



Dissecting horizontal and vertical gene transfer of antibiotic resistance plasmid in bacterial community using microfluidics

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ABSTRACT

The spread of antibiotic resistance genes (ARGs) has become an emerging threat to the global health. Although horizontal gene transfer (HGT) is regarded as one of the major pathways, more evidence has shown the significant involvement of vertical gene transfer (VGT). However, traditional cultivation-based methods cannot distinguish HGT and VGT, resulting in often contradictory conclusions. Here, single-cell microfluidics with time-lapse imaging has been successfully employed to dissect the contribution of plasmid-mediated HGT and VGT to ARG transmission in an environmental community. Using *Escherichia coli* with an ARG-coded plasmid pKJK5 with trimethoprim resistance as the donor, we quantified the effects of three representative antibiotics (trimethoprim, tetracycline and amoxicillin) on the ARG transfer process in an activated sludge bacterial community. It was found that HGT was influenced by the inhibitory mechanism of an antibiotic and its targets (donor, recipient alone or together), whereas VGT contributes significantly to the formation of transconjugants and consequently ARG spreading. Trimethoprim is highly resisted by the donor and transconjugants, and its presence significantly increased both the HGT and VGT rates. Although tetracycline and amoxicillin both inhibit the donor, they showed different effects on HGT rate as a result of different inhibitory mechanisms. Furthermore, we show the kinetics of HGT in a community can be described using an epidemic infection model, which in combination with quantitative measure of HGT and VGT on chip provides a promising tool to study and predict the dynamics of ARG spread in real-world communities.

1. Introduction

Antibiotic resistance has become an emerging threat to the global health. Among the various routes for the spread of antibiotic resistance genes (ARGs), horizontal gene transfer (HGT) by plasmid-mediated conjugation is regarded as one of the major mechanisms (Soucy et al., 2015). Through conjugation, DNA can be transferred by mobile plasmids between genera, phyla and even major domains (Sørensen et al., 2005). In the events of HGTs, primary transconjugants form after conjugation with original donors. The formed transconjugants can further promote ARG dissemination via both secondary HGT and cell growth – a process known as vertical gene transfer (VGT) (Seoane et al., 2011). It is known HGT and VGT coexist in the natural environments (Qiu et al., 2018), thus ARGs dissemination in a community normally comes from diverse pathways. Currently, study of HGT processes largely depend on various formats of mating assays, such as filter-mating, flasks or plate culture based mating (Guo et al., 2015; L. Li et al., 2018;

Klümper et al., 2014). With these approaches, only end-point measurements are carried out (Sørensen et al., 2005). These give the information on total plasmid transfer at the point of test but it is difficult to acquire the dynamic behavior and responses of the bacteria (Meng et al., 2017) and cannot distinguish the contributions by HGT and VGT (Lopatkin et al., 2016a; Klümper et al., 2014).

Recently, growing evidence suggests that certain groups of chemicals can select and stimulate antibiotic resistance through conjugation. These include, for example, antibiotics (Jutkina et al., 2016; Lopatkin et al., 2016b; Kim et al., 2014; Zhang et al., 2013), heavy metals (Zhang et al., 2018; Klümper et al., 2017), disinfectants (Guo et al., 2015; Zhang et al., 2016), biocides (Jutkina et al., 2018; Carey and McNamara, 2015), ionic liquids (Wang et al., 2015) and organic compounds (Jiao et al., 2017). Among these chemicals, antibiotics are directly related to the dissemination of ARGs, since natural selection by antibiotics favors the spread and maintenance of antibiotic resistance in the environment (Gillings, 2013). However, some contradictory

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findings were reported. For example, 0.50 mg/L of erythromycin was reported to increase the frequency of plasmid mediated resistance transfer between *Lactobacillus plantarum* and *Enterococcus faecalis* strains (Feld et al., 2008). However, in another study, the same concentration of erythromycin decreased the frequency of the ARG-encoding plasmid transfer in *Staphylococcus aureus* (Almasaudi et al., 1991). Furthermore, knowledge of how antibiotics modulate ARG spread is limited because of the complexity associated with the community structure, unknown inhibitory mechanisms and the existing ARGs in bacteria. A general view of the effects of antibiotics on ARG spread is that contrasting scenarios may co-exist: on the one hand, antibiotics could promote plasmid-mediated HGT of ARGs by acting as selection stresses (Lopatkin et al., 2016a); on the other hand, because of antibiotic inhibition of bacterial growth, ARGs transfer via VGT could cease. Without distinguishing VGT from HGT, it is impossible to understand how antibiotics affect these two essential routes and consequently the overall spread of ARGs.

In recent years, microfluidic technology has opened up a new avenue for applications in microbiology. Manipulation of bacteria at the single cell level has become well established, leading to the discovery of a variety of new phenomena (Grunberger et al., 2014; Yin and Marshall, 2012; Yuan et al., 2017; McIlvenna et al., 2016). It is worth noting that conjugation at the single cell level has been observed, illustrating the highly dynamic nature of this process (Seoane et al., 2011; Babi et al., 2008). However, most of these studies are based on thin gel patch culture, which could accumulate (toxic) metabolites and alter cell growth conditions (e.g. pH change over time in the gel surface) (Reinhard and van der Meer, 2010). Therefore, investigations are limited to simple systems and short experimental periods (e.g. within ~2 h). In contrast, microfluidics can precisely control a bacterial microenvironment under flow conditions, allowing long-term chemostat cultivation (Li et al., 2014a; B. Li et al., 2018). In combination with time-lapse imaging, real time monitoring of cell growth at the single cell level can be easily realized (Li et al., 2014a; Song et al., 2015). A variety of studies have demonstrated the unique abilities of single-cell microfluidics for investigation of dynamic cellular processes (Yuan et al., 2017; Li et al., 2014b, 2016a; Yuan et al., 2018). With the aid of fluorescence probe technology purposely developed for conjugation detection (Sørensen et al., 2005; Klumper et al., 2014), it is expected that microfluidics can provide a promising platform to investigate the dynamic process of ARG transfer with single cell resolution.

Here, we have employed a single-cell microfluidic approach to investigate the dynamic process of ARG transfer in a complex community, the contributions of HGT and VGT to this process, and the effects of various antibiotics. A recently developed multilayered microfluidic device was employed to maintain long-term monolayer cell culture and in situ tracking of individual cells (Yuan et al., 2017). *Escherichia coli* (*E. coli*) MG1655 harboring the ARG-encoding plasmid PKJK5 was used as the donor and the complex communities extracted from activated sludge was used as the recipients. With this platform, dynamic transfer of antibiotic resistance plasmid was monitored at the single cell level. This allowed us to discover dynamic variations in HGT and VGT of plasmids under antibiotic stresses. In addition, it has been found that the kinetics of the HGT process in the activated sludge community can be described using an epidemic infection model, indicating that ARG spread in a complex community could be treated in a similar manner to the spread of infectious disease.

2. Materials and methods

2.1. Bacterial strains and culture

The donor strain, *Escherichia coli* MG1655 (hosting the GFP-encoded pJK5 plasmid with trimethoprim resistance) was a generous gift from Professor Barth F. Smets in Technical University of Denmark. The donor strain was grown overnight in LB medium supplemented with

trimethoprim (30 mg/L) at 30 °C. This strain was genetically modified and chromosomally tagged with a gene cassette encoding constitutive red fluorescence and constitutive *lacI*^q production, which suppresses the expression of GFP (Klumper et al., 2017). Thus, the donor strains only emit red fluorescence. When a recipient cell receives the GFP-encoded plasmid pJK5, it becomes a transconjugant and emits green fluorescence. This method has been successfully used to detect gene transfer between cells in the bacterial communities from activated sludge (L. Li et al., 2018) and soil (Klumper et al., 2014).

The plasmid pJK5 was recovered from a soil environment by exogenous plasmid isolation, belonging to the subgroup of IncP-1 plasmids. It includes a Tra1 region (*tra* genes), a Tra2 region (*trb* genes), a central control module and a replication initiation module. Two discrete accessory elements of 2145 bp (load 1) and 11,678 bp (load 2) are situated between the Tra1 and Tra2 regions of pJK5. The load 1 consists of an insertion sequence ISPa17 and load 2 is a Tn402-derivative containing a class 1 integron. Moreover, the Tra2 region (*trb* genes) was involved in the formation of mating pairs. The 338 bp intergenic region between the *traJ* and *traK* genes of plasmid pJK5 contains the putative origin of conjugative transfer (*oriT*) (Bahl et al., 2007). These characters indicated that plasmid pJK5 could be transferred between bacteria through conjugation.

Escherichia coli ATCC 25922 (ATCC, USA) was used as a single strain recipient to validate the method. Bacterial communities extracted from activated sludge from a wastewater treatment plant (WWTP) in Beijing were used as community recipients. Activated sludge samples were pretreated as shown previously (Li et al., 2016a). The mixed liquid after pretreatment was cultured in LB medium at 30 °C overnight and then harvested as mixed bacterial communities. The main genera in the community were identified using 16S rRNA sequencing (Supplementary information Table S1). It should be noted that the harvest bacteria represent cultivable cells in the activated sludge community.

2.2. Microfluidic chip

Microfluidic devices were made in polydimethylsiloxane (PDMS) elastomer as described previously (Yuan et al., 2017). The device consists of eight parallel shallow micro-chambers, which are designed for trapping individual cells and enabling monolayer culture (Fig. 1). The dimensions of each chamber are 200 μm (length) × 100 μm (width) × 1.5 μm (height). On one side of the chambers, barriers with a height of 0.4 μm are used to block cells in the chamber when loading the cells from the other side. On both sides of the chambers, there is a channel (50 μm wide by 10 μm high) for delivering medium during bacterial cultivation.

2.3. Mating assays on the chip

The monolayer cultivation of bacteria was carried out for the mating assays on the microfluidic chip at room temperature (26 °C). The donors and recipients were washed with PBS three times and diluted in LB medium to ~10⁸ cells/mL. After this, they were mixed in suspension at a ratio of 1:1. The mixed bacterial suspension was inoculated into the chambers through the loading channel at a flow rate of 1 μL/min. After loading, fresh medium was supplied to flow through both sides of the chambers at a suitable flow rate to remove non-trapped cells. Thereafter, the flow rate was changed to 1.8 μL/min to maintain on-chip cultivation. The continuous flow provided constant nutrient supply to the cells and simultaneously removed metabolic waste – akin to chemostatic culture. Images were taken during the cultivation. Bacteria overgrew in chambers after about 3–4 h depending on the initial loaded cell density. Excessive bacteria were removed from the chambers by the sheath flow in the channel.

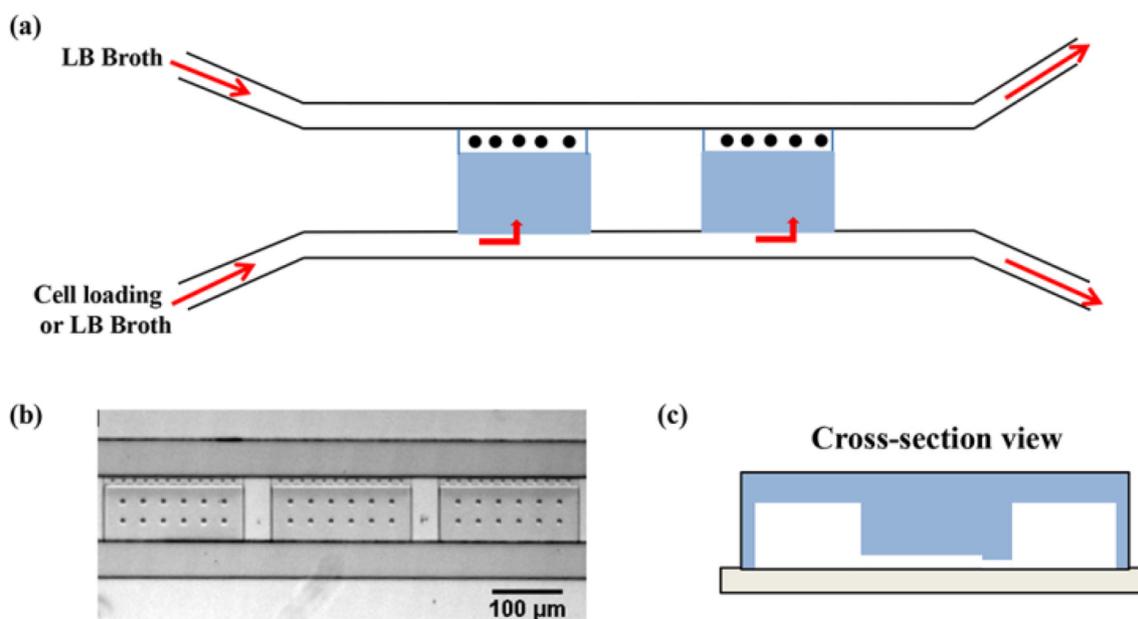


Fig. 1. (a) Schematic drawing of the chip and cell loading at the start of the experiment. (b) Optical image of the chambers on the chip; (c) the cross-section view of the chip.

2.4. Antibiotics and chemicals

Three antibiotics, tetracycline (TET), amoxicillin (AMO) and trimethoprim (TMP) were selected to test their influence on ARG transmission in bacterial communities. A computerized incubator (Bioscreen C; Lab Systems, Helsinki, Finland) was used to obtain bacterial inhibition curves under the antibiotic treatments as described before (Li et al., 2016a). For the donor strain, the minimum inhibition concentrations (MIC) were determined from the inhibitory curves (Supplementary Information, Fig. S1) as 5 mg/L for TET and 10 mg/L for AMO. There was no inhibition from 10 mg/L of TMP on the donor since the PKJK5 plasmid carries the TMP resistance gene. 10 μg/L TET and 1 mg/L AMO were selected as inhibitory antibiotics to exert 10% inhibition on the growth of the donor. 10 mg/L TMP was used as the positive control (i.e. a kind of resisted antibiotic) and LB broth without antibiotics was used as negative control for the donors. The recipient cells from activated sludge were not inhibited by 10 μg/L TET and 1 mg/L AMO, but were inhibited by 10 mg/L TMP with 25% reduction in their growth rate (Supplementary information, Fig. S1). Experiments were repeated at least three times under each condition.

2.5. Microscopy and image analysis

Time-lapse imaging of the on-chip mating experiments was carried out on an inverted microscope (Ti-E, Nikon Corp., Japan) equipped with a 40×/NA0.60 objective lens and a CCD camera (CCD, iXon X3 897, Andor Company, UK). Bright field and fluorescence images of each chamber were taken at defined time intervals (e.g. every 15 min or every 1 h). Green fluorescence protein (GFP) was detected at excitation of 488 ± 20 nm and emission of 525 ± 40 nm, whereas *dsRed*-based fluorescence was detected at excitation of 561 ± 25 nm and emission of 650 ± 60 nm. All the bright field images were processed and analysed using ImageJ as described before (Li et al., 2014a). From these, the specific growth rates of the donors and activated sludge recipients cultured on chip were determined to be 0.44 h^{-1} and 0.61 h^{-1} respectively (Supplementary information, Table S2). Transconjugants formed were identified based on the changes of the green fluorescence area from the time-lapse images (Supplementary information, Fig. S2). The first randomly appearing green fluorescence spot in an image was regarded as a primary HGT event. The fluorescence extension from this

spot via cell division is due to VGT (Supplementary information, Fig. S3).

2.6. Calculation of horizontal and vertical gene transfer rates

Previously, HGT transfer frequency was determined by counting the number of fluorescence transconjugants colonies at the end of filter mating assays and then dividing it by the number of originally added recipient cells (Musovic et al., 2010). A connected colony was considered as one HGT event which in reality could be a combination of several HGT and VGTs. In this study, the ability to monitor the transfer process at single cell resolution allows the identification of the on-set of HGT events as well as cell growth. As shown previously, bacteria grew as a monolayer in the chambers, therefore cell mass is in a linear relationship with their area (Li et al., 2014a). The areas occupied by transconjugants within a defined area of a confluent cell layer represent the total percentage of transconjugants in a population. Therefore, its change with time (i.e. the total transconjugant increment rate (R_T) can be calculated as shown in Eq. (1). The transconjugants formed by primary HGT events can be identified based on the first appearance of a green spot in the time-lapse images. Therefore, the transconjugant formation rate via primary HGT (denoted as HGT rate, R_H) and that via cell growth (i.e. vertical gene transfer rate, denoted as VGT rate, R_V) can also be estimated using Eqs. (2) and (3).

$$R_T(t) = \frac{A_G(t) - A_G(t - \Delta t)}{aA_0 \Delta t} \times 100\% \quad (1)$$

$$R_H(t) = \frac{\Delta A_{Gh}(t)}{aA_0 \Delta t} \times 100\% \quad (2)$$

$$R_V(t) = R_T(t) - R_H(t) \quad (3)$$

where $A_G(t)$ is the green fluorescence area (i.e. the transconjugant area) in a series of time-lapse images at a given time t ; Δt is the defined time interval (e.g. 1 h); $A_G(t - \Delta t)$ is the green fluorescence area at the time $t - \Delta t$; A_0 is the total area of one chamber, which has a fixed value of 0.2 mm^2 . The parameter a is a correction factor for a confluent monolayer. The loading density is $\sim 65\%$ of a chamber, and thus $a = 0.65$ at $t = 0$ h. It should be noted that a confluence monolayer of bacteria was normally formed in the chamber by hour 1 or 2. $\Delta A_{Gh}(t)$ is the area of transconjugants formed via HGT within Δt and is determined manually

based on the first appearance of a green spot. Calculation of $A_G(t)$ and $\Delta A_{Gh}(t)$ was described in the Supplementary information (Supplementary Fig. S3).

Since conjugation is triggered randomly with the prerequisite of contact between a donor and a recipient cell, the spread of ARGs via plasmid-mediated HGT can be considered to transmit through networks of susceptible individuals linked by a range of contacts. Therefore, plasmid dissemination in a population can follow an epidemic spread model that describes the kinetics of infection distribution in networks (Avramov, 2007). The model is adjusted to describe the kinetics of HGT rate (R_H) as shown in Eq. (4).

$$W(t) = A \cdot t^d \cdot \exp(-t^m) \quad (4)$$

where $W(t)$ represents the intensity of HGT rate (R_H) at time t ; A is a coefficient associated with experimental conditions; d reflects the possibility of fractal dimension of the network; m is defined as $d + 1$ originally in the function but here we consider m and d as independent constants that represent unknown complexity of the network in the environmental bacterial community.

3. Results and discussion

3.1. Plasmid transfer routes at the single cell level

Since bacteria grew as a monolayer in the chamber, transconjugants can be readily identified at the single cell level. As shown in Fig. 2, the occurrence of an HGT event from a donor resulted in a primary transconjugant (note, this time point was defined as 0 min). After that, the transconjugant continued its growth and divided into two individual cells (at $t = 45$ min), which then kept on growing to the full size of their parents. This demonstrated a typical VGT after HGT process. At $t = 60$ min, another primary HGT event happened in the chamber, resulting in a new transconjugant. At $t = 120$ min, more transconjugants were formed via HGT and randomly scattered in the chamber. By $t = 180$ min, the majority of transconjugants had divided into new cells and the green fluorescence area has significantly expanded via VGT. These results show that in situ monitoring of transconjugant formation

on chip can successfully dissect HGT and VGT events, and therefore provide a powerful means to study gene transfer in bacteria communities.

3.2. Determination of the contribution of HGT and VGT to ARG spread

The bacteria communities extracted from activated sludge were used to represent the complex communities in real world. High density of cells was loaded in the chambers as shown in the bright-field image (Fig. 3, $t = 0$ h) to enable close contact between the donor and recipient cells. Time-lapse images of another chamber were also shown in Fig. S2. Within the first hour ($t = 1$ h), several transconjugants appeared randomly in the chamber. This is typical formation via primary HGT. It should be noted that these HGT events were observed in each chamber due to high loading density. Between 2 and 3 h, the primary transconjugants grew into colonies via VGT. At the same time, more primary transconjugants appeared at random locations in the chamber. In the later stage ($t = 4-6$ h), very few primary transconjugants appeared, indicating that plasmid-mediated HGT reached saturation. This saturation was likely caused by the spatial distribution of the donor and recipient colonies on chip, which prevented close contact between the donor and recipient cells.

Fig. 4a shows the accumulative transconjugant formation over time in LB broth. Contributions due to primary HGT and VGT at any given time are identified through image analysis (Supplementary information Fig. S3). Up to 4 h, more transconjugants were formed via HGT than that formed via VGT. After that, negligible new transconjugants were formed via HGT whereas the number of transconjugants formed via VGT still increased. At 6 h, the total transconjugants formed via HGT and those via VGT were 3.0% and 4.3% respectively, suggesting their similar contributions to the overall transconjugant formation within 6 h. Furthermore, dynamic changes of HGT rate (R_H) were monitored as shown in Fig. 4b. R_H peaked to $\sim 1.0\%/h$ around 3 h (Fig. 4b). However, by 6 h, the interfaces between the donors and recipients were substantially reduced as the result of expansion of the recipient and transconjugant colonies (Fig. 3). Therefore, it is not surprising that hardly any new conjugation occurred at hour 6.

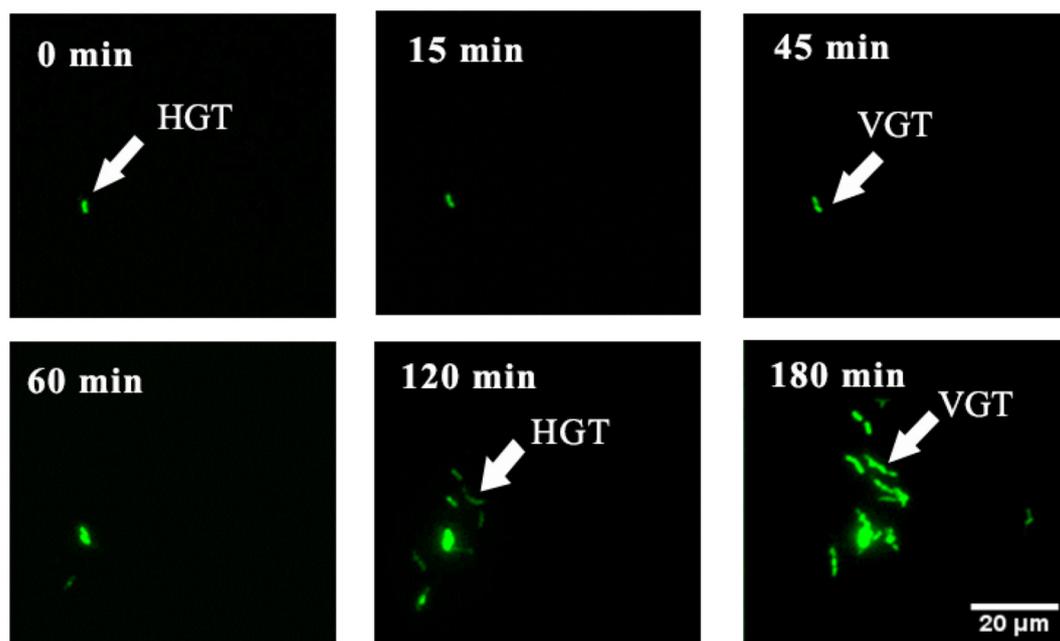


Fig. 2. Time-lapse images of the *E. coli* MG1655 donor and *E. coli* (ATCC 25922) recipient system. A green fluorescence transconjugant appeared randomly in the chamber at $t = 0$ (white arrow), indicating a typical formation via HGT. This transconjugant showed cell division by $t = 45$ min (white arrow), which represents a typical formation via VGT. More transconjugant formed either via HGT or VGT are indicated by the white arrows in the later stages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

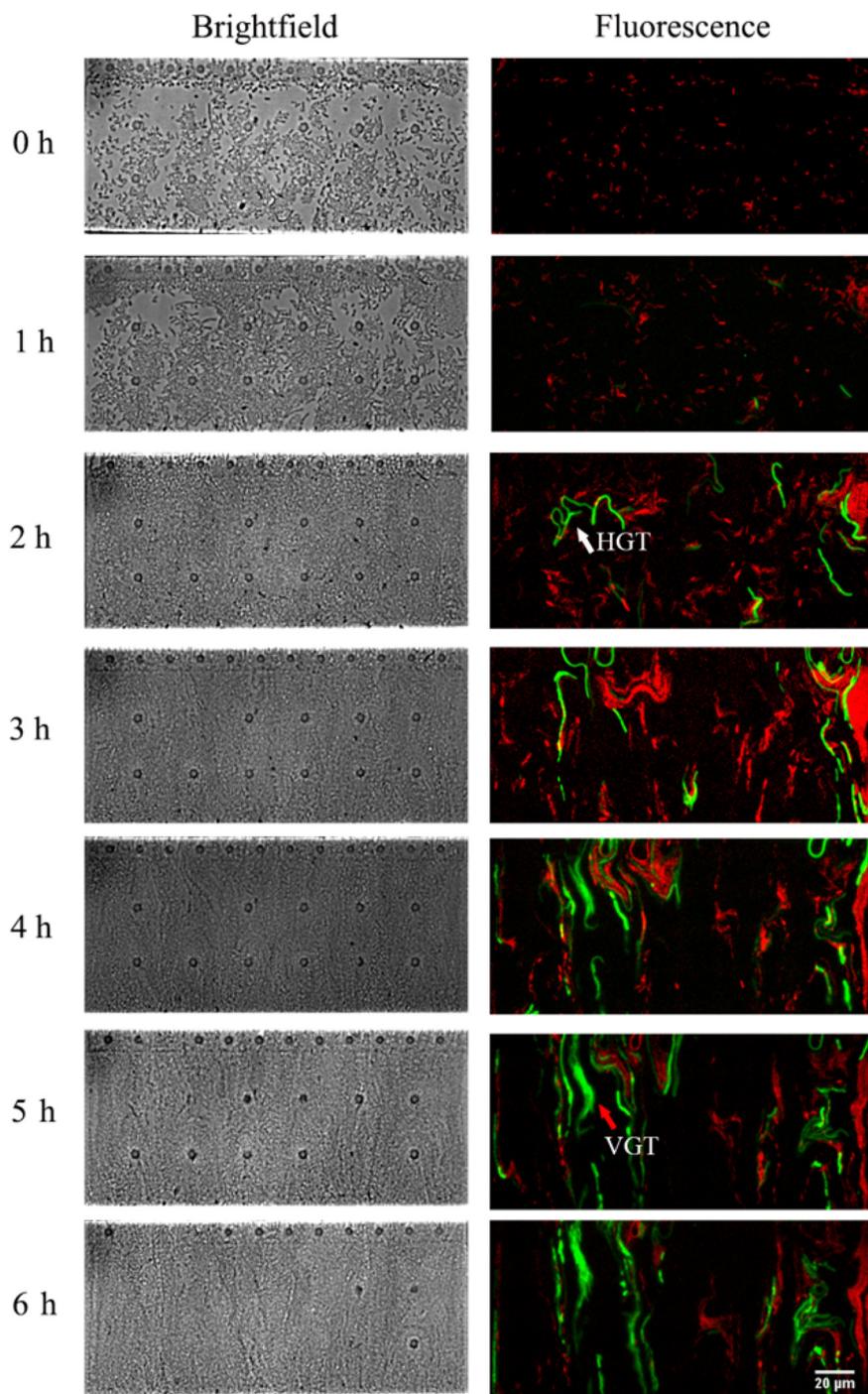


Fig. 3. Time-lapse images of the on-chip mating assays of the donor and activated sludge community in LB broth. Bacteria with red and green fluorescence were donors and transconjugants respectively. White arrow – transconjugants formed via HGT. Red arrows – the expansion of transconjugants via VGT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is worth noting that the open-end chamber is akin to real-world bacterial communities in the form of clusters or granules that consist of a confined core (i.e. the chamber area) and a free growing periphery (i.e. shed away bacteria from the edge of the chamber). Bacteria growing in the confined space widely exist in different environments, such as carriers for wastewater treatment, indwelling devices for medical treatment and so on (Hachulla et al., 2004). Therefore, the quantitative measure of HGT rate in the “core” area (i.e. chamber) may provide valuable data to describe the kinetics of primary transconjugant formation in a complex community. Despite the apparent difference, ARG spread via plasmid-mediated conjugation has many similar

features to epidemic distribution of diseases, such as in random directions in a spatial-temporary manner and through a network of susceptible individuals. Therefore, the kinetics of HGT rate (R_H) may be described using similar equations for propagation of other disturbances in a population, for example, the epidemic model (e.g. various SIR model) of infectious disease (Pastor-Satorras and Vespignani, 2001).

Here, the modified epidemic spread model derived from the Kolmogorov–Mehl–Avrami method (Avramov, 2007) was employed to fit the time dependence of HGT rates (Eq. (4)). An excellent match was observed between the experimental data and the fit to the model with R^2 of 0.996 (Fig. 4b). The fitting gave three characteristic parameters to

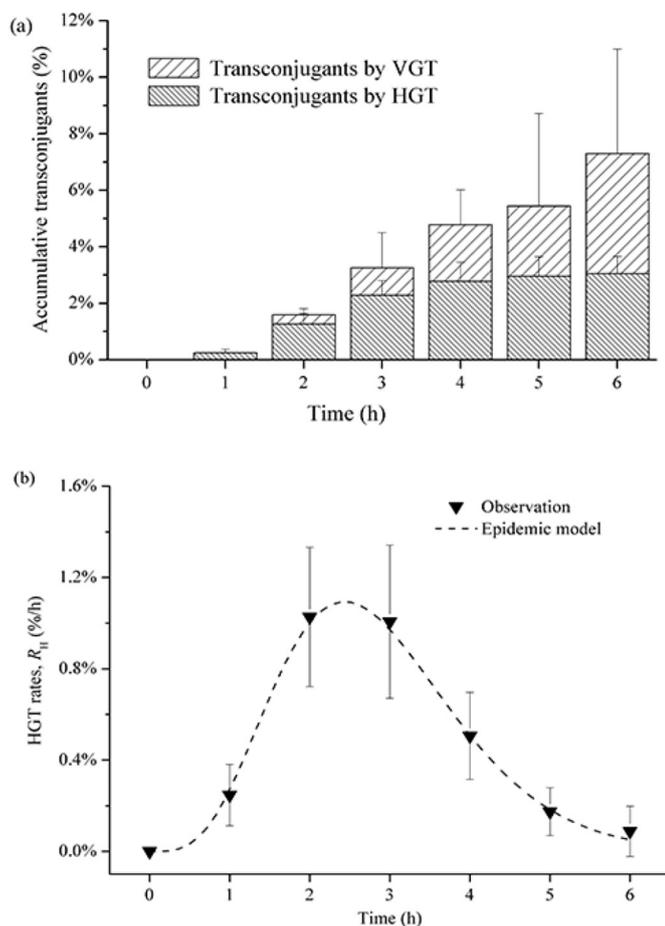


Fig. 4. Transconjugant formation between the donor and activated sludge community recipient in LB broth. (a) Accumulative transconjugant formation via HGT and via VGT; (b) HGT rate (R_H) versus time. The dashed line shows the fit to the epidemic spread model (Eq. (4), $R^2 = 0.996$).

describe the dynamics of the HGT process with the donors and bacterial community recipient in LB: 1) A found to be 0.61%/h – a coefficient associated with the maximum increment rate of transconjugants; 2) d was 4.2 – indication of the fractal dimension of the network; and 3) m was 1.3 – representing unknown complexity. These parameters can be used as quantitative measures to compare different effects of antibiotics on the kinetics of ARG spread via HGT within a community.

With the single-cell microfluidic approach, we have revealed three novel aspects of plasmid-mediated conjugation: (1) The HGT process evolves dynamically in a population in time course. Real-time monitoring of transconjugant formation on chip allows the kinetics of this process to be measured. (2) The VGT route contributes significantly to the formation of transconjugants and consequently ARG spread. Previously, it was assumed that all the transconjugants found in the mating assays were generated via HGT events. However, our results showed this is not the case. Instead, VGT can be the main driver for ARG spread in wastewater treatment systems as suggested previously (Backhaus and Grimme, 1999). (3) Direct observation of primary transconjugant formation provides a more accurate measure for horizontal transfer rates than the previous concepts of transfer frequency or permissiveness (Klümper et al., 2014). The concept of permissiveness assumes that only one HGT event exists for one colony. However, since the tests are endpoint assays, several HGT events can merge into one colony via VGT during the dynamic process.

The influence of environmental factors on HGT is quite complicated, and can lead to over-estimation when using endpoint assays (Lopatkin et al., 2016b), or under-estimation when using permissiveness assays.

However, with the capability of tracking dynamic changes in HGT and VGT rates, a more accurate and better understanding of the environmental influence is achievable. The approach is further applied to study the effects of antibiotics on ARG transmission.

3.3. The effect of antibiotics on ARG transmission in bacterial community

Because of different cell susceptibility to antibiotics, the effects of antibiotics on plasmid transmission in a population vary significantly when using traditional mating assays (Lopatkin et al., 2016a; Kim et al., 2014; Zhang et al., 2013; Jutkina et al., 2018; Guo et al., 2018). TMP (10 mg/L), AMO (1 mg/L) and TET (10 μ g/L) have different levels of inhibition on the donor and recipient cells (Supplementary information, Fig. S1), and thus enable systematic evaluation of different effects of antibiotics on donor or recipients. Fig. 5a shows the time-lapse images of the donor and activated sludge community under different antibiotic stresses. In all cases, few scattered transconjugants appeared at 1 h and then more transconjugant areas appeared, either via HGT or via VGT. This phenomenon is similar to that in LB (Fig. 3). However, the total areas of transconjugants and the donor cells under different antibiotics varied significantly and followed the order of TMP > AMO > TET. Furthermore, since donor cells contained plasmid with TMP resistance, TMP treatment created large donor colonies whereas small and scattered colonies of the donors were formed under the AMO and TET treatments.

Quantification of the total transconjugants formed via HGT and via VGT illustrated interesting variations caused by different antibiotic treatments (Fig. 5b). Within the first 6 h of the experiment, TMP exposure resulted in the largest amount of transconjugants via either HGT (10%) or VGT (26%). They were 3 and 5 times higher than those in LB broth (3% and 4.8% respectively). Although both AMO and TET inhibited the donor cells, AMO exposure had a similar transconjugant formation via HGT (3.2%) to the LB control and ~ half of the transconjugant formation via VGT (2.7%). In contrast, TET treatment significantly reduced transconjugant formation both via HGT (1.6%) and VGT (1.7%).

The drastically different effects from three antibiotics highlighted the importance of the inhibitory mechanism of an antibiotic and its targets (i.e. donors or recipients). Firstly, the antibiotic resistance gene carried by a plasmid plays an important part in either HGT or VGT process under antibiotic stresses, which is clearly demonstrated in the contrasting phenomena between TMP and TET treatments. Donor cells contain a TMP resistance gene. Therefore, not only they are highly resistant to TMP but their growth is stimulated by the presence of TMP (Fig. 5a and Supplementary information, Table S2). This results in more donor colonies that further enhanced cell-to-cell contacts between recipients and donors, and hence initial plasmid transfer. As a consequence, despite the recipient cells are inhibited by TMP, the overall HGT rates were still significantly increased. In contrast, inhibition of the donor cells by TET significantly reduced the HGT rates, despite it not inhibiting the recipient cells.

Secondly, the inhibitory mechanism of an antibiotic on the donor cells modulates its effects on HGT. TET is one kind of tetracycline antibiotics, which inhibit protein synthesis by disrupting amino acid chain elongation (Li et al., 2016b), but do not induce SOS and act upon the outer membrane, which is related to transmission of resistance genes (Hastings et al., 2004). Therefore, tetracycline antibiotics hardly promote HGT. Since AMO belongs to β -lactam antibiotics, it inhibits the development of bacterial cell wall by interfering with transpeptidase enzymes. Exposure under low concentrations of AMO usually results in cell elongation (Li et al., 2014a,b). Previous studies suggested that elongated recipients tend to receive the plasmid due to increases surface contacts and mating frequency (Seoane et al., 2011). This is also confirmed by cell length (Supplementary information Fig. S4). Donor and recipient cells showed similar length under TET, TMP and control treatments. However, cell length of transconjugants was longer than

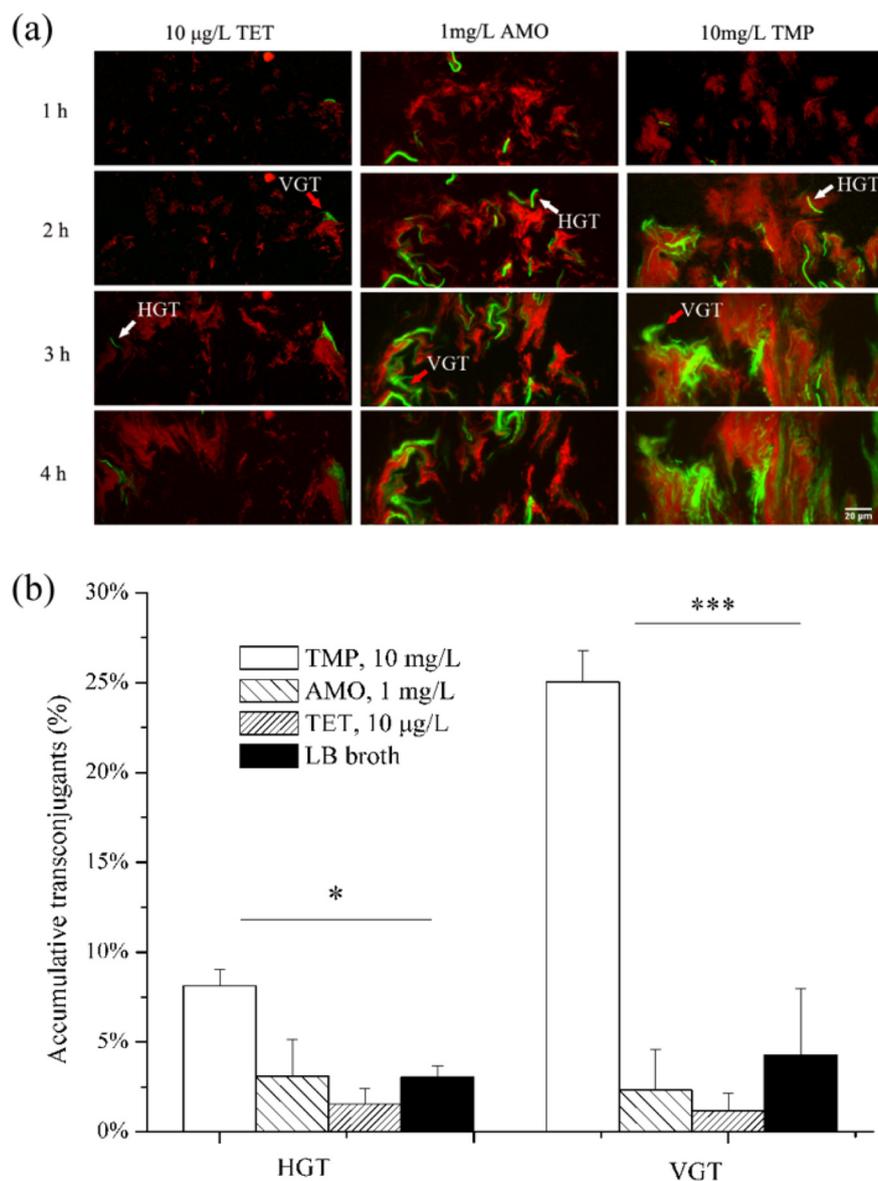


Fig. 5. (a) Time-lapse images of transconjugant formation under three antibiotic treatments. White arrows – transconjugants via HGT. Red arrows – transconjugants via VGT. (b) The total transconjugants formed via HGT and via VGT within 6-hour experiments under different antibiotic conditions (*: $p < 0.05$, **: $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recipients, especially under AMO treatment, which was significantly longer than the others.

Thirdly, VGT rates not only depend on the inherent properties of the recipients but also can be altered by the mobile genes coded in the plasmid. Under the unstressed LB condition, VGT rate is determined by the recipient growth rate. As a result, the VGT rate is slightly higher than the HGT rate in LB control because of relatively faster growth of the recipient cells (Supplementary information Table S2). However, despite the fact that 10 mg/L TMP reduces the recipient cells' growth by 25%, it still significantly enhanced its VGT rate by a factor of 5 in comparison to that in LB. On one hand, this shows that the primary transconjugants have successfully integrated the transferred TMP resistant gene into its own and developed new traits. Plasmids are more stable in bacterial cells under TMP stress. On the other hand, since TMP inhibits the growth of the recipients only, the recipients are out of competition in terms of space. This could also contribute to the increased vertical transfer rates in transconjugants. Furthermore, the VGT rate was 2.5 times higher than the HGT rate in the presence of TMP, suggesting that VGT can play a dominant role in disseminating ARGs in

communities when the resisted antibiotic is present.

3.4. The dynamics of ARG spread under different antibiotics

The dynamic variations of the transconjugant formation rate further demonstrated diverse effects of the three antibiotics (Supplementary information Fig. S5). In the case of HGT rate (R_H), within 1 h, no significant difference was observed between AMO, TMP and LB, whereas R_H under TET treatment was significantly lower. Between 2 h and 3 h, R_H under TMP treatment was substantially higher than the others, possibly due to the significantly enhanced growth of the donor (Supplementary Information, Table S2). After 4 h, no significant difference was observed among all the conditions and all of the HGT rates were below 1%. In the case of the VGT rate (R_V), apart from the first hour where VGT has not occurred, the R_V under TMP is significantly higher than the others throughout the whole period of the experiment.

The epidemic spread model (i.e. Eq. (4)) was also used to fit the dynamic variations of HGT rates under different antibiotic stresses (Fig. 6) and characteristic parameters were derived (Table 1). In terms

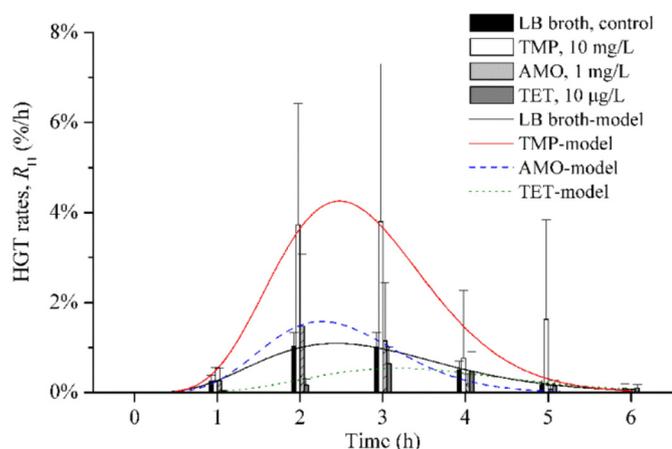


Fig. 6. HGT rates under different antibiotics. The data are fitted to the epidemic spread function (Eq. (4)). The derived parameters are listed in Table 1.

Table 1

The epidemic spread model fitting for HTG rates under antibiotic exposure.

Antibiotics	LB	TMP	AMO	TET
Concentration	–	10 mg/L	1 mg/L	10 µg/L
A	0.007	0.0144	0.007	0.0007
d	3.9	5.2	5.2	5.6
m	1.3	1.4	1.5	1.3
R ²	0.9975	0.8683	0.9931	0.9148

Note: The epidemic spread model function is $W(t) = A \cdot t^d \cdot \exp(-t^m)$. R² is the correlation coefficient.

of coefficient A, TMP treatment shows an obvious enhancement (i.e. $A_{TMP} = 0.014$ versus $A_{LB} = 0.007$ for the LB control); AMO treatment has a negligible effect ($A_{AMO} = 0.007$) and TET treatment has significant inhibition ($A_{TET} = 0.0007$). The changes agree well with variations in HGT rates caused by each antibiotic. Furthermore, a right shift of the peak position was observed in the TET treatment, indicating a significantly delayed conjugation process. The combination of the model with quantitative measurement of transconjugant formation provides a promising method to study and predict the kinetics of ARG spread in complex communities.

4. Conclusion

Here, single-cell microfluidics in conjunction with time-lapse imaging has been successfully employed to dissect the contribution of plasmid-mediated HGT and VGT to ARG transmission in a community. The platform enabled real-time monitoring of the dynamic changes in transconjugant formation via HGT and VGT under treatments of three representative antibiotics (TET, AMO and TMP). Contrasting effects were observed, showing that the effects depend on both the inhibitory mechanism of an antibiotic and its targets (donor, recipient alone or together). For the *E. coli* MG1655 donor (hosting the PKJK5 plasmid with TMP resistance) and activated sludge community system, substantial enhancement in HGT rate was observed by TMP treatment, whereas negligible effect and reduction in HGT rate were found for AMO and TET. Furthermore, transconjugants develop new traits from the acquired ARG gene and become the dominant driver for ARG transmission via VGT.

Interestingly, the dynamic HGT rates in a community can be described using an epidemic spread model. Excellent match between the data and the fit to model was found. The combination of the model with quantitative measure of transconjugant formation provides promising method to study and predict the kinetics of ARG spread in complex

communities.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.105007>.

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