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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.

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