co-occurrence of two or three *mob* genes, which often was *mob*_{F12} in combination with either *mob*_{P51} or *mob*_{Qu} (Appendix 2.1) resulted in a higher proportion of increased MAR in the resistant *E. coli* population (Figure 2.2).

Similar to the *mob* genes, there was also a very significant difference ($P < 0.0001$) in distribution of *intI* genes among plasmids of isolates across MAR number (Figure 2.3). A total of 64 (46%) plasmids of 139 isolates were detected to harbor integron genes which were subdivided into class 1 integron ((31% isolates); class 2 integron (2% isolates); and both class 1 and 2 integron ((14% isolates) (Appendix 2.2). The occurrence of integrons, particularly class 1 integrons, alone or in combination, shifted the distribution proportion of *E. coli* isolates such that a more of the population possessed larger MAR numbers (MAR 3 to MAR 5 or 6) (Figure 2.3).

Across MAR number, there was a significant difference ($P < 0.0001$) in distribution of *sul* genes among plasmids of isolates (Figure 2.4). Plasmids from a total of 99% of the isolates were positive for at least one *sul* gene, whereas 105 (75%) isolates displayed phenotypic resistance to sulfamethoxazole leading to a discrepancy between phenotypic and genotypic resistance, i.e. isolates did not show resistance but harbored *sul* genes, for 39 (24%) isolates. A

total of 74% isolates was redundant in encoding sulfamethoxazole resistance with coexistence of *sul1*, *sul2*, and/or *sul3* genes on plasmids from a single isolate. Coexistence of all 3 *sul* genes (*sul1*, *sul2*, and *sul3*) on plasmids from a single isolate accounted for 60 (43%) of the total 139 isolates (Appendix 2.3). This coexistence of all three *sul* genes was more likely to occur in plasmids of *E. coli* resistant to four, five, or six antibiotics than if there was only one or two *sul* genes (Figure 2.4). The occurrence of single *sul* gene and coexistence of two *sul* genes, on the other hand, were distributed similarly across MAR number compared to each other and were unlikely to be detected on the isolates resistant to five or six antibiotics.

Similar to *sul* genes, there was also a significant difference ($P < 0.0001$) of distribution of *dfr* genes among plasmids of isolates (Figure 2.5). A higher proportion of isolates (89%) harbored one or more genes for resistance to trimethoprim than had exhibited phenotypic resistance to the antibiotic (Figure 2.5). A total of 59% isolates was redundant in encoding trimethoprim resistance with coexistence of two to five *dfr* genes on plasmids from a single isolate. There were 84 (60%) isolates exhibiting phenotypic resistance leading to a discrepancy of phenotypic and genotypic resistance for 39 (29%) isolates. Similar to the results for sulfamethoxazole, the proportion of isolates exhibiting phenotypic resistance (60%) was similar to the proportion of the populations exhibiting redundancy in resistance genes against the trimethoprim. Three or four *dfr* genes were commonly detected in plasmids from a single isolate accounting for 19% or 20% isolates, correspondingly, of the total *E. coli*. The *dfrA1* gene was the most prevalent, followed by *dfrA12*, *dfrA8* and, *dfrA17*, whereas *dfrB3* and *dfrA14* gene were less often detected accounting for 10 (7%) and 5 (3.5%) of total isolates, respectively (Appendix 2.4). While single *dfrA1*, *dfrA8*, *dfrA12*, and *dfrA14* were detected in 30% of the total isolates, 17 combinations of two to five different *dfr* genes were

detected in 59% of total plasmids (Appendix 2.4). Plasmids conferring two to five coexisting *df_r* genes were likely to be harbored in a larger proportion of the isolate population showing phenotypic resistance to four, five, or six antibiotics (Figure 2.5).

E. Discussion

Prevalence of MAR isolates, and the association of their antibiotic resistance genes with mobile genetic elements such as plasmids and integrons, is increasingly leading to significant implications in both clinical and non-clinical settings. In the present study, the presence of multiple transmissible plasmids or integrons shifted the distribution of multiple antibiotic resistant bacteria toward larger multiple antibiotic resistant numbers. Antibiotic resistance genes which are potentially disseminated throughout the environment as transmissible (conjugative or mobilizable) plasmids play a major role in the spread of antibiotic resistance genes (Laroche-Ajzenberg et al., 2015). Integrons have also been found to be prevalent in multiple antibiotic resistant gram-negative and gram-positive bacteria (Ishikawa, 2011). Furthermore, the results of this study linked increased redundancy of sulfamethoxazole and trimethoprim resistance genes to isolates with increased multiple antibiotic resistance.

Almost half of the total plasmids in this study are characterized as transmissible plasmids indicated by the occurrence of *mob* genes. Similarly, approximately 50% of plasmids recovered from γ -proteobacteria including *E. coli* were found to be potentially transmissible (Garcillán-Barcia et al., 2009). Conjugative plasmids were considered a major means for the spread of antibiotic resistance genes from MAR *E. coli* coming from human fecal contamination (Laroche-Ajzenberg et al., 2015). Furthermore, the present study also

demonstrated that more than half of the positively detected transmissible plasmids were identified as *mob*_{F12}. The *mob*_{F12} genes were detected alone or in coexistence with other *mob* genes in plasmids of isolates conferring resistance from one to six antibiotics. These results are in agreement with the previous finding signifying *mob*_{F12} gene as the most frequently found *mob* gene among different genera of *Enterobacteriaceae* (Alvarado et al., 2012). Moreover, *mob*_{F12} was found on more than 90% of plasmids isolated from clinical multi-resistant *E. coli* (Garcillán-Barcia et al., 2015). Additionally, *mob*_{F12} was the most abundant *mob* gene found on plasmid of *E. coli* isolated from farm animal and human origins that were resistant to third-generation cephalosporin (de Been et al., 2014).

Perhaps more significantly were the occurrences of multiple relaxases within plasmids of the same isolate that resulted in the shift to increased proportion of isolates with larger MAR numbers. The occurrence of multiple plasmids within isolates was supported by the plasmid profile analysis in the current study, results of which confirmed a previous determination (Akiyama, 2009). The presence of multiple plasmids in isolates may account for redundancy in resistance genes in at least some of the *E. coli*, and may contribute to the persistence and dissemination of isolates.

In addition to *mob* genes, almost half of the isolates possessed integrons. In terms of MAR number, the present study demonstrated the positive relationship between increasing MAR number and class 1 integron existing singly or in coexistence with class 2 integron. That the presence of class 1 with or without class 2 integrons resulted in a shift towards a greater proportion of the population with higher MAR numbers may indicate the role of integrons in facilitating multiple antibiotic resistance (Kotlarska et al., 2015). Previous researchers have also indicated that bacteria harboring integron are generally resistant to at least three different

antibiotics (Xu et al., 2011, Koczura et al., 2012). Integrons can potentially be used as a tool to predict the presence of multiple antibiotic resistant bacteria in environmental samples (Ishikawa, 2011) as well as to determine anthropogenic pollution since integrons commonly carry a wide range of antibiotic resistance genes within their structure in a broad variety of pathogenic and nonpathogenic bacteria associated with human waste streams and agriculture (Gillings et al., 2014).

The prevalence of plasmid-mediated class 1 integron (46 % of the plasmids) corroborates previous findings. Class 1 and class 2 integron occurred in 40% and 10%, respectively, of multiple antibiotic resistant *E. coli* isolated from aquaculture water in Iran (Tajbakhsh et al., 2015) and 32% and 3%, of the antibiotic resistant *E. coli* isolated from two WWTPs and their receiving waters in Poland, respectively (Kotlarska et al., 2015). The discrepancy of occurrence between more predominantly found class 1 integron than class 2 integrons in all of these studies might be related to the association of class 1 integrons with multiple antibiotic resistance determinants since integrons confer greater diversity of ARG embedded within the gene cassette compared to the class 2 integrons (Koczura et al., 2014).

Sulfamethoxazole resistance (*sul*) genes and trimethoprim resistance (*df_r*) genes were plasmid-mediated in these *E. coli* isolates. Among *sul* genes, *sul2* gene was detected more frequently than *sul1* and *sul3* genes in plasmids of *E. coli* isolates in this study, supporting previous investigations demonstrating a gene-frequency distribution of *sul2*>*sul1*>*sul3* (Blahna et al., 2006). However, others found distributions of *sul1*>*sul2*>*sul3* (Phuong Hoa et al., 2008, Gündoğdu et al., 2011, Lu et al., 2015). Also, in the current study *sul3* genes were almost as frequently detected as *sul1* genes, and *sul3* genes were commonly detected in coexistence with two *sul* genes (detected in 21 plasmids) and/or three *sul* genes (detected in 60

plasmids) suggesting the emergence of *sul3* genes in the aquatic environment. Previously, *sul3* genes were the least common gene or were not detected in wastewater and shrimp ponds in North Vietnam (Phuong Hoa et al., 2008). The presence of the *sul3* gene was earlier reported in *E. coli* isolated from animal (Perreten and Boerlin 2003) and human (Grape et al., 2003) origins.

Regarding the MAR number, the current study signified the redundancy of plasmid-mediated *sul* genes existing among three *sul* genes in association towards the increasing MAR number. The occurrence of *sul1* genes are predominantly found in high levels in sulfonamide-resistant bacteria (Antunes et al., 2005) and are highly associated with class 1 integron as *sul1* gene is classically encoded within 3' conserved regions of class 1 integron (Fluit and Schmitz, 1999). The presence of *sul3* gene is emerging due to its association with integron that was recently identified as being encoded within the conserved segment (*sul3*-CS) of class 1 integron of plasmids recovered from commensal *E. coli* (Moran et al., 2016).

More than 89% of the plasmids in the present study conferred trimethoprim genes with a great diversity of different *dfr* genes detected in isolates, often within the same isolate, ranging from a single gene to up to five *dfr* genes. The four most predominant *dfr* genes in this study were *dfrA1*, followed by *dfrA12*, *dfrA8*, and *dfrA17* genes which is in accord with previous research demonstrating that *dfrA1* was the most predominant *dfr* gene, followed by *dfrA12*, in commensal *E. coli* from pigs in Poland (Mazurek et al., 2015). Both *dfrA1* and *dfrA12* genes were present in coexistence among two to four trimethoprim resistance genes. Others reported that *dfrA1* and *dfrA17* were found most frequently in clinical isolates, especially in sulfamethoxazole-trimethoprim resistant isolates from urine specimens (Brolund et al., 2010, Shin et al., 2015). Similar to *sul* genes, the occurrence of redundant *dfr* genes was

also positively correlated with the increasing MAR number. This signified the prevalence of *dfr* genes among multiple antibiotic resistant bacteria that might be also a result of associations with integrons. Previously, integron-associated trimethoprim resistant *E. coli* have been isolated from humans and animals in Lithuania (Šeputienė et al., 2010) or in Syria (Al-Assil et al., 2013).

Despite the prevalence and diversity of *sul* and *dfr* genes, a discrepancy between phenotypic and genotypic resistance occurred in the present study accounting for 24% and 28%, respectively. This finding indicates those isolates that did not show resistance phenotypically, but harbor corresponding antibiotic resistance genes (also known as silent/cryptic resistance) might act a reservoir of ARG in antibiotic resistome (Perry et al. 2014). These genes do not contribute to the phenotypic resistance in their original genetic state, yet their potential determinants might be expressed by mutation, mobilization, or alteration of the expression (Perry et al. 2014). Additionally, the diversity and the abundance of the potential genes in the resistome might be accelerated along with horizontal gene transfer induced by the selective pressure of antibiotics in the environment (Gillings 2013).

The prevalence and diversity of both *sul* and *dfr* genes in the present study demonstrates a redundancy among plasmid-mediated sulfamethoxazole and trimethoprim resistant genes. Isolates appear to be accumulating ARG within plasmids, which could be a result of recombination events (Bennett, 2008). Moreover, both sulfamethoxazole and trimethoprim resistance genes are highly associated with other mobile genetic elements, i.e. integron and insertion sequences (Shin et al., 2015). As a part of adaptation to the changing selective pressure of antibiotics, including at sub-inhibitory level, in the environment, bacteria may be duplicating and amplifying their antibiotic resistance genes, leading to

increasing/reversing their plasmid size (Sandegren and Andersson, 2009). Sub-inhibitory concentrations of antibiotics are likely to be present in the stream water environment. A total of 5.8 $\mu\text{g L}^{-1}$ of antibiotics have been detected in addition to various pharmaceutical and other organic chemicals in stream water including Mud Creek, the stream where the isolates harboring plasmids originally sampled for this study, in north-central and northwestern Arkansas (Haggard et al., 2006). Massey et al. (2010) detected trimethoprim at concentrations of 0.029-0.061 $\mu\text{g L}^{-1}$ and sulfamethoxazole at concentrations of 0.356-0.564 $\mu\text{g L}^{-1}$ in Mud Creek water. A complex mixture of antibiotic resistant bacteria harboring mobile genetic elements, i.e. transmissible plasmids and integron, in the WWTP is an ideal hotspot and reservoirs for antibiotic resistance genes to be widely disseminated into aquatic environments (Marti et al., 2014). Therefore, further study is needed to determine the effect of each antibiotic, particularly at sub-inhibitory level, towards the persistence of the isolates harboring different integron-*mob* designation.

F. Conclusion

Half of the total plasmids of multiple antibiotic resistant *E. coli* were transmissible as indicated by the existence of mobilization genes, with *mob*_{F12} gene the most frequently detected on the isolates conferring resistance from one to six antibiotics. While *mob* and *intI* genes did not necessarily occur in the same plasmids, about half of multiple antibiotic resistant *E. coli* also conferred plasmid-mediated integron. The current study signified the prevalence of plasmid carrying single or coexistence of three *sul* genes and carrying single or a combination up to five *dfr* genes, even in isolates not exhibiting phenotypic resistance to either antibiotic. Having two or more *mob*, one or two *intI*, three *sul*, and/or a combination of

two to five *dfr* genes each contributed to significantly increasing the proportion of the *E. coli* population exhibiting larger multiple phenotypic antibiotic resistances. Overall, these findings indicate the role of transmissible plasmid-mediated and integron-associated antibiotic (sulfamethoxazole and/or trimethoprim) resistance gene diversity among bacteria, which may contribute to persistence of antibiotic resistance in the stream environment.

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Table 2.1. Target genes and their corresponding primer sequences with amplification features of PCR

Target Gene		Primer sequence	Annealing Temperature (°C)	Product size (bp)	References
<i>sul1</i>	FW ^a RV	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	55.9	163	(Pei et al., 2006)
<i>sul2</i>	FW RV	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	55.9	191	(Pei et al., 2006)
<i>sul3</i>	FW RV	TCCGTTCAGCGAATTGGTGCAG TTCGTTACGCCTTACACCAGC	60.8	128	(Pei et al., 2006)
<i>dfrA1</i>	FW RV	GTGAAACTATCACTAATGG ACCCTTTTGCCAGATTTG	46	471	(Šeputienė et al., 2010)
47 <i>dfrA8</i>	FW RV	TTGGGAAGGACAACGCACTT ACCATTTTCGGCCAGATCAAC	56.3	382	(Šeputienė et al., 2010)
<i>dfrA12</i>	FW RV	GGTGAGCARAAGATYTTTCGC TGGGAAGAAGGCGTCACCCTC	52	309	(Šeputienė et al., 2010)
<i>dfrA14</i>	FW RV	GCBAAGGDGARCAGCT TTMCCAYATTTGATAGC	50.8	394	(Šeputienė et al., 2010)
<i>dfrA17</i>	FW RV	AAAATTTTCATTGATTTCTGCA TTAGCCTTTTTTCCAAATCT	48.4	471	(Šeputienė et al., 2010)
<i>dfrB3</i>	FW RV	GATCACGTRCGCAAGAARTC GACTCGACVGCRTASCCTTC	56	95	(Šeputienė et al., 2010)
<i>intI1</i>	FW RV	GGGTCAAGGATCTGGATTTTCG ACATGCGTGTAATCATCGTCG	62	483	(Mazel et al., 2000)

Table 2.1. Target genes and their corresponding primer sequences with amplification features of PCR (Con't.)

Target Gene		Primer sequence	Annealing Temperature (°C)	Product size (bp)	References
<i>intI2</i>	FW RV	CAAGCATCTCTAGGCGTA AGAAGCATCAGTCCATCC	50	233	(Mazel et al., 2000)
<i>mob_{P11}</i>	FW RV	CGTGCGAAGGGCGACAARACBTAYCA CCCTTGCCTGGTGYTSNACCCA	60	180	(Alvarado et al., 2012)
<i>mob_{P14}</i>	FW RV	CGCAGCAAGGACACCATCAAAYCAYTAYRT CCCTTGCCTGGTGYTSNACCCA	50	174	(Alvarado et al., 2012)
<i>mob_{P51}</i>	FW RV	TACCACGCCCTATGCGAARAARTAYAC CCCTTGCCTGGTGYTSNACCCA	58	167	(Alvarado et al., 2012)
<i>mob_{F11}</i>	FW RV	GCAGCGTATTACTTCTCTGCTGCCGAYGAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	53	234	(Alvarado et al., 2012)
<i>mob_{F12}</i>	FW RV	AGCGACGGCAATTATTACACCGACAAGGAYAAAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	55	234	(Alvarado et al., 2012)
<i>mob_{Q11}</i>	FW RV	CAATCGTCCAAGGCGAARGCNGAYTA CGCTCGGAGATCATCAYYTGYCAYTG	50	331	(Alvarado et al., 2012)
<i>mob_{Qu}</i>	FW RV	AGCGCCGTGCTGTCCGCBGCNTAYCG CTCCGCAGCCTCGRCSGCRRTCCA	64	179	(Alvarado et al., 2012)

^aFW: forward; RV: reverse

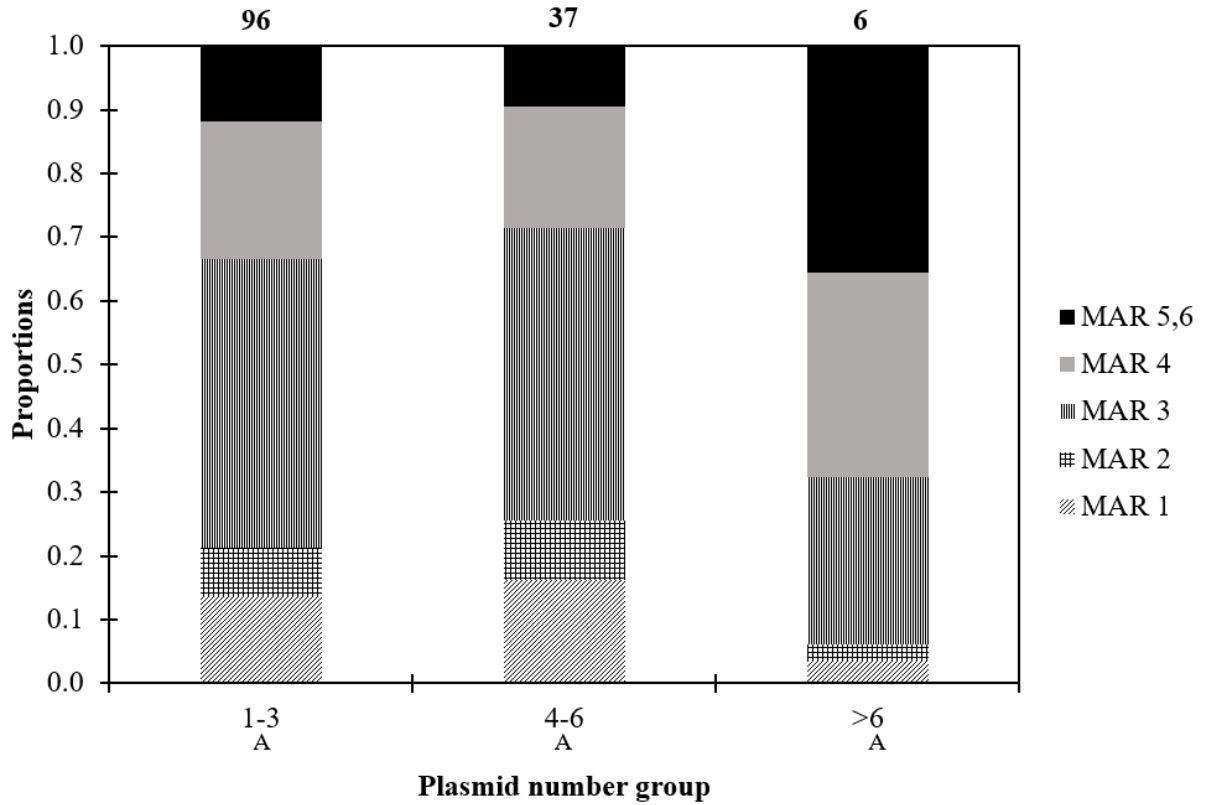


Figure 2.1. Proportion of plasmid number among multiple antibiotic resistant *E. coli* isolated from Mud Creek in Fayetteville, Arkansas. The same letters at the bottom of plasmid group number are not significantly different using an overall test for equality of distributions on the cumulative logit scale contrasts procedure ($P \leq 0.05$). Numbers at the top represent the sum of occurrences by row or column.

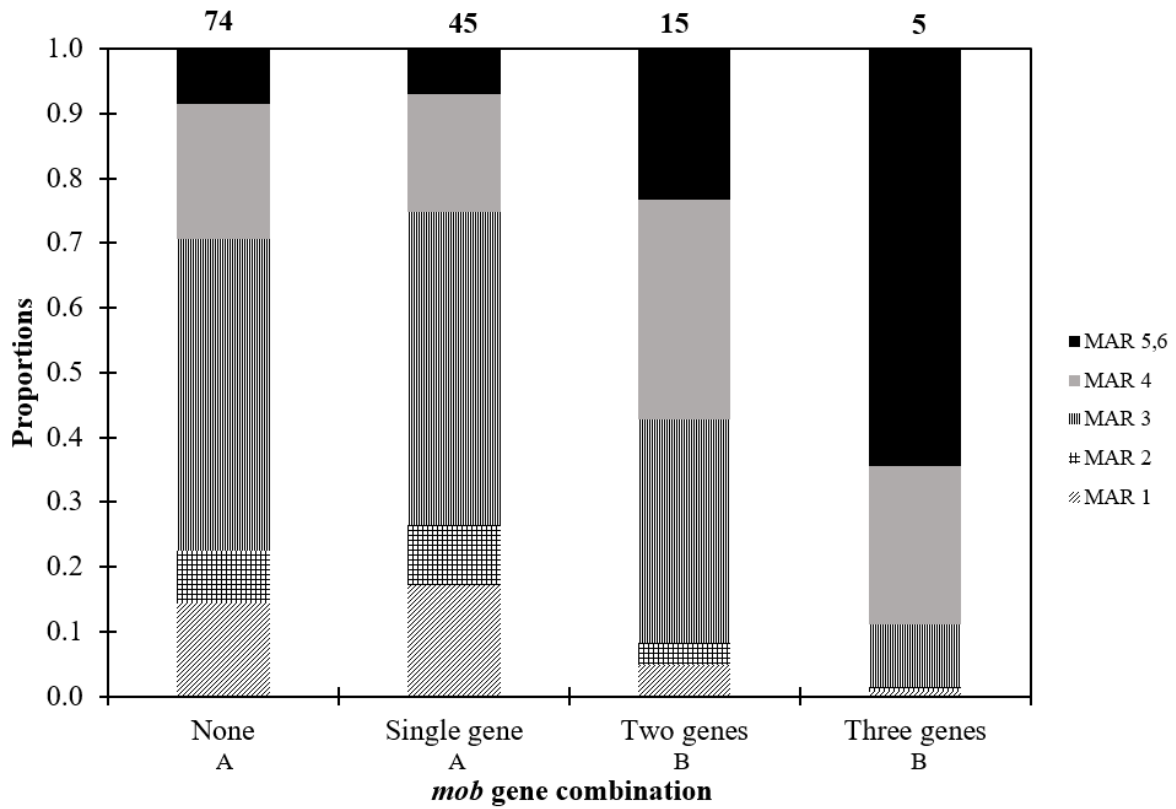


Figure 2.2. Proportion of *mob* genes among multiple antibiotic resistant *E. coli* isolated from Mud Creek in Fayetteville, Arkansas. The same letters at the bottom of gene combinations are not significantly different using an overall test for equality of distributions on the cumulative logit scale contrasts procedure ($P \leq 0.05$). Numbers at the top represent the sum of occurrences by row or column.

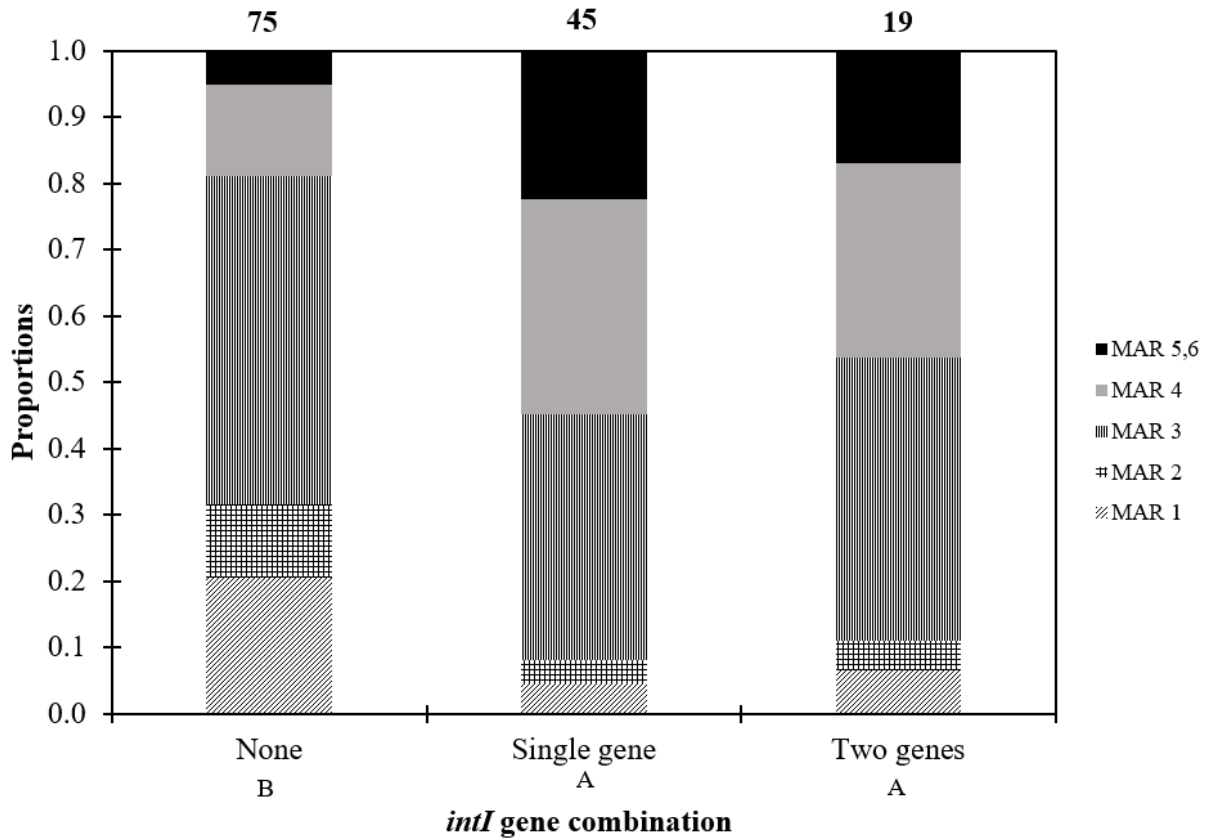


Figure 2.3. Proportion of *intI* genes among multiple antibiotic resistant *E. coli* isolated from Mud Creek in Fayetteville, Arkansas. The same letters at the bottom of gene combinations are not significantly different using an overall test for equality of distributions on the cumulative logit scale contrasts procedure ($P \leq 0.05$). Numbers at the top represent the sum of occurrences by row or column.

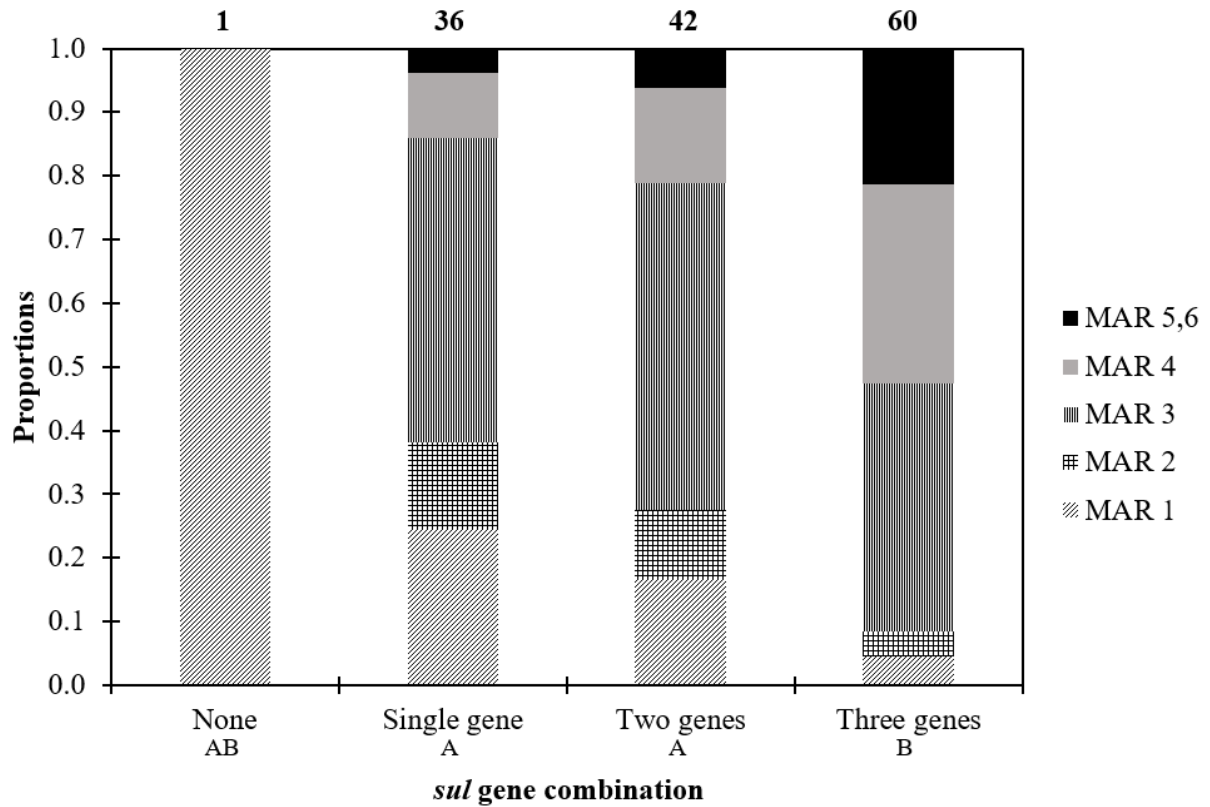


Figure 2.4. Proportion of *sul* genes among multiple antibiotic resistant *E. coli* isolated from Mud Creek in Fayetteville, Arkansas. The same letters at the bottom of gene combinations are not significantly different using an overall test for equality of distributions on the cumulative logit scale contrasts procedure ($P \leq 0.05$). Numbers at the top represent the sum of occurrences by row or column.

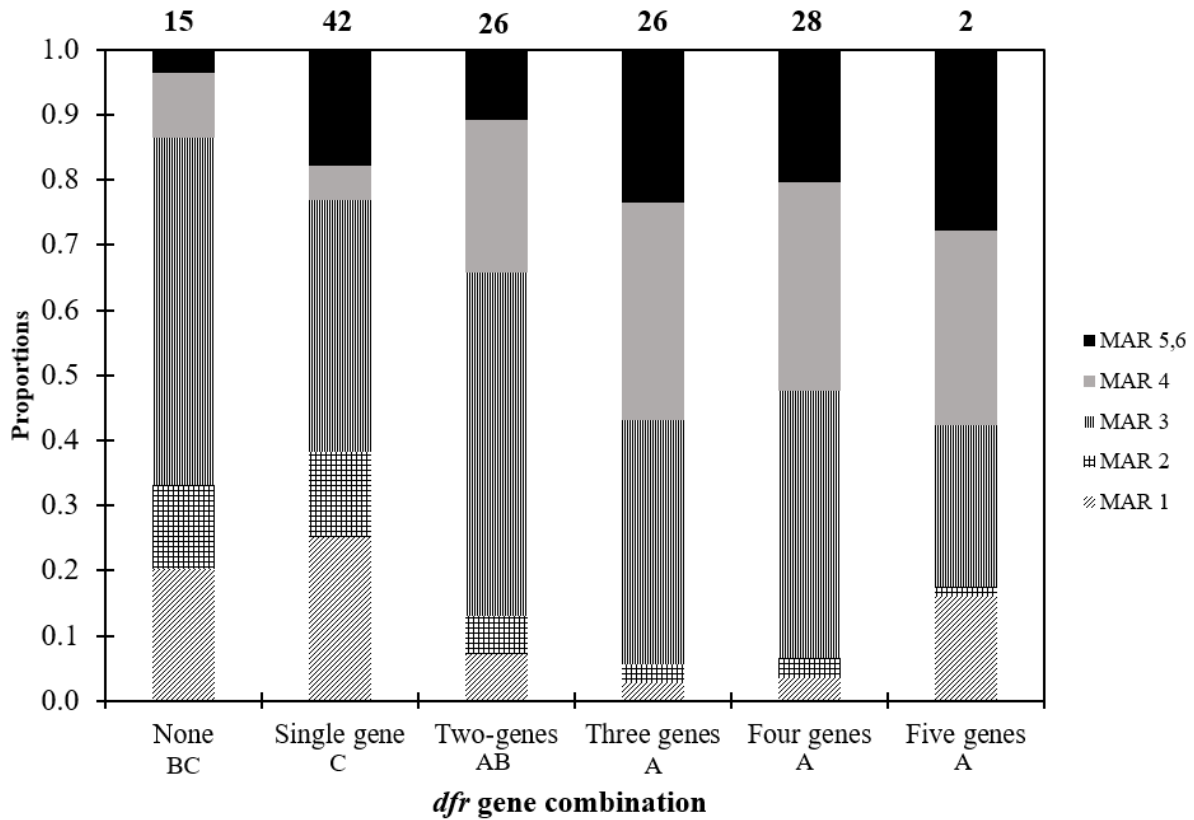


Figure 2.4. Proportion of *dfr* genes among multiple antibiotic resistant *E. coli* isolated from Mud Creek in Fayetteville, Arkansas. The same letters at the bottom of gene combinations are not significantly different using an overall test for equality of distributions on the cumulative logit scale contrasts procedure ($P \leq 0.05$). Numbers at the top represent the sum of occurrences by row or column.

CHAPTER 3. GENETIC REDUNDANCY AND PERSISTENCE OF PLASMID-
MEDIATED TRIMETHOPRIM/SULFAMETHOXAZOLE RESISTANT *Escherichia coli*
ISOLATED FROM WASTEWATER TREATMENT PLANT EFFLUENT AND STREAM
WATER IN NORTHWEST ARKANSAS

Genetic redundancy and persistence of plasmid-mediated trimethoprim/sulfamethoxazole resistant *Escherichia coli* isolated from wastewater treatment plant effluent and stream water in Northwest Arkansas

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A. Abstract

Antibiotic resistant bacteria may persist in effluent receiving surface water in the presence of sub-inhibitory antibiotic concentrations if the bacteria possess multiple genes encoding resistance to the same antibiotic. This redundancy of antibiotic resistance genes may occur in plasmids conferring conjugation and mobilization (*mob*) and integrase (*intI*) genes. Plasmids extracted from 76 sulfamethoxazole-trimethoprim resistant *E. coli* originally isolated from an effluent-receiving stream were used as DNA template to identify sulfamethoxazole (*sul*) and trimethoprim (*dfr*) resistance genes plus detect the presence of *intI* and *mob* genes using PCR. Sulfamethoxazole and trimethoprim resistance was plasmid-mediated with three *sul* (*sul1*, *sul2* and *sul3* genes) and four *dfr* genes (*dfrA12*, *dfrA8*, *dfrA17*, and *dfrA1* gene) the most prevalently detected. Approximately half of the plasmids conferred class 1 and/or 2 integron and, although unrelated, half were also transmissible. Sampling site in relationship to effluent input significantly affected the number of *intI* and *mob* but not the number of *sul* and *dfr* genes. In the presence of sub-inhibitory sulfamethoxazole concentration, isolates persisted regardless of integron and mobilization gene designation, whereas in the presence of trimethoprim, the presence of both integron and mobilization genes made isolates less persistent than in the absence of both or the presence of a gene from either group individually. Regardless, isolates persisted in large concentrations throughout the experiment. Treated effluent containing antibiotic resistant bacteria may be an important source of integrase and mobilization genes into the stream environment. Sulfamethoxazole-trimethoprim resistant bacteria may have a high degree of genetic redundancy and diversity conferring resistance to each antibiotic, although the role integrase and mobilization genes towards persistence is unclear.

B. Introduction

Antibiotic resistance is an alarming global issue. Extensive use of antibiotics in human, veterinary, and agricultural practice is increasing concern about the spread of antibiotic resistance throughout the environment. The genes responsible for the antibiotic resistance determinants, termed as antibiotic resistant genes (ARGs), are predominantly encoded in mobile genetic elements, such as plasmids. In spite of generating metabolic costs, conjugative plasmids contribute to the persistence of bacterial host cells across generations by means of providing fitness advantages (Dionisio et al., 2005). Conjugative plasmids contain ‘backbone modules’ conferring genes, such as mobilization (*mob*) genes, for replication, stability, and propagation; and ‘adaptation modules’ conferring translocative and operative elements, such as integron, allowing bacterial hosts to succeed within environments (Norman et al., 2009). A mobilization gene encodes for relaxase, a protein responsible for DNA processing reactions in conjugation or mobilization (De La Cruz et al., 2010, Smillie et al., 2010). An integron is a genetic unit that is able to acquire, integrate, rearrange, and express resistant determinants within a gene cassette (Deng et al., 2015).

Since the 1990s, sulfamethoxazole and trimethoprim have been first-line antibiotics of choice administered in combination to treat bacterial infections, particularly in urinary tract infections (UTIs) (Nicolle, 2003). Both antibiotics disrupt folic acid synthesis in bacteria; disruption occurs by means of inhibition of dihydropteroate synthetases (DHPS) in the presence of sulfamethoxazole and inhibition of dihydrofolate reductase (DHFR) in the presence of trimethoprim (Eliopoulos and Huovinen, 2001). However, resistance of bacterial pathogens to both sulfamethoxazole and trimethoprim altering the efficacy of UTIs’ treatment has been reported (Guneysel et al., 2009). This is mainly as consequence of the

occurrence of insensitive DHPS and/or DHFR enzymes to the drugs resulting from natural or mutational or acquired changes of the enzymes (Eliopoulos and Huovinen, 2001). To date, there are three different genes associated with insensitive DHPS enzyme, namely *sul1*, *sul2*, and *sul3* (Gündoğdu et al., 2011), and more than 30 different genes associated with insensitive DHFR enzymes (Šeputienė et al., 2010). Genes encoding insensitive DHPS and/or DHFR enzymes are transferable and highly associated with mobile genetic elements, such as integrons and insertion sequence common regions (ISCRs)(Shin et al., 2015).

Wastewater treatment plants (WWTPs) can serve as input sources to streams of ARGs, bacteria, and antibiotics, including the aforementioned sulfamethoxazole and trimethoprim (Göbel et al., 2007). The WWTPs receive inflow containing antibiotic resistant bacteria originating from various anthropogenic sources, such as hospitals, residences, industries, and agriculture. Despite different disinfection protocols in different WWTPs, *Escherichia coli* and broad-host-range (BHR) plasmids (Akiyama et al., 2010), and ARG (MacLeod and Savin, 2014) remain in discharged WWTP effluents, which lead to inputs of corresponding plasmids into receiving streams. Previous investigations recovered a number of *E. coli* possessing ARG (Akiyama and Savin, 2010) and plasmids (Akiyama et al., 2010) from one site upstream (20 m upstream), WWTP effluent discharge, and two sites downstream (640 and 2000 m) of the pipe discharging water from the Fayetteville, Arkansas WWTP into Mud Creek (Akiyama and Savin, 2010). Antibiotics, including sulfamethoxazole and trimethoprim, pharmaceuticals, and other organic compounds have also been detected in northwestern and north-central Arkansas streams (Haggard et al., 2006, Massey et al., 2010).

It was hypothesized that a greater proportion of plasmids of sulfamethoxazole-trimethoprim resistant *E. coli* isolated from effluent and downstream water would also harbor

mob genes and/or integrons compared to *E. coli* isolated from upstream water. Furthermore, it was hypothesized that *E. coli* isolates containing plasmids harboring *sul* and *dfr* and *mob* genes and/or integrons would demonstrate greater persistence in artificial wastewater containing low levels of antibiotics than *E. coli* isolates containing plasmids harboring *sul* and *dfr* genes in the absence of *mob* genes and integrons. The objectives of the research were to determine if treated wastewater effluent and receiving stream water sampling location impacted the detection of integrase and mobilization genes with sulfamethoxazole and trimethoprim resistance genes on plasmids in *E. coli* and to determine the influence of those integrase and mobilization genes towards the persistence of the isolates.

C. Material and methods

Initial environmental sources were 76 isolates of phenotypically sulfamethoxazole (80 $\mu\text{g mL}^{-1}$) and trimethoprim (4 $\mu\text{g mL}^{-1}$) resistant *E. coli* harboring plasmids recovered from four collection sites of an effluent-driven stream Mud Creek, a tributary of the Illinois River located in Fayetteville, Arkansas (36.090° latitude, 94.111° longitude). Sample locations included WWTP effluent (ME), 20 meters upstream (M1), and receiving stream water 640 meters (M2) and 2000 meters (M3) downstream of effluent discharge (Akiyama and Savin, 2010). Mud Creek tributary has a 293 ha of catchment area with 57.7%, 42.3%, and less than 1% of its land use for forests, urban, and pasture, correspondingly (Haggard et al., 2007). See Akiyama et al. (2010) for description of original sampling, water properties, and bacterial isolation and characterization.

The *E. coli* isolates were stored in glycerol solution at -76 °C and grown overnight at 37 °C on selective Luria-Bertani (LB) agar plates containing both sulfamethoxazole (80 μg

mL⁻¹) and trimethoprim (4 µg mL⁻¹) based on their minimum inhibitory concentration (MIC). Single colonies were inoculated in 10 mL of selective LB broth (Mobio, Carlsbad, CA) containing antibiotics similar to LB agar plates and incubated overnight at 37 °C with shaking at 150 rpm. Plasmid extraction was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer's instruction.

The occurrences of sulfamethoxazole (*sul1*, *sul2*, and *sul3*) and trimethoprim (*dhfrA1*, *dhfrA8*, *dhfrA12*, *dhfrA14*, *dhfrA17*, and *dhfrB3*) resistance genes plus integrons (*intI1* and *intI2*), and mobilization genes (*mob_{P11}*, *mob_{P14}*, *mob_{P51}*, *mob_{F11}*, *mob_{F12}*, *mob_{Q11}*, and *mob_{Qu}*) were determined using PCR amplification with primer sets outlined in Table 1. All PCR amplifications were performed in 20 µL reactions containing 1× PCR buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 400 ng/µL bovine serum albumin (Merck KGaA, Darmstadt, Germany), 0.5 µM of each primer, 1 µL of template DNA, and 0.5 U of GoTaq DNA polymerase (Promega, Madison, WI). DEPC-treated water (EMD Millipore, Darmstadt, Germany) was used as no template control (NTC) run in parallel with samples. The PCR reactions were carried out using PTC-200 thermocycler (MJ Research, Waltham, MA) under conditions as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing (varied temperature, see Table 1) for 30 s, and 72°C for 60 s, with a final extension at 72°C for 8 min. The PCR products were analyzed on 1.5 % (w/v) agarose gels with ethidium bromide at 100V for 50 min in 0.5X Tris-borate-EDTA (TBE) buffer and visualized using Kodak EDAS 290 gel documentation and analysis system (Eastman Kodak Co., Rochester, NY) to assess bands of the expected size. Additional confirmation of the PCR products was performed through DNA sequencing (Eurofin Genomics, Kansas City, KS).

The influence of plasmid-mediated *mob* and *int* genes on persistence of *E. coli* isolates over time was tested in 500-mL sterile Erlenmeyer flasks containing 200 mL synthetic wastewater made from components as described by McKinney (1962) supplied with antibiotics (either 0.19 $\mu\text{g L}^{-1}$ trimethoprim or 0.5 $\mu\text{g L}^{-1}$ sulfamethoxazole). Isolates were placed into one of four groups according to *intI* and *mob* gene presence/absence combinations: group I (*mob*⁺*intI*⁺), group II (*mob*⁻*intI*⁺), group III (*mob*⁺*intI*⁻), or group IV (*mob*⁻*intI*⁻). The flasks were maintained at 23°C for 11 days, with 3 mL removed from each flask after 1, 3, 5, 7, 9 and 11 days of incubation. The colony forming unit (CFU) number on day 1, 7, 9, and 11 was determined using plate count assay on selective tryptic soy agar media supplemented with either sulfamethoxazole (80 mg L⁻¹) or trimethoprim (4 mg L⁻¹).

A statistical analysis was performed to evaluate the effects of occurrence of sulfamethoxazole resistance genes, and trimethoprim resistance genes, integron, and mobilization genes, towards the sites, with 95% confidence intervals using GLIMMIX procedure on SAS 9.4 (Cary, North Carolina, USA). The data were analyzed based on a multinomial logit model with a cumulative logit link function and the results were back-transformed to the proportion scale for presentation of the results. Following the preliminary overall test for treatment effects contrasts were used to compare individual pairs of treatments ($P \leq 0.05$) on the cumulative logit scale.

An analysis of variance (ANOVA) was performed to evaluate the effects of mobilization and/or integron presence or absence, days of incubation, and the combination of gene presence/absence and time on bacterial concentration in the presence of each antibiotic. When appropriate, means were separated by Fisher's least significant difference (LSD) at $\alpha =$

0.05. Analysis was performed using GLM procedure with 95% confidence intervals on SAS 9.4 (Cary, NC).

D. Results

The occurrence of *sul* and *dfr* genes among sulfamethoxazole-trimethoprim resistant isolates per site of collection (upstream (M1); WWTP effluent (ME); first downstream (M2); and second downstream (M3)) is listed in Figure 3.1 and 3.2, respectively. While *E. coli* isolates were selected based on phenotypic expression of sulfamethoxazole-trimethoprim resistance, PCR confirmed the genetic basis, and confirmed the location of resistance specifically to plasmid DNA for at least one, and frequently more than one, *sul* and *dfr* gene. The proportion of isolates conferring *sul* genes distributed across the sites did not change as number of *sul* genes present in the plasmids changed ($P = 0.4310$). A total of 96% isolates in the present study was redundant in encoding sulfamethoxazole resistance with coexistence of *sul1*, *sul2*, and/or *sul3* genes on plasmids from a single isolate. Coexistence of all 3 *sul* genes (*sul1*, *sul2*, and *sul3*) on plasmids from a single isolate accounted for 56 (74%) of the total 76 isolates across all sites (Figure 3.1). Plasmids conferring all three *sul* genes were also positive for class 1 integron, class 2 integron, or both class 1 and class 2 integrons in 14 (18%), 2 (3%), and 12 (16%) of the total isolates, respectively. Plasmids encoding both *sul1* and *sul3* genes or *sul1* and *sul2* genes from a single isolate were recovered from almost all sites and accounted for 12 (16%) and 5 (7%) isolates, respectively.

Similar to *sul* genes, the proportion of isolates conferring *dfr* genes distributed across the sites did not change as number of *dfr* genes present in the plasmids changed ($P = 0.9990$). A total of 96% isolates in the present study was redundant in encoding trimethoprim

resistance with coexistence of two to five *dfr* genes on plasmids from a single isolate (Figure 3.2). Five *dfr* genes were detected within a single isolate from both the effluent and the first downstream location. Three *dfr* genes (*dfrA1* + *dfrA8* + *dfrA12*; *dfrA1* + *dfrA8* + *dfrA17*; *dfrA1* + *dfrA12* + *dfrA17*; *dfrA8* + *dfrA12* + *dfrA17*; and *dfrA8* + *dfrA12* + *dfrB3*) or four *dfr* genes (*dfrA1* + *dfrA8* + *dfrA12* + *dfrA17*; *dfrA1* + *dfrA8* + *dfrA12* + *dfrB3*; and *dfrA8* + *dfrA12* + *dfrA14* + *dfrA17*) were commonly detected in plasmids from a single isolate at all locations accounting for 26 (34%) or 28 (37%), correspondingly, of the *E. coli* isolated among the sites.

The proportions of isolates conferring *intI* genes were significantly different across sites ($P = 0.0176$) (Figure 3.3). A total of 38 (50%) plasmids harbored integrase genes which were further subdivided into class 1 integron (18 isolates); class 2 integron (2 isolates); and both class 1 and 2 integron (18 isolates). Plasmids conferring class 1 integron were prevalent on isolates recovered from effluent, whereas plasmids harboring class 2 integron alone were detected upstream only. Fourteen (18%) *E. coli* isolated from effluent contained plasmids harboring class 1 integron and eight contained plasmids harboring both class 1 and 2 integrons. By the second downstream location, if integrons were detected in plasmids, plasmids harbored both class 1 and 2 integrons.

There was also a significantly different proportion of isolates conferring *mob* genes in plasmids across sites ($P = 0.0035$). Similar to the percentage of isolates that did not harbor integrons in plasmids, half of the isolates did not harbor *mob* genes (Figure 3.4), although these were not necessarily the same isolates lacking integrase. All but three stream water isolates in which a *mob* gene was detected revealed only one *mob* gene, which was most frequently *mob*_{F12}. The single *mob* gene, *mob*_{F12}, was detected on plasmids from 20 (26.3%)

isolates. The *mob* genes on plasmids recovered from the effluent contained one-*mob* gene, i.e. *mob*_{F12} or *mob*_{Qu} from two isolates each; two-*mob* genes, i.e. *mob*_{P51} + *mob*_{F11} in one isolate, *mob*_{P51} + *mob*_{F12} in one isolate, and *mob*_{F12} + *mob*_{Qu} in five different isolates; or three-*mob* genes, i.e. *mob*_{P51} + *mob*_{F11} + *mob*_{F12} in one isolate; *mob*_{P51} + *mob*_{F12} + *mob*_{Q12} in one isolate, and *mob*_{P51} + *mob*_{F12} + *mob*_{Qu} in one isolate, and *mob*_{F12} + *mob*_{Q12} + *mob*_{Qu} in two isolates.

In terms of persistence, there was significant effect of incubation time on bacterial concentration when grown in the presence of sub-inhibitory concentration of sulfamethoxazole regardless the *mob*-integron designation (Table 6). Despite its significant decrease on day 11, isolates persisted during incubation such that concentrations remained at almost 1 billion CFU per mL. In the presence of trimethoprim, there was a significant interaction of integrase by mobilization gene presence or absence with incubation time affecting bacterial growth (Figure 3.5). Bacterial concentration harboring plasmids with both integron and mobilization genes decreased over time; however, after 11 days, bacterial concentrations in all treatments remained over 1 billion CFU per mL. Isolates harboring plasmids absent in either or both integron and mobilization genes did not significantly decrease in concentration during the experiment.

E. Discussion

Increasing occurrence of sulfamethoxazole and trimethoprim resistance among both environmental and clinical isolates, followed by the association of antibiotic resistance genes with mobile genetic elements, such as plasmids and integrons, have human health, agricultural management and ecological implications. The present study confirmed not just occurrence of *sul* and *dfr* genes among sulfamethoxazole and trimethoprim resistant *E coli* isolates

recovered from upstream, effluent, and downstream of WWTP effluent input into stream water in Fayetteville, AR, but a diversity of resistance genes to the same antibiotic from bacteria grown from a single isolate. Plasmids of the all isolates tested possessed at least one *sul* and *dfr* gene; however, the majority possessed more than one of each gene, indicating more redundancy and greater diversity of plasmid-encoded-sulfamethoxazole and trimethoprim resistance genes among the isolates of resistant bacteria within WWTP and the receiving water than expected. The occurrence of multiple *dfr* and *sul* genes in the same isolate has been reported at low frequency (Brolund et al., 2010, Šeputienė et al., 2010). However, the results in this study are substantiated by previous findings demonstrating multiple *dfr* and *sul* genes in single strains are not uncommon and accounted for 71 (20%) of 352 *E. coli* isolates from pigs (Mazurek et al., 2015). Additionally, it was reported that plasmid-borne *sul1* and *sul2* genes in combination with *dfrA17* and *dfrA12* genes could concomitantly lead to high rates of sulfamethoxazole-trimethoprim resistance (Hu et al., 2011). Others have also found diversity of sulfamethoxazole and trimethoprim resistance genes in clinical isolates of gram-negative *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter* spp., and *Pseudomonas aeruginosa* collected from urine specimens in Korea (Shin et al., 2015), commensal *E. coli* isolated from pigs in Poland (Mazurek et al., 2015), and *E. coli* from human and food-producing animal in Hong Kong (Ho et al., 2009). These findings suggest that sulfamethoxazole-trimethoprim resistant bacteria conferring corresponding ARG are likely to survive in WWTP as previously reported (Ziemińska-Buczyńska et al., 2015).

There was greater detection of *sul1*, *dfr1*, and ARG at the effluent and downstream of WWTP in Nykvarnsverket, Sweden (Berglund et al., 2015). Czekalski et al. (2014) using spatial mapping, found the high abundance of sulfamethoxazole resistance (*sul1*) gene

accumulated around the discharge pipe of WWTP in Lausanne, Switzerland. In contrast to those two studies, both *sul* and *dfr* genes were prevalent at all sampling locations in this study, including upstream, effluent, and downstream of effluent input. Commonly, within the integron structure, sulfamethoxazole resistance, particularly *sulI* genes, are encoded in 3'-conserved segments, whereas trimethoprim resistance (*dfr*) genes are frequently found on the variable regions (gene cassette array) (Barbolla et al., 2004, Deng et al., 2015). However, in the present study the occurrences of *sul* and *dfr* genes were not necessarily associated with class 1 integron indicating both resistance genes might be encoded within larger mobile genetic elements than integron, i.e. insertion sequences (IS) or plasmids. There were several reports demonstrating lack of association with class 1 integron among sulfamethoxazole or trimethoprim resistant isolates conferring *sulI*-positive as well as *dfr*-positive isolates (Chung et al., 2015). Others signified the association of *sul* and *dfr* genes in insertion sequences (IS) (Shin et al., 2015) and plasmids (Hu et al., 2011). Despite the lack of effect of sampling location on the *sul* and *dfr* antibiotic resistance genes, class 1 integrons were predominantly detected alone or in combination with class 2 integron, in effluent and downstream from upstream. These results support previous research demonstrating the proportion of class 1 integron detected in effluent (38.9%) was almost as twice that in influent (20.4%) of sewage treatment plant in Nanjing, China (Ma et al., 2011). Class 1 integrons were found with highest abundance from antibiotic resistant bacteria isolated from the closest site of the industrial-driven effluent discharge in the Savannah River Site, Georgia (McArthur et al., 2011). Also, interestingly, Marathe et al. (2013) isolated 96 bacterial strains from an Indian industrial WWTP that receives bulk waste input from pharmaceutical manufacturers. The 96 bacterial strains showed resistance to 20 or more antibiotics with 95% of the bacteria possessing at least

one type of integron. The present study and previous findings signify effluent as an important source of bacteria conferring integron-borne ARG. This might increase the likelihood of emergence of integron-mediated antibiotic resistance among bacteria into the stream environment since integrons, especially class 1 integron, possess key potential in genetic exchange by means of horizontal dissemination (Domingues et al., 2012).

Similar to the integrase genes, there was significant effect of site origin of isolates on the proportion of transmissible plasmid conferring *mob* genes. The transmissible plasmids were mostly detected in plasmids of the isolates recovered from effluent and first downstream site. This result supports that of a previous study demonstrating the occurrences of *E. coli* strains harboring conjugative plasmids carrying numerous resistances isolated from a WWTP effluent-driven river in Haute-Normandie (France) (France) (Laroche-Ajzenberg et al., 2015). Similar findings were also demonstrated by Moura et al. (2012) who confirmed the occurrence of conjugative plasmids among 56 bacteria belonging to *Aeromonas* and *Enterobacteriaceae* isolated from urban and slaughterhouse WWTP in Mirandela, Portugal in which of 73% of the plasmids were able to transfer their resistance.

As has been found in previous investigations specifying *mob_{F12}* as the most frequently found *mob* gene among different genera of *Enterobacteriaceae* (Alvarado et al., 2012), as many as half of the total plasmids (n = 38) in this study were confirmed as transmissible plasmids with most possessing *mob_{F12}* gene singly or in combination with other *mob* genes. Furthermore, *mob_{F12}* was found on more than 90% of plasmids isolated from clinical multi-antibiotic resistant *E. coli* (Garcillán-Barcia et al., 2015). This result might indicate a significant role of WWTP effluents as a transmissible plasmid reservoir conferring ARG that potentially introduce them to the water environment through conjugations and/or

mobilizations. Two or three *mob* genes were detected in 13% (n = 10) and 6.6% (n = 5), respectively, of the total isolates, suggesting the occurrences of multiple relaxases. It is likely that those isolates hosted more than one plasmid (Smillie et al., 2010). Plasmid profile analysis (Akiyama, 2009, Suhartono et al., 2016) suggested the occurrence of multiple plasmids, up to seven plasmids in several isolates. The presence of multiple plasmids in isolates may account for redundancy in resistance genes in at least some of the *E. coli*, and may contribute to the persistence of isolates in the presence of sub-inhibitory concentrations of antibiotics.

Sub-inhibitory concentrations of antibiotics are likely to be present in the stream water environment. A total of 5.8 $\mu\text{g L}^{-1}$ of antibiotics have been detected in addition to various pharmaceutical and other organic chemicals in stream water including Mud Creek, the stream sampled for this study, in north-central and northwestern Arkansas (Haggard et al., 2006). Massey et al. (2010) detected trimethoprim at concentrations of 0.029-0.061 $\mu\text{g L}^{-1}$ and sulfamethoxazole at concentrations of 0.356-0.564 $\mu\text{g L}^{-1}$ in Mud Creek water. Incubation of bacteria in the presence of sulfamethoxazole antibiotic at the sub-inhibitory concentration (0.5 $\mu\text{g L}^{-1}$) had a significant effect on the bacterial CFU concentrations over time regardless of the *mob*-integron designation. In spite of a significant decrease, the viable bacterial CFU concentrations remained at greater than the eighth order of magnitude indicating the persistence of the bacterial isolates over the time period of study. Very low levels of antibiotics, in most cases along with low heavy metal concentrations, might contribute to long-term persistence of the bacterial cells (Andersson and Hughes, 2014). Concentrations would have to be sufficient to balance the fitness cost of maintaining resistance and induce accumulation of small-step mutations, leading to increasing variability of genotypic and

phenotypic determinants, and increasing horizontal gene transfer and recombination (Andersson and Hughes, 2014). This low level of antibiotic-mediated persistence generates potential implications for spread of resistance since the concentration of sulfamethoxazole in the water environment is commonly at sub-inhibitory levels. Concentrations of sulfamethoxazole detected in Mud Creek (Massey et al., 2010) were similar to concentrations detected (0.07 to 0.438 $\mu\text{g L}^{-1}$) in European rivers (Johnson et al., 2015). Sulfamethoxazole has a low degradation rate in aquatic environment (Straub, 2015) and does not absorb strongly to soil and sediments, so the antibiotic is likely to be retained in stream water as it flows (Huang et al., 2011).

In the presence of sub-inhibitory level of trimethoprim, the combination of integron-mobilization gene designation and time of incubation significantly impacted bacterial persistence. Bacterial cells harboring plasmids conferring both integron and mobilization genes were less persistent over time as opposed to their counterparts, although there was little significant distinction among the four groups. The variability in experimental results highlight the difficulty of detecting small differences in results given experimental and temporal variability, even under controlled conditions. On average, isolates harboring plasmids conferring both integron-mobilization genes (group I) possessed 3.25 *dfr* genes as opposed to 2.95, 3.24, and 3.05 *dfr* genes for group II, III, and IV, respectively, also indicating little difference among isolates. The plasmid-mediated antibiotic resistance positively elevates its fitness cost for the host cells with the increasing number of multi-drug resistance to which those plasmids are resistant (Vogwill and MacLean, 2015). Despite its decreased persistence, the viable cell number of bacterial isolates of group I, remained high at the ninth order of magnitude. Similar to sulfamethoxazole, the presence of sub-inhibitory concentrations of

trimethoprim might contribute to continued persistence and generate significant implications in aquatic environments since the concentration of trimethoprim is commonly found at low levels. In addition to being detected at 0.029-0.061 $\mu\text{g L}^{-1}$ in Mud Creek in northwest Arkansas (Massey et al., 2010), the origin of these bacteria, trimethoprim ranged from 0.128 - 0.271 $\mu\text{g L}^{-1}$ in European rivers (Johnson et al., 2015).

F. Conclusion

About half of the plasmids of sulfamethoxazole and trimethoprim resistant *E. coli* in this study conferred integron: predominantly class 1 integron, or both class 1 and 2 integron. Additionally, half of the plasmids were transmissible as indicated by the existence of mobilization genes, with *mob_{F12}* gene the most frequently detected. Resistance to sulfamethoxazole and trimethoprim was plasmid-mediated with three *sul* genes (*sul1*, *sul2* and *sul3* genes) and sixteen-*dfr* gene combinations detected (including the prevalent *dfrA1+dfrA8+dfrA12+dfrA17* and *dfrA1+dfrA8+dfrA12* combinations). In the presence of sub-inhibitory concentrations of sulfamethoxazole, isolates persisted regardless of integron and mobilization gene designation, whereas in the presence of trimethoprim, isolates harboring plasmids with both integron and mobilization genes decreased in concentration during an 11-day experiment. However, there was little significant differentiation in persistence among the four groups designating presence and absence of integron and mobilization genes. Overall, these findings might indicate that treated effluent containing antibiotic resistant bacteria may be an important source of integrase and mobilization genes. Sulfamethoxazole- trimethoprim resistant bacteria may have a high degree of genetic redundancy and diversity conferring resistance to each antibiotic which may lead to

persistence of the bacteria in the stream environment, although the role integrase and mobilization genes towards persistence is unclear.

G. Acknowledgements

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Table 3.1. Target genes and their corresponding primer sequences with amplification features of PCR

Target Gene		Primer sequence	Annealing Temperature (°C)	Product size (bp)	References
<i>sul1</i>	FW ^a RV	CGCACCGGAAACATCGCTGCAC TGAAGTTCGCGCCGAAGGCTCG	55.9	163	(Pei et al., 2006)
<i>sul2</i>	FW RV	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	55.9	191	(Pei et al., 2006)
<i>sul3</i>	FW RV	TCCGTTACAGCGAATTGGTGCAG TTCGTTACGCCTTACACCAGC	60.8	128	(Pei et al., 2006)
<i>dfrA1</i>	FW RV	GTGAAACTATCACTAATGG ACCCTTTTGCCAGATTTG	46	471	(Šeputienė et al., 2010)
<i>dfrA8</i>	FW RV	TTGGGAAGGACAACGCACTT ACCATTTTCGGCCAGATCAAC	56.3	382	(Šeputienė et al., 2010)
<i>dfrA12</i>	FW RV	GGTGAGCARAAGATYTTTCGC TGGGAAGAAGGCGTCACCCTC	52	309	(Šeputienė et al., 2010)
<i>dfrA14</i>	FW RV	GCBAAGGDGARCAGCT TTMCCAYATTTGATAGC	50.8	394	(Šeputienė et al., 2010)
<i>dfrA17</i>	FW RV	AAAATTTTCATTGATTTCTGCA TTAGCCTTTTTTCCAAATCT	48.4	471	(Šeputienė et al., 2010)
<i>dfrB3</i>	FW RV	GATCACGTRCGCAAGAARTC GACTCGACVGCRTASCCTTC	56	95	(Šeputienė et al., 2010)
<i>intI1</i>	FW RV	GGGTCAAGGATCTGGATTTTCG ACATGCGTGAAATCATCGTCCG	62	483	(Mazel et al., 2000)

Table 3.1. Target genes and their corresponding primer sequences with amplification features of PCR (Con't.)

Target Gene		Primer sequence	Annealing Temperature (°C)	Product size (bp)	References
<i>intI2</i>	FW RV	CAAGCATCTCTAGGCGTA AGAAGCATCAGTCCATCC	50	233	(Mazel et al., 2000)
<i>mob_{P11}</i>	FW RV	CGTGCGAAGGGCGACAARACBTAYCA CCCTTGCCTGGTGYTSNACCCA	60	180	(Alvarado et al., 2012)
<i>mob_{P14}</i>	FW RV	CGCAGCAAGGACACCATCAAAYCAYTAYRT CCCTTGCCTGGTGYTSNACCCA	50	174	(Alvarado et al., 2012)
<i>mob_{P51}</i>	FW RV	TACCACGCCCTATGCGAARAARTAYAC CCCTTGCCTGGTGYTSNACCCA	58	167	(Alvarado et al., 2012)
<i>mob_{F11}</i>	FW RV	GCAGCGTATTACTTCTCTGCTGCCGAYGAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	53	234	(Alvarado et al., 2012)
<i>mob_{F12}</i>	FW RV	AGCGACGGCAATTATTACACCGACAAGGAYAAAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	55	234	(Alvarado et al., 2012)
<i>mob_{Q11}</i>	FW RV	CAATCGTCCAAGGCGAARGCNGAYTA CGCTCGGAGATCATCAYYTGYCAYTG	50	331	(Alvarado et al., 2012)
<i>mob_{Qu}</i>	FW RV	AGCGCCGTGCTGTCCGCBGCNTAYCG CTCCGACGCTCGRCSGCRRTCCA	64	179	(Alvarado et al., 2012)

^aFW: forward; RV: reverse

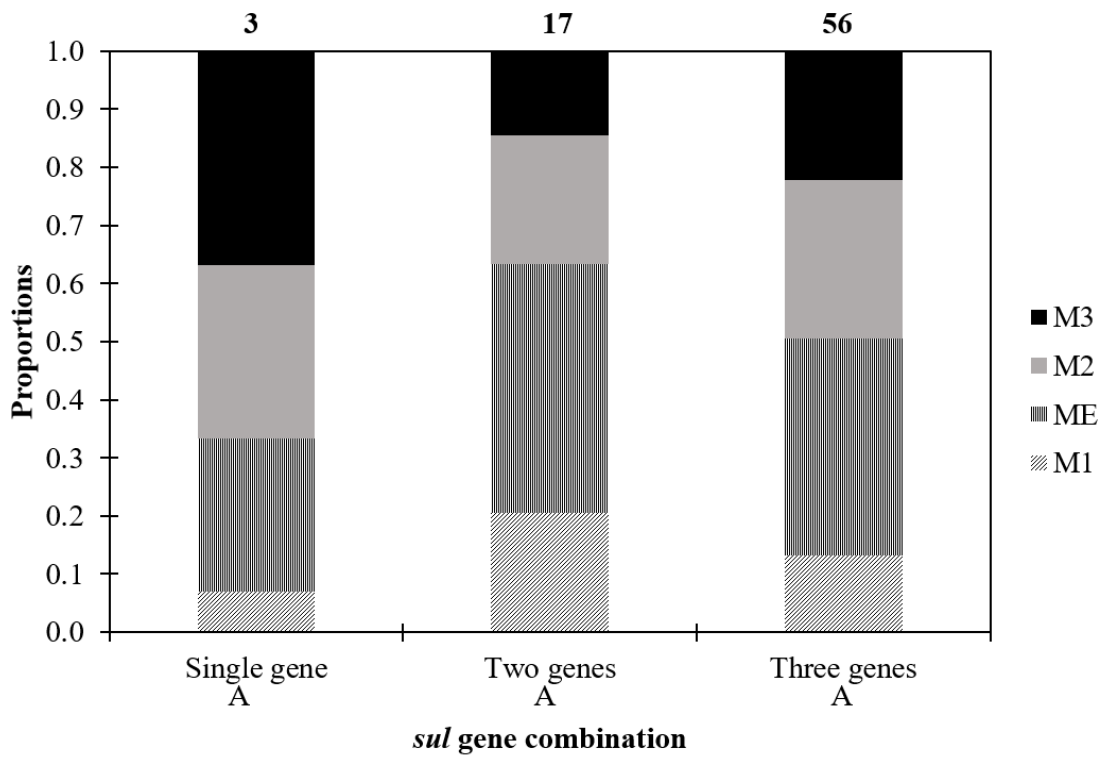


Figure 3.1. Proportion of *sul* genes among sulfamethoxazole-trimethoprim resistant *E. coli* (n = 76) isolated from Mud Creek in Fayetteville, Arkansas. The gene combination followed by the same letter are not significantly different using contrasts tested on the cumulative logit scale ($P \leq 0.05$). M1, ME, M2, and M3 are the sampling site of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input. Numbers at the top represent sum of the occurrences by row or column.

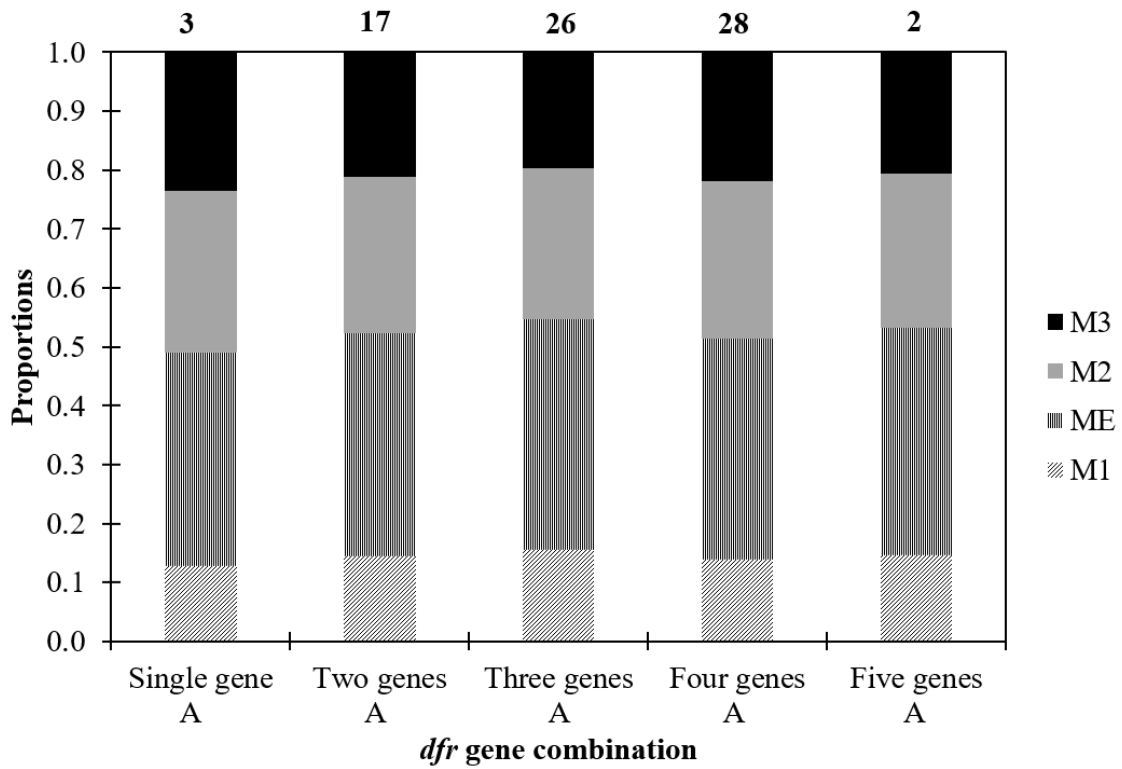


Figure 3.2. Proportion of *dfr* genes among sulfamethoxazole-trimethoprim resistant *E. coli* ($n = 76$) isolated from Mud Creek in Fayetteville, Arkansas. The gene combination followed by the same letter are not significantly different using contrasts tested on the cumulative logit scale ($P \leq 0.05$). M1, ME, M2, and M3 are the sampling site of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input. Numbers at the top represent sum of the occurrences by row or column.

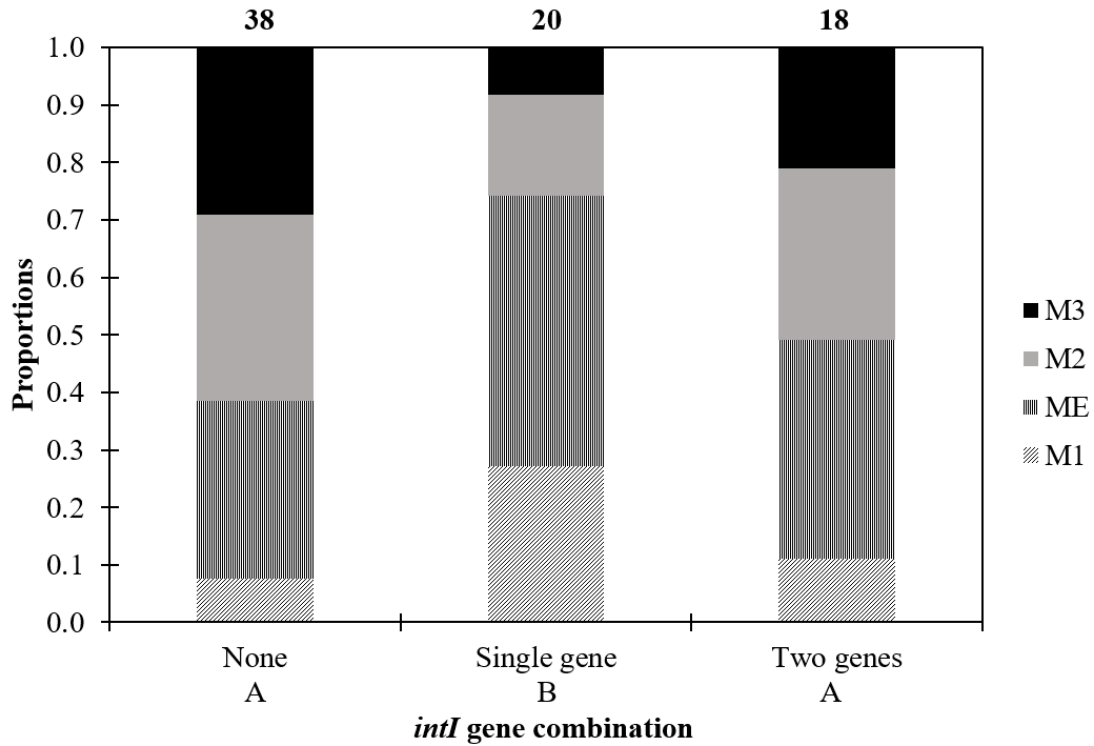


Figure 3.3. Proportion of *intI* genes among sulfamethoxazole-trimethoprim resistant *E. coli* ($n = 76$) isolated from Mud Creek in Fayetteville, Arkansas. The gene combination followed by the same letter are not significantly different using contrasts tested on the cumulative logit scale ($P \leq 0.05$). M1, ME, M2, and M3 are the sampling site of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input. Numbers at the top represent sum of the occurrences by row or column.

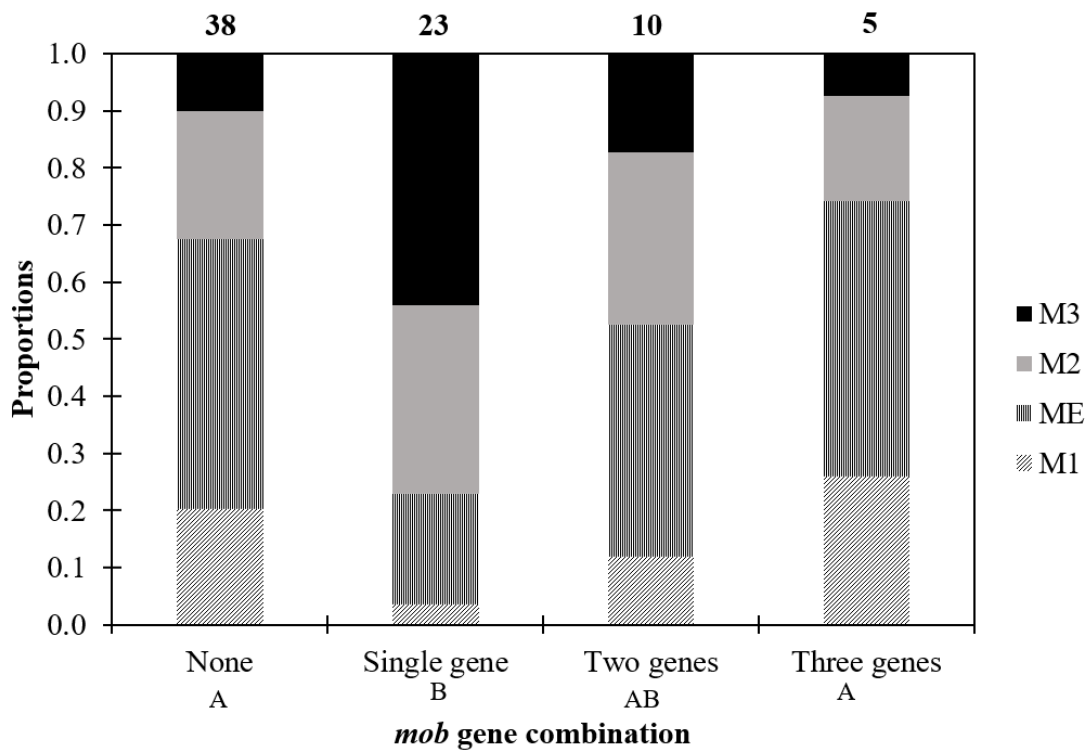


Figure 3.4. Proportion of *mob* genes among sulfamethoxazole-trimethoprim resistant *E. coli* ($n = 76$) isolated from Mud Creek in Fayetteville, Arkansas. The gene combination followed by the same letter are not significantly different using contrasts tested on the cumulative logit scale ($P \leq 0.05$). M1, ME, M2, and M3 are the sampling site of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input. Numbers at the top represent sum of the occurrences by row or column.

Table 3.2. Means of cell density (log CFU mL⁻¹) grown on sulfamethoxazole based on time of incubation (n = 76)

Day of incubation	Mean of log CFU/ml
1	9.168 ^a
7	9.112 ^a
9	9.092 ^{ab}
11	8.994 ^b

Least square means followed by the same letter are not significantly different at $\alpha=0.05$ using a protected LSD procedure (LSD = 0.1033).

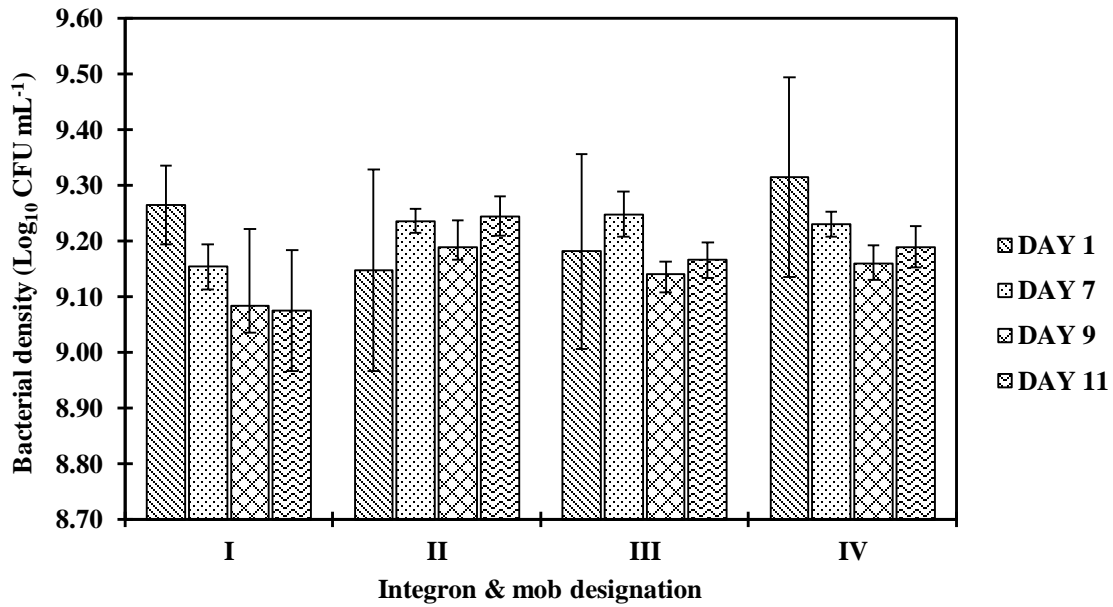


Figure 3.5. Means of cell density ($\log \text{CFU mL}^{-1}$) with error bars of isolates grown on trimethoprim ($0.19 \mu\text{g L}^{-1}$) from group I: mob^+intI^+ ($n= 17$); group II: mob^-intI^+ ($n= 21$); group III: mob^+int^- ($n= 21$); group IV: mob^-int^- ($n= 17$) on trimethoprim based on presence or absence of integron (*IntI*) and mobilization (*mob*) genes, after 1, 7, 9, and 11 days of incubation. LSD to compare concentrations within and between groups I and IV is 0.1590; LSD to compare concentrations within and between groups II and III is 0.1431; LSD to compare concentrations between groups I and II, I and III, II and IV, and III and IV is 0.1513.

CHAPTER 4. HIGH FREQUENCY OF CONJUGATION-BASED DISSEMINATION OF
TRANSMISSIBLE PLASMIDS HARBORING CLASS 1 AND 2 INTEGRONS OF MULTI-
DRUG RESISTANT BACTERIA ISOLATED FROM WASTEWATER TREATMENT
PLANT EFFLUENT AND STREAM WATER IN NORTHWEST ARKANSAS

High frequency of conjugation-based dissemination of transmissible plasmids harboring class 1 and 2 integrons of multi-drug resistant bacteria isolated from wastewater treatment plant effluent and stream water in Northwest Arkansas

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A. Abstract

The rapid emergence of antibiotic resistance genes (ARG) may be in part due to their association with mobile genetic elements, such as plasmids and integrons. The objective of the research was to determine the effects of multiple antibiotic resistance (MAR), site of origin, integrons, sulfamethoxazole resistance (*sul*) genes, and trimethoprim resistance (*dfr*) genes on the transfer rate of multiple antibiotic resistant *Escherichia coli* harboring transmissible plasmids. The transfer rate was measured using a conjugation assay and was determined using ratios between transconjugants/donors (T/D) or transconjugants/recipients (T/R). Of 51 isolates, there were 38 *E. coli* demonstrating the ability to transfer plasmids, with a mean transfer rate of 1.60×10^{-3} (T/D) or 5.89×10^{-3} (T/R). Integrons, MAR number (T/R only), *sul* gene combination (T/R only), and *dfr* gene combination had no statistically significant difference in terms of the transfer rate of the isolates. However, site of origin (T/D), MAR number (T/D), and *sul* gene combination (T/D) had significant impact on the transfer frequency of the isolates; isolates recovered from further downstream and those possessing plasmid-mediated-resistance to three antibiotics with three-*sul* genes had significantly higher transfer frequency than their counterparts. The remarkably high in vitro gene transfer rate suggests that horizontal gene transfer of conjugative plasmid mediated-antibiotic (*sul*) resistance genes among bacteria exhibiting MAR may be significant in the stream environment.

B. Introduction

Antibiotic resistant bacteria (ARB) are one of the alarming global threats that rapidly emerge and generate considerable potential risks, primarily in clinical settings. These ARB are able to survive in the presence of prescribed antibiotics and may double the rates of adverse outcomes, i.e. mortality rates, in hospital compared to their susceptible counterparts at the similar infection. For example, the number of deaths resulting from methicillin-resistant *Staphyococcus aureus* bacteremia infections doubled than susceptible *S. aureus* (Cosgrove et al., 2003). Direct adverse outcomes include increasing rate of morbidity and mortality, incremental utilization of hospital-related resources and costs, and reduced hospital activities due to unit closures and surgery cancellations (Friedman et al., 2015). It is estimated that the economic impact of ARB exceeds \$55 billion, comprising \$20 billion in medical and \$35 billion in societal costs per year in the U.S. alone (Roberts et al., 2009; Smith and Coast, 2013).

The widespread of ARB is mainly owing to the presence of antibiotic resistance genes (ARG) that are embedded within mobile genetic elements, including plasmids. Plasmids are extra-chromosomal circular double-stranded DNA that autonomously replicate with widely varying copy number and size per bacterial cell (Carattoli, 2009). Plasmids, depending on their host range, conjugative apparatus, and mobility, play a major role in horizontal gene transfer (HGT), promoting dissemination of ARG among bacteria, not only in the same species or genus but also among distantly related bacteria (Thomas and Nielsen, 2005). One of the most promiscuous mechanisms of HGT is through bacterial conjugation. The bacterial conjugative transfer or conjugation occurs from a plasmid-bearing donor cell to a recipient cell. The donor will transfer its plasmids to the recipient through a pilus, an appendage of the

donor cell that allows physical contact between both cells. This process requires a viable and metabolically capable donor cell to transfer its genetic material to the recipient cell.

There are two classes of plasmids according to their transmissibility by conjugation, namely conjugative plasmids and mobilizable plasmids. Conjugative plasmids contain a full set of conjugation genes comprising 1) MOB module, which carries genetic information essential for the processing of conjugative DNA and 2) mating pair formation (MPF) module encoding a membrane-associated complex, which is a type 4 secretion system that forms the mating channel (Smillie et al., 2010). An example of this plasmid is the AbR IncW plasmid R388 of *Enterobacteriaceae* (Garcillan-Barcia et al., 2011). Mobilizable plasmids, on the other hand, contain only a minimal set of genes that allow them to be mobilized by conjugation when they coexist in the same donor cell with a conjugative plasmid. These non-self-transmissible plasmids carry only the MOB module, and their transfer requires an MPF provided by another genetic element (Smillie et al., 2010). An example of a mobilizable plasmid is the IncQ1 plasmid RSF1010 (Garcillan-Barcia et al., 2011).

In addition to plasmids, ARG, including sulfamethoxazole resistance (*sul*) genes, and trimethoprim resistance (*dfp*) genes, might be encoded within another genetic element called an integron. An integron is a bacterial genetic element containing a site-specific recombination system that is able to efficiently acquire, rearrange, and express genes embedded within a gene cassette (GC) (Fluit and Schmitz, 1999). Structurally, an integron consists of three major components: the *IntI* gene (*intI*); the recombination site (*attI*); and the promoter (P_c). The *intI* gene encodes for an enzyme called integrase that allows specific excision and integration of gene cassette(s) adjacent to the recombination site (*attI*) followed by the transcription and expression of the gene cassettes containing ARG and other genes through the integron

promoter (Pc) (Fluit and Schmitz, 1999; Stalder et al., 2012; Gillings, 2014). The mobility of plasmids combined with a highly efficient natural gene-capture and expression system plus highly heterogeneous gene cassettes of an integron may allow ARG to be widely spread and exchanged among diverse bacteria (Carattoli, 2001).

Previous investigations indicated that ARG might emerge in water and sediment of northwest Arkansas streams. It was reported that a number of multiple antibiotic resistant *Escherichia coli* plus plasmids were recovered in wastewater treatment plant (WWTP) effluent and receivingstream water in Northwest Arkansas (Akiyama et al., 2010, Akiyama and Savin, 2010). These MAR determinants might be associated with conjugative and/or mobilizable plasmids and integrons promoting dissemination of the antibiotic resistance determinants. Hence, the objective of the research was to determine the effects of sampling origin, integrons, multiple antibiotic resistances (MAR), sulfamethoxazole resistance (*sul*) genes, and trimethoprim resistance (*dfr*) genes towards the transfer rate of isolates harboring transmissible plasmids.

C. Material and methods

Donor cells were environmental *E. coli* isolated from treated effluent and receivingstream water and characterized according to Akiyama and Savin (2010). *E. coli* were originally isolated from water samples collected from one of four sampling locations in Mud Creek (36.090° latitude, 94.111° longitude). Mud Creek is a tributary of the Illinois River, located in Fayetteville, Arkansas, and has been described in Haggard et al. (2007) with the water quality at the time of sampling and sampling locations described in Akiyama and Savin (2010). Fifty-one donor *E. coli* were selected based on the presence of at least one *mob* gene in plasmids extracted using the Wizard® Plus SV Minipreps DNA Purification System

(Promega, Madison, WI, USA). The extracted plasmid DNA was used as template to determine the occurrences of mobilization genes (*mob_{P11}*, *mob_{P14}*, *mob_{P51}*, *mob_{F11}*, *mob_{F12}*, *mob_{Q11}*, and *mob_{Qu}*) using primer sets and PCR conditions outlined Chapter 2 (Suhartono et al., 2016a).

Eleven *E. coli* were isolated from upstream (M1), 12 *E. coli* were isolated from the effluent (ME), 12 *E. coli* were isolated from the first downstream (M2), and 16 *E. coli* were isolated from second downstream sampling location (M3). *E. coli* characterization, including phenotypic MAR number for each isolate, was determined as described in Akiyama and Savin (2010). Six isolates were resistant to one-antibiotic, six isolates were resistant to two antibiotics, 25 isolates were resistant to three antibiotics, 12 isolates were resistant to four antibiotics, and two isolates were resistant to five antibiotics. Antibiotics resistances were determined for the environmental *E. coli* at the following concentrations for a total of six different antibiotics: 32 µg/mL ampicillin, 16 µg/mL gentamicin, 16 µg/mL tetracycline, 8 µg/mL ofloxacin, 4 µg/mL trimethoprim, and 80 µg/mL sulfamethoxazole (Akiyama and Savin, 2010).

Environmental *E. coli* were grouped based on the following determinants: MAR number, site of origin, integron number (10 isolates harbored *intI1* or *intI2*, 9 isolates harbored both *intI1* and *intI2*, and 32 isolates harbored no integrons); number of sulfamethoxazole resistance (*sul*) genes (15 isolates conferred a single *sul* gene, 13 isolates conferred two *sul* genes, 22 isolates conferred three *sul* genes, and one isolate conferred no *sul* genes); and number of trimethoprim resistance (*drf*) genes (17 isolates conferred one *drf* gene, nine isolates conferred two *drf* genes, 10 isolates conferred three *drf* genes, seven isolates conferred four *drf* genes, one isolate conferred five *drf* genes, and seven isolates conferred no *drf* gene).

Recipient cells were *E. coli* DH5 CGSC# 12383 (The Coli Genetic Center at Yale, New Haven, Connecticut) resistant to nalidixic acid (20 mg L⁻¹), *E. coli* K12 JM109 #E4107S (New England Biolabs, Ipswich, Massachusetts) resistant nalidixic acid (20 mg L⁻¹), and *E. coli* TOP10 (Invitrogen, Carlsbad, California) resistant to streptomycin (20 mg L⁻¹). All strains of *E. coli* were grown on selective Luria-Bertani agar plates containing antibiotic(s) which the isolates were originally determined to be resistant and incubated at 37 °C for overnight. Single colonies were inoculated into 10 mL of selective LB broth (Mobio, Carlsbad, California) containing antibiotic(s) similar to LB agar plates which were incubated overnight at 37 °C with shaking at 150 rpm.

Conjugation assays were performed using a modification of the protocol in Moura et al. (2012). Donor and recipient cells were incubated separately overnight at 37 °C with shaking at 165 rpm in liquid culture containing 5 mL LB broth. Donor and recipient cells were spread and enumerated using plate count assay on Mueller-Hinton (MH) agar before both cells were mixed in ratio of 1:1 and precipitated by centrifugation at room temperature for 5 min at 6700 g. Supernatants were removed and replaced by 1 mL fresh LB followed by incubation at 28 °C for 24 h without shaking. After incubation, cells were then centrifuged at 6700 g for 5 min and washed in 0.9% NaCl solution. The mixture was then serially diluted in 0.9% NaCl solution and 100 µL of aliquots were spread on MH agar supplemented with nalidixic acid (20 mg L⁻¹) and tetracycline (16 mg L⁻¹) for mixture of donor cells and *E. coli* DH5; or supplemented with nalidixic acid (20 mg L⁻¹) and sulfamethoxazole (80 mg L⁻¹) for mixture of donor cells and *E. coli* K12, or streptomycin (20 mg L⁻¹) and tetracycline (16 mg L⁻¹) for mixture donor cells and *E. coli* TOP10. Aliquots (100 µL) were also spread on MH agar supplemented with nalidixic acid (20 mg L⁻¹) or streptomycin (20 mg L⁻¹) as a control for

recipient cells. All plates were incubated at 37 °C for 48 h. Assays were performed in duplicate. Plasmid transfer was measured by the ratio of number of the transconjugant (T) cells divided by the initial donor (D) or number of transconjugant (T) cells divided by the initial recipient (R) cells (Fernandez-Astorga et al., 1992).

A statistical analysis was performed to evaluate the effects of MAR number, site of origin, integron, *mob* gene, *sul* and/or *dfr* gene number towards the transfer rate of transmissible plasmids with 95% confidence intervals using analysis of variance (ANOVA) test procedure using XLSTAT 2016 (New York, NY). Fisher's least significant difference (LSD) test was performed to compare means ($P \leq 0.05$).

D. Results

Of 51 multiple antibiotic resistant isolates harboring transmissible plasmids, 38 (74.5%) isolates were able to transfer plasmid-mediated antibiotic resistant determinants and no transconjugants were obtained from 13 (25.5%) isolates. Overall, among 51 isolates, the mean transfer rates were 1.60×10^{-3} (T/D) or 5.89×10^{-3} (T/R), whereas among the transconjugant-generating *E. coli* (38 isolates), the mean transfer rates were 2.14×10^{-3} (T/D) or 7.90×10^{-3} (T/R). Based on ANOVA results, MAR number had a statistically significant impact on the conjugal rate of the isolates affecting the T/D ratio (Figure 4.1a, $P = 0.007$), yet there was no significant effect of MAR number on the T/R ratio (Figure 4.1b, $P = 0.612$). The site of origin where the isolates were recovered did have a significant impact on the conjugal rate for T/D (Figure 4.2a, $P < 0.0001$), although no significant difference among sites was observed on the T/R (Figure 4.2b, $P = 0.183$). Based on the T/D ratio, *E. coli* recovered from the second downstream site (M3), which was 2 km downstream of the effluent input, had

significantly higher transfer frequency compared to *E. coli* recovered from upstream, effluent, or approximately 640 m downstream of effluent input into the stream.

In terms of sulfamethoxazole resistance, the number of *sul* genes in plasmids within an isolate had a statistically significant effect on the transfer rate of plasmids based on the ratio of T/D (Figure 4.3a, $P = 0.048$). Isolates possessing three plasmid-mediated *sul* (*sul1*+*sul2*+*sul3*) genes had a significantly greater mean transfer rate (2.56×10^{-3} T/D) than the isolates possessing only one *sul* (*sul2*) gene (3.56×10^{-4} T/D). The transfer rate (1.55×10^{-3} T/D) for *E. coli* conferring two *sul* resistance genes was intermediate, and not different from, those conferring one or three *sul* genes. Based on the T/R ratio, however, *sul* gene combination had no significant effects on the transfer rate (Figure 4.3b, $P = 0.435$).

The number of *dfr* genes conferring trimethoprim resistance detected in plasmids from environmental *E. coli* did not have a statistically significant effect on the transfer rate of the plasmids based on either the T/D (Figure 4.4a, $P = 0.062$) or T/R (Figure 4.4b, $P = 0.165$) ratio. Similar to *dfr* genes, the presence of no, one or two *intI* genes had no statistical difference in transfer rate of plasmids among *E. coli* isolates based on either ratio, T/D (Figure 4.5a, $P = 0.714$) or T/R (Figure 4.5b, $P = 0.194$).

E. Discussion

There are potentially three major factors explaining the prevalence of antibiotic resistant bacteria in aquatic environment, namely greater input (effluent) containing antibiotics from WWTP, the persistence of the bacteria in the aquatic system, and dissemination of the resistance through horizontal gene transfer (Fernandez-Astorga et al., 1992). Conjugation is a promiscuous mechanism of horizontal gene transfer that allows plasmids containing genes, such as antibiotic resistance genes, to be widely disseminated among bacterial populations. In

the present study, conjugation rate or transfer rates were measured based on ratios of the observed transconjugants over either the donor cells or recipient cells. Since both ratios are widely used to measure conjugation efficiency, both measurements were applied in this study. Because of differential variation in cell density or growth rates of donor and recipient cells, the measurement rates T/D versus T/R may differ and the factors that significantly influence ratios, i.e. MAR, site of origin, number of *sul* genes, may differ. In fact, the ratio of T/D was ten-fold lower than T/R as growth rate of donors was higher by ten-fold than recipients.

Approximately three quarters of the multiple antibiotic resistant *E. coli* possessing conjugative plasmids recovered from an effluent driven stream demonstrated a high rate of plasmid transfer under the experimental conditions utilized. Previous investigations demonstrated a transfer frequency at 10^{-4} to 10^{-2} transconjugants per recipient cells of integron-harboring multidrug-resistant *Enterobacteriaceae* isolated from clinical settings (Leverstein-van Hall et al., 2002). Different results, however, were observed by Moura et al. (2007) in which the rate was one to three orders of magnitude lower at 3.79×10^{-5} and 5.46×10^{-5} (T/R), respectively, for *Aeromonas sp.* and *E. coli* possessing integrons in which bacteria were isolated from slaughterhouse WWTP in Mirandela, Portugal. Transfer frequencies of *E. coli* possessing class 1 integron isolated from cooked meat products in China were measured at 10^{-6} to 10^{-4} (T/R) (Yu et al., 2016). *Salmonella* harboring class 1 integron transferred plasmids to *E. coli* through conjugation at the rates of 10^{-6} and 10^{-5} (T/R) (Meng et al., 2011, Yu et al., 2014). The multiple antibiotic resistant environmental *E. coli* isolates in this experiment showed a high plasmid transfer rate regardless of the presence or absence of class 1 or class 2 integrons. While the possibility of conjugal transfer of antibiotic resistance might increase as a result of increased MAR number and the presence of class 1 integron (Sunde and

Norström, 2006), results did not support that conjugation rate was a factor for integron in this experiment.

However, in the present study it was observed that as many as one quarter of the total isolates failed to generate transconjugants in spite of possession of transmissible plasmids, predominantly *mob_{F12}* gene. Gene transfers may occur through a different mechanism other than bi-parental conjugation as performed in this experiment. Mobilizable plasmids are not able to promote their own transfer as they lack of a membrane-associated MPF protein that is commonly found on self-transmissible or conjugative plasmids (Smillie et al., 2010). In order to have the gene transfer to the recipient cells, mobilizable plasmids require the co-existence with conjugative plasmids, called helpers, in the same host cells; that is commonly set up as tri-parental mating in vitro (Sana et al., 2014). Another plausible explanation for the lack of transconjugants able to grow on the specific antibiotics was the possibility that the ARG were located among different plasmids within an isolate. As many as 69% (9 out of the 13 which did not produce transconjugants) harbored multiple plasmids in individual organisms (Akiyama and Savin, 2010; Suhartono et.al., 2016a).

Notably, the isolates possessing resistance to three antibiotics had significantly higher transfer rate (T/D) than the isolates possessing any other MAR numbers. It was speculated that isolates possessing MAR 3 tended to be predominantly found accounting for 49% of the isolates used in this study. Additionally, as many as 96% of these three-antibiotic resistant isolates predominantly harbored conjugative plasmids with *mob_{F12}*. Not surprisingly, the isolates possessing higher transfer rate as *mob_{F12}* as the most frequently found *mob* gene among different genera of *Enterobacteriaceae* (Alvarado et al., 2012) and predominantly detected on the plasmids of clinical multi-resistant *E. coli* (Garcillán-Barcia et al., 2015)

suggesting the important role of *mobF12* gene in the transmissibility of the plasmids among bacteria.

Among the *E. coli* transferring ARG into recipient cells, those recovered from the second downstream sampling location (2 km downstream from the effluent input) exhibited significantly higher transfer frequency (T/D) as compared to isolates recovered from any location upstream of that site. Antibiotics, including sulfamethoxazole and trimethoprim, have been detected in the water column of Mud Creek, the source stream for these bacteria, and the concentration of antibiotics remained consistent or decreased within the length of stream sampled for these bacteria, except sulfamethoxazole concentration which was variable at the 2 km downstream location (Haggard et al., 2006; Massey et al., 2010). Despite the fact that antibiotics do not significantly influence conjugation within bacteria (Lopatkin et al., 2016), antibiotics, especially at the sub-inhibitory level, may play an important role in the survival and persistence of antibiotic resistant bacteria in the aquatic environment (Suhartono et al., 2016b).

Environmental factors, i.e. temperature, nutrient availability and pH, might also contribute to the variability of the conjugation efficiency of isolates from different sites of origin by impacting the bacteria surviving in the stream. Water properties in Mud Creek were strongly influenced by effluent input and changed as water flowed downstream. For example, in Massey et al. (2010), the Mud Creek sampling sites, i.e. upstream, effluent, 640 m downstream, and 2 km downstream of effluent input had pH values of 7.22, 7.61, 7.50, and 7.35 and temperatures (in °C) of 17.2, 23.2, 22.9, and 23.9, respectively, during seasonal base flow conditions in August and September 2006. Moreover, it was observed that water downstream was more similar to effluent rather than upstream with dissolved nutrient

concentrations, organic carbon and nitrate/nitrite nitrogen, significantly increased downstream of effluent input at the time of sampling in July, August, and September 2007 (Akiyama and Savin, 2010). Nutrient availability positively increased conjugation rates in vitro; addition of glucose, N and P significantly increased conjugative transfer by more than four orders of magnitude as opposed to transfer without nutrient addition (Guo et al., 2015). Furthermore, Akiyama and Savin (2010) also demonstrated increasing bacterial concentrations from effluent to downstream indicating growth and/or resuspension of bacteria in the sediments into the water column.

The *E. coli* isolates conferring three *sul* genes (*sul1+sul2+sul3*) had higher gene transfer rates than those with only one *sul* gene (*sul2* gene). This finding might signify that the presence of the *sul3* gene as well as the *sul1* gene in isolates conferring a total of three *sul* genes is an indicator of the presence of conjugative plasmids. Recently, it was observed that all *sul3* genes residing in plasmids of *E. coli* from pigs, pig carcasses, and humans were conjugative (Wu et al., 2010). The presence of *sul3* gene is being recognized for its association with integron and has recently been identified as being encoded within the non-classical conserved segment (*sul3*-CS) of class 1 integron of plasmids recovered from commensal *E. coli* (Moran et al., 2016). The *sul1* gene is classically encoded within 3' conserved regions of class 1 integron (Fluit and Schmitz, 1999). While integron was not related to plasmid transfer rate, perhaps multiple *sul* genes are better indicators of the placement of integrons within conjugative plasmids, and thus contributed to the increased rate of transfer compared to the presence of a single *sul* gene, although further investigation is required to support this.

F. Conclusion

This study confirmed the high gene transfer in vitro among multiple antibiotic resistant *E. coli* isolates harboring transmissible plasmids, regardless of integrons, or trimethoprim resistance genes. The presence of three compared to only a single sulfamethoxazole resistance gene increased plasmid transfer rate. Further investigation is warranted to determine if the presence of three *sul* genes indicates a greater likelihood that antibiotic resistance genes are located on conjugative plasmids, or on mobile elements such as integrons within conjugative plasmids and thus are linked to increased transfer rates. Sampling site in the stream impacted plasmid transfer frequency among bacterial populations, indicating that environmental conditions, such as temperature or nutrient availability, are also likely to significantly increase dissemination of antibiotic resistance genes. Taken together, these results demonstrate conjugation rate to be influenced by both factors related to the resistance genes within the bacteria and the environmental conditions where bacteria reside.

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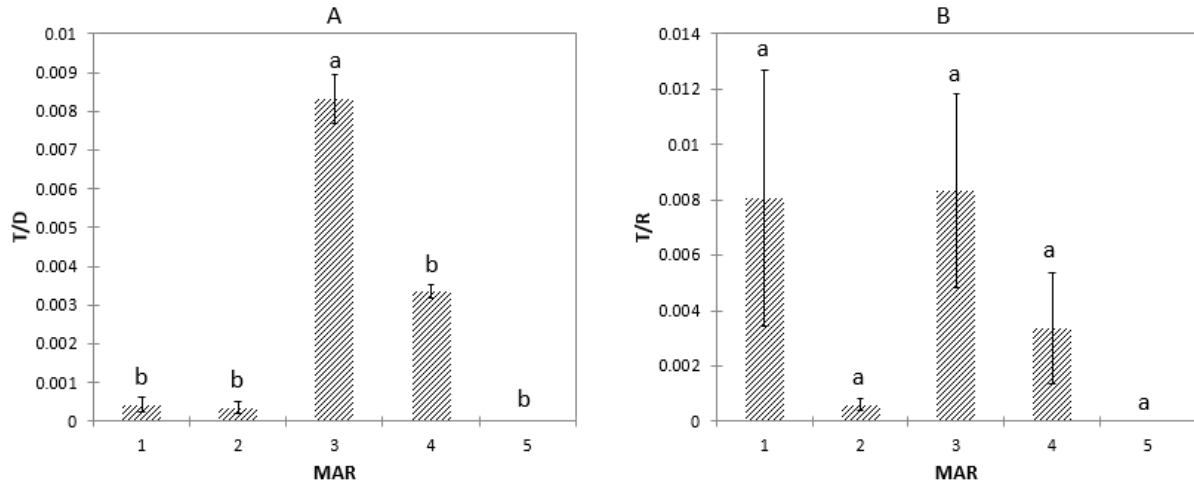


Figure 4.1. The effect of MAR number on the mean (+ standard error) transfer rate of plasmids from 51 isolates (n per MAR; MAR1=12; MAR2= 12; MAR3 = 50; MAR4 = 24; MAR5 = 4) recovered from Mud Creek, Fayetteville, Arkansas based on (a) ratio of transconjugants/donors (T/D) and (b) transconjugants/recipients (T/R). The transfer rates followed by the different letters are significantly different at $P \leq 0.05$.

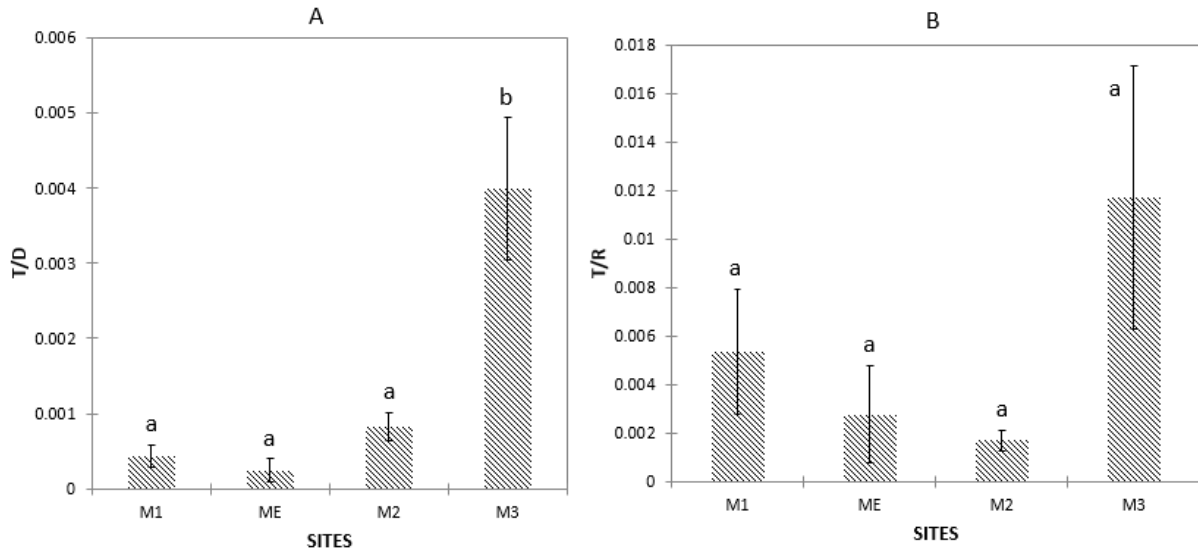


Figure 4.2. The effects of sites of origin on the mean (+ standard error) transfer rate of plasmids from 51 isolates (n per site, M1 = 22; ME= 24; M2= 24; and M3=32) recovered from Mud Creek, Fayetteville, Arkansas based on (a) ratio of transconjugants/donors (T/D) and (b) transconjugants/recipients (T/R). M1: upstream, ME: effluent, M2: 640 m downstream from effluent input, and M3: 2000 m downstream from effluent input. The transfer rates followed by the different letters are significantly different at $P \leq 0.05$.

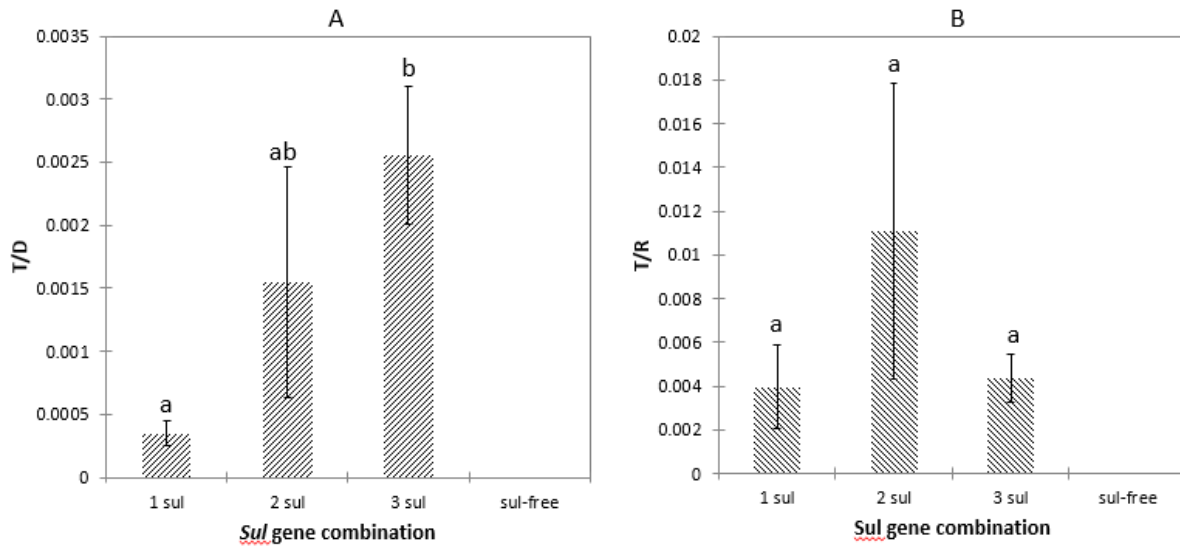


Figure 4.3. The effect of the number of *sul* genes on the mean (+ standard error) transfer rate of plasmids from 51 isolates (n per *sul*: one *sul*= 30; two-*sul*= 26; three-*sul* = 44; and *sul*-free = 2) recovered from Mud Creek, Fayetteville, Arkansas based on (a) ratio of transconjugants/donors (T/D) and (b) transconjugants/recipients (T/R). The transfer rates followed by the different letters are significantly different at $P \leq 0.05$.

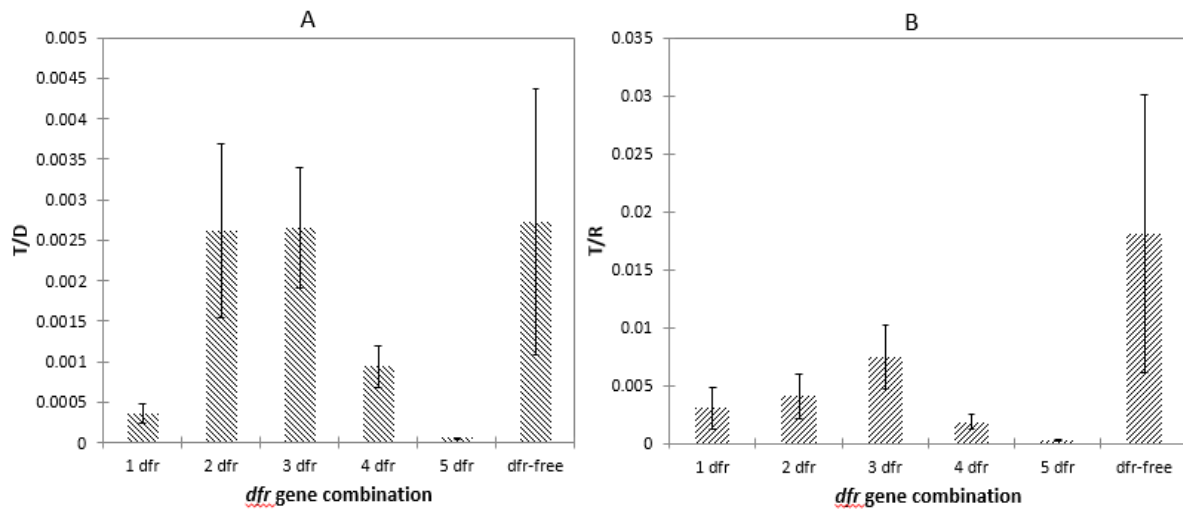


Figure 4.4. The effect of the number of *dfr* genes on the transfer rate of plasmids from 51 isolates (n per *dfr*: one *dfr*= 34; two-*dfr*= 18; three-*dfr*= 20; four-*dfr*= 14; five-*dfr*= 2; and *dfr*-free = 14) recovered from Mud Creek, Fayetteville, Arkansas based on (a) ratio of transconjugants/donors (T/D) and (b) transconjugants/recipients (T/R). Rates are not significantly different at $P \leq 0.05$

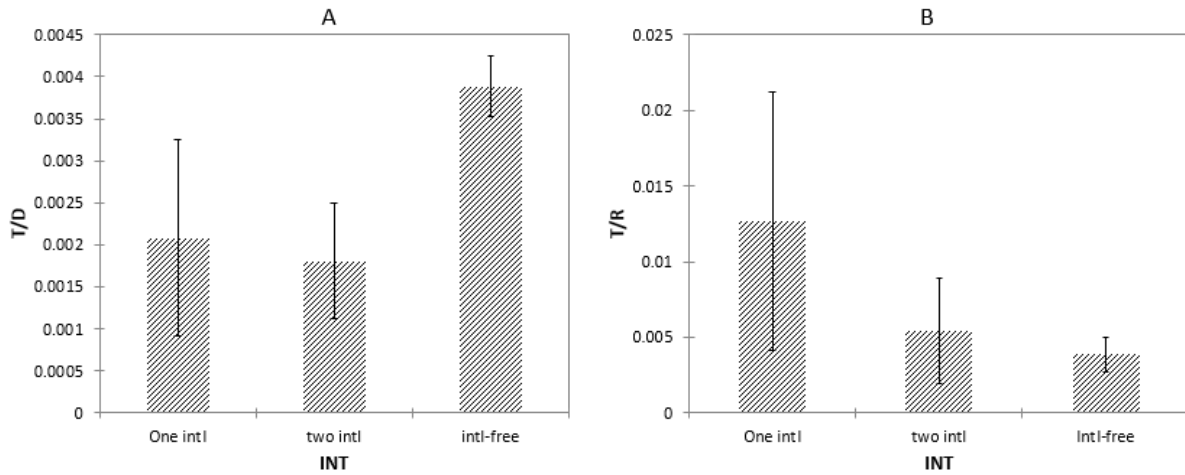


Figure 5. The effects of *intI* gene combination on the transfer rate of plasmids from 51 isolates (n per intI: one *intI* = 20; two-*intI* = 18; and *intI*-free = 64) recovered from Mud Creek, Fayetteville, Arkansas based on (a) ratio of transconjugants/donors (T/D) and (b) transconjugants/recipients (T/R). Rates are not significantly different at $P \leq 0.05$

CHAPTER 5. CONCLUSION

Almost half of the total plasmids (47%) in the present study were transmissible with *mob_{F12}* gene as the most frequently detected mobilization gene. When two or three *mob* genes were detected in plasmids of isolates, there was a significant shift in the population towards larger multiple antibiotic resistance (MAR) number. Plasmid-borne class 1 and/or 2 integrons were prevalent (46%). Integron presence also significantly shifted the isolate population towards larger MAR number. More isolates possessed plasmid DNA carrying single or coexistence of two or three *sul* genes (99.3%), and single or a combination up to five *df_r* genes (89.3%) than had exhibited phenotypic resistance to the respective antibiotics (Chapter 2).

In the persistence study (Chapter 3), the presence of low (sub-inhibitory) levels of sulfamethoxazole, isolates persisted regardless of integron and mobilization gene designation, whereas in the presence of trimethoprim, isolates harboring plasmids with both integron and mobilization genes were less persistent than in the absence of both or presence of a gene from either group individually. In the dissemination study (Chapter 4), overall, the mean transfer rates of isolates harboring transmissible plasmids were 1.60×10^{-3} (transconjugants /donors) or 5.89×10^{-3} (transconjugants/recipients). These findings may support transmissible plasmids and integron as a mechanism for accumulating antibiotic resistance genes and thus contribute to the spread and persistence of multiple antibiotic resistances among bacteria in the stream environment.

Notably, the current study also signified the redundancy of plasmid-mediated *sul* genes existing among three *sul* genes in association towards the increasing MAR number. These three *sul* genes reside within 60 (43%) transmissible plasmids and integrons allowing the multiple antibiotic resistant isolates to persist in the presence of sub-inhibitory concentration of sulfamethoxazole and disseminate their resistance determinants with significantly greater

transfer rate of 2.56×10^{-3} (T/D) compared to *E. coli* possessing a single *sul* gene (3.56×10^{-4} (T/D)).

Overall, this research indicates that treated effluent containing antibiotic resistant bacteria may be an important source of integrase and mobilization genes in multiple antibiotic resistant bacteria entering the stream environment. Transmissible plasmid-mediated and integron-associated antibiotic (sulfamethoxazole and/or trimethoprim) resistance gene diversity among bacteria, in particular sulfamethoxazole resistance, may play a significant role in contribution to persistence and dissemination of antibiotic resistance in the stream environment.

CHAPTER 6. APPENDICES

Appendix 2.1. Distribution of *mob* genes among multiple antibiotic resistant *E. coli* isolates (N = 139) from Mud Creek in Fayetteville, Arkansas

<i>mob</i> gene combination ^ψ		Number of <i>E. coli</i> isolates containing <i>mob</i> genes based on their MAR number				
		1 (n=19)	2 (n=11)	3 (n=62)	4 (n=30)	5,6 (n=17)
Single gene ^A	<i>mob</i> _{P51}	2	0	0	0	0
	<i>mob</i> _{F11}	0	1	0	0	0
	<i>mob</i> _{F12}	3	2	24	5	2
	<i>mob</i> _{Q11}	0	1	0	0	0
	<i>mob</i> _{Q12}	1	0	0	0	0
	<i>mob</i> _{Qu}	0	0	2	1	1
Two-genes ^B	<i>mob</i> _{P51} + <i>mob</i> _{F11}	0	1	0	1	0
	<i>mob</i> _{P51} + <i>mob</i> _{F12}	0	0	2	3	1
	<i>mob</i> _{P51} + <i>mob</i> _{Qu}	0	1	0	0	0
	<i>mob</i> _{F12} + <i>mob</i> _{Qu}	0	1	0	6	0
Three-genes ^B	<i>mob</i> _{P51} + <i>mob</i> _{F11} + <i>mob</i> _{F12}	0	0	0	1	0
	<i>mob</i> _{P51} + <i>mob</i> _{F11} + <i>mob</i> _{Q12}	0	0	0	0	1
	<i>mob</i> _{P51} + <i>mob</i> _{F12} + <i>mob</i> _{Qu}	0	0	0	4	0
	<i>mob</i> _{F12} + <i>mob</i> _{Q12} + <i>mob</i> _{Qu}	0	0	0	0	2
None ^A		13	5	34	12	10
Total	<i>mob</i> _{P11}	0	0	0	0	0
	<i>mob</i> _{P14}	0	0	0	0	0
	<i>mob</i> _{P51}	2	1	2	6	2
	<i>mob</i> _{F11}	0	1	0	2	0
	<i>mob</i> _{F12}	3	3	26	16	6
	<i>mob</i> _{Q11}	0	1	0	0	0
	<i>mob</i> _{Q12}	1	0	0	0	3
	<i>mob</i> _{Qu}	0	2	2	8	3
Total of isolates positive for <i>mob</i> genes	6	6	28	18	7	

^ψ*mob* gene combination followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$)

Appendix 2.2. Distribution of *intI* genes among multiple antibiotic resistant *E. coli* isolates (N = 139) from Mud Creek in Fayetteville, Arkansas

<i>IntI</i> gene combination ^ψ		Number of <i>E. coli</i> isolates containing <i>intI</i> genes based on their MAR number				
		1 (n=19)	2 (n=11)	3 (n=62)	4 (n=30)	5,6 (n=17)
Single gene ^A	<i>intI1</i>	5	0	15	12	11
	<i>intI2</i>	0	0	0	2	0
Two-genes ^A	<i>intI1</i> + <i>intI2</i>	0	1	10	5	3
None ^B		14	10	37	11	3
Total	<i>intI1</i>	5	1	25	17	14
	<i>intI2</i>	0	1	10	7	3
Total of isolates positive for <i>intI</i> genes		5	1	25	19	14

^ψ*intI* gene combination followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$)

Appendix 2.3. Distribution of *sul* genes among multiple antibiotic resistant *E. coli* isolates (N = 139) from Mud Creek in Fayetteville, Arkansas

<i>sul</i> gene combination ^ψ		Number of <i>E. coli</i> isolates containing <i>sul</i> genes based on their MAR number				
		1 (n=19)	2 (n=11)	3 (n=62)	4 (n=30)	5,6 (n=17)
Single gene ^A	<i>sul1</i>	0	1	1	1	1
	<i>sul2</i>	10	6	9	5	2
	<i>sul3</i>	0	0	0	0	0
Two-genes ^A	<i>sul1+sul2</i>	8	1	10	2	0
	<i>sul1+sul3</i>	0	0	7	4	2
	<i>sul2+sul3</i>	0	1	7	0	0
Three-genes ^B	<i>sul1+sul2+ sul3</i>	0	2	28	18	12
None ^{AB}		1	0	0	0	0
Total	<i>sul1</i>	8	4	46	25	15
	<i>sul2</i>	18	10	54	25	14
	<i>sul3</i>	0	3	42	22	14
Total of isolates positive for <i>sul</i> genes		18	11	62	30	17

^ψ*sul* gene combination followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$)

Appendix 2.4. Distribution of *dfr* genes among multiple antibiotic resistant *E. coli* isolates (N = 139) from Mud Creek in Fayetteville, Arkansas

<i>dfr</i> gene combination ^ψ		Number of <i>E. coli</i> isolates containing <i>dfr</i> genes based on their MAR number				
		1 (n=19)	2 (n=11)	3 (n=62)	4 (n=30)	5,6 (n=17)
Single gene ^C	<i>dfrA1</i>	13	6	16	3	1
	<i>dfrA8</i>	0	1	0	0	0
	<i>dfrA12</i>	0	0	1	0	0
	<i>dfrA14</i>	0	0	0	1	0
Two- genes ^A _B	<i>dfrA1+dfrA8</i>	2	0	1	0	0
	<i>dfrA1+dfrA12</i>	1	1	6	1	0
	<i>dfrA8+dfrA12</i>	0	0	3	0	0
	<i>dfrA8+dfrA17</i>	0	0	1	1	1
	<i>dfrA12+dfrA14</i>	0	0	0	1	0
	<i>dfrA12+dfrA17</i>	0	0	1	2	3
	<i>dfrA1+dfrA8+dfrA12</i>	0	0	0	2	0
Three- genes ^A	<i>dfrA1+dfrA8+dfrA17</i>	0	0	1	1	0
	<i>dfrA1+dfrA12+dfrA17</i>	0	0	4	3	1
	<i>dfrA8+dfrA12+dfrA17</i>	0	0	6	1	4
	<i>dfrA8+dfrA12+dfrB3</i>	0	0	1	1	1
	<i>dfrA1+dfrA8+dfrA12+dfrA17</i>	0	0	10	5	5
Four- genes ^A	<i>dfrA1+dfrA8+dfrA12+dfrB3</i>	0	0	4	2	0
	<i>dfrA8+dfrA12+dfrA14+dfrA17</i>	0	0	0	2	0
	<i>dfrA1+dfrA8+dfrA12+dfrA14+dfrA17</i>	0	0	0	1	0
Five- genes ^A	<i>dfrA1+dfrA8+dfrA12+dfrA17+dfrB3</i>	0	0	0	1	0
	<i>dfrA1+dfrA8+dfrA12+dfrA17+dfrB3</i>	0	0	0	1	0
None ^{BC}		3	3	6	2	1
Total	<i>dfrA1</i>	16	7	43	19	7
	<i>dfrA8</i>	2	1	27	17	11
	<i>dfrA12</i>	1	1	36	22	14
	<i>dfrA14</i>	0	0	0	5	0
	<i>dfrA17</i>	0	0	24	17	14
	<i>dfrB3</i>	0	0	5	4	1
	Total of isolates positive for <i>dfr</i> genes	16	8	56	28	16

^ψ*dfr* gene combination followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$)

Appendix 3.1. Distribution of *sul* genes among sulfamethoxazole-trimethoprim resistant *E. coli* isolates (N = 76) from four locations of Mud Creek in Fayetteville, Arkansas

<i>sul</i> gene combination [‡]		Number of <i>E. coli</i> isolates from a sites of origin containing <i>sul</i> genes			
		M1 (n=11) [‡]	ME (n=29)	M2 (n=20)	M3 (n=16)
Single gene ^A	<i>sul1</i>	0	1	1	0
	<i>sul2</i>	0	0	0	1
	<i>sul3</i>	0	0	0	0
Two-genes ^A	<i>sul1+sul2</i>	0	2	2	1
	<i>sul1+sul3</i>	4	4	3	1
	<i>sul2+sul3</i>	0	0	0	0
Three-genes ^A	<i>sul1+sul2+ sul3</i>	7	22	14	13
Total	<i>sul1</i>	11	29	20	15
	<i>sul2</i>	7	24	16	15
	<i>sul3</i>	11	26	17	14
Total of isolates positive for <i>sul</i> genes		11	29	20	16

[‡]*sul* gene combinations followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$)

[‡]M1, ME, M2, and M3 are the sampling site of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input.

Appendix 3.2. Distribution of *sul* genes versus integrons among sulfamethoxazole-trimethoprim resistant *E. coli* (N = 76) isolated from Mud Creek in Fayetteville, Arkansas

<i>sul</i> gene combination		Number of <i>E. coli</i> isolates from a sites of origin containing <i>sul</i> genes			
		<i>intI1</i> (n=18)	<i>IntI2</i> (n=2)	<i>intI1+intI2</i> (n=18)	Integrase-free (n=38)
Single gene	<i>sul1</i>	0	0	1	1
	<i>sul2</i>	0	0	0	1
	<i>sul3</i>	0	0	0	0
Two-genes	<i>sul1+sul2</i>	0	0	2	3
	<i>sul1+sul3</i>	4	0	3	5
	<i>sul2+sul3</i>	0	0	0	0
Three-genes	<i>sul1+sul2+ sul3</i>	14	2	12	28
Total	<i>sul1</i>	18	2	18	37
	<i>sul2</i>	14	2	14	32
	<i>sul3</i>	18	2	15	33
Total of isolates positive for <i>sul</i> genes		18	2	18	38

Appendix 3.3. Distribution of *dfr* genes among sulfamethoxazole-trimethoprim resistant isolates (N = 76) from four locations of Mud Creek in Fayetteville, Arkansas

<i>dfr</i> gene combination [‡]		Number of <i>E. coli</i> isolates from a site of origin containing <i>dfr</i> genes			
		M1 (n=11) [‡]	ME (n=29)	M2 (n=20)	M3 (n=16)
Single gene ^A	<i>dfrA1</i>	0	1	0	0
	<i>dfrA12</i>	0	0	0	1
	<i>dfrA14</i>	0	1	0	0
Two-genes ^A	<i>dfrA1+dfrA8</i>	0	0	0	1
	<i>dfrA1+dfrA12</i>	3	0	0	0
	<i>dfrA8+dfrA12</i>	0	0	2	1
	<i>dfrA8+dfrA17</i>	0	1	2	0
	<i>dfrA12+dfrA14</i>	0	1	0	0
	<i>dfrA12+dfrA17</i>	0	3	2	1
Three-genes ^A	<i>dfrA1+dfrA8+dfrA12</i>	0	1	0	1
	<i>dfrA1+dfrA8+dfrA17</i>	1	0	1	0
	<i>dfrA1+dfrA12+dfrA17</i>	3	4	1	0
	<i>dfrA8+dfrA12+dfrA17</i>	1	3	3	4
	<i>dfrA8+dfrA12+dfrB3</i>	0	1	1	1
Four-genes ^A	<i>dfrA1+dfrA8+dfrA12+dfrA17</i>	1	10	6	3
	<i>dfrA1+dfrA8+dfrA12+dfrB3</i>	2	0	1	3
	<i>dfrA8+dfrA12+dfrA14+dfrA17</i>	0	2	0	0
Five-genes ^A	<i>dfrA1+dfrA8+dfrA12+dfrA14+dfrA17</i>	0	1	0	0
	<i>dfrA1+dfrA8+dfrA12+dfrA17+dfrB3</i>	0	0	1	0
Total	<i>dfrA1</i>	10	17	10	8
	<i>dfrA8</i>	5	19	17	14
	<i>dfrA12</i>	10	26	17	15
	<i>dfrA14</i>	0	5	0	0
	<i>dfrA7</i>	6	24	16	8
	<i>dfrB3</i>	2	1	3	4
Total of isolates positive for <i>dfr</i> genes		11	29	20	16

[‡]*dfr* gene combinations followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$).

[‡]M1, ME, M2, and M3 are the sampling sites of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input.

Appendix 3.4. Distribution of *intI* genes among sulfamethoxazole-trimethoprim resistant *E. coli* (n = 76) isolated from Mud Creek in Fayetteville, Arkansas

<i>IntI</i> gene combination [¥]		Number of <i>E. coli</i> isolates from a site of origin containing <i>intI</i> genes			
		M1 (n=11) ^Ψ	ME (n=29)	M2 (n=20)	M3 (n=16)
Single ^A gene	<i>intI1</i>	1	14	3	0
	<i>intI2</i>	2	0	0	0
Two- genes ^B	<i>intI1</i> + <i>intI2</i>	1	8	6	3
None ^A		7	7	11	13
Total	<i>intI1</i>	2	22	9	3
	<i>intI2</i>	3	8	6	3
Total of isolates positive for <i>intI</i> genes		4	22	9	3

[¥]*intI* gene combinations followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$).

^ΨM1, ME, M2, and M3 are the sampling sites of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input.

Appendix 3.5. Distribution of *mob* genes among sulfamethoxazole-trimethoprim resistant isolates (N = 76) from four locations of Mud Creek in Fayetteville, Arkansas

<i>mob</i> gene combination [‡]		Number of <i>E. coli</i> isolates from a site of origin containing <i>mob</i> genes			
		M1 (n=11) ^Ψ	ME (n=29)	M2 (n=20)	M3 (n=16)
Single gene ^B	<i>mob</i> _{F12}	0	2	8	10
	<i>mob</i> _{Qu}	1	2	0	0
Two-genes ^{AB}	<i>mob</i> _{P51} + <i>mob</i> _{F11}	0	1	0	0
	<i>mob</i> _{P51} + <i>mob</i> _{F12}	0	1	0	3
	<i>mob</i> _{F12} + <i>mob</i> _{Qu}	0	5	0	0
Three-genes ^A	<i>mob</i> _{P51} + <i>mob</i> _{F11} + <i>mob</i> _{F12}	0	1	0	0
	<i>mob</i> _{P51} + <i>mob</i> _{F11} + <i>mob</i> _{Q12}	0	1	0	0
	<i>mob</i> _{P51} + <i>mob</i> _{F12} + <i>mob</i> _{Qu}	0	1	0	0
	<i>mob</i> _{F12} + <i>mob</i> _{Q12} + <i>mob</i> _{Qu}	0	2	0	0
None ^A		10	13	12	3
Total	<i>mob</i> _{P11}	0	0	0	0
	<i>mob</i> _{P14}	0	0	0	0
	<i>mob</i> _{P51}	0	5	0	3
	<i>mob</i> _{F11}	0	2	0	0
	<i>mob</i> _{F12}	0	13	8	13
	<i>mob</i> _{Q11}	0	0	0	0
	<i>mob</i> _{Q12}	0	3	0	0
	<i>mob</i> _{Qu}	1	10	0	0
Total of isolates positive for <i>mob</i> genes		1	16	8	13

[‡]*mob* gene combinations followed by the same letter are not significantly different using a contrasts procedure ($P \leq 0.05$).

^ΨM1, ME, M2, and M3 are the sampling sites of isolate origins: M1, 20m upstream of effluent discharge pipe; ME, effluent input; M2, 1st site 640m downstream of effluent; M3, 2nd site 2000m downstream of effluent input.