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The effects of antibiotics on the biofilm formation and antibiotic resistance gene transfer

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ABSTRACT

Recently, a number of microcontaminants were found in aquatic environment and it raises the concerns about their effects to ecosystem. This study investigated if low levels of antibiotics can trigger the biofilm formation and result in the enhanced antibiotic gene transfer. For this purpose, the biofilm formation of enteric bacteria (*Escherichia coli*), environmental microorganism (*Pseudomonas aeruginosa*), and their mixture by ppb level of antibiotics were investigated. In addition, the effects on the conjugation of *E. coli* with *P. aeruginosa* in the biofilm structure were also evaluated using biofilm colony-forming unit assay in 96-well plates and ELISA. Interestingly, at 100–1000 ppb, the mixed culture was able to reach its highest biofilm biomass and also form the highest number of transconjugants, which is greater than negative controls. This experiment shows that ppb levels of tetracycline and cephradine can alter the transfer rate of the pB10 plasmid among the biofilm biomass at rates 2–5 times faster than without antibiotics, which indicated the facilitated Antibiotic Resistance Gene (ARG) spread by the presence of low-antibiotic residues in the environment.

Keywords: Biofilm; ARG; Antibiotics; *Escherichia coli*; *Pseudomonas aeruginosa*

1. Introduction

Environment is at risk with the accumulation of pharmaceuticals and personal care products (PPCPs) [1–3]. This involves numerous classes of substances and micropollutants with different chemical properties, biological activities, and removal efficiencies [4]. Some of the most representative PPCPs found in Sewage Treatment Plants are estrogens, fragrances, lipid regulators, anti-inflammatories, antiepileptics, tranquilizers, contrast media, and contraceptives with very different chemical structures [1], that can build up in the environment such as land and bodies of water [3,5,6]. Not only that PPCPs can be toxic or cause pollution [7] but can also contaminate and can persist in the environment for a long period of time [8], thereby affecting the organisms living in it. And these include a vast number of antibiotics at low levels.

A number of different types of antibiotic residues are found in the environment [1,9,10] because of

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continuous input from various human activities [6]. Until now, little is known for the effect of ppb levels of antibiotics [11]. Antibiotics can select persistent micro-organisms, but killing susceptible ones [12]. This is because the use of antibiotics may accelerate the development of ARGs [13], or act directly on DNA triggering the SOS response and the expression of error-prone DNA polymerases [14]. There is a subtle change of micro-organism behavior caused by antibiotics. Studies showed that low quantities of antibiotics and other pharmaceutical drugs can either induce or repress growth of micro-organisms [11,15]; thereby, altering their genes which leads the micro-organisms to acquire antibiotic resistance [16].

One of the concerns of these changes is the either induced or reduced biofilm formation by low levels of antibiotics [17]. Recent studies showed antimicrobial effects to *in vitro* biofilm by examining the biofilm inhibition model [18]. Biofilms are well known to be resistant to antimicrobial agents [19], in which they survive in the presence of high concentrations at 10^2–10^4 times the minimum inhibitory concentration (MIC) than free-swimming bacteria of the same species [20]. This results to micro-organisms surviving harsh conditions of the environment. One of the factors of the increased resistance of biofilms is the protection by a self-produced extracellular polymeric substance (EPS), which is a matrix made up of proteins, polysaccharides, lipids, and nucleic acids [20,21]. This substance interacts with the antimicrobial agents, reduces their penetration and weakens their negative effects on the biofilm biomass [22].

Researches showed horizontal gene transfer (HGT) and biofilm formation are connected to one another [23]. Biofilms are made up of complicated networks which are believed to be the reason why HGT is higher in biofilm than in planktonic states. Biofilms are the hot spots for HGT because they provide high-population densities and close proximity of cells [24]. These networks channel nutrients to go in and wastes to go out of the system [23]; and also allowing exchange of genetic materials within species and among other species alike.

Our previous study showed antibiotic resistance plasmid transfer rate was increased in the presence of ppb levels of antibiotics [25] in pure and activated sludge cultures. It has been observed that HGT can be affected by the conditions of the environment, especially the antimicrobial agents, such as antibiotics and other chemicals, present in the media or substrate [2,16]. This can turn-on and turn-off certain genes of micro-organisms and can cause natural selection within and between species that makes them stronger and less susceptible to antibiotics.

The purposes of this study were to investigate whether antibiotic resistance can be attributed to: (1) biofilm formation caused by ppb levels of antibiotics; and (2) how gene transfer was facilitated in the biofilm. For these purposes, we conducted tests to determine what type and what concentration of antibiotics can induce biofilm formation in two different micro-organisms and in mixture, and to investigate the pB10 transfer rate in the biofilm under the different doses of antibiotics.

2. Materials and methods

2.1. Preparation of micro-organisms

In this study, *Escherichia coli* DH5α, containing the multidrug resistance plasmid pB10, was selected as the plasmid donor. The complete 64,508 bp nucleotide sequence of the *IncP-1* plasmid pB10 was originally isolated from a wastewater treatment plant in Germany [26] and mediates resistance against the antimicrobial agents; amoxicillin, streptomycin, sulfamethoxazole, tetracycline, and metallic mercury. Gentamicin-resistant *Pseudomonas aeruginosa* reference strain P680 PAK exoT Gm, obtained from Laboratory of Molecular Immunology and Microbial Pathogens, was used for conjugation experiments.

Prior to use, bacterial strains were first grown in liquid medium in test tubes. For each strain, one colony was transferred from the 24-h solid culture into 3-ml lysogeny broth (LB) test tubes. *E. coli* and *P. aeruginosa* were incubated for 12 and 14 h, respectively, under orbital agitation (Vision Scientific Co., Ltd, KMC-8480S) at 150 rpm at 37˚C, to obtain a planktonic culture in exponential growth phase. After incubation period, optical densities were checked using spectrophotometer (Bio-Rad SmartSpec^{TM} Plus Spectrophotometer), approximately 5 AU taken at 600 nm wavelength. These bacterial suspensions were used as the inocula at a concentration of 10^8 colony-forming units (CFU) ml^{-1}. Hereafter, *E. coli* was called the donor, while *P. aeruginosa* was called the recipient. LB was used for growing bacterial strains and for dilution of inocula prior to plating in wells. LB agar plates supplemented with 2 ppm tetracycline were used to grow *E. coli*, while plates supplemented with 100 ppm gentamicin were used for *P. aeruginosa*. For the determination of the pB10 transfer rate in the biofilm, LB agar plates supplemented with 2 ppm tetracycline and 10 ppm gentamicin were used.

2.2. Preparation of antibiotics

Micronized cephradine (Sigma–Aldrich), ciprofloxacin (Fluka), gentamicin (Duchefa-Biochemie), streptomycin (Sigma–Aldrich), amoxicillin (Sigma–Aldrich), and tetracycline (Sigma–Aldrich) were used. Stock solutions of all antimicrobial agents were prepared in sterile water, syringe-filtered, and stored at –20°C until use.

2.3. Preparation of polystyrene plates and quantitative determination of biofilm formation

Inocula were prepared by diluting the organisms to 1:100 using LB. One hundred microliter of inocula of *E. coli* and *P. aeruginosa* were added to the wells of 96-well plates (200 µl well^{-1} from Sarstedt, Germany) separately. Antibiotics were then added to the wells using final concentrations of 1, 10, 100, 1,000, and 10,000 ppb. Plates were sealed with unbreatheable plastic (Thermo Scientific) and were incubated at 37°C for 24 h in CO_2 incubator.

Quantitative determination of biofilm formation was performed by the spectrophotometric method, which measures the total biofilm biomass including bacterial cells and EPS. Absorbance was first obtained using ELISA (Sunrise-Basic Tecan, Tecan Inc., Austria, GmBH) at a wavelength of 595 nm. This was recorded as cell density. During the test, cell densities were relatively similar in each well. Then the liquid medium with the bacteria was aspirated from the wells, and the wells were washed with sterile distilled water three times, without disturbing the adherent film. The biofilms were stained with 200 µl of 0.3% crystal violet and incubated at room temperature for 20 min. After incubation, excess stains were removed by gently washing with sterile distilled water three times. The biofilms were then air dried for 10 min. After the biofilms were dried, crystal violet was solubilized by adding 200 µl 99.9% ethanol. Biofilm formation was then determined by measuring the absorbance using ELISA at a wavelength of 540 nm. The results were averaged and standard deviations were calculated. Crystal violet staining was adapted from the method previously described by Stepanović et al. [27].

2.4. Quantification of plasmid transfer in biofilm by means of plate counting

Using the same procedure of preparation of polystyrene plates, a combination of 50 µl of *E. coli* and 50 µl of *P. aeruginosa* was used instead. After incubation for 24-h, each well was washed three times with sterile distilled water. One hundred microliter of phosphate buffer was injected into each well. The biofilms were scraped thoroughly, with particular attention to the well edges. The contents of the wells were pipetted and transferred into 2 ml microcentrifuge tubes. Total number of CFU ml^{-1} of transconjugants was determined by serial dilution method and plating on antibiotic supplemented plates.

3. Results and discussion

3.1. Biofilm formation under the presence of ppb levels of antibiotics

The quantitative results (Fig. 1) of biofilm formation by *P. aeruginosa* were lower than those produced by *E. coli* and mixed cultures (data not shown) of *E. coli* and *P. aeruginosa.* In addition, 100 ppb and 1,000 ppb of tetracycline greatly favored biofilm formation, which was unobservable in the case of *P. aeruginosa*. Bendouah et al. [28] explained that *P. aeruginosa* produces a biofilm limited to the air-liquid interface; thus, after washing, only the biofilm formed around the wall remains available for staining.

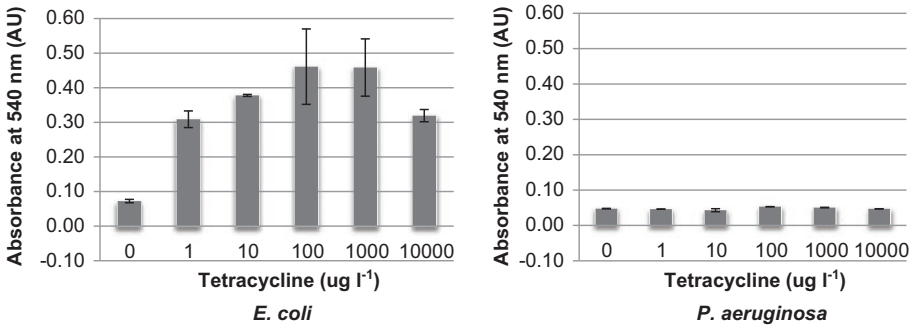

Fig. 1. Dose response of biofilm formation in the 24-h biofilm pure cultures of *E. coli* (left) and *P. aeruginosa* (right), to different concentrations of tetracycline. AU, absorbance unit.

For *E. coli*, biofilm formation was evenly distributed over the well surface; thus, higher absorbance values after crystal violet staining.

Previous studies suggested that mixed cultures were less susceptible to stress than a single species because there was a change in the EPS matrix constituents due to the presence of a second species and can lead to viscosity and permeability changes that reduced diffusion of antibiotics in the biofilm [29].

Fig. 2 shows a microphotograph of the wells with biofilm formation of mixed cultures of *E. coli* and *P. aeruginosa* with increasing concentrations of tetracycline. It shows that cluster formation stained with crystal violet, is observed to be more on 100 and 1,000 ppb concentrations of the antibiotic than other concentrations. The introduction of the antibiotic into the culture affected the transconjugation represented by multicell clusters. Thus, increasing cluster number means increasing transconjugation formation.

In the following experiment, combinations of two antibiotics were used to investigate the synergic effects of antibiotics. Fig. 3 shows dose response of biofilm formation to the different concentrations of tetracycline, cephradine, and tet-cep combinations. There was a significant effect of the different concentrations of antibiotics on the biofilm formation at the $p < 0.05$ level for the two trials conducted on six concentrations [$F(1,5) = 9.23$, $p = 0.027$] for tet; while $p < 0.5$ [$F(1,5) = 0.50$, $p = 0.50$] and [$F(1,5) = 0.97$, $p = 0.36$] for cep and tet-cep combination, respectively. The combinations of cephradine and tetracycline show the highest absorbance values at 540 nm, that is, biofilm formation is highest than any other combination of two antibiotics used in this study.

3.2. pB10 transfer rate comparison in the biofilm

Biofilm colonies are well known to be resistant to MICs of antibiotics [30]. During the recent years the concept of antibiotics as solely killing agents has been reconceptualized due to the findings that low concentrations of antibiotics may play a regulatory function

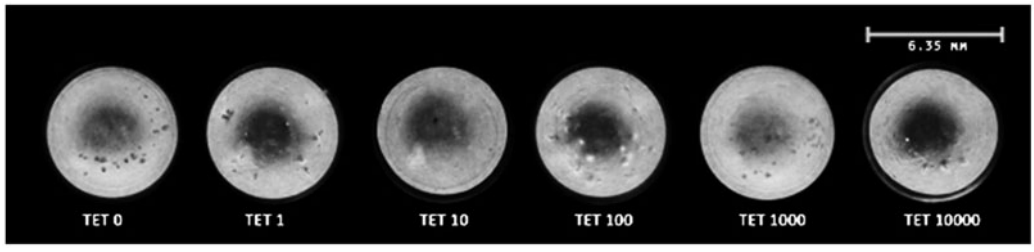

Fig. 2. Effects of increasing concentrations (TET0–TET1000, 0 $\mu g\,l^{-1}$ to 10,000 $\mu g\,l^{-1}$) of tetracycline (TET) on the biofilm formation of mixed cultures of *E. coli* and *P. aeruginosa* after 24-h incubation.

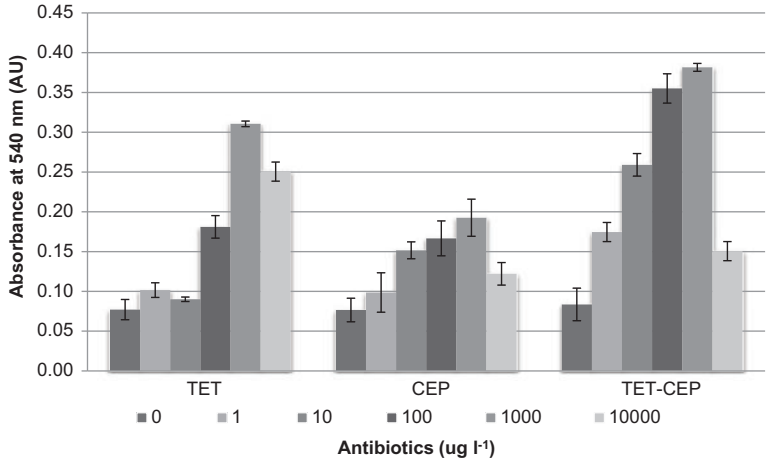

Fig. 3. Dose response of biofilm formation in the 24-h biofilm of mixed cultures of *E. coli* and *P. aeruginosa*, to TET (tetracycline), CEP (cephradine), and TET-CEP combinations at different concentrations (averages in duplicate experiments). AU, absorbance unit.

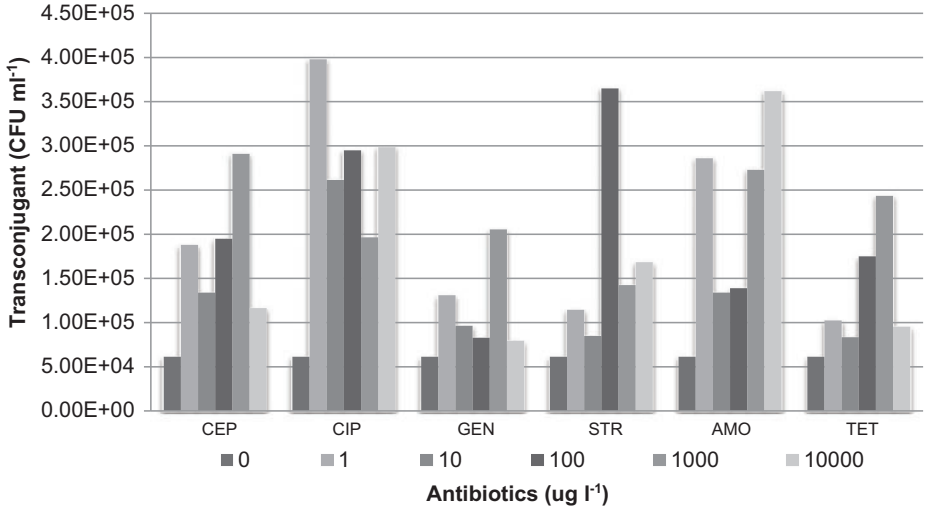

Fig. 4. Dose response of transconjugant population in the 24-h biofilm biomass of mixed cultures of *E. coli* and *P. aeruginosa*, to different antibiotics: cephradine (CE), ciprofloxacin (CIP), gentamicin (GEN), streptomycin (STR), amoxicillin (AMO), and tetracycline (TET) at different concentrations (averages in duplicate experiments).

in the natural ecosystem [24]. To determine the effect on the gene transfer rate involved in the biofilm biomass during biofilm formation, we added different concentrations of antibiotics in the wells. Sub-inhibitory concentrations of antibiotics may significantly increase the frequency of horizontal transfer of many of mobile genetic elements (MGEs) [24].

In this experiment, mixed cultures of *E. coli* and *P. aeruginosa* were inoculated in 96-well microtiter plates in the presence of increasing concentrations of cephradine, ciprofloxacin, gentamicin, streptomycin, amoxicillin, and tetracycline. Based on the results (Fig. 4), transconjugant formations were positively affected by ppb levels of antibiotics, that the values were higher enumeration of transconjugants with the samples of antibiotics compared to the control, which had no antibiotic. It was characterized by low-dose stimulation of biofilm formation and high-dose inhibition in which either 100 ppb and 1,000 ppb had the highest transconjugant population on cephradine, streptomycin, gentamicin, and tetracycline. For ciprofloxacin and amoxicillin, 1 and 10,000 ppb had the highest transconjugant population, respectively.

The gene transfer rate was affected by the different concentrations of antibiotics used on the mixed cultures of *E. coli* and *P. aeruginosa* biofilm formation experiment. It shows that transconjugation is varied from different antibiotic used.

The plausible explanation about the increased HGT in the biofilm is not clear. However, the ability of the micro-organisms to form biofilm might increase the fitness with exposure to antimicrobial agents [31]. This occurs while activating the transcription of a corresponding resistance gene and, concurrently, the genes involved in the mobility of MGEs. In this regard, the interaction of an antibiotic and antibiotic resistance gene (ARG) resembles a positively regulated switch, with an antibiotic possessing a signaling function, ultimately leading to the activation of HGT in microbiota [24].

In the environmental situation where a number of micropollutants are present, different rates of HGT can be facilitated by the different rates of biofilm formation as well as the different microbial diversity. Enhancement of the biofilm formation due to these stress factors also leads to increased antibiotic resistance aforementioned earlier on this study, especially when transferred genetic materials are needed for their survival.

4. Conclusions

This study showed that antibiotics at ppb levels can reduce or induce biofilm formation, as well as ARG transfer rate depending on the concentration. It can be concluded that biofilm formation is related to the antibiotic resistance study of the previous experiment done by Kim et al. [25]. Among the antibiotics used in this study, ciprofloxacin, amoxicillin, and streptomycin increased the transfer rate of pB10 plasmid at different concentrations. At 1,000 ppb of cephradine and tetracycline, biofilm formation was the highest both in number of transconjugants in the biofilm biomass and the formation of clusters. The

cephradine and tetracycline combination gives the highest biofilm formation, 23% higher than tetracycline alone and 98% higher than cephradine alone, at 1,000 ppb.

The results of this study provide information on how micropollutant levels in the environment affect biofilm formation as well as gene transfer. Further studies are needed to investigate the reason why some antibiotics have no direct effect on the biofilm formation and antibiotic gene transfer. Additional experiment is needed to find out the mechanism of how antibiotics induce or reduce biofilm formation and antibiotic gene transfer rate, and the estimation of HGT frequencies.

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