Induction of Multidrug Resistance Mechanism in *Escherichia coli* Biofilms by Interplay between Tetracycline and Ampicillin Resistance Genes

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Running Title: Multidrug Resistance Mechanism in *E. coli* Biofilms

Keywords: Efflux pumps, Antibiotic resistance, β-lactamase, pBR322, TetA(C), *bla*TEM-1, EmrY/K, EvgA/S

Abbreviations: MIC, Minimal Inhibitory Concentration. ABC, ATP Binding Cassette. MFS, Major Facilitator Superfamily. RND, Resistance Nodulation Division. SMP, Small Multidrug Resistance.

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Abstract

Biofilms gain resistance to various antimicrobial agents, and the presence of antibiotic resistance genes is thought to contribute to a biofilm-mediated antibiotic resistance. Here we showed the interplay between the tetracycline resistance efflux pump TetA(C) and the ampicillin resistance gene (blaTEM-1) in biofilms of Escherichia coli harboring pBR322 in the presence of the mixture of ampicillin and tetracycline. E. coli in the biofilms could obtain the high-level resistance to ampicillin, tetracycline, penicillin, erythromycin and chloramphenicol during biofilm development and maturation, as a result of the interplay between the marker genes on the plasmids, the increase of plasmid copy number, and consequently the induction of the efflux systems on the bacterial chromosome especially the EmrY/K and EvgA/S pumps. In addition, we characterized the over-expression of TetA(C) pump that contributed to osmotic stress response and was involved in the induction of capsular colanic acid production, promoting formation of mature biofilms. However, this investigated phenomenon was highly dependent on the addition of the subinhibitory concentrations of antibiotic mixture, and the biofilm resistance behavior was limited to aminoglycoside antibiotics. Thus, marker genes on plasmids played an important role in both resistance of biofilm cells to antibiotics and in formation of mature biofilms as they could trigger specific chromosomal resistant mechanisms to confer a high-level resistance during biofilm formation.
Introduction

Bacteria that adhere to abiotic and biotic surfaces, including medical implants and human tissues, can become the cause of refractory infections (60). The microscopic observations of these refractory infections have revealed bacteria growing as biofilms (49). Recently, it has been observed that resistance of biofilms to antibiotics is greater than what is normally seen with planktonic cells (29, 41). Several mechanisms including the failure of the antibiotic penetration into biofilms have been proposed for the resistance to antibiotics, biofilm acting as a barrier (41, 60). However, the exopolysaccharide matrix does not always act as an impenetrable barrier to the diffusion of antibiotics, especially to \( \beta \)-lactams or tetracyclines, and thus other mechanisms must be sought to explain the biofilm resistance (1, 2, 15, 61). In addition, it has been suggested that high-cell density stationary-phase characteristics and/or quorum sensing molecules play an important role in resistance to antibiotics, but again, their exact roles are not clear. Conversely, resistance mechanisms of planktonic cells such as the production of antibiotic-degrading enzymes (\( \beta \)-lactamase for \( \beta \)-lactams) and the increase in antibiotic-efflux pumps (Tet pump for tetracyclines) have been well studied. The TetA(C) efflux pump and the \( \beta \)-lactamase (\( \text{bla} \)) genes are often encoded on transmissible elements, such as plasmid, transposon or intergron, which can spread rapidly among bacteria. Since the efflux pumps can extrude antibiotics from the cell, the induction of the pumps is thought to be one of the key alterations conferring resistance to biofilm cells (66). In Escherichia coli, a putative multidrug resistance pump YhcQ was reported to be involved in antibiotic resistance of biofilms (39, 69), and Mar, Sox and AcrAB-ToIC efflux pumps encoded genes have been found to be up-regulated under stress conditions, such as a stationary-growth condition, growth in biofilms, and exposure to several antimicrobial agents (5, 14, 68). Furthermore, inactivation of efflux pumps by a number of efflux pump inhibitors reduces biofilm formation, indicating the efflux system is required for biofilm formation (32). However, the exact role of efflux pumps in biofilm formation and their importance in biofilm-mediated antibiotic resistance remain under investigation.
The rapid spread of antibiotics resistance among bacteria is mainly due to the localization of antibiotic resistance marker genes on plasmids (46, 58). Recently, it has been reported that there is a connection between the antibiotic marker genes on the plasmid and bacterial biofilm formation. The presence of the β-lactamase gene reduced the amount of biofilm formed by *E. coli* strains harboring β-lactamase encoded plasmid, while the presence of either gentamicin or tetracycline resistance gene did not impair biofilm formation (4, 18). This indicates that the resistance to antibiotics is linked to biofilm development and maturation. Therefore, biofilms of bacteria that harbor marker genes on the plasmid may gain resistance to some antibiotics at the expense of other protective phenotypes, and the effect of marker genes on bacterial biofilm phenotypes must be investigated.

In this study, we studied the effect of the marker genes, an ampicillin and a tetracycline resistance gene on pBR322 plasmid, on *E. coli* biofilm formation in relation to the resistance of biofilms to antimicrobial agents. Furthermore, we investigated the interplay among the genes on pBR322 which effectively promoted *E. coli* biofilm development and maturation. A global transcriptional approach was also used to analyze the connection between genes on the plasmid and bacterial chromosome regarding the induction of efflux pumps during biofilm formation.
Materials and Methods

Bacterial strains and growth conditions. A pBR322 plasmid, which encodes the \textit{bla}_{TEM-1} and \textit{tetA(C)} genes conferring resistance to ampicillin and tetracycline, respectively, was used in this study (7). The laboratory \textit{E. coli} strain MG1655 was used in all experiments. The \textit{E. coli} g62 (genotype; 1655sp rpsL polA12 Zih::Tn10 \Delta(emrY-ddg)::kan) was obtained from National Institute of Genetics (Japan). OCL62 is a medium deletion strain, an mutant of \textit{emrY}, \textit{emrK}, \textit{evgA}, and \textit{evgS} operons, in which the related and neighboring genes were removed counterclockwisely with respect to the \textit{oriC} position (22). In this study, OCL62 was genetically modified by replacing the downstream \textit{kan} gene with \textit{tetR} gene by using the method of Datsenko and Wanner (12). Thus, the modified OCL62 is \Delta(emrY-ddg)::tetR that has a counteraction of the constitutive \textit{tetA(C)} gene by the expression of the \textit{tetR} repression gene. The modified OCL62 was then used to investigate the counteraction of TetA(C) pump and the loss-of-function in the EmrY/K and EvgA/S pumps on bacterial chromosome. Luria-Bertani, LB, (Difco, USA) broth or agar was used for standard cultivation. Appropriated antibiotics (ampicillin, tetracycline, penicillin, erythromycin, chloramphenicol, kanamycin and gentamicin) and chemicals (NiCl\textsubscript{2}, NaCl, MgCl\textsubscript{2}) were added when required. All antibiotics and chemicals were purchased from Wako Chemical (Japan).

Biofilm formation assay and flow cell experiment. Biofilms were cultured by using LB broth on the polystyrene, PS, (TPP, Switzerland) or polyvinylchloride, PVC, (Costar, USA) 96 well-plates, which were inoculated with early stationary growing cultures of \textit{E. coli} strains diluted to 0.1 OD\textsubscript{600}, and incubated at 37°C for 24 h without shaking. Antibiotic(s) was diluted and was then challenged directly to the wells without washing for an additional 24 h. The conditions and concentrations of antibiotics used in this study are shown in \textbf{Table 1}. The biofilm formation assay protocol was the modified Reisner’s method (54). Briefly, the culture plates were washed with 0.85% NaCl, and the attached cells were then strained with Crystal-Violet (CV) by semi-automatic staining using microplate washer, ImmunoWash 1575 (Biorad, USA). The OD\textsubscript{570} absorbances of CV binding-biofilms were measured with a microplate reader, ARVO 1420 Multilabel Counter (PerkinElmer,
USA). The average values of CV stained biofilms were obtained from at least twenty-four independently grown biofilms. The error bars represent the standard deviations from these averages. The bacterial growth activity was determined by subculturing (1:100) the relevant strain into the medium without shaking. The OD$_{600}$ readings were taken over time using the microplate reader.

For flow cell conditions, biofilms were cultivated in the three-channel flow cell reactors (Stovall, USA) by using M9 minimal medium (Difco, USA) supplemented with 20% LB broth to optimize the biofilm biomass of MG1655. Flow cells were inoculated with early stationary growing cultures of *E. coli* strains diluted to 0.1 OD$_{600}$. After inoculation, the medium flow was arrested for 2 h to allow the injected bacteria to attach to the glass surface. After bacterial cells attached to the glass surface, the medium, which supplemented with appropriate concentration of antibiotic(s), was fed to the flow cell chamber for another 48 h. Medium flow was controlled at a constant rate of 0.20 ml min$^{-1}$ using an Ismatec IPC8 peristaltic tubing pump (Ismatec, Switzerland).

**Determination of minimal inhibitory concentration (MIC).** Comparative MICs were performed according to the standard protocols (34, 52). The conditions and concentrations of antibiotic(s) used in this study are shown in Table 1. Briefly, the planktonic MICs were determined in 96-well PS microtitration plates, which contain an array of 12 by 8 wells with a series of tenth-fold dilution of antibiotic(s), administered separately or in combination. These antibiotic(s), when added into the wells, were placed at room temperature for 1 h. Next, the *E. coli* cultures were inoculated into the wells, and the plates were then incubated aerobically for 24 h at 37°C. The planktonic MICs were determined as the lowest antibiotic concentration causing the greatest diminution of growth. The biofilm MICs were determined in 96-well PVC microtitration plates. Biofilms were cultured without shaking for 24 h at 37°C, and then the antibiotic(s) were challenged directly to the wells without washing. The plates were incubated for an additional 24 h before the CV straining of biofilm formation assay as described above. The biofilm MICs were determined as the lowest antibiotic concentration causing the greatest inhibition of biofilm formation. Statistical analysis was conducted to evaluate the significance of each MIC using *T*-test with *P*-value $\leq 0.05$. 

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**Microscopes and image analyses.** Microscopic observation was performed with LSM 510 Confocal Laser Scanning Microscope (CLSM, Zeiss, Germany). In order to study the spatial localization of the cells in biofilms using the CLSM, the strains were genetically marked by insertion of green fluorescent protein (GFP) into the chromosome according to the Diederich’s protocol (13). Images were obtained using the IMARIS software package (Bitplane AG, Switzerland). For biofilm quantification, CSLM image stacks were analyzed by using the computer program COMSTAT (24).

For electron microscopy, cells were fixed with 1 M sodium cacodylate buffer (pH 7.2) containing 1% glutaraldehyde. The samples were then washed with the same buffer and post-fixed in 1% osmium tetroxide in the cacodylate buffer. The post-fixed samples were dehydrated in a graduated ethanol series (30-100%), and then treated with the HCP-2 critical point dryer (HITACHI, Japan) and observed with a Hitachi S4000 scanning electron microscope (SEM, Hitachi, Japan).

**Determination of plasmid copy number.** The genomic DNA and plasmid DNA were extracted by using QIAGEN DNeasy Tissue Kit and Plasmid Mini Kit (Qiagen, USA), respectively, according to manufacturer’s manuals. The protocol was the modified Lee’s method (36). Briefly, DNA was used directly as template with Power SYBR Green PCR Master Mix (ABI, USA). The blaTEM-1 gene were used for monitoring the copy number of the pBR322 plasmid and normalized with the ftsZ house-keeping gene of the genomic DNA. List of PCR primers is shown in the Table 2. The copy number of plasmid was then calculated as the number of copies per chromosome.

**Gene expression analysis and reverse transcription qPCR analysis.** Samples were taken at 0.5 OD$_{600}$ from suspended culture (planktonic growth) and/or directly from flow-cell (biofilm growth). The *E. coli* Antisense GeneChip Array (Affymetrix, USA) was used to study the global gene expression pattern, according to the Affymetrix Expression Analysis Technical Manual. For each experiment, three biological replicates were analyzed. Statistical analysis was carried out by using the computer program, DNA-Chip Analyzer (dChip) (37). The data were normalized using the invariant set method. The model-based expression value was calculated. After the statistical filtering process, the transcripts were subjected to cluster analysis. The data reported in this paper have
been deposited in the ArrayExpress database, http://www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-953).

For reverse transcription qPCR experiment, RNA was converted to cDNA using PrimeScript™ RT reagent Kit (Takara Bio, Japan) as described by the manufacturer. Remaining RNA was digested with RNase H, and first-strand cDNA was used directly as template with SYBR® Premix Ex Taq™ (Takara Bio, Japan) using an Applied Biosystems 7000 Sequence Detection System (ABI, USA). List of RT-PCR primers is shown in the Table 2. The ftsZ house-keeping gene on the host-cell chromosome was used as a standard curve, equal in quantity to the test article RNA. The mRNA expression level was calculated as the number of relative cDNA copies per host genome copy detected for each of eight independent dilutions of the RNA samples.
Results and Discussion

Subinhibitory concentrations of the antibiotic mixture promoted *E. coli* biofilm formation.

Tetracycline resistance gene on pBR322 vector, a source of tetA(C) in this study, provides for its constitutive expression of TetA(C) efflux pump in most *E. coli* strains due to a lack of tetR repressor on the plasmid (45, 66). It carries along with an ampicillin resistance gene (bla<sub>TEM-1</sub>) that encodes for an ampicillin-degrading enzyme, β-lactamase. The purified β-lactamase of pBR322 is an ubiquitous TEM-1 enzyme (62), which has been reported to decrease the ability of *E. coli* to form biofilms (18).

However, the effect of both the tetracycline resistance TetA(C) efflux pump and the β-lactamase encoded gene on *E. coli* biofilm formation in the presence of the mixture of ampicillin and tetracycline have never been elucidated. Therefore, the antibiotic susceptibility and the biofilm formation ability of *E. coli* harboring pBR322 were investigated and compared with those of a plasmid-free *E. coli* strain (Fig. 1 and Table 3).

The biofilm formation ability of plasmid-free MG1655 in the absence of antibiotic was defined as a baseline, so called a wild-type biofilm phenotype (Fig. 1A). The planktonic MICs for ampicillin and tetracycline were 0.5 µg/ml. When supplemented with ampicillin, we found the concentration range from 0.0000005 to 0.5 µg/ml slightly reduced biofilm formation, but the concentration range from 5 to 500 µg/ml inhibited biofilm formation, indicating by the biofilm MIC at 5 µg/ml. Similar MIC trends were obtained for planktonic cells when supplemented with either tetracycline alone or the antibiotic mixture (both ampicillin and tetracycline). However, the biofilm formation was inhibited at much higher concentrations (the biofilm MICs were 500 µg/ml for either tetracycline or the mixture). Since the antibiotics were supplemented after biofilms are formed in this study, our results indicate that the biofilms were 1,000 times more tolerable to tetracycline and the antibiotic mixture treatment than planktonic cells. In contrast, for ampicillin, the resistance of biofilms over planktonic cells is only 10 times. These results suggested that bla<sub>TEM-1</sub> is not having much effect, and the response to the exposure to the mixture is dominated by the response to TetA(C).
For MG1655 harboring the plasmid, MG1655[pBR322] (Fig. 1B), in the absence of antibiotic, the adherence to the surface was reduced as compared with a plasmid-free strain, showing that the presence of the constitutive TetA(C) efflux pump did not counteract the effect of TEM-1 β-lactamase. In the presence of antibiotic, the planktonic MICs for both antibiotics were at 500 µg/ml. The presence of ampicillin inhibited biofilm formation because the addition of ampicillin induces β-lactamase production. β-lactams, such as ampicillin, inhibits bacterial growth by inactivating penicillin-binding proteins that are involved in synthesis of peptidoglycan (65). Peptidoglycan is thought to play an important role in biofilm formation and is required for optimal assembly of surface molecules. The biofilm MICs for either tetracycline or the mixture were at concentration more than 500 µg/ml. Surprisingly, the tetracycline concentration from 0.5 to 50 µg/ml promoted biofilm formation. When treated with the antibiotic mixture, the biofilm formation was further promoted at the concentration ranges from 0.05 to 50 µg/ml, so called the subinhibitory concentration levels (Table 3). Thus, the mixture of ampicillin and tetracycline had the strongest biofilm promotion effect than tetracycline alone. In addition, we also tested that E. coli strain harboring the β-lactamase encoding gene alone clearly delayed biofilm formation as compared with a wild-type. E. coli strain harboring the TetA(C) encoding gene alone had no effect on biofilm formation (data not shown). In contrast, only E. coli strain harboring both the genes, like pBR322, promoted the biofilm formation. Therefore, the subinhibitory concentration of the antibiotic mixture at 5 µg/ml each (which gave the highest relative biofilm formation ability) was used for the following flowcell experiments.

**Biofilm structures of E. coli harboring pBR322 in the presence of the antibiotic mixture.**

To confirm that the subinhibitory level of the antibiotic mixture promote cell attachment and subsequent biofilm formation, we cultured biofilms using flowcell systems and monitored the biofilm formation at 24 h and 48 h after addition of antibiotic(s) (Fig 2A). The biofilm architecture was quantitatively analyzed using COMSTAT software (Fig 2B). In the absence of antibiotic, E. coli MG1655 strain formed rather uniform biofilm at 48 h, which is defined as a flowcell wild-type biofilm phenotype. On the other hand, the same strain harboring pBR322 formed less biofilm due to the presence of blaTEM-1 gene and subsequent β-lactamase expression. In addition, the amount of...
biofilms was obviously reduced when supplemented with ampicillin, which induces high-level of β-lactamase production and consequently inhibits the biofilm development under the flowcell condition. The presence of β-lactamase makes bacteria resistant to ampicillin by enzymatic activity in mainly planktonic condition. However, the expression of β-lactamase harms the peptidoglycan remodeling and assembly on the bacterial cell wall, resulting in the lack of coherence ability to form biofilm (56). In contrast, addition of tetracycline could restore a wild-type biofilm phenotype showing similar substratum coverage and average thickness to MG1655 in the absence of antibiotics. Notably, tetracycline is a broad-spectrum bacteriostatic antibiotic that inhibits bacterial protein synthesis by preventing aminoacyl-tRNA from binding to the bacterial ribosome (10, 55). From the result, the activation of TetA(C) pump by addition of tetracycline did not interrupt construction of bacterial cell surface structures because they still could form mature biofilms. As we expected, the antibiotic mixture enhanced biofilm formation of E. coli harboring pBR322. Its mature biofilm at 48h had a higher roughness coefficient and biofilm thickness than a wild-type biofilm (Fig. 2B). The expression of TetA(C) seemed to interfere the β-lactamase activity. Therefore, the interplay between marker genes in the presence of subinhibitory level of antibiotic mixture might contribute to the development of mature biofilms.

**Copy number of pBR322 was stably maintained in biofilms.** Although the promotion of biofilm formation by the presence of plasmids has been investigated in several studies (38, 44, 53, 54), it is still not known whether the copy number of plasmid per cell in biofilms is different from those in planktonic cells. The effect of antibiotics on the plasmid copy number and its contribution to biofilm formation are also unclear. Therefore, we determined the copy numbers of pBR322 in biofilms (Fig. 3A). Normally, the copy number of pBR322 is about 40 copies per cell in both minimal and complex media (23, 35, 36). In this study, the plasmid was stably maintained around 40 copies per cell in biofilms in the absence of antibiotic, while it was only about 20 copies per cell in the planktonic culture. The presence of each antibiotic could sustain the copy number of pBR322 at around 40 copies per cell in both the biofilm and planktonic conditions. Surprisingly, when supplemented with the antibiotic mixture, the copy number significantly increased up to 60.2±7.6
copies per cell in biofilms, but not in planktonic cultures. This indicates that biofilm is able to increase the relative fitness of plasmid and enhance the propagation of resistant plasmid. Thus, the presence of antibiotic markers in association with an addition of subinhibitory concentrations of the antibiotic mixture is a great selective pressure to keep the resistant genes in the biofilm community.

Interplay between the marker genes; the high-level TetA(C) reduced the expression of β-lactamase in biofilm. There are two marker genes on pBR322, the ampicillin (blaTEM-1) and tetracycline (tetA(C)) resistance genes, and one plasmid control (rop) gene (11). Rop protein is the plasmid copy number regulator and serves to decrease the copy number (3). To understand the interplay mechanism between the marker genes, we quantified mRNA expression levels of those genes (Fig. 3B). The rop gene was more expressed in planktonic conditions than in biofilms, supporting the notion of the stable maintenance of plasmid during biofilm formation. In contrast, both tetA(C) and blaTEM-1 genes were more expressed in biofilms. This indicates that E. coli harboring pBR322 produced more β-lactamase inside biofilm cells than in planktonic culture even in the absence of antibiotic. It might be due to the mature biofilms maintained more copy of plasmid than in the planktonic cells. Addition of ampicillin further increased the expression of the blaTEM-1 gene. Therefore, E. coli biofilm formation is impaired by β-lactamase that could cause damages to peptidoglycan. These results could confirm that the horizontal acquisition of ampicillin resistance enzymes damages the bacterial cell surfaces and leads to the reduction of biofilm formation.

However, blaTEM-1 could protect planktonic cells, but reduces biofilm formation, which is a clear-cut distinction between a biofilm and planktonic phenotype. Interestingly, the antibiotic mixture further increased the expression of the tetA(C) gene but repressed the expression of the blaTEM-1 gene, indicating the reduction of β-lactamase production. Notably, addition of tetracycline resulted in high-level expression of the TetA(C) efflux pump in biofilms. In addition, the antibiotic mixture also reduced the expression of the rop gene, which could explain the increased copy number of pBR322 inside mature biofilms (Fig. 3A). However, this interplay mechanism only occurred when ampicillin is added together with tetracycline at the subinhibitory levels. Therefore, the interplay between marker genes on pBR322 led to the expression of a high-level of TetA(C) efflux pump, the
interruption of the ampicillin-degradation enzymatic activity, and the increase in the plasmid copy number and its stability in biofilms.

High-level tetracycline efflux pump stimulated several efflux systems on *E. coli* chromosome. Since TetA(C) efflux pump is mainly responsible for tetracycline-resistant mechanism (19), we analyzed whether the biofilms can be resistant to ampicillin without the production of β-lactamase. Recently, the possibility of the cooperation between the β-lactam efflux pump and β-lactamase has been suggested by Masuda & Church (42). This cooperation may effectively decrease the concentration of β-lactams in the periplasmic spaces of Gram-negative bacteria to the point where penicillin-binding proteins are no longer saturated. These bacteria might then display the high-level resistance without producing high-level of β-lactamase (33, 43). Thus, we hypothesized that the interplay between marker genes on the plasmid was stimulated by the presence of subinhibitory concentrations of the antibiotic mixture, which consequently promotes other resistance mechanisms on *E. coli* bacterial chromosome. To verify this hypothesis, we analyzed a global gene expression with DNA microarray technology. We first analyzed the relationship between the interruption of β-lactamase and the expression of *E. coli* biofilm-specific genes. Second we investigated the chromosomal efflux mechanisms, which were activated when the high-level TetA(C) pump is present during the biofilm development of *E. coli* harboring pBR322.

First, since the morphology of MG1655 [pBR322] biofilms is similar to the wild-type biofilm (MG1655) when the antibiotic mixture is supplemented (Fig 2A), we assumed that the presence of both the plasmid and subinhibitory level of the antibiotic mixture must have impacts on *E. coli* gene expression. Based on the clustering analysis, we found similar gene expression profiles to a wild-type biofilm (Fig 4A). We found that *E. coli* harboring pBR322 in biofilms also induced the genes involved in several bacterial surface structures such as flagella, fimbriae, autotransporter proteins, colanic acid and exopolysaccharide poly-N-acetyl-glucosamine (see Table S1 in the supplemental material). Since β-lactamase affects assembly and function of large macromolecular bacterial surface structures (18, 56), the interplay between marker genes could remodify the cell wall
structures and reproduce necessary bacterial cell surface adhesins that are important for biofilm formation.

Secondly, we screened the regulated genes encoding chromosomal efflux pumps in the MG1655 [pBR322] biofilm in the presence of antibiotic mixture, but not in MG1655 in the planktonic conditions and biofilms. Generally, the antibiotic efflux pumps in bacteria are classified into four superfamilies according to their energy source, sequence alignment and substrate specificity; the ABC superfamily (ATP Binding Cassette), the MFS superfamily (Major Facilitator Superfamily), the RND superfamily (Resistance Nodulation Division), and the SMP superfamily (Small Multidrug Resistance) (48, 66). TetA(C) pump on pBR322 belongs to the MFS superfamily (55). In this study, we found 49 bacterial chromosomal efflux pumps related genes. Most of the genes belonging to the MFS and RND superfamilies were repressed (except for araJ), whereas the genes belonging to the SMR superfamily were up-regulated. Nine out of 49 genes were overexpressed more than 2-fold change (in which b1484, gltK, ycbO, yhdX and emrK were up-regulated, while b1486, b1690, acrE and ybdE were down-regulated (Fig 4B and Table S2 in the supplemental material). This indicates that the interplay between the marker genes on pBR322 stimulated several chromosomal efflux pumps, resulting in multidrug resistance of biofilms. For this reason, the production of β-lactamase on pBR322 was repressed by the high-level expression of TetA(C) pump during biofilm development (Fig 3B), and then other effective resistance mechanisms on the E. coli chromosome were stimulated instead.

Since the genes belonging to the SMR superfamily were clearly up-regulated, we believed that the EmrY/K pumps were involved in the antibiotic resistance. Recently, it has been reported that subinhibitory concentrations of tetracycline effectively induced the EmrY/K operon (64), and the tetracycline-dependent promoter of EmrY/K was located between the emrKY and evgAS operons (16, 40). The response of the EvgA/S system to environmental signals is likely to be an important factor of EmrK/Y drug resistance (30). It has been reported that EmrK/Y system could efflux various antibiotic(s) and chemical(s) when EvgA/S two-component system was initiated (16, 25). Therefore, we examined the contribution of the EmrY/K and EvgA/S systems to the antibiotic resistance in
biofilms (Fig. 4C-D) using the modified strain OCL62, in which *emrY*, *emrK*, *evgA* and *evgS* genes are deleted (31) and has counteracted with TetA(C) pump. The disruption of these genes abolished biofilm formation (Fig. 4C and Table 3). The modified OCL62 harboring pBR322 formed patchy biofilms when the antibiotic mixture was added, similar to MG1655 harboring pBR322 when only ampicillin, but not the antibiotic mixture, was supplemented (Fig. 3D & 2A). These results indicate that the EmrY/K and EvgA/S efflux systems also play an important role in the antibiotic resistance during biofilm formation, which are induced by the high-level TetA(C) expression. Thus, *E. coli* harboring the pBR322 plasmid encoding antibiotic resistance genes triggered other chromosomal resistance mechanisms to confer the high-level resistance within biofilms.

**Induction of multidrug resistance in biofilms.** The EmrY/K and EvgA/S efflux systems played an important role in the antibiotic resistance during the biofilm formation, in association with the high-level TetA(C) efflux pump on the plasmid. Both the EmrY/K and EvgA/S pumps are drug/metabolite transporters or proton-drug antiporters coupling substrate removal to electrochemical proton gradients in the opposite direction (17, 20). The overexpression of these genes could make bacteria become resistant to a wide variety of cationic hydrophobic compounds such as tetracycline, erythromycin, β-lactam, macrolide, surfamide, as well as other antiseptics and intercalating dyes (26, 50, 66). Therefore, we investigated whether the activated efflux pumps confer resistance to other antibiotics on the biofilm. We assayed other 5 antibiotics, penicillin, erythromycin, chloramphenicol, kanamycin and gentamicin (Fig. 5A-E and Table 3). The antibiotic was separately added after the biofilms were cultured for 12 h and treated with 5 μg/ml of the mixture of ampicillin and tetracycline for 12 h, and then the plate(s) was incubated for another 24 h. We found that β-lactam antibiotic, penicillin, when given in combination with ampicillin and tetracycline, promoted biofilm formation (Fig 1B). Similarly, the biofilms could resist to the protein synthesis inhibitor, erythromycin, and bacteriostatic antibiotic, chloramphenicol. In contrast, two aminoglycoside antibiotics, kanamycin and gentamicin, inhibited biofilm formation. Therefore, the resistance mechanisms by TetA(C) in combination with EmrY/K and EvgA/S pumps in biofilm cells are likely to be solely due to inhibition of specific protein synthesis or interaction with cell wall. However, they
were sensitive to the aminoglycoside antibiotics because these drugs might accumulate inside the cell (19, 21).

Efflux pumps responded to osmotic stress, which is responsible for colanic acid production and biofilm maturation. We investigated how the high-level TetA(C) and additional EmrY/K-EvgA/S pumps promote the development and maturation of *E. coli* biofilms. Both the pumps mediated energy-dependent efflux of the antibiotic from the bacterial cell (47, 67). It has been reported that TetA(C) protein facilitates the uptake of nickel ions and aminoglycoside antibiotics into the cell (51), therefore the increasing copy number of the *tetA(C)* gene might make cells more susceptible to NiCl$_2$ and aminoglycoside antibiotics. The results shown in Fig. 6A and Fig. 5D-E indicate that the influx and accumulation of toxic metal salts and aminoglycoside antibiotics increased inside bacterial cells. In addition, when present at high levels, the tetA(C) gene product significantly alters the structure of the inner membrane and this confers an additional phenotype of osmotic sensitivity (59). This was investigated by determining the growth sensitivity and the biofilm formation ability of the cultures to increasing concentrations of NaCl and MgCl$_2$. As shown in Fig. 6B-C, biofilm formation was promoted by NaCl (at concentration 0.005-5,000 mM) and MgCl$_2$ (at concentration 100-1000 mM). Thus, there was a positive correlation between the enhanced efflux system and the increased osmotic pressure in biofilms.

We, therefore, hypothesized that the induced efflux pumps in response to osmotic stress contribute to development of a three-dimensional (3D) mature biofilms. Accordingly, it has been reported that the osmotic stress induced the production of exopolysaccharide such as capsular colanic acid and/or poly-N-acetyl-glucosamine, which generally act as an important cell-to-cell adhesin during biofilm development and maturation (9, 28, 44, 57). To confirm this possibility, we observed the morphology and compositions of the biofilms using SEM (Fig. 6D & 7B). As we expected, the MG1655 [pBR322] biofilms had depositions of colanic acid in association with other ultramicroscopic bacterial adhesins when supplemented with the antibiotic mixture, while there were no such depositions in any other tested conditions. The identification of the bacterial cell surfaces structures was based on the our previous study conducted with the specific strains (44). Therefore,
changes in bacterial surface properties including the production of essential exopolysaccharide such as colanic acid promoted the formation of thick mature biofilms. We also found the up-regulation of fimbriae (mot, yad), poly-N-acetyl-glucosamine (aga), and colanic acid (wca) biosynthesis genes, but none of colanic acid transporters such as csp genes in *E. coli* harboring pBR322 biofilm (see Table S1 in the supplemental material).

We further analyzed the mRNA expression levels of *cspE* gene in the MG1655 biofilm and found that its level was higher than one in MG1655 [pBR322] biofilm in the absence of antibiotic (Fig. 7A). As we expected, the highest *cspE* gene expression was obtained in the biofilms when 50 mM NaCl was added. This indicates that the high-level TetA(C) pumps and the induced SMR pumps responded to osmotic stress, which subsequently promoted production of colanic acid and biofilm maturation. In addition, the expression level of *cspE* gene in the modified OCL62 biofilm was low, supporting that the development of *E. coli* harboring pBR322 biofilm is specifically attributed to the presence of the high-level tetracycline efflux pump and the induced SMR pumps. The further maturation process is related to the secondary characteristics of these pumps, which induces effective bacterial adhesins such as colanic acid (Fig. 7B). Overall, these results indicate that subinhibitory concentrations of antibiotics could result in biofilm maturation, leading to high-level antibiotic resistance.

In summary, there is growing evidence that bacteria respond specifically and defensively to subinhibitory concentrations of antibiotics (6, 8, 27, 63). The evidence presented in this study clearly demonstrated that *E. coli* harboring marker genes on the plasmid, which encodes an efflux pump gene, could gain high-level resistance to various antimicrobial agents by stimulating other efflux pump systems on host-cell chromosome and by forming a biofilm when supplemented with the subinhibitory level of the antibiotic mixture. Additionally, the interplay between the marker genes also stimulated high osmotic pressure and then directly promoted the progressive development and maturation of biofilms, which makes the biofilm more difficult to treat. Therefore, care should be taken with regards to handing of the antibiotics and their spread in the environment.
Acknowledgements

We thank Andreas Reisner of the University of Graz in Austria and Maarten Leyssen of the Glaxosmithkline Biologicals S.A. in Belgium, for critical comments on the original manuscript, Søren Molin from Biocentrum-DTU in Denmark for supplying the GFP plasmids, and Keiko Ito of the National Institute of Genetics (NIG) in Japan for kindly providing *E. coli* strains and pBR322 plasmid.

Supplementary materials

Table S1. List of regulated genes when compared MG1655 [pBR322] biofilms in the presence of antibiotics with those of in planktonic cultures (Fold change ≥ 2), and then filtered the genes only regulated in MG1655 [pBR322] biofilms but not in MG1655 biofilms.

Table S2. List of the significantly regulated chromosomal antibiotic efflux pump genes when compared MG1655 [pBR322] biofilms in the presence of antibiotics with those of in planktonic cultures and MG1655 biofilms. The genes that were regulated more than 2-fold change are highlighted in gray.
References


Figure legends

Figure 1. Subinhibitory concentration of ampicillin and tetracycline promoted biofilm formation by *E. coli* harboring pBR322. Quantification of biofilm biomass of MG1655 (A) and MG1655 [pBR322] (B) in the absence of antibiotic, in the presence of ampicillin (Ap), tetracycline (Tc) and ampicillin and tetracycline (Ap+Tc). Biofilms were treated with the antibiotic(s) at different concentrations from 0.0000005 to 500 µg/ml for 24 h after the initial attachment, and then the growth activity of the total bacterial cells, which include both the suspended cells and biofilm cells, (line-graph, indicated as “Growth”) and the biofilm biomass, which attached to the wells (bar-graph, indicated as “Biofilm”), were quantified. Results are averages of 8 replicates ± SD and are representative of 4 independent experiments. Photographs of biofilms formed on PVC wells, which were stained with crystal violet, in each condition were captured and represented in the top panels. w/, with. w/o, without. The statistically comparative MICs in each condition are shown in the Table 3.

Figure 2. The morphology of early (24 h) and late (48 h) biofilms formed by the GFP tagged strains of a plasmid-free MG1655 (a wild-type biofilm) and MG1655 [pBR322] in the absence of antibiotic or in the presence of the mixture of ampicillin and tetracycline at 5 µg/ml each under flowcell condition (A). Antibiotics were supplemented directly to the medium after the bacterial cells were allowed to attach to the glass surface for 12 h, and the biofilm photographs were taken at 24 h and 48 h, respectively, after the addition of antibiotic(s). All experiments were repeated 3 times and representative images are shown. The substratum coverage, average thickness, and roughness coefficient of the biofilms were quantified using the COMSTAT (B). Values are means of data from 20 image stacks (ten image stacks from two independent channels). The error bars in the individual columns represent the standard deviations. w/, with. w/o, without.

Figure 3. The interplay between antibiotic marker genes on pBR322 during *E. coli* biofilm formation resulted in the increase of plasmid copy number, the high-level expression of TetA(C) pump, and the interruption of β-lactamase expression. (A) The plasmid copy numbers of pBR322 in
the suspended cultures and in biofilms were determined. (B) The mRNA expression levels of the ampicillin resistance gene (blaTEM-1), the tetracycline resistance gene (tetA(C)), and the copy number regulator (rop) on the pBR322 were analyzed using quantitative RT-PCR. All experiments were repeated 3 times and the average expression values with their standard derivations are shown. w/, with. w/o, without.

**Figure 4.** The interplay between the marker genes on the plasmid induced chromosomal SMR efflux pumps during biofilm development and maturation. (A) Dynamic hierarchical clustering analysis of *E. coli* gene expression during biofilm formation. Each column represents one gene. Red, up-regulated; White, no change; and Blue, down-regulated. The sample profiles were grouped into two clusters: MG1655 planktonic growth versus MG1655 (plasmid-free) and MG1655 [pBR322] biofilms. For each experiment, three biological replicates were analyzed. (B) Forty-nine regulated genes, which encode chromosomal antibiotic efflux pumps, during the biofilm formation of MG1655 [pBR322] in the presence of antibiotics but not in MG1655 neither in suspended cultures nor biofilms. Nine significantly regulated genes (fold change ≥ 2) were labeled with asterisk. The classification of efflux systems in this study was based on the gene information via Affymetrix's NetAffx database. (C) Quantification of the total bacterial growth activity (line-graph, indicated as “Growth”) and biofilm formation (bar-graph, indicated as “Biofilm”) by the modified OCL62 strain, in which the *emrY, emrK, evgA* and *evgS* are removed and has counteracted with TetA(C), with or without pBR322 plasmid, in the absence or in presence of antibiotics. Results are averages of 8 replicates ± SD and are representative of 4 independent experiments. (D) CSLM photographs of the modified OCL62 [pBR322] biofilms in the presence of antibiotic mixture at 24h and 48 h, respectively. The experiment was duplicated and representative images are shown.

**Figure 5.** The high-level resistance phenomenon was found in the *E. coli* harboring pBR322 biofilms in the presence of subinhibitory concentrations of ampicillin and tetracycline, which confer the resistance to penicillin, erythromycin and chloramphenicol, but not to kanamycin and gentamicin. Quantification assay for the total bacterial growth activity (line-graph, indicated as “Growth”) and
biofilm formation (bar-graph, indicated as “Biofilm”) of MG1655 [pBR322] in the presence of the antibiotic mixture, in association with separately supplemented of following antibiotic; (A) penicillin (Pc), (B) erythromycin (Em), (C) chloramphenicol (Cp), (D) kanamycin (Km) and (E) gentamicin (Gm), respectively. Results are averages of 8 replicates ± SD and are representative of 4 independent experiments. The statistically comparative MICs in each condition are shown in the Table 3.

**Figure 6.** Characteristics of the induced efflux pumps in *E. coli* harboring pBR322 contribute to the increase in osmotic stress response and the production of capsular colanic acid. Quantification assay for the total bacterial growth activity (line-graph, indicated as “Growth”) and biofilm formation (bar-graph, indicated as “Biofilm”) by MG1655 [pBR322] at various concentrations of NiCl₂ (A), NaCl (B) and MgCl₂ (C), respectively, in the presence of antibiotic mixture. Results are averages of 8 replicates ± SD and are representative of 4 independent experiments. (D) SEM micrographs of the structure of biofilms developed in the flowcells. The presence of capsular exopolysaccharide colanic acid (indicated by white arrows) was found only in MG1655 [pBR322] biofilms. The experiment was duplicated and representative images are shown.

**Figure 7.** The induced efflux pumps cause a change in bacterial surface properties during biofilm formation. (A) The mRNA expression levels of *cpsE* show over production of colanic acid by *E. coli* harboring pBR322 in response to osmotic stress. All experiments were repeated 3 times and the average expression values with their standard derivations are shown. (B) SEM micrographs of *E. coli* harboring pBR322 biofilm compared with a wild-type biofilm. Arrows point at the deposition of colanic acid. The results are the means of 20 SEM images from two independent experiments and representative images are shown.
Table 1. The conditions and concentrations of antibiotics used in this study.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Conditions of antibiotic(s)</th>
<th>Concentration range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exposure to ampicillin and tetracycline (administered separately or in combination) by MG1655[pBR322]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>Ampicillin/Tetracycline</td>
<td>0.0000005-500</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0000005-500</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Ampicillin/Tetracycline</td>
<td>0.0000005-500</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0000005-500</td>
</tr>
<tr>
<td><strong>Exposure to ampicillin (administered separately or in combination with tetracycline) by OCL62[pBR322]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCL62</td>
<td>Ampicillin</td>
<td>0.0005-50</td>
</tr>
<tr>
<td>OCL62[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0005-50</td>
</tr>
<tr>
<td><strong>Exposure to penicillin, erythromycin, chloramphenicol, kanamycin and gentamicin (administered separately or in combination with the mixture of ampicillin and tetracycline, 5 µg/ml each) by MG1655[pBR322]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Penicillin/Erythromycin/Chloramphenicol/Kanamycin/Gentamicin</td>
<td>0.0005-50</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Penicillin</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gentamicin</td>
</tr>
<tr>
<td><strong>Exposure to Ni&lt;sup&gt;2+&lt;/sup&gt;, Na&lt;sup&gt;+&lt;/sup&gt; and Mg&lt;sup&gt;2+&lt;/sup&gt; salts (administered separately or in combination with the mixture of ampicillin and tetracycline, 5 µg/ml each) by MG1655[pBR322]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NiCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NaCl</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The antibiotic(s) were administered separately or in combination, depending on each condition.

<sup>b</sup> The mixture of ampicillin and tetracycline was diluted at concentrations from 0.0000005-500 µg/ml each.

<sup>c</sup> The mixture of ampicillin and tetracycline was fixed at concentrations of 5 µg/ml each.

<sup>d</sup> With a series of tenth-fold dilution
Table 2. List of PCR/RT-PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ftsZ</td>
<td>Forward 5'-ATGGAACTTACCAATGACGCG-3'  &lt;br.Reverse 5'-TCAACACCTTCAATGCGCTC-3'</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>blatem-1</td>
<td>Forward 5'-GCATCTTTACGGATGGCATGA-3'  &lt;br.Reverse 5'-GTTCCTCGATCGTTGTCAGAA-3'</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>tetA(C)</td>
<td>Forward 5'-TCTAAACAATGCCGCTCATCGTCATCC-3'  &lt;br.Reverse 5'-GGAATGGAGCATATCCCGCA-3'</td>
<td>61</td>
<td>109</td>
</tr>
<tr>
<td>rop</td>
<td>Forward 5'-CAGGAAAAAACCGCCCTTAACATG-3'  &lt;br.Reverse 5'-ATGTCTGCCTGTTATTCATCCGC-3'</td>
<td>59</td>
<td>101</td>
</tr>
<tr>
<td>cpsE</td>
<td>Forward 5'-CTATTTCTCGACGCCAATCAACG-3'  &lt;br.Reverse 5'-CCTGCAAAGAACATGCGCTCT-3'</td>
<td>59</td>
<td>104</td>
</tr>
</tbody>
</table>

a Primers were used for determination of pBR322 plasmid copy number and mRNA gene expression levels. CpsE; colonic acids biosynthesis gene, blatem-1; TEM-1 β-lactamase gene, tetA(C); tetracycline efflux pump gene, rop; pBR322 plasmid stabilization gene, and ftsZ; cell division protein FtsZ protein (E. coli essential gene).

b Primers were designed by using Primer Express Software (Applied Biosystems, USA). The primer conditions were determined according to dyes manufacturer.
Table 3. Comparative MICs (µg/ml) (in both planktonic cells and biofilm cultures) of all antibiotics against all *E. coli* strains used in this study.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Conditions of antibiotic(s)</th>
<th>MIC (µg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Planktonic</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exposure to ampicillin and tetracycline (administered separately or in combination) by MG1655[pBR322] (Fig. 1A-B)</strong></td>
<td>Ampicillin</td>
<td>-</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>-</td>
<td>0.5</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline</td>
<td>-</td>
<td>0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>500&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MG1655[pBR322]</td>
<td>Ampicillin</td>
<td>-</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>-</td>
<td>500</td>
<td>&gt;500&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline</td>
<td>-</td>
<td>500&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;500&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Exposure to ampicillin (administered separately or in combination with tetracycline) by OCL62[pBR322] (Fig. 4C)</strong></td>
<td>Ampicillin</td>
<td>-</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>OCL62[pBR322]</td>
<td>Ampicillin</td>
<td>-</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline</td>
<td>-</td>
<td>5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Exposure to penicillin, erythromycin, chloramphenicol, kanamycin and gentamicin (administered separately or in combination with the mixture of ampicillin and tetracycline, 5 µg/ml each) by MG1655[pBR322] (Fig. 5A-E)</strong></td>
<td>Penicillin</td>
<td>-</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>-</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>-</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>-</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>-</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td>MG1655 [pBR322]</td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Penicillin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Erythromycin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chloramphenicol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>(0.0005-0.05 promoted biofilm)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Kanamycin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0.00005</td>
</tr>
<tr>
<td></td>
<td>Mixture of ampicillin and tetracycline&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gentamicin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0.00005</td>
</tr>
</tbody>
</table>

<sup>a</sup> The mixture of ampicillin and tetracycline was fixed at concentrations of 5 µg/ml each.

<sup>b</sup> When penicillin, erythromycin, chloramphenicol, kanamycin or gentamicin was supplemented in combination with the mixture of ampicillin and tetracycline, the representative MICs were determined against the penicillin, erythromycin, chloramphenicol, kanamycin or gentamicin.

<sup>c</sup> Statistical analysis was applied to evaluate the “clear-cut” of each MIC using *T-Test* with *P-value* ≤ 0.05.

<sup>d</sup> The representative MICs were at the same concentration of ampicillin and tetracycline each.

<sup>e</sup> The subinhibitory concentration levels of antibiotic(s) that promoted biofilm formation.
[Figure 1] (Width of page 169 x 100 mm)
[Figure 2] (Width of page 169 x 122 mm)
[Figure 3] (Half of page 80 x 87 mm)
Figure 4: (Width of page 169 x 123 mm)
[Figure 6] (Width of page 169 x 74 mm)
[Figure 7] (Half of page 80 x 74 mm)