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Bacterial Concentrations and Water Turbulence Influence the Importance of Conjugation Versus Phage-Mediated Antibiotic Resistance Gene Transfer in Suspended Growth Systems

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ABSTRACT: Despite the abundance of phage-borne antibiotic resistance genes (ARGs) in the environment, the frequency of ARG propagation via phage-mediated transduction (relative to via conjugation) is poorly understood. We investigated the influence of bacterial concentration and water turbulence level [quantified as Reynold's number (Re)] in suspended growth systems on the frequency of ARG transfer by two mechanisms: delivery by a lysogenic phage (phage λ carrying gentamycin-resistance gene, $genR$) and conjugation mediated by the self-transmissible plasmid RP4. Using *Escherichia coli* (*E. coli*) as the recipient, phage delivery had a comparable frequency ($1.2 \pm 0.9 \times 10^{-6}$) to that of conjugation ($1.1 \pm 0.9 \times 10^{-6}$) in suspensions with low cell concentration (10^4 CFU/mL) and moderate turbulence ($Re = 5 \times 10^4$). Turbulence affected cell (or phage)-to-cell contact rates and detachment (due to shear force), and thus, it affected the relative importance of conjugation versus phage delivery. At 10^7 CFU/mL, no significant difference was observed between the frequencies of ARG transfer by the two mechanisms under quiescent water conditions ($2.8 \pm 0.3 \times 10^{-5}$ for conjugation vs $2.2 \pm 0.5 \times 10^{-5}$ for phage delivery, $p = 0.19$) or when Re reached 5×10^5 ($3.4 \pm 1.5 \times 10^{-5}$ for conjugation vs $2.9 \pm 1.0 \times 10^{-5}$ for phage delivery, $p = 0.52$). Transcriptomic analysis of genes related to conjugation and phage delivery and simulation of cell (or phage)-to-cell collisions at different Re values corroborate that the importance of phage delivery relative to conjugation increases under either quiescent or turbulent conditions. This finding challenges the prevailing view that conjugation is the dominant ARG transfer mechanism and underscores the need to consider and mitigate potential ARG dissemination via transduction.

KEYWORDS: ARG propagation, conjugative plasmid, lysogenic phage, horizontal gene transfer

INTRODUCTION

The global spread of antibiotic resistance has resulted in significant societal and economic costs of dealing with resistant infections,[1] which annually exceeds $4.6 billion in just the United States.[2] Thus, elucidating the origins and system-specific dissemination pathways of the associated antibiotic resistance genes (ARGs) has become a research priority.[3,4] Of the three main horizontal gene transfer (HGT) mechanisms (i.e., conjugation, transduction, and transformation), conjugation is often considered to have the greatest influences on ARG dissemination in the environment due to the high transfer efficiency[5,6] and general abundance of plasmid-borne ARGs.[6,7] Transformation generally has a minor influence on ARG dissemination due to limited species of naturally transformable bacteria in the environment (about 80 species[8]) and low probability of incorporating ARG-bearing extracellular DNA strands into the bacterial chromosome or plasmids.[4] In contrast, little is known about the overall contribution of ARG delivery by phages to antibiotic resistance propagation despite increasing attention to this mechanism in recent years (Figure 1a).[9]

Phages are the most abundant and diverse entities in the biosphere,[10] and phage-mediated transduction can result in genetic exchange between distantly related bacterial species.[9,11] Errors in phage replication may incidentally incorporate bacterial DNA (including ARGs) into the phage genome and transfer it to another bacterium upon the next infection.[3] Recently, mounting evidence suggests that phages are a potentially significant ARG reservoir and serve as vectors in numerous environments including wastewater,[12] sludge,[13] rivers,[14] sediment,[15] soils,[16] fresh vegetables,[17] air,[18] and
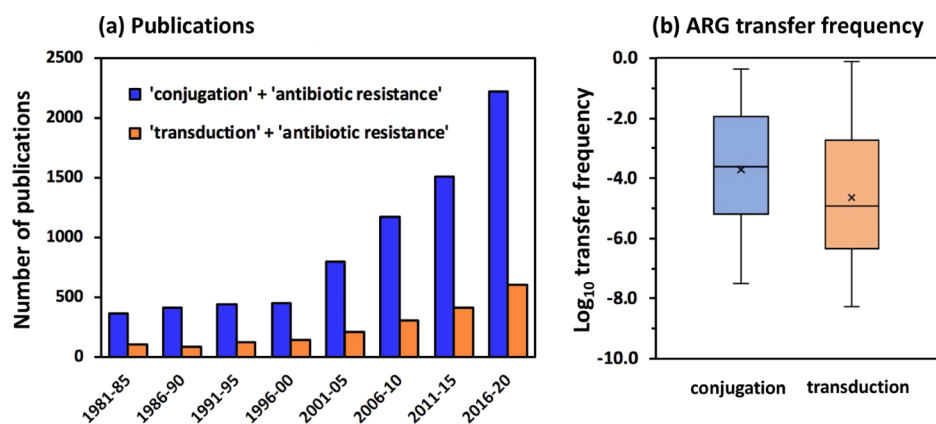
Figure 1. Publication trends (a) and the reported ARG transfer frequencies (b) of plasmid-mediated conjugation and phage-mediated transduction. (a) Publications (https://pubmed.ncbi.nlm.nih.gov/) from 1981 to 2020 on conjugation and transduction-associated antibiotic resistance both show an exponential increase, with the number of papers related to conjugation 3−5 times higher than that of transduction. (b) Reported frequencies of ARG transfer via conjugation and transduction both showed high variability. Average frequencies are indicated by "×".

animal and human feces.[19,20] The abundance of phage-borne ARGs is sometimes comparable or even higher than that found inside bacteria, as determined by quantitative polymerase chain reaction (qPCR) or metagenomic analyses,[15,16,21] although qPCR may sometimes detect unfunctional fragments of phage-borne ARGs. Therefore, it is important to discern conditions under which functional ARG delivery by phages is as significant as conjugation and assess the magnitude of their relative HGT contributions to prioritize associated mitigation and risk management efforts.

The contributions of conjugative plasmids and phages to ARG dissemination depend not only on the abundance of plasmid- or phage-borne ARGs but also on the ARG transfer frequency,[5] which is highly variable (Figure 1b)[6,22−45] and dependent on system-specific conditions.[46−52] However, the frequency of ARG transfer via conjugation versus phage delivery has rarely been systematically compared under the same environmental conditions.[53]

In this study, we compare ARG dissemination in suspended growth systems that prevail in some wastewater treatment processes and natural water bodies, via conjugation versus phage delivery. We investigate how two system-specific factors known to influence HGT frequency[51] (i.e., bacterial concentration and water turbulence) affect the relative importance of these two ARG delivery mechanisms. The conjugative plasmid RP4 and lysogenic phage λ harboring *genR* were selected for conjugation and phage delivery assays, respectively, using *Escherichia coli* as the recipient. Frequency of ARG dissemination was quantified as the percentage of recipient *E. coli* receiving *genR*, determined by plate counting methods and qPCR analysis. Transcriptomic analysis was performed to mechanistically explore the influence of bacterial concentration and water turbulence on the relative importance of conjugation versus phage delivery in ARG transfer. Collision theory to assess how turbulence affects donor-recipient contact frequency was also considered. Overall, this study advances the understanding of prevalent ARG dissemination pathways and rates as a function of system-specific conditions to inform potential mitigation and control strategies.

■ MATERIALS AND METHODS

Experimental Approach

ARG transfer experiments were conducted with commonly used vectors to facilitate comparison with other studies. Conjugation tests were run with plasmid RP4, which is a model for plasmid biology (i.e., for plasmid maintenance and stability, synthesis of type IV secretion pili, and regulation of conjugation-related genes[54,55]) and is a representative plasmid of the incompatibility group P (IncP). Experiments involving ARG delivery by phages were run with phage λ, which belongs to the *Siphoviridae* family[56] and is representative of long-tailed phages.[57] Both conjugation and phage delivery experiments were conducted with *genR*, which is a well-studied ARG prevalent in both clinical and natural environments.[58,59]

Two system-specific factors, bacterial concentration and water turbulence (represented by *Re*), were selected as they exhibited significant influence on HGT frequency with great variability in the aquatic environments.[51] *E. coli* concentration was varied between 10^3 and 10^8 CFU/mL, which represents a wide range of conditions—from 0 in some drinking water systems[60] to about 10^6 CFU/mL in sewage[61] and 10^8 CFU/mL in activated sludge.[62] The range of *Re* in this study was varied from 0 to 5×10^5 which are typical values in groundwater (1 to at least 10^5),[63−65] lakes, and streams (0 to at least 5×10^6)[66−68] and some wastewater transport and treatment processes (10 to 10^6).[69−72] *Re* was calculated in shaking flasks as[73]

$$Re = \frac{\rho \cdot n \cdot D^2}{\eta}$$

(1)

where ρ is the fluid density (kg/m³), n is the shaking frequency (s⁻¹), D is the maximum inner diameter of the flasks (m), and η is the dynamic viscosity (Pa·s). Parameter values are provided in Table S1.

When investigating the influence of bacterial concentration on conjugation and phage delivery frequencies, *Re* was fixed at 5×10^4. In the study of turbulence level, bacterial concentration was fixed at 10^7 CFU/mL which is representative of some systems where nutrients are abundant such as sewage and wastewater treatment plants.[74]

Bacterial Strains, Plasmids, and Phage Construction

Conjugative plasmid RP4 was obtained from Addgene (plasmid 79813). Plasmid RP4 contains a set of transfer genes enabling horizontal transmission[75] and has been genetically modified to confer resistance to ampicillin, gentamycin, and tetracycline (Amp^R, Gm^R, and Tc^R), as previously reported.[76] *E. coli* DH5α (Invitrogen, USA) harboring plasmid RP4 was selected as the donor strain, and *E. coli* MG1655 (a kind gift from Dr. Lauren Stadler, Rice University) carrying chloramphenicol resistance gene (*cat*) in the chromosome as the recipient (Table 1). The λ phage was obtained from Agilent Technologies, USA. *GenR* was amplified from plasmid RP4 by PCR

Table 1. Plasmid, Phage, and Bacterial Strains Used in This Study

plasmid or phage or strain	purpose	selective marker	ref. or source
plasmid RP4	self-transmissible plasmid for conjugation	Amp[R], Gm[R], and Tc[R]	Addgene[76]
λ phage	lysogenic phage for phage delivery assays	Gm[R]	Agilent Technologies
E. coli DH5α	donor strain carrying plasmid RP4 for conjugation		Invitrogen
E. coli MG1655	recipient strain for both conjugation and phage delivery assays	Chl[R]	a gift from Dr. Lauren Stadler
E. coli Y1088	propagation of λ phage	Amp[R] and Tc[R]	Agilent Technologies

with the EcoRI endonuclease restriction site added at both ends. After EcoRI digestion, genR was ligated into the genome of λ gt11 (Agilent Technologies, USA) according to the manufacturer's instructions and electroporated into E. coli strain Y1088 (Agilent Technologies, USA) using Gene Pulser Xcell Electroporation Systems (Bio-Rad, USA). Phage plaques were screened to identify recombinant λ phage by PCR assays and gene sequencing. Further details of phage engineering and plaque screening are available in the Supporting Information (Text S1).

Conjugation and Phage Delivery Assays

E. coli strains DH5α and MG1655 were grown to the stationary phase at 37 °C in Luria broth (LB) medium (BD Biosciences, USA) supplemented with gentamycin (10 mg/L) and chloramphenicol (25 mg/L), respectively. Bacteria were centrifuged at 14,000g for 2 min and washed three times with phosphate-buffered saline (PBS) to remove extracellular DNA and nutrients (thus obviating potential confounding effects by transformation) and then resuspended in the M9 mineral salt medium.[77] Viable bacteria were counted by a plate assay using standard Difco plate count agar (BD Biosciences, USA) and expressed as colony-forming units (CFUs). Recombinant λ phage was propagated in E. coli Y1088 at 37 °C overnight in a LB medium. Bacterial cells were removed via centrifugation (4 °C, 8000g, 8 min), followed by filtration through 0.22 μm PES filter membranes (Millipore, USA). Phages were further concentrated and purified by PEG precipitation and suspended in PBS or SM buffer stored at 4 °C. Phage concentration was determined using the double-layer plaque assay and expressed as plaque-forming units (PFUs).[78]

The donor (E. coli DH5α harboring plasmid RP4 for conjugation assays or λ phage for assays using phages as gene delivery vectors) and recipient (E. coli MG1655) of genR transfer were mixed at a 1:1 initial ratio[79−83] in all tests to facilitate fair comparisons. ARG donors and recipient cells were mixed in an M9 minimal medium spiked with 3 g/L glucose and shaken at targeted Re representative of a wide range of environmental conditions.[38] Conjugation and phage delivery assays were performed under the same experimental conditions but in different flasks to separately quantify conjugation and phage delivery events. Assays were performed at room temperature (20 °C) for 1 h,[79] without significant bacterial replication (i.e., no vertical genR transfer) (Figure S1), plasmid lost (Figure S2), or phage decay (Figure S3). Samples were taken and recovered afterward following previously reported procedures with minor adjustments.[77,84] Briefly, samples were vigorously vortexed to stop ARG transfer events and washed with M9 medium three times. Recovered bacteria were well-dispersed using vortex and then plated onto LB agar. Plates containing both gentamycin (5 mg/L) and chloramphenicol (25 mg/L) were used to count transconjugants (i.e., chloramphenicol-resistant E. coli MG1655 that were recipients of plasmid-borne genR) or transductants (i.e., recipients of phage-borne genR) because only recipients of genR could offer resistance to gentamycin and grow on these plates. Colony PCR was used to verify that plasmid RP4 or phage λ carrying genR had been transferred into the recipients. Detailed procedures for colony PCR are included in the Supporting Information (Text S2).

To correct for conjugation and phage delivery events that could occur on the agar plates, donor and recipient were mixed in a 1:1 ratio at different concentrations and then immediately transferred on agar plates as a control. The number of transconjugants or transductants colonies were subtracted from the counting results mentioned above.

Quantification of Prophage Integration

After 1 h of phage delivery assays, bacteria were centrifuged at 8000g for 40 s, and microbial DNA was extracted from the pellet using a QIAamp DNA Micro Kit (Qiagen, USA) following the manufacturer's instructions. The qPCR analysis was performed to quantify E. coli DNA and λ prophage integration using primers as previously reported[85] (Table 2). The qPCR mixture contained 7.5 μL of PowerUp SYBR Green Master Mix (Applied Biosystems, USA), 1 μL of DNA template, 0.3 μM of each forward and reverse primers, and DNA-free water to a total volume of 15 μL. Triplicate qPCR reactions were conducted on a CFX96TM real-time system (Bio-Rad, USA) with the temperature setup provided in the Supporting Information (Text S3).

Differential Gene Expression Analysis

Bacteria samples were collected by centrifugation after 1 h of conjugation and phage delivery assays. Total bacterial RNA was extracted from the pellets using a PureLink RNA mini kit (Invitrogen, USA) with on-column DNase treatment (PureLink DNase Set, Invitrogen, USA) to remove residual DNA. The quality and quantity

Table 2. Primers for qPCR Analysis

target genes	primer sequence	annealing temp. (°C)	purposes	refs
attB-upstream	5′-GCCGACAACAAAGTCAGGTT-3′ 5′-AAAAGAAGCGCAGAATTTCG-3′	59.5	detecting E. coli DNA	85
attB-attP	5′-AGACGGGAAACTGAAAATGTG-3′ 5′-CTGATAGTGACCTGTTCGTTGC-3′	59.5	detecting the integration of λ phage genome	85
traI	5′-TTGAACTCTGCTGTGCCGTTGAC-3′ ATCACGAAGAAGGGAACCATCATC-3′	58.0	transcriptomic analysis of conjugation	a
traJ	5′-GACGTGCTCATAGTCCA-3′ 5′-TGTACTGCCTTCCAGAC-3′	58.0		a
trfAp	5′-GAAGCCCATCGCCGTCGCCTGTAG-3′ 5′-GCCGACGATGACGAACTGGTGTGG-3′	58.0		84
lamB	5′-GGTGGTTCTTCCTCTTTC-3′ 5′-CGACACCCAGTTCTAATG-3′	59.0	transcriptomic analysis of phage delivery assays	a
malT	5′-GCAGGCCGGACGTAAAAGTGAC-3′ 5′-ATGCTGTTCCAGTTCCGGCA-3′	59.0		a

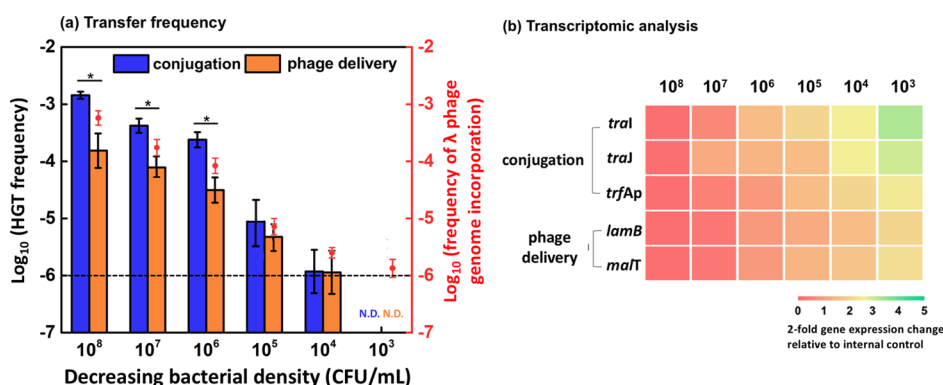[a]Primers were custom designed and ordered from Integrated DNA Technology (IDT, USA).

Figure 2. Lower frequency but higher relative importance of *genR* transfer via phage delivery (relative to conjugation) occurs at lower bacterial concentration. (a) Transfer frequency was determined after 1 h of conjugation and phage delivery assays in minimal medium with 3.0 g/L glucose and shaken at an *Re* of 5×10^4. The *genR* donor (*E. coli* DH5α harboring plasmid RP4 for conjugation assays or λ phage for phage delivery assays) and recipient (*E. coli* MG1655) were mixed at a 1:1 ratio. Frequency determined by the plate counting method is presented as bars with the detection limit (10^{-6}) indicated by the horizontal dashed line; orange dots represent the frequency determined by qPCR analysis. Asterisks (*) indicate significant differences ($p < 0.05$) between conjugation and phage delivery frequency based on Student's *t*-test. "N.D." refers to "not detected". (b) Upregulation of conjugation-related genes (*tra*I, *tra*J, and *trb*Ap) and phage delivery-related genes (*lam*B and *mal*T) at lower bacterial concentration (twofold gene expression change). The top *X*-axis depicts decreasing bacterial concentration (CFU/mL). The expression level at a bacterial concentration of 10^8 CFU/mL was used as the control. Error bars depict ± one standard deviation from the mean of at least three independent replicates.

of RNA were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). RNA was transcribed to cDNA by reverse transcription PCR (RT-PCR) using a High-Capacity RNA-to-cDNA kit (Invitrogen, USA).

qPCR was performed to quantify the transcribed cDNA of conjugation-related genes (*tra*I, *tra*J, and *trb*Ap) and phage delivery-related genes (*lam*B and *mal*T) (Table 2). 16S rRNA gene was included as a reference gene.[86] Differential gene expression relative to the 16S rRNA gene was quantified by the $2^{-\Delta\Delta C_T}$ method,[87] and the cycle threshold (C_T) values used were the means of independent triplicates. Heatmap presenting the overall RT-PCR and qPCR data of targeted genes was performed in Origin Pro 2021. Details of the RT-PCR and qPCR protocols are available in the Supporting Information (Text S3).

Simulation of Cell (or Phage)−Cell Collision Frequency

The frequency of collision Z (total collision event/s) between donor (*E. coli* cells for conjugation or phages) and recipient (*E. coli* cells) at different water turbulence levels was simulated according to the collision theory[72,74] with several assumptions: (1) bacterial cells and phages all have a spherical shape with hydrodynamic diameters of 1.2×10^{-6}[88] and 7.0×10^{-8} m;[89] (2) Brownian motion prevails under static conditions ($Re = 0$) and neither cells nor phages move by themselves, although *E. coli* strains used in this study have flagella; (3) the concentrations of the donor and recipient cells are constant through the assays; and (4) under nonstatic conditions ($Re > 0$), the velocities (m/s) of cells and phages (u) are the same as water velocity, which is simplified as

$$u = 2\pi n r \tag{2}$$

where r is the distance from cells (or phages) to the center of the flask (m).

Under static conditions ($Re = 0$), the collision between donors and recipients resulted from Brownian motions (Z_B). Collision frequency Z (total collision event/s) was determined as[90]

$$Z = Z_B = \alpha \frac{2kT}{3\eta} \frac{(d_d + d_r)^2}{d_d d_r} N_d N_r V \tag{3}$$

where α is the collision efficiency (assumed as 1), k is the Boltzmann constant (J/K), T is the thermodynamic temperature (K), μ is the fluid viscosity (Pa·s), d_d is the hydrodynamic diameter of donor (m), d_r is the hydrodynamic diameter of recipient (m), N_d is the donor

concentration [CFU (or PFU)/m³], N_r is the recipient concentration (CFU/m³), and V is the fluid volume (m³).

Under nonstatic conditions ($Re > 0$), the collision between ARG donor and recipient resulted from both Brownian motion (eq 3) and shear force. The simulation of shear force-related collision frequency,[88] Z_S (total collision event/s), was determined as

$$Z_S = \frac{\sqrt{2}}{24} \pi^3 (d_d + d_r)^2 n N_d N_r H D^3 \tag{4}$$

where H is the height of the fluid (m) calculated from the fluid volume and flask diameter.

Details of formula derivation and the values of aforementioned parameters are provided in the Supporting Information (Text S4 and Table S1).

Statistical Analyses

ANOVA analysis was performed to compare the differences between conjugation and phage delivery frequencies under various experimental settings. Pearson correlation analysis was also conducted to characterize the relationship between the system-dependent factors and corresponding *genR* transfer frequency using SPSS 10.0 software. Differences were considered to be significant at the 95% confidence level ($p < 0.05$).

■ RESULTS AND DISCUSSION

Phages Deliver ARGs at a Comparable Frequency to Conjugation at Low Bacterial Concentration

Both conjugation and phage delivery frequencies (determined by plate counting) were positively correlated with bacterial concentration, ranging from 10^4 to 10^8 CFU/mL (Pearson correlation coefficient $r = 0.95$, $p = 0.013$ for conjugation; $r = 0.92$, $p = 0.027$ for phage delivery) (Figure 2a), which facilitates contact between ARG donor and recipient, as previously reported.[79,91] The relative importance of conjugation versus phage delivery was also affected by bacterial concentration. At high bacterial concentration, the frequency of conjugation was significantly higher than that of *genR* delivery by phages, exceeding it by 9.3-, 5.6-, and 5.9-fold at 10^8, 10^7, and 10^6 CFU/mL, respectively. There was no significant difference between conjugation and phage-mediated transfer frequencies when bacterial concentration was at 10^5 or

10^4 CFU/mL ($8.8 \pm 3.2 \times 10^{-6}$ vs $5.9 \pm 2.4 \times 10^{-6}$ and $1.2 \pm 0.9 \times 10^{-6}$ vs $1.1 \pm 0.9 \times 10^{-6}$, respectively), indicating that the contribution of phages to ARG dissemination can rival that of conjugation at low bacterial concentration.

No ARG recipients were detected by plate counting at a bacterial concentration of 10^3 CFU/mL, which contrasts previous reports that bacterial concentration thresholds for phage infection can be as low as 10^2 CFU/mL.[82] Thus, further analysis using qPCR was needed to evaluate the phage delivery frequency at the genetic level. The frequency of ARG delivery by phages was determined as the percentage of recipient *E. coli* that was infected by λ phage and incorporated phage genome in the chromosome (Figure 2a) and was generally a little higher (0–0.5 log) than that determined by the plate counting method. The difference in frequency detected by these two methods might be due to insufficient accumulation of *genR*-coded gentamycin acetyltransferase[92] in some of the phage-infected recipients. The λ phage genome incorporation was detected at a bacterial concentration of 10^3 CFU/mL, indicating that ARG delivery by phages might be more frequent than by conjugation at such low bacterial concentrations.

The expression of genes related to conjugation and phage delivery was upregulated as bacterial concentration decreased, possibly due to more abundant nutrients per cell at lower bacterial concentration to support energy-dependent gene expression[93] (Figure 2b). The expression levels of conjugation-related genes *tra*I (coding DNA relaxosome required for conjugation[55]), *tra*J (coding positive regulator of plasmid transfer operon[94]), and *trf*Ap (coding positive regulator of plasmid transfer and replication system[95]) at a bacterial concentration of 10^3 CFU/mL were 9.21-, 7.82-, and 5.51-fold higher than that at 10^8 CFU/mL, respectively. Phage delivery-related gene *lam*B (coding the receptor of λ phage[96]) and *mal*T (coding the positive regulator of *lam*B[97]) were overexpressed by 4.3- and 4.8-fold. The upregulation of conjugation-related genes was more significant than that of phage delivery, possibly due to quorum sensing regulation.[98] The regulation of gene expression did not explain the influence of bacterial concentration on the relative importance of conjugation versus phage delivery. The frequency of ARG delivery by phages was less dependent on bacterial concentration than that of conjugation, which collaborates previous studies,[38,82] although the reason for this observation remains unclear.

Water Turbulence Significantly Affects the Relative Frequency of ARG Conjugation Versus Phage Delivery

Water turbulence, quantified as *Re*, had a considerable effect on the frequency of ARG dissemination by conjugation versus phage delivery, and shaped their relative importance (Figure 3). The transfer frequency of *genR* peaked at *Re* of 5×10^4 for both conjugation and phage delivery, with a conjugation frequency 5.6-fold higher than that via phages. As *Re* decreased or increased from the peak value, the frequencies of ARG delivery by both conjugation and phages decreased due to lower collision frequency or higher detachment rate, respectively.[88] However, the importance of phage delivery relative to conjugation increased under either quiescent or turbulent conditions. Specifically, the frequency of *genR* transfer mediated by phages was comparable to conjugation at *Re* of 0 (static) and 5×10^5 as no significant difference was observed. This implies that ARG transfer by phages should
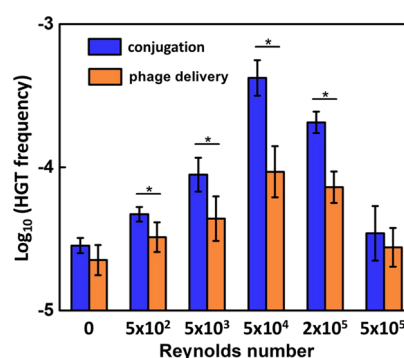

Figure 3. Turbulence (represented by Reynolds number) significantly impacts the relative frequency of conjugation vs phage delivery for the horizontal transfer of *genR* in a bell-shaped fashion. The transfer frequency was determined after 1 h of conjugation and phage delivery assays in minimal medium containing 3.0 g/L glucose with bacterial concentration at 10^7 CFU/mL. The *genR* donor (*E. coli* DH5α harboring plasmid RP4 for conjugation assays or λ phage for phage delivery assays) and recipient (*E. coli* MG1655) were mixed at a 1:1 ratio. Asterisks (*) indicate significant differences ($p < 0.05$) between conjugation and phage delivery frequency based on Student's *t*-test. Error bars depict $\pm$ one standard deviation from the mean of at least three independent replicates.

receive increasing attention in turbulent aquatic systems where *Re* can exceed 5×10^5 or in quiescent systems where a high level of phage-borne ARGs is detected.

Transcriptomic analysis indicated that genetic regulation was not as influential as turbulence in shaping the relative importance of conjugation versus phage delivery, as no significant difference was observed for the expression level of selected conjugation- and phage delivery-related genes among different turbulence levels (Figure S4).

Collision theory might help explain how hydrodynamic conditions influence ARG delivery because they greatly influence cell (or phage)-to-cell contact frequency (initiating gene transfer) and the stability of mating pairs (for the completion of ARG transfer).[99] Under static conditions (*Re* = 0, no turbulence shear), the simulated frequency of collision between the conjugation donor (*E. coli* cells) and recipient (*E. coli* cells) was lower than that of phage delivery (phages as the donor and *E. coli* cells as the recipient), which helps explain the insignificant difference between conjugation and phage delivery frequencies (Figure 4a). Collision under static conditions was assumed to result only from the Brownian motion,[100,101] according to which the smaller particles (i.e., phages) diffuse faster than the larger ones (i.e., *E. coli* cells) and therefore lead to higher collision frequency. The self-motion of *E. coli* cells powered by flagella[100] was not included in this simple model.

Water turbulence leads to a trade-off between increased cell (or phage)-to-cell collision rate and higher propensity for cell (or phage)–cell detachment due to the shearing force,[100] which explains the bell-shaped ARG transfer data (Figure 3). Elevated turbulence levels resulted in higher collision frequency and enhanced both conjugation and phage delivery at *Re* ranging from 0 to 5×10^4 (Figure 4a). The cell-to-cell collision frequency was higher than that of phage-to-cell collision because *E. coli* cells have a larger hydrodynamic radius,[88,89] which contributed to higher peak conjugation frequency than phage delivery frequency (Figure 3). Decreased ARG transfer frequency at *Re* higher than 5×10^4 was probably
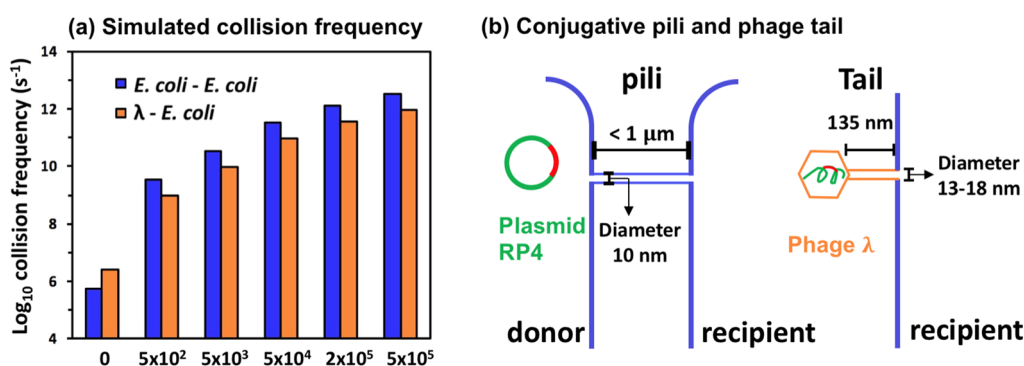
Figure 4. Higher water turbulence increases the theoretical cell (or phage)-to-cell collision frequency and interferes with the stability of ARG transfer channels. (a) Collision frequency (total collision events/s) under static ($Re = 0$) and dynamic conditions ($Re > 0$) was simulated based on the Brownian motion (eq 3) and turbulence shear (eq 4), respectively. Brownian motion was not considered in the simulation under turbulent conditions because its induced collision has a frequency that is over 3 orders of magnitude lower than that of shear-induced collision (eq S7). (b) Shear-induced detachment frequency would be higher for conjugating cells than for attached phages due to postulated weaker links formed by thinner and longer multiprotein, hair-like links.[57,102−105]

due to an increase in the detachment rate. A lack of literature data on the stability of RP4 conjugation mating pairs and λ phages attached to *E. coli* under different Re values precludes accurate simulation of detachment. Nevertheless, the higher likelihood of detachment of conjugating cells than phages adsorbed onto cells at high Re values is supported by the fact that conjugative pili produced by the RP4 conjugal system would be a more fragile link than that formed by the phage tails that attach to bacteria (Figure 4b). Specifically, both links consist of multiprotein hair-like structures, and while RP4 pili typically measure 10 nm in diameter[102] and under 1.0 μm in length (retractable),[103,104] the λ phage tails are only about 135 nm long[105] and have a thicker diameter of 13−18 nm.[57,105]

Further studies should corroborate the observed influence of hydrodynamic conditions on the relative importance of conjugation versus transduction using lower recipient bacterial concentrations representative of natural aquatic systems (e.g., 10^2–10^5 CFU/mL[106,107]), which would advance the understanding of ARG transfer from wastewater treatment plants or animal agriculture sources to natural environments (e.g., freshwater).

Environmental Implications

ARG dissemination mediated by phages is receiving increasing attention due to the abundance of phage-borne ARGs. This work demonstrates that both bacterial concentration and water turbulence can be influential in determining the relative importance of conjugation versus phage delivery of ARGs, with the latter reaching comparable transfer frequencies at relatively low cell concentrations under either quiescent or turbulent conditions that hinder conjugation. Accordingly, ARG transfer via phages may become relatively important in oligotrophic aquatic systems (Figure S5) not only due to low bacterial concentration but also because phage transfer is less energy intensive than conjugation[93,108−112] and thus it is potentially less susceptible to lower energy source availability.

The fact that ARG transduction may be important in some systems underscores the need to control phage-borne ARGs, especially at hotspots that may serve as sources for environmental dissemination. For example, municipal wastewater treatment plants are widely acknowledged as potential reservoirs of ARGs,[113] and high concentrations of phage-borne ARGs have been detected in the raw wastewater and activated sludge—sometimes comparable to intracellular ARG

concentrations.[13] Phages can persist and even propagate in activated sludge processes,[114] sometimes leading to higher phage concentrations in the effluent (8.4×10^7 virion/mL) compared to raw wastewater (2.2×10^7 virion/mL).[115] Effluent disinfection processes such as chlorination usually have a high efficacy of bacteria removal but could be less effective on phages.[12,116,117] The phages discharged from ARG hotspots may migrate to new environments and might transfer antibiotic resistance to some indigenous bacteria, enhancing resistance propagation. Therefore, the efficacy of disinfection strategies on phage removal ought to be assessed.[118]

We recognize that the environmental factors considered by this study may affect ARG transfer differently in biofilms that prevail in many systems. Furthermore, other system-specific factors such as pH, ionic strength, chemical agents, and solar ultraviolet radiation might also influence the relative importance of conjugation versus phage delivery to ARG dissemination due to their differential effects on HGT vectors and recipients.[51] Nevertheless, this study underscores the importance of transduction as an ARG propagation mechanism under hydrodynamic and cell concentration conditions that favor phage infection, which may include some groundwater systems,[63−65,119] rivers and streams,[66−68] and wastewater treatment processes.[69−72] The data also suggest the importance to address hotspots with high concentrations of phage-borne ARGs, which may require additional attention to mitigate this relatively overlooked, potential antibiotic resistance dissemination pathway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsenvironau.1c00027.

> Phage manipulations, genetic analysis, collision simulation, and complementary results (PDF)

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