RESEARCH PAPER

Conjugative transmission of antibiotic-resistance from stream water *Escherichia coli* as related to number of sulfamethoxazole but not class 1 and 2 integrase genes

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ABSTRACT

A conjugation assay was used to determine the effects of phenotypic resistance to one to up to 5 antibiotics, sampling site of origin, presence or absence of class 1 and/or class 2 integrase (*intI*) genes (*intI1* and *intI2*), and the number of sulfamethoxazole resistance (*sul*) and trimethoprim resistance (*dfr*) genes on the transfer frequencies of plasmids from environmental, antibiotic-resistant *Escherichia coli*. Of 51 sulfamethoxazole and trimethoprim-resistant *E. coli* isolates conferring at least one *mob* gene (*mob*_{P51}, *mob*_{F11}, *mob*_{F12}, *mob*_{Q11}, *mob*_{Q12}, or *mob*_{Qu}), 38 produced transconjugants with an overall mean frequency of 1.60×10^{-3} transconjugants/donors (T/D) or 5.89×10^{-3} transconjugants/recipients (T/R). The presence or absence of *intI1* and *intI2* and the presence or absence of different targeted *dfr* genes (*dfrA1*, *dfrA8*, *dfrA12*, *dfrA14*, *dfrA17*, and/or *dfrB3*) were not statistically related to plasmid transfer frequencies as determined by ANOVA ($P \geq 0.05$). However, *E. coli* isolates recovered 2 km downstream of wastewater treatment plant effluent input, and those possessing resistance to 3 antibiotics had significantly greater plasmid transfer frequency than their counterparts when calculated as T/D (ANOVA followed by Fisher's least significant difference means comparison, $P < 0.05$). Greater plasmid transfer frequency calculated as T/D was also measured for *E. coli* possessing 3 compared to a single *sul* gene. The in-vitro frequency suggests that horizontal gene transfer of conjugative mediated-antibiotic (*sul*) resistance genes may be significant among resistant, stream bacteria.

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Introduction

Antibiotic resistant bacteria (ARB) are an alarming global threat rapidly emerging and generating considerable potential risks, primarily in clinical settings. These ARB are able to survive in the presence of antibiotics and may drastically increase mortality rates compared to susceptible bacteria causing the same infection in hospitals. For example, the number of deaths resulting from methicillin-resistant *Staphylococcus aureus* bacteremia infections doubled compared to susceptible *S. aureus*.¹ Direct adverse outcomes include an 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.² The economic impact of ARB is estimated to exceed \$55 billion, comprising of \$20 billion in medical and \$35 billion in societal costs per year in the US alone.^{3,4}

The spread of ARB is associated with mobile genetic elements, including plasmids. Plasmids are extra-chromosomal, linear or circular DNA with widely varying copy number and size per bacterial cell that autonomously replicate⁵ and may have antibiotic resistance genes (ARG) embedded. Plasmids, depending on their host range, conjugative apparatus, and mobility, play a major role in horizontal gene transfer, promoting dissemination of ARG among bacteria, not only in the same species or genus but also among distantly related bacteria.⁶ One of the most promiscuous mechanisms of horizontal gene transfer 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 plasmid 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 2 classes of transmissible 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 may be a type 4 secretion system that forms the mating channel.⁷ An example of this plasmid is the AbR IncW plasmid R388 of Enterobacteriaceae.⁸ 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.⁷ An example of a mobilizable plasmid is the IncQ1 plasmid RSF1010.⁸

Integrations are not self-mobilizing genetic elements; however, previous research has indicated the likelihood of resistance to multiple antibiotics associated with integrons,⁹ and resistance to multiple antibiotics, integrons, and promiscuous plasmids co-occurring in wastewater treatment effluent.¹⁰ 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).¹¹ Structurally, an integron consists of 3 major components: the integrase gene (*intI*); the recombination site (*attI*); and the promoter (Pc). The *intI* gene allows specific excision and integration of gene cassette(s) adjacent to the recombination site (*attI*) which can be followed by the transcription and expression of the gene cassettes containing ARG and other genes through the integron promoter (Pc).¹¹⁻¹³ The mobility of plasmids combined with a highly efficient natural gene-capture and expression system plus highly heterogeneous gene cassettes of an integron may facilitate ARG exchange among diverse bacteria.¹⁴

Previously, *Escherichia coli* containing plasmids were recovered from wastewater treatment plant (WWTP) effluent and receiving stream water in Northwest Arkansas that were phenotypically resistant to one to up to 5 different antibiotics.^{15,16} A subset of isolates expressing resistance to sulfamethoxazole and trimethoprim were

found to possess redundancy and a diversity among sulfamethoxazole resistance (*sul*) or trimethoprim resistance (*dfr*) genes, respectively.¹⁷ These *sul* and *dfr* and other antibiotic resistance genes might be associated with conjugative and/or mobilizable plasmids and integrons, which were also found to be abundant among resistant isolates,¹⁸ promoting dissemination of the antibiotic resistance determinants. Hence, the objective of the research was to determine the differences among sampling site of origin, *intI* presence, phenotypic resistance to number of different antibiotics, and number of sulfamethoxazole resistance (*sul*) or trimethoprim resistance (*dfr*) genes with respect to transfer frequencies measured among isolates.

Results and discussion

Of 51 antibiotic resistant *E. coli* isolates harboring transmissible plasmids, 38 (74.5%) isolates were able to transfer plasmid-mediated antibiotic resistance and no transconjugants were obtained from 13 (25.5%) isolates. Potential mechanisms explaining the prevalence of antibiotic resistant bacteria in aquatic environments include greater inputs (e.g. effluent containing antibiotics and/or antibiotic resistant bacteria from WWTP), the persistence of the bacteria in the aquatic system, and dissemination of the resistance through horizontal gene transfer.¹⁸ 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.

Ratios of transconjugant to donor (T/D) or recipient (T/R) CFU are commonly used to measure conjugation efficiency. Because of differential variation in cell density or growth rates of donor and recipient cells, the measurement frequencies of T/D versus T/R may differ and the factors that significantly influence ratios,¹⁸ i.e. antibiotic resistance number, sampling site of origin, number of *sul* genes, may differ. In fact, the ratio of T/D was lower than T/R as growth rate of donors was greater than recipients. Overall, among 51 isolates, the overall mean transfer frequencies were 1.60×10^{-3} (T/D) or 5.89×10^{-3} (T/R). Furthermore, plasmid transfer frequency was high regardless of the presence or absence of class 1 and/or class 2 integrase genes ($P = 0.714$ for T/D, $P = 0.194$ for T/R, data not shown).

Other investigations have demonstrated a transfer frequency at 10^{-4} to 10^{-2} transconjugants per recipient CFU of integron-harboring multi-drug-resistant *Enterobacteriaceae* isolated from clinical settings.¹⁹ Different results, however, were observed by Moura et al.²⁰ in which the frequency was one to 3 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).²¹ *Salmonella* harboring class 1 integron transferred plasmids to *E. coli* through conjugation at the frequencies of 10^{-6} and 10^{-5} (T/R).^{22,23} While the possibility of conjugative transfer of antibiotic resistance might increase as a result of increased number of antibiotic resistances and the presence of class 1 integron,²⁴ results did not support that integron was a factor for conjugation frequencies in this experiment.

Based on ANOVA results, number of antibiotic resistances had a statistically significant impact on the conjugation frequency of the isolates affecting the T/D ratio (Fig. 1a, $P = 0.007$), although there was no significant effect of antibiotic resistance number on the T/R ratio (Fig. 1b, $P = 0.612$). Isolates possessing resistance to 3 antibiotics, accounting for 49% of the isolates investigated, had significantly higher T/D transfer frequency than the isolates possessing resistance to any other number of antibiotics (from one to

5). As much as 96% of these 3-antibiotic resistant isolates harbored conjugative plasmids with *mob*_{F12}. Isolates possessing *mob*_{F12}, which was the most frequently found *mob* gene among different genera of *Enterobacteriaceae*, and is predominantly detected on the plasmids of clinical multi-resistant *E. coli*,²⁵ had high transfer frequencies,²⁶ suggesting an important role of *mob*_{F12} gene in the transmissibility of plasmids conferring ARG among bacteria.

Isolates for this experiment were selected based on the detection of at least one *mob* gene, either *mob*_{P51}, *mob*_{F11}, *mob*_{F12}, *mob*_{Q11}, *mob*_{Q12}, or *mob*_{Qu}. In contrast to the high frequency of transmission among 38 isolates, as many as one quarter of the total isolates failed to generate transconjugants in spite of possession of transmissible plasmids, predominantly *mob*_{F12} gene, while 2 isolates possessed *mob*_{P51} and one possessed *mob*_{Q12} (data not shown). Gene transfers may occur through a different mechanism other than bi-parental conjugation as performed in this experiment. Although conjugation assays were attempted 3 times, the lack of transconjugants able to grow on the specific antibiotics may have resulted from the possibility that some ARG were located within the chromosome or 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.¹⁶ Otherwise, the isolates had little in common, the *E. coli* exhibited between one and 4 phenotypic resistances, were sampled from

Figure 1. The effect of phenotypic antibiotic resistance (AR) number (i.e. number of different antibiotics that an isolate expressed resistance to) detected on the mean ($\pm$ standard error) plasmid transfer frequency ($N = 51$) from *Escherichia coli* isolates expressed as a transconjugant/donor CFU (T/D) ratio in Fig. 1A and as a transconjugant/recipient CFU (T/R) ratio in Fig. 1B. Transfer frequencies within a graph followed by a different letter are significantly different ($P \leq 0.05$). Transfer was evaluated from stream water *E. coli* isolates (n per AR; AR1 = 6; AR2 = 6; AR3 = 25; AR4 = 12; AR5 = 2) recovered from Mud Creek, Fayetteville, Arkansas (36.090° latitude, 94.111° longitude). Antibiotic resistances were determined for the stream water (donor) *E. coli* at the following concentrations for a total of 6 different antibiotics: 32 $\mu\text{g/mL}$ ampicillin, 16 $\mu\text{g/mL}$ gentamicin, 16 $\mu\text{g/mL}$ tetracycline, 8 $\mu\text{g/mL}$ ofloxacin, 4 $\mu\text{g/mL}$ trimethoprim, and 80 $\mu\text{g/mL}$ sulfamethoxazole.¹⁶

Figure 2. The effect of original sampling site (site of origin) on the mean ($\pm$ standard error) plasmid transfer frequency from *Escherichia coli* isolates ($N = 51$) expressed as a transconjugant/donor CFU (T/D) ratio in Fig. 2A and as a transconjugant/recipient CFU (T/R) ratio in Fig. 2B. The transfer frequencies within a graph followed by a different letter are significantly different ($P \leq 0.05$). Sites of sampling origin are labeled M1: upstream, ME: effluent, M2: 640 m downstream from effluent input, and M3: 2000 m downstream from effluent input as described in Akiyama and Savin.¹⁶ Stream water *E. coli* isolates (n per site, M1 = 11; ME = 12; M2 = 12; and M3 = 16) were recovered from Mud Creek, Fayetteville, Arkansas (36.090° latitude, 94.111° longitude).

effluent and both downstream locations, conferred between zero and 2 *intI*, zero to 3 *sul*, and one to 4 *dfr* genes across the group of 13 isolates.

Bacteria were originally recovered from different locations in the stream environment: upstream, WWTP effluent, 640 m, and 2 km downstream of effluent input into the stream.¹⁶ The site of origin where the isolates were recovered had a significant impact on the conjugation frequency for T/D (Fig. 2a, $P < 0.0001$), although no significant difference among sites was observed on the T/R (Fig. 2b, $P = 0.183$). The *E. coli* recovered from 2 km downstream of effluent input had significantly higher T/D transfer frequency compared to *E. coli* recovered from upstream, effluent, or approximately 640 m downstream of effluent input into the stream.

While environmental factors, e.g., temperature, nutrient availability and pH, may 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. For example, in Massey et al.,²⁷ 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. Water downstream was more similar to effluent rather than upstream with dissolved organic carbon and nitrate/nitrite nitrogen significantly greater downstream of

effluent input at the time of sampling in July, August, and September 2007.¹⁶ Thus, water quality parameters influencing which bacteria survive and are sampled, and thus transconjugation frequencies, do not appear to explain the significant difference between upstream, effluent and the first downstream site compared to the second downstream site.

Antibiotics, including sulfamethoxazole and trimethoprim, have been detected at low concentrations of 0.356 – 0.564 $\mu\text{g L}^{-1}$ and 0.029 – 0.061 $\mu\text{g L}^{-1}$, respectively, in the water column of Mud Creek, the source stream for these bacteria.^{27,28} 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.^{27,28} Despite the fact that antibiotics do not significantly influence conjugation within bacteria,²⁹ antibiotics, at the sub-inhibitory level, may play an important role in the survival and persistence of antibiotic resistant bacteria in the aquatic environment.¹⁷

In terms of sulfamethoxazole resistance, the number of *sul* genes in plasmids within an isolate had a statistically significant effect on the transfer frequencies of plasmids based on the ratio of T/D (Fig. 3, $P = 0.048$). Isolates possessing 3 plasmid-mediated *sul* (*sul1* + *sul2* + *sul3*) genes had a significantly greater mean transfer frequency (2.56×10^{-3} T/D) than the isolates possessing only one *sul* (*sul2*) gene (3.56×10^{-4} T/D). The transfer frequency (1.55×10^{-3} T/D) for *E. coli* conferring 2 *sul* resistance

Figure 3. The effect of the number of *sul* genes on the mean ($\pm$ standard error) plasmid transfer frequency from *Escherichia coli* isolates ($N = 51$) expressed as a transconjugant/donor CFU (T/D) ratio in Fig. 3A and as a transconjugant/recipient CFU (T/R) ratio in Fig. 3B. The transfer frequencies within a graph followed by a similar letter are not significantly different ($P \leq 0.05$, *sul*-free was not included in statistical analysis). Stream water *E. coli* isolates (n per *sul*: 1 *sul* = 15; 2 *sul* = 13; 3 *sul* = 22; and *sul* free = 1) were recovered from Mud Creek, Fayetteville, Arkansas (36.090° latitude, 94.111° longitude).

genes was intermediate, and not different from, those conferring one or 3 *sul* genes. Based on the T/R ratio, however, *sul* gene combination had no significant effects on the transfer frequency (Fig. 3b, $P = 0.435$). In contrast, the number of *df*r genes (from zero to 5) conferring trimethoprim resistance detected in plasmids from environmental *E. coli* did not have a statistically significant effect on the transfer frequency ($P = 0.062$ for T/D, $P = 0.165$ for T/R, data not shown).

Recently, it was observed that all *sul3* genes residing in plasmids of *E. coli* from pigs, pig carcasses, and humans were conjugative.³⁰ 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*.³¹ The *sul1* gene is classically encoded within 3' conserved regions of class 1 integron.¹¹ While integron was not related to plasmid transfer frequency, perhaps multiple *sul* genes are better indicators of the placement of integrons within conjugative plasmids, and thus contributed to the increased frequency of transfer compared to the presence of a single *sul* gene, although further investigation is required to determine mechanisms of facilitated transfer of multiple *sul* genes.

Materials and methods

Donor cells were environmental, antibiotic-resistant *E. coli* isolated from treated effluent and receiving stream water upstream and at 2 locations (640 and 2000 m) downstream of effluent input in Mud

Creek and characterized according to Akiyama and Savin.¹⁴ Mud Creek is a tributary of the Illinois River, located in Fayetteville, Arkansas (36.090° latitude, 94.111° longitude) with the watershed described in Haggard et al.³² and the water quality at the time of sampling and sampling locations described in Akiyama and Savin.¹⁶ Phenotypic antibiotic resistances were determined for the environmental *E. coli* at the following concentrations for a total of 6 different antibiotics: 32 $\mu\text{g/mL}$ ampicillin, 16 $\mu\text{g/mL}$ gentamicin, 16 $\mu\text{g/mL}$ tetracycline, 8 $\mu\text{g/mL}$ ofloxacin, 4 $\mu\text{g/mL}$ trimethoprim, and 80 $\mu\text{g/mL}$ sulfamethoxazole.¹⁶

E. coli isolates had been stored in glycerol solution at -76°C . *E. coli* strains were grown on selective 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 instructions. 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 used as templates to determine the occurrences of mobilization genes (*mob_{P51}*, *mob_{F11}*, *mob_{F12}*, *mob_{Q11}*, *mob_{Q12}*, 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 1. All PCR amplifications were performed in 20 μ L reactions containing 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 200 μ M dNTPs, 400 ng/ μ L bovine serum albumin (Merck KGaA, Darmstadt, Germany), 0.5 μ M of each primer, 1 μ L 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 a 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 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).

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

Table 1. Target genes and their corresponding primer sequences with amplification features of PCR.

Target Gene		Primer sequence	Annealing Temp. (°C)	Product size (bp)	Reference
<i>sul1</i>	FW ^a	CGCACCGGAAACATCGCTGCAC	55.9	163	33
	RV	TGAAGTTCCGCCGCAAGGCTCG			
<i>sul2</i>	FW	TCCGGTGGAGGCCGTATCTGG	55.9	191	33
	RV	CGGGAATGCCATCTGCCTTGAG			
<i>sul3</i>	FW	TCCGTTCAAGCAATTGGTGCAG	60.8	128	33
	RV	TTCGTTCAAGCCTTACACCAGC			
<i>dfrA1</i>	FW	GTGAACTATCACTAATGG	46	471	34
	RV	ACCCCTTTGCCAGATTG			
<i>dfrA8</i>	FW	TTGGGAAGGACAACGCACTT	56.3	382	34
	RV	ACCATTTCCGCCAGATCAAC			
<i>dfrA12</i>	FW	GGTGAGCARAAGATYTTTCGC	52	309	34
	RV	TGGGAAGAAGGCGTCACCCTC			
<i>dfrA14</i>	FW	GCBAAAGGDGARCAGCT	50.8	394	34
	RV	TTTMCAYATTGATAGC			
<i>dfrA17</i>	FW	AAAATTTCTGATTCTGCA	48.4	471	34
	RV	TTAGCCTTTTTTCCAAATCT			
<i>dfrB3</i>	FW	GATCACGTRCGCAAGAARTC	56	95	34
	RV	GACTCGACVGCRTASCCTTC			
<i>intI1</i>	FW	GGGTCAAGGATCTGGATTTCTG	62	483	35
	RV	ACATGCGTGTAATCATCGTCG			
<i>intI2</i>	FW	CAAGCATCTCTAGGCGTA	50	233	35
	RV	AGAAGCATCAGTCCATCC			
<i>mobp₅₁</i>	FW	TACCACGCCCTATGCGAARAARTAYAC	58	167	36
	RV	CCCTTGCTGCTGGTGYTSNACCA			
<i>mob_{F11}</i>	FW	GCAGCGTATTACTCTCTGCTGCCGAYGAYTAYTA	53	234	36
	RV	ACTTTTGGGCGCGGARAABTGSAGRTC			
<i>mob_{F12}</i>	FW	AGCGACGCAATTATTACACCGCAAGGAYAYTAYTA	55	234	36
	RV	ACTTTTGGGCGCGGARAABTGSAGRTC			
<i>mob_{Q11}</i>	FW	CAATCGTCCAAGGCGAARGCNGAYTA	50	331	36
	RV	CGCTCGGAGATCATCAYYTGCACTG			
<i>mob_{Q12}</i>	FW	CTGGAATATACTGAACACGNAAYATGCC	52	341	36
	RV	ATCCTTGGTGTAGCACGTTTAAARWAYTG			
<i>mob_{Qu}</i>	FW	AGCGCCGTGCTGCTCCGBCGNTAYCG	64	179	36
	RV	CTCCGCAGCCTCGRCSGCRITCCA			

Note. ^aFW: forward; RV: reverse

Conjugation assays were performed using a modification of the protocol in Moura et al.²⁰ 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 μ L 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 μ L) 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, or triplicate if no transconjugants were observed. Conjugation, or transfer, frequency was measured based on ratio of the observed transconjugant CFU (T) divided by the initial donor CFU (T/D) or divided by the recipient CFU (T/R).¹⁸

Fifty-one donor *E. coli* were selected based on the presence of at least one *mob* gene, predominantly, but not necessarily, *mob*_{F12}. Environmental *E. coli* were diverse in characteristics evaluated and were grouped to perform statistical analyses. Analysis of variance (ANOVA) with 95% confidence intervals using XLSTAT 2016 (New York, NY) was conducted to evaluate the following toward the transfer frequency of transmissible plasmids: phenotypic resistance to the number of different antibiotics (e.g., antibiotic resistance number), sampling site of origin, integron number; number of sulfamethoxazole resistance (*sul*) genes, and number of trimethoprim resistance (*drf*) genes. Fisher's least significant difference (LSD) test was performed to compare means where appropriate ($P \leq 0.05$).

Conclusion

Conjugative transfer of plasmids from environmental *E. coli* isolates exhibiting single to multiple antibiotic resistances harboring transmissible plasmids occurred at a high frequency in vitro, regardless of integrons, or trimethoprim resistance genes. The presence of 3 compared to a single sulfamethoxazole (*sul*) resistance gene increased plasmid frequency. Further investigation is warranted to determine if the presence of 3 *sul* genes indicates a greater likelihood that antibiotic resistance genes are located on conjugative plasmids, or on mobile elements within conjugative plasmids and thus are linked to increased transfer frequencies. Sampling site of bacterial isolation from the stream impacted plasmid transfer frequency among bacterial populations, indicating the potential for environment to significantly increase dissemination of antibiotic resistance genes, although the mechanism was unclear. Taken together, these results indicate conjugation frequencies to be influenced by both factors related to the resistance genes within the bacteria and the environment.

Abbreviations

ARB	antibiotic resistant bacteria
ARG	antibiotic resistance genes
LB	Luria-Bertani
MH	Mueller-Hinton
PCR	polymerase chain reaction
WWTP	wastewater treatment plant

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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