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Effects of Nutrient Level and Growth Rate on the Conjugation Process That Transfers Mobile Antibiotic Resistance Genes in Continuous Cultures

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ABSTRACT Bacteria in the effluent of wastewater treatment plants (WWTPs) can transfer antibiotic resistance genes (ARGs) to the bacteria in receiving water through conjugation; however, there is a lack of quantitative assessment of this phenomenon in continuous cultures. Our objective was to determine the effects of background nutrient levels in river water column and growth rates of bacteria on the conjugation frequency of ARGs from effluent bacteria to river bacteria, as well as on the resulting resistance level (i.e., MICs) of the river bacteria. Chemostats were employed to simulate the discharge points of WWTPs into rivers, where effluent bacteria (donor cells) meet river bacteria (recipient cells). Both donor and recipient cells were *Escherichia coli* cells, and the donor cells were constructed by filter mating with bacteria in the effluent of a local WWTP. Results showed that higher bacterial growth rate (0.45 h^{-1} versus 0.15 h^{-1}) led to higher conjugation frequencies (10^{-4} versus 10^{-6} transconjugant per recipient). The nutrient level also significantly affected the conjugation frequency, albeit to a lesser extent than the growth rate. The MIC against tetracycline increased from 2 mg/L in the recipient to 64 to 128 mg/L in transconjugants. In comparison, the MIC only increased to as high as 8 mg/L in mutants. Whole-genome sequencing showed that the *tet*-containing plasmid in both the donor and the transconjugant cells also occur in other fecal bacterial genera. The quantitative information obtained from this study can inform hazard identification related to the proliferation of wastewater-associated ARGs in surface water.

IMPORTANCE WWTPs have been regarded as an important hot spot of ARGs. The discharge point of WWTP effluent, where ARGs may be horizontally transferred from bacteria of treated wastewater to bacteria of receiving water, is an important interface between the human-dominated ecosystem and the natural environment. The use of batch cultures in previous studies cannot adequately simulate the nutrient conditions and growth rates in receiving water. In this study, chemostats were employed to simulate the continuous growth of bacteria in receiving water. Furthermore, the experimental setup allowed for separate investigations on the effects of nutrient levels (i.e., simulating background nutrients in river water) and bacterial growth rates on conjugation frequencies and resulting resistance levels. The study generates statistically sound ecological data that can be used to estimate the risk of wastewater-originated ARGs as part of the One Health framework.

KEYWORDS tetracycline resistance, conjugation, chemostat, *E. coli*, growth rate, nutrient level

The decreased effectiveness of antibiotics to treat bacterial infections due to antibiotic resistance poses a serious threat to public health (1). Recently, the role of the environment as a medium in disseminating antibiotic resistance has been recognized. The

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"one-health" concept, which integrates humans, animals, and the environment, has been proposed as a strategy to address the emergence and spread of antibiotic resistance (2). Wastewater treatment plants (WWTPs), an important interface between human and the environment, are deemed "hot spots" for antibiotic resistance genes (ARGs) (3). Effluent from WWTPs can carry microbes containing mobile genetic elements and ARGs (4). Further, effluent from WWTPs can also contain pollutants such as antibiotics, heavy metals, surfactants, nanoparticles, etc. (5–7). These contaminant residues can create selective pressures for horizontal gene transfer (HGT) of ARGs in receiving water and soil (8). Consequently, WWTP effluent, particularly effluent without disinfection, could be a major contributor to the emergence and spread of antibiotic resistance in receiving water.

Two mechanisms, chromosomal mutation and HGT, may be involved in the emergence of antibiotic resistance in previously susceptible cells as a consequence of WWTP discharge into receiving water. Mutation can be induced by selective pressure in the environment, such as the presence of antibiotics (9, 10). Although mutations conferring resistance impose an initial fitness cost to bacterial hosts (11, 12), this fitness cost is often temporary and can be alleviated by compensatory mutations in later generations (10, 13). Therefore, mutational resistance against antibiotics can emerge and persist under selective pressure, which can occur in surface waters receiving WWTP effluent containing low levels of antibiotics (9, 10).

HGT can occur through conjugation, transformation, and transduction. Conjugative plasmids can self-replicate and carry the genes needed for self-transmission. Most broad-host-range conjugative plasmids have been found carrying ARGs (14). Consequently, conjugation is considered responsible for the spread of the majority of antibiotic resistance (15). Environmental factors, such as nutrient availability, temperature, and pH (16), and the characteristics of donor and recipient cells, such as their growth rate, can affect conjugation frequencies (17). However, most of the conjugation studies were conducted in batch cultures (18–20), which cannot simulate microbial communities residing in natural environments such as rivers. Different from microbes growing in batch cultures, where the nutrient contents are very high initially before gradually depleting, microbes face relatively constant, low nutrient levels over a long segment of a river (21), although the nutrient levels can vary significantly among river systems (22). Also, in contrast to microbes growing in batch reactors, where cells go through four distinctive growth phases (i.e., lag, exponential, stationary, and death phases), microbes in river systems may maintain a relatively stable growth rate (23). Hence, in order to assess the risk of ARG proliferation in surface water receiving WWTP effluent, it is important to examine how environmental and microbial factors affect conjugation frequencies in continuous cultures.

The objective of this study was to determine the effects of background nutrient levels in river water column and growth rates of bacteria on the conjugation frequency of ARGs from bacteria in WWTP effluent to river bacteria, as well as on the resulting resistance level of the river bacteria. Two *Escherichia coli* strains were used as donor and recipient cells to simulate bacteria in WWTP effluents and river bacteria, respectively. Chemostats were used to grow continuous cultures simulating river waters receiving WWTP effluents and were operated under different growth rates and nutrient levels (Fig. 1). The antibiotic tetracycline was also present in the chemostat reactors at an environmentally relevant concentration (i.e., 10 μg/L) (6, 24). Both culture-based and whole-genome sequencing (WGS) approaches were used to evaluate phenotypic and genotypic changes, respectively, resulting from conjugation events under continuous culture conditions. The findings from this study can improve our understanding of the transfer of ARGs from WWTP effluents to surface water.

RESULTS AND DISCUSSION

Effects of growth rate and nutrient level on conjugation frequency after 16 h of conjugation. It took 1.5 and 3.0 days, respectively, for the number of recipient cells to reach steady state in the chemostat reactors under high (0.45 h^{-1}) and low (0.15 h^{-1}) growth rates (see Fig. S1 in the supplemental material). After the recipient cells

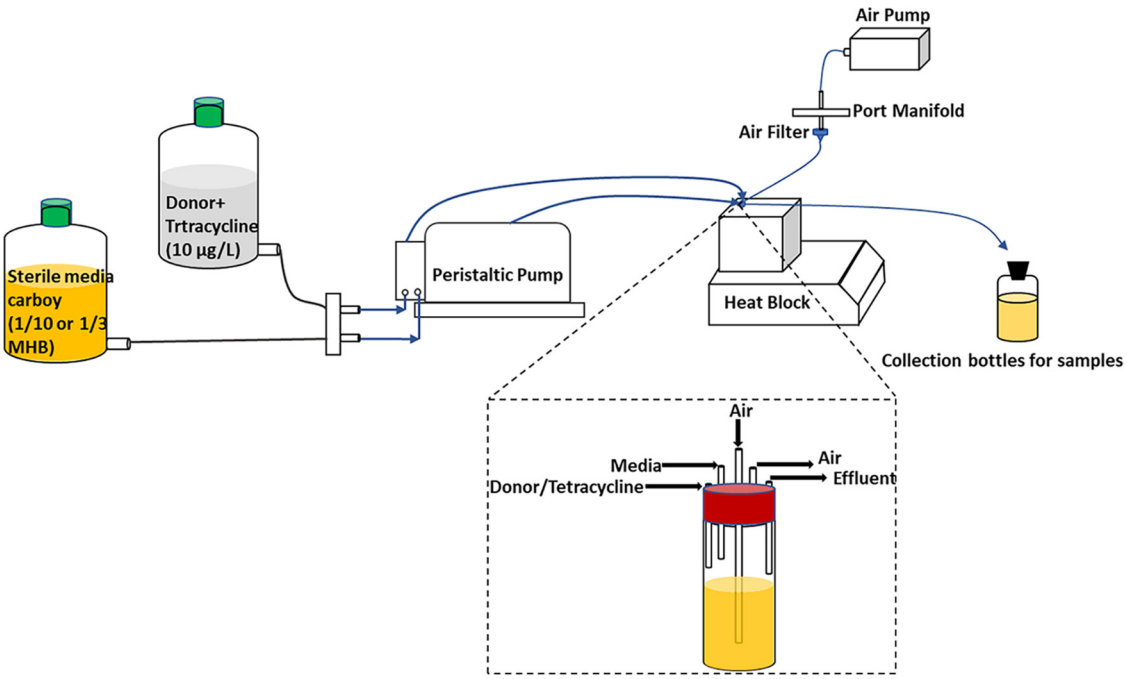

FIG 1 Experimental setup of the chemostat reactor system.

reached steady state, donor cells were introduced to the chemostat reactor (time 0). By analyzing the conjugation frequency measured at 16 h, the analysis of variance (ANOVA) results showed that both the nutrient level and the growth rate had significant effects on conjugation frequency (*P* values in Table S1). Tukey's *post hoc* test results showed a significant main effect for the growth rate under both nutrient levels (Fig. 2; F = 20, $P < 0.001$ for 1/10 MHB; F = 133, $P < 0.001$ for 1/3 MHB), while the nutrient level had a significant effect on the conjugation frequency only under a growth rate of 0.45 h^{-1} (Fig. 2; F = 17, $P < 0.05$).

A higher growth rate resulted in a higher conjugation frequency (Fig. 2). Under the nutrient level of 1/10 Mueller-Hinton broth (MHB), the conjugation frequencies were $(8.08 \pm 4.41) \times 10^{-4}$ and $(1.81 \pm 2.21) \times 10^{-6}$ after 16 h for the 0.45 h^{-1} and 0.15 h^{-1} growth rates, respectively. In comparison, under a nutrient level of 1/3 MHB, the conju-

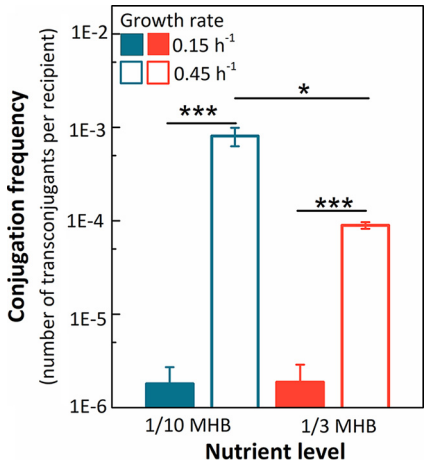

FIG 2 Conjugation frequency under different growth rates (0.45 h^{-1} and 0.15 h^{-1}) and different nutrient levels (1/10 MHB and 1/3 MHB) at 16 h. Asterisk represents statistically significant differences (* and *** indicate $P < 0.05$ and $P < 0.001$, respectively [Tukey's *post doc* test]). Error bars represent standard deviations ($n = 6$).

gation frequencies after 16 h were (8.94 ± 1.76) × 10^{-5} for bacteria grown at 0.45 h^{-1} and (1.87 ± 2.48) × 10^{-6} bacteria grown at 0.15 h^{-1}.

Several factors can affect conjugation frequencies, such as growth phase, cell density, donor-to-recipient ratio, nutrient concentrations, temperature, pH, and mating time (25). Batch reactors are often used to measure conjugation frequencies; however, they do not allow for separate controls of growth phase (i.e., resource-dependent growth rate) and cell densities. By using batch reactors, Lopatkin et al. reported that conjugation frequency significantly increased when the concentration of glucose was increased in the medium (26). In batch reactors, higher initial nutrient levels result in higher cell densities and higher growth rates, which makes it difficult to compare the relative importance of cell density and growth rate to conjugation frequency. In contrast to batch reactors, chemostat reactors are operated to grow continuous cultures, which can better simulate the river environment (27) and allow for separate controls of cell density (through nutrient level in chemostat influent) and growth rate (through dilution rate) (28). As shown in the result (Fig. 2), the growth rate significantly affected the conjugation frequency, regardless of the cell densities, in chemostat reactors.

Effects of growth rate and nutrient level on the number of transconjugants during the 96-h conjugation experiment. Conjugation experiments often use a mating window of 16 h because subsequent growth of transconjugants may cause overestimation of the conjugation frequency (29). In reality, bacteria from WWTP effluent can co-reside with indigenous river bacteria in receiving water for days or longer, resulting in longer mating times. Therefore, the number of transconjugants were also monitored for the time points beyond 16 h.

ANOVA tests show significant main effects of both nutrient level (see Table S2, $F_{4, 20}$ = 7.991, P = 0.037) and growth rate ($F_{4, 20}$ = 18.508, P = 0.008) on the number of transconjugants. Tukey's *post hoc* tests showed significantly higher concentrations of transconjugants under higher nutrient levels ($P < 0.05$) and at higher growth rates ($P < 0.001$). There was a significant interaction effect between nutrient level and growth rate (ANOVA: $F_{4, 20}$ = 7.170, P = 0.040). Tukey's *post hoc* tests showed significantly higher concentration of transconjugants at a 0.45 h^{-1} growth rate and 1/3 MHB than under the other treatment combinations ($P < 0.05$). Conjugation time had a significant effect on the number of transconjugants after 96 h of conjugation (see Table S2).

Under the 1/10 MHB nutrient level, the number of transconjugants increased from (9.83 ± 1.95) × 10^1 to (4.89 ± 4.37) × 10^3 CFU mL^{-1} at the 0.45 h^{-1} growth rate and increased from (5.08 ± 5.32) × 10^1 to (3.69 ± 3.38) × 10^2 CFU mL^{-1} for the 0.15 h^{-1} growth rate (Fig. 3a). Similarly, under the 1/3 MHB nutrient level, the number of transconjugants showed an increase from (2.10 ± 0.32) × 10^2 to (4.19 ± 2.88) × 10^4 CFU mL^{-1} for

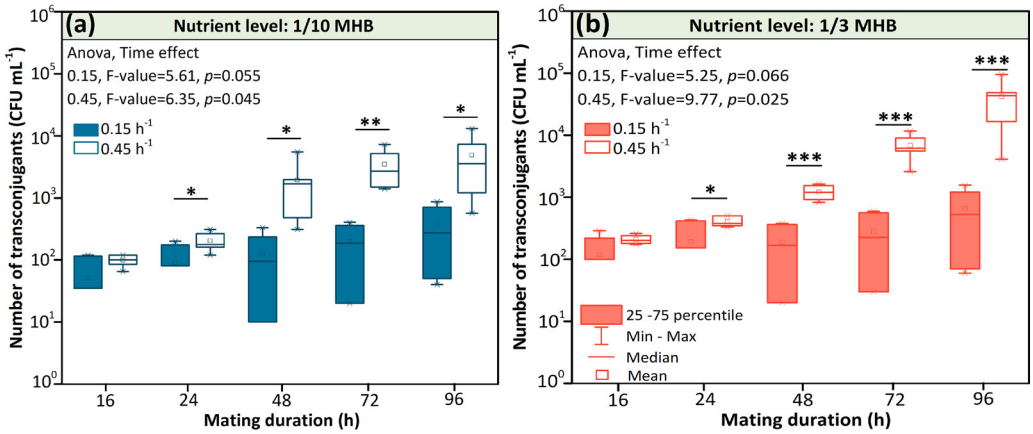

FIG 3 Concentrations of transconjugants (n = 6) at 0.45 h^{-1} and 0.15 h^{-1} growth rates with nutrient levels of 1/10 MHB (a) and 1/3 MHB (b). Asterisks represents statistically significant differences (i.e., *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively [Tukey's *post doc* test]).

cultures grown under 0.45 h^{-1} and (1.18 ± 1.21) × 10^2 to (6.61 ± 6.18) × 10^2 CFU mL^{-1} for the 0.15 h^{-1} growth rate (Fig. 3b).

For a given experimental duration, a higher growth rate means a larger number of generations. Therefore, the number of generations of the cultured cells associated with the growth rate of 0.45 h^{-1} was always three times that associated with the growth rate of 0.15 h^{-1}. Interestingly, the number of transconjugants at the same generation numbers were the same for the same nutrient conditions, regardless of growth rate (see Fig. S2). Therefore, slow-growing bacteria can have a similar number of transconjugants as fast-growing bacteria, if they reach the same generation numbers.

In addition, the presence of antibiotic also likely had an impact on the conjugation frequency reported here. Unlike batch reactors, where the concentration of antibiotics may decrease over time due to degradation and adsorption to bacterial cells, the tetracycline concentration in the chemostat reactors was maintained at 10 μg L^{-1} by continuously pumping freshly prepared antibiotic solutions. The antibiotic concentration used in this study is an environmentally relevant concentration. A previous study showed that 10 μg L^{-1} of tetracycline increased the transfer frequency of ARGs by 2-fold compared to a condition without tetracycline (18). Furthermore, although higher concentrations of tetracycline may favor selection of a tetracycline-resistant phenotype, such concentrations could also lead to a decline in the number of detectable transfer events.

MIC change. The minimum inhibitory concentrations (MICs) of transconjugants were substantially higher than those of the original recipients. The MIC of the original recipient cells for tetracycline was 2 mg L^{-1}. Under the 1/10 MHB nutrient level, for the cells with a growth rate of 0.45 h^{-1}, the MIC of transconjugants increased to 64 mg L^{-1} at 16 h and eventually reached 128 mg L^{-1} at 96 h (Fig. 4a). For the cells with the growth rate of 0.15 h^{-1}, the MIC of transconjugants increased to 64 mg L^{-1} at 16 h and remained at that level until the end of the conjugation experiments at 96 h (Fig. 4a). Under the 1/3 MHB nutrient level, the MIC of the transconjugants with 0.15 h^{-1} growth rate increased to and maintained at 64 mg L^{-1}, while the MIC of the transconjugants with the 0.45 h^{-1} growth rate eventually increased to 128 mg L^{-1} at the end of the conjugation experiment at 96 h (Fig. 4b). It is worth noting that the MIC of the donor increased from 2 mg L^{-1} (for strain CV601) to 256 mg L^{-1} (for strain CW) after the filter-mating experiment with treated WWTP effluent.

According to Kruskal-Wallis tests, the growth rate had a significant impact on the MICs of transconjugants (see Table S3, $P < 0.05$), while the nutrient level did not have significant impact. The difference in MICs between the two growth rates was likely caused by the difference in the development of compensatory adaptation under the two growth rates. Newly acquired genes often function inefficiently within existing

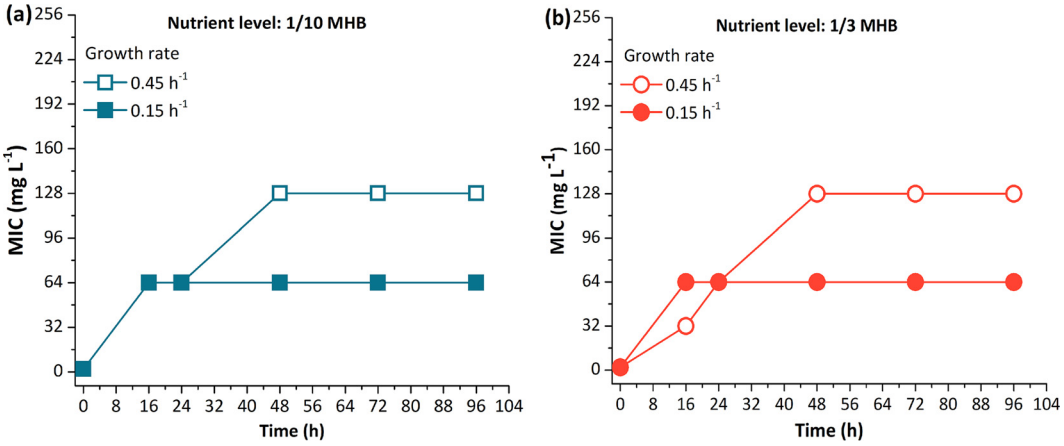

FIG 4 Change in tetracycline MIC relative to the J53 control strain after 4 days of the conjugation experiment at 0.45 h^{-1} and 0.15 h^{-1} growth rates with nutrient levels of 1/10 MHB (a) and 1/3 MHB (b). The results are averages from six replicate chemostats, and error bars are too small to be visible.

genomic background (30). Because plasmids often only harbor loci that are necessary for their own replication, the fitness costs could be high for gene disruption, energetic requirements, and specific protein level interactions (31). Prensky and coworkers reported that new transconjugants grew significantly slower and/or with longer lag times than lineages that had been replicating for several generations (32). This observation is consistent with our findings where, at any given time, transconjugants from later generations (i.e., the ones under higher growth rate) had higher MIC values than transconjugants from earlier generations (i.e., the ones under lower growth rate).

In actual riverine environment, the antibiotic residues in wastewater effluent could also cause the emergence of antibiotic resistance in river bacteria by causing mutations. In order to study the effects of antibiotics alone on the emergence of antibiotic resistance in riverine consortia, mutation experiments were also conducted in our simplified chemostat systems. In the mutation experiment, the increases in MIC were much smaller than those from the conjugation experiment. Under a 1/10 MHB nutrient level, the MIC of the mutant increased from 2 to 8 mg/L and to 4 mg/L under growth rates of 0.45 h^{-1} and 0.15 h^{-1}, respectively, after 96 h of mutation experiment (see Fig. S3a). Under a 1/3 MHB nutrient level, the MIC of the mutant increased from 2 to 4 mg/L under both growth rates of 0.45 h^{-1} and 0.15 h^{-1} (see Fig. S3b).

The MICs of the mutants were much lower than those of the transconjugants. This suggests that cells acquiring resistance through conjugation could pose a higher hazard (i.e., a higher MIC) than those acquiring resistance through mutation. Furthermore, the MIC of mutants were not significantly affected by either growth rate or nutrient level (see Fig. S3). Among the four treatment scenarios tested, the combination of 0.45 h^{-1} growth rate and 1/10 MHB resulted in the highest MIC at the end of the mutation experiment. Resistance mutations are often costly, and there is a significant difference in the fitness cost between antibiotic classes and bacterial species (9). Further, through meta-analysis, Melnyk and coworkers found that the relative fitness of resistance mutation is negatively correlated with the fold-incase in MIC conferred by the mutation.

Whole-genome sequencing. To confirm the transfer of plasmids carrying tetracycline resistance genes, whole-genome sequencing was conducted to compare the genomes of the donor, recipient, transconjugants, and mutants. The sequence of all possible plasmids was examined in the donor (CW) to identify tetracycline resistance genes (see Table S4). Of the seven plasmids identified, only plasmid CW_60 contained the known tetracycline-resistant genes *tet*(A) and *tet*(R) (see Fig. S4). The CW_60 plasmid shared more than 98% nucleotide sequence identity with plasmids found in *E. coli*, *Klebsiella pneumonia*, *Salmonella enterica*, *Shigella flexneri*, and *Shewanella algae* strains. Table S5 shows four plasmid entries, out of all BLAST hits, containing sequences similar to the CW_60 plasmid conferring resistance to tetracycline and other antibiotics (33–35). The presence of similar plasmids in different bacterial genera suggests that the plasmid can be transferred between different species via HGT.

CW_60 was also detected in the transconjugants grown with the 0.45 h^{-1} growth rate (i.e., T45) with 8 bases shorter in the transconjugants (see Fig. S5). CW_60 was not detected in the transconjugants grown under the growth rate of 0.15 h^{-1} (i.e., T15). However, other plasmids that were present in donor and T45 were found in T15. CW_60 in T15 was probably not detected because of the fragmentation of predicted open reading frames as a result the inability of short read lengths to bridge certain genomic regions (36). These results proved that a mobile plasmid containing tetracycline resistance genes were transferred from WWTP effluent to donor and then later from donor (i.e., CW) to transconjugants.

The discrepancy in MIC level between donor and transconjugants could be due to several factors, such as heterogeneity of MIC of cells in the culture and compatibility of the plasmid (37). Further studies are needed to better elucidate the reasons for the difference in MIC between transconjugants and donor, as well as between transconjugants grown at different growth rates. Attempts were made to identify underlying

TABLE 1 Mutations in recipients grown at 0.45 and 0.15 h^{-1}

Sample[a]	Gene	Mutation(s)	Description
M45	*yaaA*	A→E 73; D→A 242	Peroxide stress resistance protein YaaA
	cbrA	T→A 243; T→A 292; V→M 345	Colicin M resistance protein
	yfgI	L→F 5; I→V 54	Nalidixic acid resistance protein YfgI
	tehA	A→T 60	Tellurite resistance protein
	rclC	W→R 28	Reactive chlorine species resistance protein C
	hslJ	A→P 14; N→I 94; L→V 134	Lipoprotein implicated in novobiocin resistance
M15	*nohD*	E*82; E→Q 84	DLP12 prophage putative DNA-packaging protein NohD
	insH-5	I→V 83; A→P 117; K→E 185; G→S 187; H→N 190	Rac prophage IS*5* transposase and transactivator
	pinQ	E→G 38; K*82; D→A 194	Qin prophage putative site-specific recombinase

[a]The two mutants are denoted as M45 and M15.

mechanisms causing this difference using the WGS data. However, no convincing findings were obtained.

The genomes of mutants grown under different growth rates were assembled (i.e., M45 and M15 for mutants grown under 0.45 h^{-1} and 0.15 h^{-1} growth rates, respectively). There were only three mutation events in M15 compared to J53 (Table 1). The first mutation in M15 was found in the *nohD* gene. This gene is typically near a site of targeted chromosome cleavage by lambda terminase that introduces double-strand cleavages in DNA (38). The *nohD* gene is often involved in DNA recombination. The second mutation in M15 was on the *insH-5* gene, which interacts with the termini of the IS*5* sequence (39). IS*5* can enhance gene transcription when it is placed on either side of the promoter for a target gene (40). Hence, the interaction between *insH-5* and the IS*5* sequence can play a key role in transcription enhancement. The third mutation was on the *pinQ* gene. There is no known involvement of *pinQ* in antibiotic resistance. No mutation in M15 was directly related to tetracycline resistance, which might be the reason for the similar MICs between J53 and M15 (2 mg L^{-1} versus 4 mg L^{-1}). Gene knockout or monitoring of expression levels of these genes would be needed to further confirm their roles for the elevated MICs.

Six mutations were identified in the M45 genome (Table 1). Of those mutations, *yaaA* encodes a peroxide stress resistance protein, which prevents oxidative damage to both DNA and proteins by diminishing the amount of unincorporated iron within the cell and may play a role in tetracycline resistance (41). The mutation in *yaaA* likely occurred due to a stress response in reaction to tetracycline presence. Tetracycline produces lethal oxidative stress to kill the bacteria (42). Therefore, when facing oxidative stress induced by tetracycline, cells produce antioxidant enzymes to survive (43). Consequently, the higher MIC of M45 (8 mg L^{-1}) compared to J53 (2 mg L^{-1}) was likely due to the mutation in the *yaaA* gene.

Environmental implications. Conjugation frequencies vary widely (10^{-8} to 10^{-1} transconjugants per recipient [T/R]) for different bacterial donor-recipient pairs and different plasmids. They can also be affected by the test methods (batch versus continuous cultures; Table 2). The transfer frequencies (10^{-6} to 10^{-4}) obtained in our study were comparable to other studies (18, 20, 44) and were much lower than in other studies reporting conjugation frequency between 10^{-3} and 10^{-1} (19, 23, 29, 45–47). Cells in batch systems, with a cultivation period of 12 to 48 h, may undergo growth stage regulation (48), where some plasmids were significantly downregulated in conjugative transfer during stationary phase. Similar to this study, an *in situ* study (49), which reported the plasmid transfer frequencies of 10^{-6} to 10^{-4} in seawater, also suggested that the transfer frequencies from batch tests might have been overestimated (45).

The antibiotics in WWTP effluent may act as a selective force that drives the HTG of ARGs and may cause the mutation of river bacteria (46, 50, 51). In the simplified model system tested here (i.e., *E. coli* and tetracycline as model bacteria and antibiotic), our results demonstrate that the antibiotic residues in WWTP effluent would result in a greater ecological and public health implications through driving conjugation than through causing mutations.

TABLE 2 Plasmid transfer frequencies between pure strains and bacterial communities of various environments

E. coli strain						
Donor	Recipient	Source	Plasmid	Method	Frequency (T/R)[a]	Source or reference
MG1655		Activated sludge	pKJK5	Batch	1×10^{-3} to 4.3×10^{-2}	19
CV601		Wastewater		Batch	2.2×10^{-6} to 8.7×10^{-6}	18
MG1655		Activated sludge	pKJK5	Batch	5.05×10^{-4}	20
MG1655		Activated sludge	pKJK5	Batch	3.0×10^{-3} to 1.4×10^{-2}	23
MG1655		Wastewater	pKJK5	Batch	2.0×10^{-2} to 1.0×10^{-1}	45
K-12		Activated sludge	RP4	Bioreactor	2.76×10^{-5}	44
C600		Activated sludge	RP4	Batch	4.6×10^{-3} to 1.3×10^{-2}	47
CV601	J53	Sediment		Batch	8.48×10^{-7} to 7.48×10^{-1}	29
CV601	J53-2			Batch	8.46×10^{-5} to 1.02×10^{-1}	46
CV601	J53	Wastewater	CW_60	Chemostat reactor	1.81×10^{-6} to 8.08×10^{-4}	This study

[a]T/R, transconjugants/recipient.

MATERIALS AND METHODS

***E. coli* strains.** Two *E. coli* strains, CV601 and J53, obtained from Holger Heuer of the Julius Kühn Institute were used as donor and recipient cells, respectively, for the conjugation experiments. These strains have been used as model bacteria to study ARG proliferation in several other studies (29, 52, 53). *E. coli* strain CV601 is resistant to both kanamycin and rifampin and is tagged with green fluorescent protein, which makes the cells appear green under UV light (54). *E. coli* strain J53 is resistant to sodium azide (55).

Construction of donor cells by acquiring *tet*-carrying plasmid(s) from WWTP effluent. Filter mating was used to capture the conjugative plasmids according to a previously published procedure with minimal modifications (54). Initially, *E. coli* CV601 was cultured in lysogeny broth (LB; i.e., 1 L of LB medium contains 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride) containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin at 180 rpm and 30°C overnight. The cell culture was then diluted 20-fold in fresh LB broth and grown for 10 h to reach the exponential growth phase. The cells were washed twice in phosphate-buffered saline (PBS) to remove the trace of antibiotics and were resuspended in 15 mL of PBS.

The Theresa Street WWTP in Lincoln, NE, has a treatment capacity of treating 28 million gallons of municipal wastewater per day. The secondary effluent (i.e., the wastewater was treated by the activated sludge process and was not disinfected by the UV system) from the WWTP was filtered through S-Pak filters with a 0.45-μm pore size (Millipore Corp., Bedford, MA). Bacteria were removed from the filters by vortexing for 15 min in 25 mL of PBS. The bacterial suspension was decanted to a new tube and centrifuged for 15 min at 2,700 $\times$ *g*. The pellet was washed with PBS twice and resuspended in PBS. The volume of PBS was adjusted to reach an optical density at 600 nm (OD_{600}) of 1.4.

Next, 2 mL of effluent bacteria and 2 mL of CV601 were combined and vortexed in a 10-mL centrifuge tube. A 100-μL portion of the combined suspension was loaded onto a 0.22-μm pore size mixed cellulose ester filter (Whatman, Plc, Inc., Maidstone, Kent, UK), which was placed on an LB agar plate amended with 100 mg L^{-1} cycloheximide, followed by incubation at 37°C for 2 days. Cycloheximide was used to remove undesirable organisms from WWTP effluent (56). After incubation, the cell mixture on the filter disk was resuspended in 2 mL of PBS with vortexing. The suspension was plated on LB agar plates, which contained 50 mg L^{-1} kanamycin, 50 mg L^{-1} rifampicin, 100 mg L^{-1} cycloheximide, and 10 mg L^{-1} tetracycline. As controls, CV601 cells were grown and plated under the same conditions except the presence of cells from WWTP effluent. After 2 days, CV601 transconjugants receiving plasmids from the WWTP effluent formed visible colonies on agar plates, and their identities were further verified by their green appearance under UV light. Confirmation of plasmids conferring tetracycline resistance was later verified using the MIC test. The resulting CV601 transconjugant (designated CW), which contained *tet*-carrying plasmids, was inoculated in selective LB media and stored at –80°C with glycerol. CW was later used as the donor cells in the conjugation experiments in this study. Tetracycline was used as the model antibiotic in this study, since it commonly occurs in surface waters impacted by WWTP effluent (57).

Chemostat reactor design. The experiment was conducted according to a 2 $\times$ 2 factorial design: nutrient level (1/3-strength and 1/10-strength MHB) and growth rate (0.15 and 0.45 h^{-1}). Two *E. coli* strains, CW (i.e., CV601 transconjugants) and J53, were used as the donor and recipient, respectively. First, *E. coli* J53 were cultured in LB broth in the presence of 200 mg L^{-1} sodium azide at 35°C with 180-rpm shaking overnight. The J53 culture at the late exponential phase was then diluted to an OD_{600} of 0.3. Next, 15 mL of the diluted J53 culture was added to the chemostat reactor (Fig. 1) to simulate recipient cells in surface water (i.e., river bacteria). The J53 cell number in chemostats was monitored to ensure it reached a steady state before simulated WWTP effluent (i.e., a solution containing donor CW cells and tetracycline) was introduced to the chemostat reactors.

Three replicate chemostat reactors were operated for each treatment combination (i.e., nutrient level $\times$ growth rate). The chemostat systems were established according to a published ministat manual (58). Briefly, the chemostat reactors were made of glass and the tubing was made of autoclavable Marprene. The entire experiment was conducted in a fume hood without sunlight exposure, and the chemostat reactor was operated at room temperature 22°C. Filtered air was pumped into chemostats to provide oxygen and to mix solution. Within each experimental run, all reactors had the same dilution rate (i.e., 0.15 or 0.45 h^{-1}). Given the growth rates of *E. coli* in the environment (0.17 to 0.90 h^{-1}) (59), as well as the washout rate and

the bacteriostatic effect of tetracycline, dilution rates of 0.15 h^{-1} and 0.45 h^{-1} were chosen to represent low and high growth rates, respectively, in this study. In chemostat reactors, the growth rate of a single culture was equal to the dilution ratio of the reactor at steady state. To monitor if a steady state had been reached, the cell density of recipient cells was monitored by measuring the optical density. When the density of recipient cells reached steady state in the chemostat reactors, 15 mL of the CW donor culture was directly added to reactors. Then, a simulated effluent (i.e., a solution containing CW cells and tetracycline) was pumped into the reactors with the same flow rate as the media that had been pumped to support the growth of the recipient cells in the reactors. The time that the simulated effluent was introduced was denoted as day 0. Since the flow rate into the reactors doubled and the solution volumes increased from 15 to 30 mL, the dilution ratio was kept constant. The CW cells and tetracycline in the simulated effluent reached final concentrations of 10^8 CFU mL^{-1} and 10 μg L^{-1}, respectively, in the chemostats. To account for the emergence of ARGs due to mutation, all of the chemostat experiments were repeated in the same manner by introducing a simulated effluent without CW donor (i.e., tetracycline only). The entire experiment was repeated for a second time.

Bacterial enumeration. Samples from chemostats were taken after 16, 24, 48, 72, and 96 h and plated on selective media to enumerate recipient and transconjugant cells. Donor cells inside chemostats were measured as well. Serial dilutions (1:10) were done using PBS, and diluted samples were plated in triplicate for each reactor. Donors were enumerated on LB agar supplemented with 50 mg L^{-1} kanamycin, 50 mg L^{-1} rifampicin, and 10 mg L^{-1} tetracycline, followed by incubation at 35°C for 16 h. Recipients were enumerated on LB agar supplement with 200 mg L^{-1} sodium azide and then incubated at 35°C for 20 h. Transconjugants were plated on LB agar supplemented with 10 mg L^{-1} tetracycline and 200 mg L^{-1} sodium azide, followed by incubation at 35°C for 24 h. The results were recorded as CFU mL^{-1}.

Calculating the conjugation frequency. The conjugation frequency (CF) was expressed as the number of transconjugants per recipient colonies formed (60):

$$\mathrm{CF} = \frac{\text{No. of transconjugants } \left(\frac{\text{CFU}}{\text{mL}}\right)}{\text{No. of recipients} \left(\frac{\text{CFU}}{\text{mL}}\right)}$$

MIC measurement. MIC values were assessed using MHB media containing a series of 2-fold diluted tetracycline concentrations (i.e., 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 mg L^{-1}). The MHB media were dispensed into 96-well plates (VWR Company, Monroeville, PA). Tetracycline hydrochloride (Sigma-Aldrich, St. Louis, MO) stock solution (512 mg/L) was freshly prepared and stored for no more than 7 days at 4°C. The stability of the stock solution over the storage period was checked using a high-pressure liquid chromatograph in the Water Sciences Laboratory at University of Nebraska—Lincoln (61). Inocula representing the donors, recipients, or transconjugants were diluted and then inoculated into each well of 96-well plates containing an ~110-μL final volume in each well. A negative control (no cell or antibiotic) was included on each 96-well plate. The plates were then incubated in a microplate reader (BioTek instruments, Inc., Winooski, VT) at 37°C for 16 to 20 h to obtain growth curves by continuous reading of the absorbance during the incubation period. The MIC was determined as the lowest concentration of tetracycline that did not permit any visible cell growth (62).

Whole-genome sequencing and assembly. Seven samples were selected for WGS, including strains CV601, J53, and CW, as well as two transconjugant and two mutant isolates, from growth rates 0.15 and 0.45 h^{-1} with nutrient concentrations of 1/3 MHB. DNA was extracted using a QIAamp DNA minikit (Qiagen, Inc, Germantown, MD). Samples were sequenced to obtain 150-bp, paired-end reads at the University of Nebraska Medical Center Genomics Core Facility using an Illumina NextSeq 500 (Illumina, Inc., San Diego, CA) with a midoutput flow cell. All samples have been uploaded to NCBI's Sequence Read Archive (SRA) under BioProject ID PRJNA750500.

Analysis of sequencing reads was conducted by the Center for Biotechnology at University of Nebraska—Lincoln. Initial trimming and quality control were performed using Trim Galore! (v6.0, with the default parameters except for q = 30 and retain length $\geq$ 35) and FASTQC (v0.11) to remove low-quality reads and adapter sequences (63). The remaining reads were assembled via SPAdes (v3.13) with multiple Kmer values (7 to 125 in steps of four) (64). After the assemblies, the quality of each assembly was analyzed using QUAST v5.0 (65). Genome annotation was conducted using Prokka (v1.13.3) with the following settings: –kingdom Bacteria and –gcode 11 (66). Overlap between the coding regions of genes and rRNAs region was allowed (–cdsrnaolap). In addition, noncoding RNAs were searched in annotation (–rfam). KAIJU (v1.7) was used for taxonomic classification with default parameters and database ProGenome (version May 2019) (67). The assembled contigs were assessed using Plasmid Finder (v2.0) to identify potential plasmids (68). The PLSDB database was utilized to confirm the presence of the plasmids (69). Bowtie 2 (v2.3) with default parameters was used to align samples (70). SAMtools (v1.9) and BCFtools (v1.9) were utilized to generate and filter variant coding format files (71).

Statistical analysis. Repeated-measure ANOVAs were conducted to evaluate the main and interaction effects of the nutrient level and growth rate, with time as a repeated-measure factor, on the conjugation frequency or the number of transconjugants. A Kruskal-Wallis test was conducted to evaluate the effects of conjugation times, growth rates, and nutrient levels on the MICs of mutants and transconjugants. All the statistical analysis was carried out using TIBCO Statistica (v13.3.0; Tibco Software, Palo Alto, CA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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We report that we have no conflicts of interest.

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