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Short Communication

Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative micro-contaminant concentrations

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HIGHLIGHTS

- Increased plasmid transfer in ppb levels of tetracycline and sulfamethoxazole.
- Significant increase in plasmid transfer on activated sludge with the tetracycline.
- All pB10 plasmid received bacteria were enterics in presence of tetracycline.

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ABSTRACT

The presence of antibiotics in the natural environment has been a growing issue. This presence could also account for the influence that affects microorganisms in such a way that they develop resistance against these antibiotics. The aim of this study was to evaluate whether the antibiotic resistant gene (ARG) plasmid transfer can be facilitated by the impact of 1) environmentally representative micro-contaminant concentrations in ppb (part per billion) levels and 2) donor-recipient microbial complexity (pure vs. mixed). For this purpose, the multidrug resistant plasmid, pB10, and *Escherichia coli* DH5α were used as a model plasmid and a model donor, respectively. Based on conjugation experiments with pure (*Pseudomonas aeruginosa* PAKexoT) and mixed (activated sludge) cultures as recipients, increased relative plasmid transfer frequencies were observed at ppb (μg/L) levels of tetracycline and sulfamethoxazole micro-contaminant exposure. When sludge, a more complex community, was used as a recipient, the increases of the plasmid transfer rate were always statistically significant but not always in *P. aeruginosa*. The low concentration (10 ppb) of tetracycline exposure led to the pB10 transfer to enteric bacteria, which are clinically important pathogens.

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1. Introduction

Over the past decade, the presence of antimicrobial compounds and their residues in the environment have attracted great attention because of their intrinsic bioactivity and their continuous input to the aquatic environment (Kummerer, 2009a). The commonly found antimicrobial compound concentrations in the environment are around ppt (part per trillion) or ppb (part per billion) levels (Behera et al., 2011; Hirsch et al., 1999; Watkinson et al., 2009).

Continuous input of antimicrobial agents to the environment could result in increased antibiotic resistance (Levy, 2002). This might be one of the major reasons that an increasing number of antibiotic resistant genes (ARGs) are found in the environment (Martinez, 2008). Several researchers showed that the abundance of these ARGs in the environment has been increasing because of human activities (Aminov and Mackie, 2007; Knapp et al., 2010; Zhu et al., 2013). Furthermore, the ARGs associated with pathogens have also increased (Brusselaers et al., 2011). Although a number of studies have documented positive relationships between antibiotic and the presence/persistence of antibiotic resistance in the environment (Kim et al., 2007; Merlin et al., 2011; Shakibaie et al., 2009), it is still unclear whether antibiotic

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concentrations at ppb (parts per billion) levels can reach an effective threshold concentration towards proliferating antibiotic resistance in the environment (Barr et al., 1986; Ohlsen et al., 2003). In addition, the traditional selective pressure theory about antibiotics for the proliferation of antibiotic resistance in the microbial community might not be appropriate in environmental conditions since most of the ppb level of antibiotics cannot efficiently inhibit the antibiotic sensitive microorganisms. It is therefore possible that there is an alternate mechanism such as horizontal gene transfer (HGT) for the dissemination of antibiotic resistance traits in environments with such low levels of antimicrobial compounds.

HGT is an essential step for competitive bacterial survival in the environment and is also believed to be one of the major drivers for antibiotic gene transfer (Aminov, 2011; Shakibaie et al., 2009). ARG acquisition rate by HGT can possibly be affected by various environmental contaminants and conditions. For example, contaminants such as metals or antibiotics can damage the genomic DNA and result in the induction of the SOS response; SOS response could then promote the dissemination of HGT (Aminov, 2011; Hastings et al., 2004). In addition, the rate of HGT also depends on various environmental microorganism-associated factors such as plasmid donor and recipient species (De Gelder et al., 2005; Dionisio et al., 2002). In a previous study (Ohlsen et al., 2003), the transfer of conjugative gentamicin resistant (*aacA–aphD*) plasmids of *Staphylococcus aureus* (*S. aureus*) were investigated with different antibiotic concentrations. Although most antibiotics have no effect on the transfer of plasmid, approximately 3-fold increases were observed for gentamicin at 100 ppb in one mating pair [methicillin resistant *S. aureus* (MA31) × methicillin resistant *S. aureus* (MA20)].

Based on a combination of these previous observations, it is hypothesized that the increase of ARGs in environmental conditions could be related to the increased HGT rate induced by micro-contaminants among bacteria. It is also hypothesized that HGT can be affected by the environmental system's microbial complexity. These hypotheses are worthy of careful study because the micro-contaminants' effect on HGT among various bacteria under environmental matrices is scarce. Therefore, the aim of this study is to evaluate the impact of 1) threshold environmental micro-contaminant concentrations and 2) donor-recipient microbial complexity (pure vs. mixed) in the transfer of plasmid encoded antibiotic resistant genes.

2. Materials and methods

2.1. Bacterial strains

According to previous studies (Nikaido, 1998; Kelch and Lee, 1978) gram-negative bacteria have more significance in terms of medical research and are found to be more resistant to antibiotics than gram-positive bacteria. Therefore, we assumed that *Escherichia coli* (*E. coli*) and its derived plasmid is a good model plasmid donor to the environment. Accordingly, *E. coli* DH5α, containing the multidrug resistance plasmid pB10 was selected as the plasmid donor in this study. The complete 64,508 bp nucleotide sequence of the IncP-1β plasmid pB10 was originally isolated from a wastewater treatment plant in Germany and mediates resistance against the antimicrobial agents amoxicillin, streptomycin, sulfamethoxazole, tetracycline and metallic mercury (Schluter et al., 2003). As a pure culture recipient, gentamicin resistant *Pseudomonas aeruginosa* PAKexoT was used in this study (Kaufman et al., 2000). In this study, *P. aeruginosa* was selected as a model environmental microorganism since it is found in various environmental conditions such as soil and water (Alonso et al., 1999). In evaluating the impact of donor–recipient microbial complexity in the transfer of pB10, activated sludge was used as a recipient in this study.

The donor and recipient cultures were grown separately in LB (lysogeny broth) medium, supplemented with appropriate antibiotics [donor: amoxicillin (50 μg/mL), tetracycline (20 μg/mL), streptomycin (50 μg/mL) and sulfamethoxazole (150 μg/mL), recipient: gentamicin (50 μg/mL)], and placed on a 150 rpm rotary shaker at 20 or 37 °C.

As a complex recipient, two-liter grab activated sludge samples were directly collected from an aeration basin in Cheongwon Wastewater Treatment Plant located at Osong in Chungbuk, Korea during June to July and decanted into sterile 1 L plastic bottles. Activated sludge samples were kept in an ice box, transported to the laboratory, and stored in a refrigerator. Samples were used as a recipient within 24 h after storing.

2.2. Plasmid transfer mating experiment

Each donor and recipient culture was grown in LB broth, with appropriate antibiotics in a 37 °C shaking incubator until they reached an optical density (O.D.) of 0.9 at 600 nm. Activated sludge was diluted with phosphate buffer to achieve an O.D. of 0.9. When the O.D. value was reached at 0.9, initial concentrations of potential recipients (*P. aeruginosa* PAKexoT or activated sludge) were enumerated by plate cultivation method for later transfer frequency calculation (*T*/*R*, transconjugant/(potential) recipient). After harvesting, each culture was centrifuged at 4,000*g* for 15 minutes. The most of the supernatant was then discarded and the pellets containing the donor (*E. coli* DH5α pB10) and recipient (*P. aeruginosa* PAKexoT or activated sludge) were re-suspended in the remaining supernatant and then mixed together, and inoculated on mating LB media plates containing one of the five stressors, antibiotics (amoxicillin, tetracycline, streptomycin, sulfamethoxazole) or metal (mercury), with concentrations from 0 to 1 μg/mL. After 16 h of incubation, the pellets (donor and recipient mixture) were re-suspended with 1 mL of LB broth and transferred to a tube and vortex-mixed for ten seconds. The donor–recipient mixtures were serially diluted and spread onto a transconjugant selecting LB media plates containing mixture of antibiotics depending on the recipient (*P. aeruginosa* PAKexoT or activated sludge). The recipients possessing pB10 plasmid were called transconjugants.

When *P. aeruginosa* PAKexoT was used as the recipient, the transconjugant selecting LB media plates contained tetracycline (2 mg/L) and gentamicin (10 mg/L). When the activated sludge was used as the recipient, the transconjugant selecting LB media plates contained a mixture of amoxicillin (1 ppm), tetracycline (1 ppm), streptomycin (1 ppm), sulfamethoxazole (1 ppm), and gentamicin (10 ppm). Donor and transconjugant are distinguished because of gentamicin (10 ppm) addition in selecting plate. The concentrations of four antibiotics (amoxicillin, tetracycline, streptomycin and sulfamethoxazole) were determined by preliminary study to confirm no growth of bacteria in recipients (activated sludge) (data are not shown).

After overnight incubation, the grown transconjugant colonies were counted. The colony averages were calculated using the triplicate plates. The pB10 transfer rate in this study was calculated using Eq. (1):

$$\mathrm{pB10} = \frac{\text{Transaction/Recipient ratio on slective plate with sterssor mating history}}{\text{Transaction/Recipient ratio on slective plate with sterssor mating history}} \quad (1)$$

2.3. Statistical analysis

The effects of the types and concentrations of micro-contaminants as well as the incubation times of the transconjugant colony count were tested by analysis of variance (ANOVA) using the linear fixed-effect model and linear mixed-effect model. Relative frequency (rate) of the transconjugant count over the total number of recipients is used as the response variable in the model because the large count makes the relative frequency behave as a normal distribution, while the values of relative frequency are quite small and almost nearly zero. The type and concentrations of micro-contaminants and the incubation times were considered as fixed factors in the models. Triplicated experiments were considered as random effects in the linear mixed-effect model to compensate for unwanted experimental effects which may not be

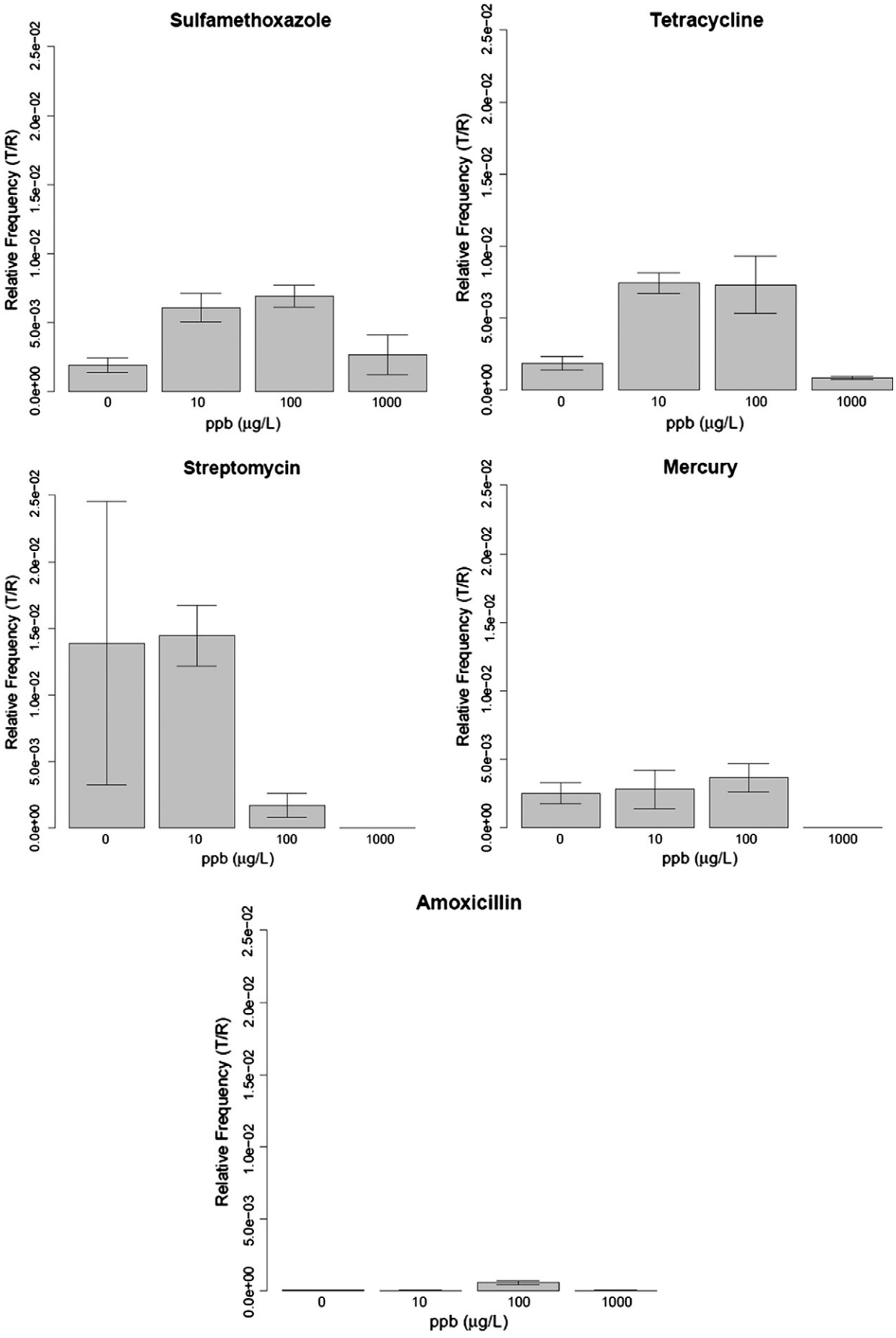

Fig. 1. Relative frequencies (*T*/*R*) of transconjugants (*P. aeruginosa* with pB10) as a function of various stressor concentrations [bar charts of averages are presented with standard error bars].

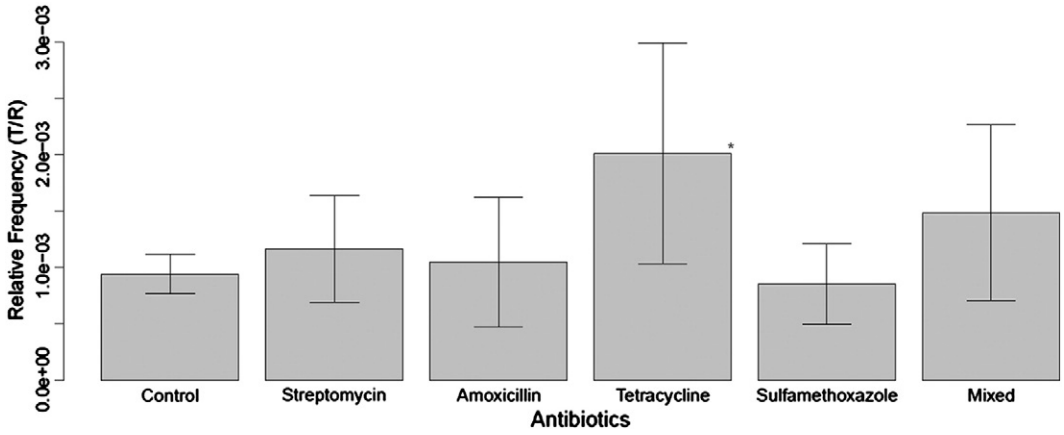

Fig. 2. Relative frequencies (*T*/*R*) of transconjugants (*P. aeruginosa* with pB10) with 2.5 ppb antibiotic concentration and antibiotic mixture [bar charts of averages are presented with standard error bars; statistical significance is denoted in the bar by * for *p*-value <0.05, ** for *p*-value <0.01, and *** *p*-value <0.001].

controllable in the experiments. Factor levels were compared using a post-hoc multiple-comparison procedure (Tukey's HSD). Statistical analyses were performed using R 2.15.1 with a significance level of 0.05.

2.4. DNA extraction and PCR

Plasmid DNA from the pB10-containing *E. coli* DH5α was isolated using a Nucleobond Kit PC100 on AX 100 columns (Macherey-Nagel) according to the manufacturer's supplied protocol. The concentration and purity of the DNA were evaluated by ultraviolet absorbance spectrophotometry at 260/280 nm. The presence of pB10 plasmid in either extract or grown DNA colony was regularly confirmed by PCR using highly specific primers (F5′-CAATACCGAAGAAAGCATGCG-3′, R5′-AGATATGGGTATAGAACAGCCGTCC-3′) (Bonot and Merlin, 2010). After any given mating experiment, 5–10 chosen colonies from the selective LB plates and *E. coli* DH5α pB10 culture (positive control) were compared via colony PCR to confirm the presence of a pB10 plasmid in transconjugant (data not shown).

2.5. 16S rRNA gene analysis in colonies and phylogenetic tree construction

To characterize the transconjugant species isolated in sludge mating experiments, 16S rRNA gene based phylogenetic analysis was conducted. Colony PCR was conducted using universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGYTACCTTGTTACG ACTT-3′), as previously described (Teyssier et al., 2003). PCR products of about 1,400 bp were sequenced in an Applied Biosystems Automatic Sequencer (Genome Express). 16S rRNA clone sequences were clustered into OTUs (operational taxonomic units) at a cut-off of 97% by an open-source software, MOTHUR (Schloss et al., 2009). Phylogenetic analysis was conducted using the neighbour-joining method with bootstrap parameters of 1000 replications and evolutionary distances were computed using Jukes–Cantor method by using MEGA version 5 (Tamura et al., 2011).

3. Results and discussion

3.1. Impact of the stressor concentration on the extent of pB10 transfer

High numbers of transconjugant colonies, 10^6 CFU/mL to 10^9 CFU/mL, were found on the final transconjugant selecting LB plates under *P. aeruginosa* PAKexoT mating experiments (data are not shown). Transfer frequencies (*T*/*R*) were calculated by getting the proportion of transconjugants with respect to the potential recipient count as presented in Figs. 1 and 2.

The transfer frequencies of pB10 from *E. coli* DH5α to *P. aeruginosa* PAKexoT showed varying responses to the five micro contaminants at four different contaminant levels. For statistical analysis, three separate experiments with duplicate samples in each experiment have been conducted. In this test, increased frequencies were observed in 10 ppb

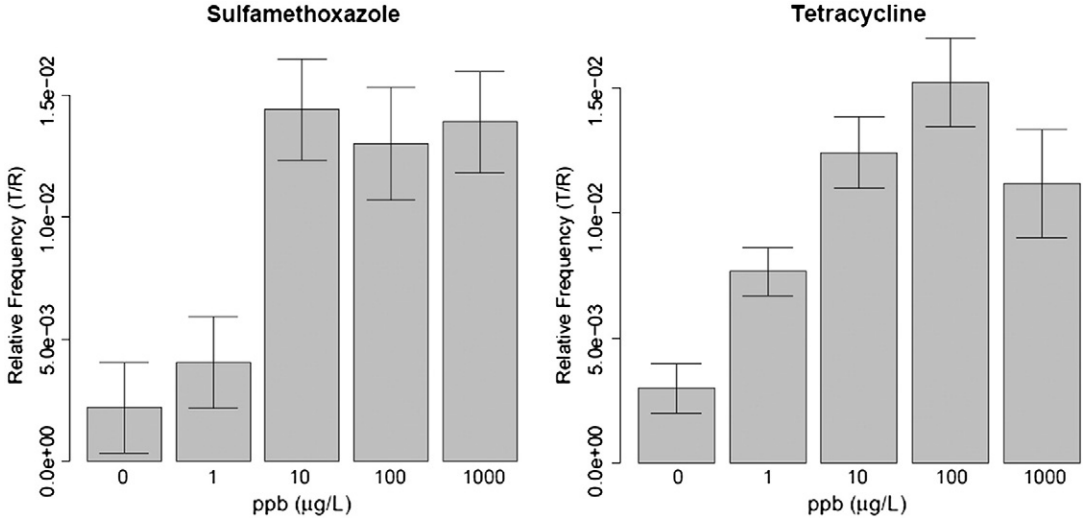

Fig. 3. Relative frequencies (*T*/*R*) of transconjugants (activated sludge with pB10) as a function of tetracycline or sulfamethoxazole concentrations [bar charts of averages are presented with standard error bars].

and 100 ppb of tetracycline and sulfamethoxazole (Tukey's HSD one-sided test, p-value $<10^{-4}$ for both 10 and 100 ppb levels) (Fig. 1). Mercury was not more considered after Fig. 1 experiment because mercury did not statistically significantly change for the pB10 transfer. In engineered environments such as wastewater treatment plants or natural environments, various antibiotics are usually found with mixed forms (Ding and He, 2010; Kummerer, 2009b). Therefore, we further tested the plasmid transfer frequencies with a lower micro-contaminant concentration (2.5 ppb) of these mixed antibiotics to mimic real aquatic systems (Fig. 2). As shown in Fig. 2, tetracycline showed a significant increase in plasmid transfer frequencies (value) while other micro-contaminant doses did not show any statistically significant increases in the plasmid transfer frequencies. Based on this result, tetracycline and sulfamethoxazole are chosen for model antibiotics for following microbial complexity on extent of pB10 transfer.

Several previous studies also reported that plasmid transfer between bacteria can be enhanced by the presence of hundred ppb levels of some antibiotics. A previous study (Almasaudi et al., 1991) reported that the transfer of a pwG613 plasmid of *S. aureus* was enhanced 10-fold after the exposure to 500 ppb gentamicin. Another study (Ohlsen et al., 2003) also observed that the transfer frequencies of pSK41 type plasmid between *S. aureus* species can be increased by a threefold at 100 ppb gentamicin. However, the rates of plasmid transfer at even lower and more environmentally relevant antibiotic concentrations (10 ppb) have not been evaluated.

Several studies reported that the presence of low level of antibiotics, sub-inhibitory concentrations of antibiotics or metal, can induce the genetic expression of microorganisms (Babic et al., 2010; Wang and Crowley, 2005; Yim et al., 2007) including those coding for the SOS response (Deneve et al., 2009; Mesak et al., 2008), virulence properties (Hacker et al., 1993), and biofilm formation (Rachid et al., 2000).

Antibiotics can damage DNA and result in the promoting the repair system of bacteria such as SOS response (Miller et al., 2004; Phillips et al., 1987). Based on the previous study (Beaber and Waldor, 2004),

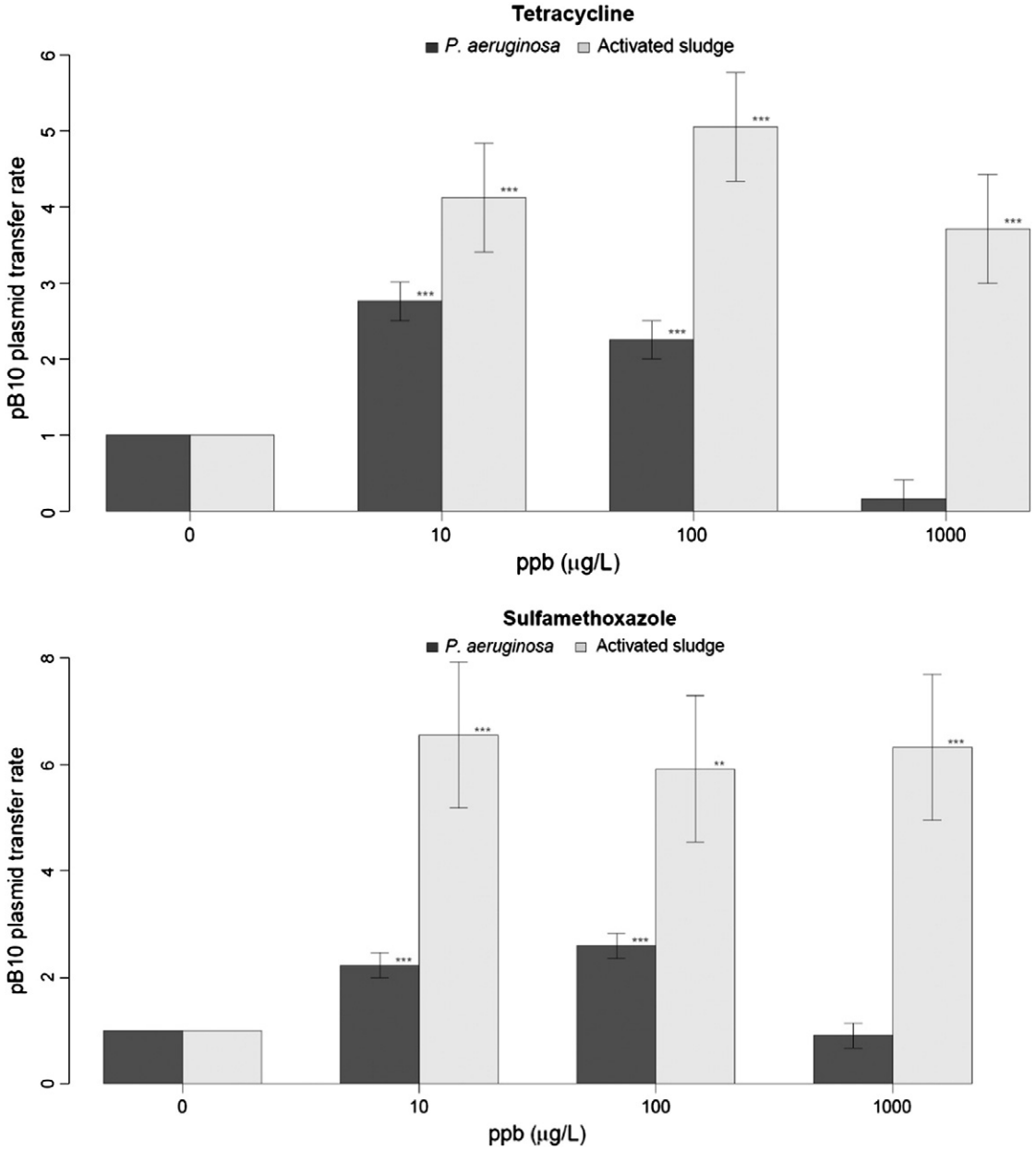

Fig. 4. Comparisons of pB10 plasmid transfer rates from *E. coli* to *P. aeruginosa* or activated sludge as a function of antibiotic concentrations [average rate in duplicate experiment; statistical significance is denoted by * for p-value <0.05, ** for p-value <0.01, and *** for p-value <0.001].

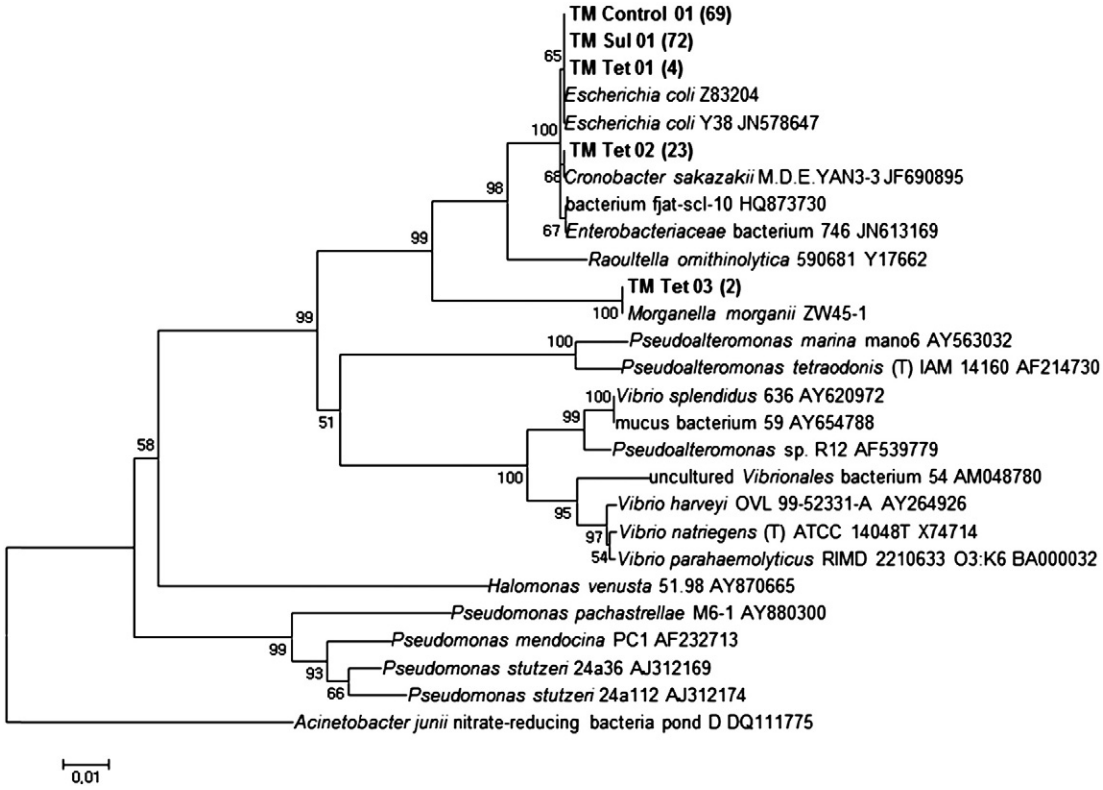

Fig. 5. Phylogenetic tree based on partial 16S rRNA gene to identify trans-conjugated microorganism species in each different antibiotic concentration in solid media [control (0 ppm; Control), tetracycline (10 ppb; Tet), sulfamethoxazole (10 ppb; Sul)]. The evolutionary distances were computed using the Jukes–Cantor method. Parentheses represent the number of clones analyzed in each experiment.

the transfer frequency of SXT, one of integrating conjugative elements (ICESs), between cell to cell can be enhanced by antibiotic-induced SOS response.

Cell to cell contact between donor and recipients might be secured by biofilm formation which is induced by the ppb concentrations of antibiotics that result in the enhanced HGT. More secretions of small proteins or peptides in the presence of certain antibiotics by the donor or recipients or both help the adhesion between bacteria or between bacteria and external surfaces (Kaplan et al., 2011) and these could result in the biofilm formation (Ong et al., 2009). Under these circumstances, plasmid transfer rate can be increased between two cells since bacterial cells in a biofilm stays in close contact since it helps the cell-to-cell gene transfer (Angles et al., 1993; Krol et al., 2011; Martiny et al., 2003).

In our experiment (Fig. 1), we also observed that relative frequencies of a transconjugant colony at a high micro-contaminant concentration (1,000 ppb) were always lower than those at 10 ppb micro-contaminant concentration (Tukey's HSD one-sided test, p-value 0.0029). A recent study (Yim et al., 2007; Linares et al., 2006) suggested that the effect of antibiotics on global bacterial transcription is concentration-dependent. Under the sub-inhibitory concentration, antibiotic can act as a signal molecule to the microorganisms or microbial community, and at higher concentrations; however, bacterial responses shift to the more stress-related ones. The loss of viability emerged at the highest antibiotic concentrations. Therefore, our observation could be explained by dual antibiotic activity: antibiotics help gene transfer at low concentrations (10–100 ppb) but inhibit or kill some of the donors and recipient cultures, as the concentration increases. To confirm this idea, both the donor (*E. coli* DH5α) and the recipient (*P. aeruginosa* PAKexoT) were separately incubated with different concentrations of tetracycline or sulfamethoxazole to account for the survival rate. The experiment showed that about 10 fold of *P. aeruginosa* PAKexoT could be reduced under the 1,000 ppb concentration of tetracycline or sulfamethoxazole (data not shown). Therefore, the reduced transconjugant concentration at 1000 ppb could be explained by the reduced recipient concentrations.

3.2. Impact of microbial complexity on extent of pB10 transfer

When activated sludge, representing more complex communities, was used as a recipient, the pB10 transfer from *E. coli* to activated sludge always increased when the microbial communities were exposed to a ppb level of tetracycline or sulfamethoxazole (ANOVA test, p-value $<10^{-4}$) (Fig. 3). The plasmid transfer rates of both *P. aeruginosa* and activated sludge were calculated using Eq. (1) and compared. For the activated sludge, the increases in the plasmid transfer rate were always statistically significant but not always for *P. aeruginosa* (Fig. 4).

As summarized in Fig. 5, with the absence of antibiotics in LB plates, all 69 of the pB10 transferred bacteria were phylogenetically related to *E. coli*. In the case of tetracycline amended plates, 4 out of 29 transconjugants were related to *E. coli*, whereas 2 transconjugants were closely related to *Morganella morganii*. However, most of the transconjugants (23/29) from tetracycline treated plates were related to *Cronobacter sakazakii*-like bacteria. In the case of sulfamethoxazole containing plates, *E. coli*-related bacteria were dominant which were similarly observed in the control plates.

In our findings, the standard deviation showed that plasmid transfer rate was more variable when a complex microbial community (activated sludge) was used as the recipient compared to a pure culture (*P. aeruginosa*), regardless of antibiotic concentrations (Fig. 4). This observed variability could be explained by the presence of the higher diversity of secondary plasmid donors in activated sludge. During *E. coli* and *P. aeruginosa* conjugation experiments, pB10 donors can be either *E. coli* (pB10) or transconjugant *P. aeruginosa* (pB10). However, when activated sludge culture was used as recipients, various types of microorganisms can become secondary pB10 donors after they receive the pB10 through primary conjugation. A previous study showed that

pB10 host diversity strongly influences the pB10 transfer rate to the complex microbial community (De Gelder et al., 2005). According to the De Gelder et al. (2005)), the *tra* gene regulation of the host, restriction-modification system for foreign DNA between donor and recipient, and non-random spatial distribution between cells in activated sludge, could result in the difference of pB10 transfer rate under host diversity. Another study also mentioned that the presence of efficient donors in heterogeneous bacterial populations can accelerate plasmid transfer and can spread by several orders of magnitude (Dionisio et al., 2002).

It can be noted that pB10 plasmids were transferred to the bacteria belonging to a potential clinically important family, the *Enterobacteriaceae* family. The high plasmid transfer frequency to the enteric bacteria implied that these microorganisms could become gene reservoirs and could play a key role in the recent rise of antibiotic resistance in an aquatic environment (Martinez, 2008). In this study, large amounts of the transconjugant colonies from tetracycline treatment were related to *C. sakazakii*-like bacteria (previously termed *Enterobacter sakazakii*) which has been identified as an opportunistic pathogen and has been linked with life-threatening infections among infants (Healy et al., 2010).

4. Conclusions

A number of studies have implicated the potential positive relationships between antibiotics and antibiotic resistant bacteria in the environment. However, little information exists regarding the threshold concentrations of antibiotics for enhancing ARGs in the environment through mechanisms such as horizontal gene transfer. Based on this study's findings, statistical increase in pB10 plasmid transfer via conjugation process was observed even in the presence of 10 ppb of antibiotics, especially tetracycline and sulfamethoxazole. It was also observed that the pB10 plasmid transfer rate to normal flora significantly can statistically increase with 10 to 100 ppb of tetracycline or sulfamethoxazole exposure rather than to *P. aeruginosa*. Although the exact mechanism for the increased pB10 transfer rate still needs further investigation, it is important to note that these results indicated that the spread of antibiotic resistance can be accelerated to the environmental microorganisms in the ppb level of antibiotics. One of the interesting findings of this study is that the low concentration (10 ppb) of tetracycline exposure led to the pB10 transfer to enteric bacteria, which are clinically important pathogens. Further study is needed to link antibiotic residue concentrations and the selection of other pathogens in mixed microbial communities such as activated sludge.

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