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Antibiotic susceptibility of *Staphylococcus aureus* with different degrees of biofilm formation

Hyo-Jung Shin[1][†], Sungtae Yang[2][†] and Yong Lim[1]*

Abstract

Staphylococcus aureus is one of the most common pathogens in biofilm-associated chronic infections. *S. aureus* living within biofilms evades the host immune response and is more resistant to antibiotics than planktonic bacteria. In this study, we generated *S. aureus* with low and high levels of biofilm formation using the *rbf* (regulator of biofilm formation) gene and performed a BioTimer assay to determine the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of various types of antibiotics. We showed that biofilm formation by *S. aureus* had a greater effect on MBC than MIC, probably due to the different growth modes between planktonic and biofilm bacteria. Importantly, we found that the MBC for biofilm *S. aureus* was much higher than that for planktonic cells, but there was little difference in MBC between low and high levels of biofilm formation. These results suggest that once the biofilm is formed, the bactericidal activity of antibiotics is significantly reduced, regardless of the degree of *S. aureus* biofilm formation. We propose that *S. aureus* strains with varying degrees of biofilm formation may be useful for evaluating the anti-biofilm activity of antimicrobial agents and understanding antibiotic resistance mechanisms by biofilm development.

Keywords: Biofilm formation, *Staphylococcus aureus*, *rbf* gene, Antibiotics, Bacterial resistance

Introduction

A biofilm is a conglomeration of one or more types of microorganisms attached to a surface, in contrast to the planktonic state in which bacteria exist as individual organisms (Flemming et al. 2016; Rumbaugh and Sauer 2020). Biofilm formation not only evades human immunosurveillance, but is also known to limit antibiotic penetration (Gebreyohannes et al. 2019; Koo et al. 2017; Sharma et al. 2019; Verderosa et al. 2019). There is a positive correlation between drug resistance and biofilm development in clinical *S. aureus* isolates (Kwon et al. 2008; Martins et al. 2007). Biofilm-forming bacteria are known to exhibit approximately 100–1000 times

higher antibiotic resistance than planktonic bacteria (Donlan 2000; Pantanella et al. 2008). The reasons for this are as follows: (1) the penetration of antibiotics into the biofilm is poor; (2) bacteria in the biofilm state are relatively slower in growth and proliferation than those in the planktonic state; (3) biofilms are different from planktonic bacteria in specific metabolic processes; and (4) biofilms appear to facilitate horizontal gene transfer of antibiotic resistance genes (Bjarnsholt et al. 2013; Hall and Mah 2017; Hoiby et al. 2010; Rose and Poppens 2009; Shapiro 1998). Infections caused by biofilm-forming pathogens are difficult to treat using conventional methods and have become a serious problem worldwide (Hall and Mah 2017; Li et al. 2020; Verderosa et al. 2019). For developing therapeutic agents for biofilm-forming bacterial infections, the susceptibility of antibiotics should be tested under biofilm conditions rather than in the planktonic state. Bacterial strains with varying degrees

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of biofilm formation are thought to contribute significantly to the search for novel antimicrobial agents effective against chronic biofilm-associated infections and to understand antibiotic resistance mechanisms due to biofilm development.

Staphylococcus aureus, a major cause of community-acquired infections and nosocomial infections, can form biofilms on the surfaces of various medical devices used in hospitals, leading to chronic and persistent infections (Ando et al. 2004; Baltch et al. 2008; Cirz et al. 2007; Villain-Guillot et al. 2007). Treatment of *S. aureus* biofilm-associated infections is very limited because of reduced antibiotic susceptibility. One of the most significant problems with biofilm formation by *S. aureus* is the development of resistance to antibiotics (Kranjec et al. 2021; Lin et al. 2019). Although the mechanism of *S. aureus* biofilm formation remains unclear, it is believed that bacteria deposit on solid surfaces, accumulate multi-layered cell clusters, and then evolve into mature biofilms, as illustrated in Fig. 1 (Moormeier and Bayles 2017; Otto 2013). We previously found that the *rbf* (regulator of biofilm formation) gene contributes significantly to biofilm formation by *S. aureus* on polystyrene or glass by interfering with the multicellular aggregation step without affecting the initial stage of biofilm formation (Lim et al. 2004). The *rbf* gene is also known to play an important role in biofilm formation in vivo (Luong et al. 2009). Therefore, different degrees of *S. aureus* biofilm formation can be generated by targeting the *rbf* gene.

In this study, we prepared a *rbf*-inactivated strain (CYL1135) and a *rbf*-complemented strain (CYL1106) of *S. aureus* with low and high degrees of biofilm formation, respectively, and investigated the antimicrobial activities of various antibiotics against planktonic and biofilm *S. aureus* in vitro. Additionally, we evaluated the ability of the antibiotics to detach the biofilm matrix using a high biofilm-forming CYL1106 strain. We believe that *S. aureus* with different degrees of biofilm formation could be useful for the discovery of novel antibiotics and substances that can effectively inhibit and/or eliminate biofilm formation.

Methods

Materials

The antibiotics used in this study were as follows: bacampicillin, moxifloxacin, norfloxacin, and amoxicillin-clavulanate from Chong Kun Dang Bio Co. (Seoul, Korea); ciprofloxacin, cefaclor, and amikacin from Ildong Pharm. Co. (Seoul, Korea); cefotaxime and quinupristin–dalfopristin from Handok Pharm. Co. (Seoul, Korea); ampicillin-sulbactam and ceftriaxone from Hanmi Pharm. Co. (Seoul, Korea); kanamycin from Donga Pharm. Co. (Seoul, Korea); tobramycin from Daewoong Pharm. Co. (Seoul, Korea); vancomycin from Cheiljedang Co. (Seoul, Korea); teicoplanin from Sanofi-Aventis Korea Co. (Seoul, Korea); erythromycin from Fluka Biochemika (Buchs, Switzerland); and fosfomycin from Pharmbio Korea Co. (Seoul, Korea). Antibiotics were of analytical grade.

Preparation of *S. aureus* with different degrees of biofilm formation

CYL1135, in which the *rbf* gene of *S. aureus* was inactivated, whereas CYL1106, in which *the rbf* gene was complemented with a multicopy plasmid, was generated as described previously (Kwon et al. 2013; Lim et al. 2004). Briefly, an internal fragment of the *rbf* gene (724 base pairs) from the 8325-4 strain was amplified by polymerase chain reaction (PCR) with two primers 5′-CACTCA TAAAAGCTTCTTC-3′ and 5′-TGGTGATTTGCGAGA
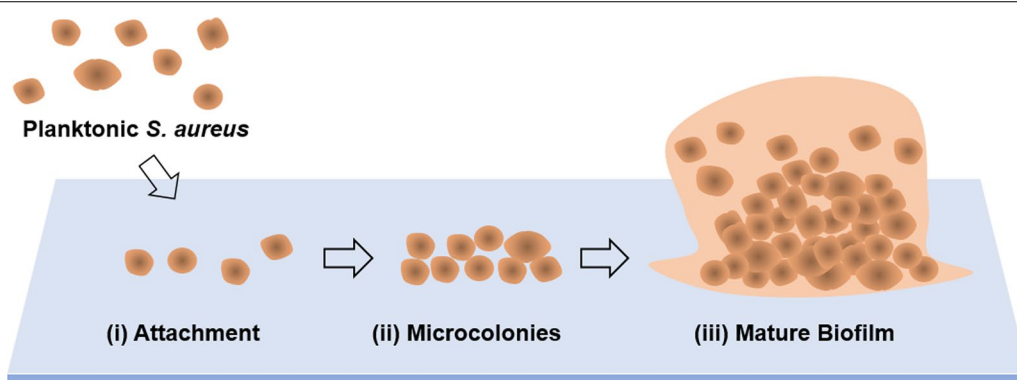

Fig. 1 Model of *S. aureus* biofilm development: (1) During the attachment phase, planktonic *S. aureus* adheres to a biotic surface and forms a monolayer. (2) After the attachment is accomplished, microcolonies are formed with multilayering cells. (3) The microcolonies evolve into a mature biofilm

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TGAGCV-3′, and then cloned into pCL53. The resulting pYL8564 plasmid was transformed into the tetracycline resistant RN4220 strain and verified by PCR.

Antibiotics susceptibility test on planktonic *S. aureus*

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of antibiotics were determined following the microdilution procedure of the Clinical and Laboratory Standard Institute (CLSI) guidelines. In brief, CYL1135 strains were inoculated into Mueller Hinton broth (MHB) medium (Becton Dickinson, NJ, USA), cultured in a 37 °C shaking incubator for 24 h, and diluted with MHB to a bacterial count of 5×10^5 CFU ml^{-1}. Samples were prepared for serial two-fold dilutions in equivalent volumes for various concentrations of antibiotics distributed on the microplates. The plates were incubated overnight at 37 °C in an incubator, and the absorbance was measured at 620 nm. The minimum concentration at which the bacteria did not grow by more than 90% was determined as the MIC. To measure MBCs, the culture medium in which the bacteria did not grow was transferred to a tryptic soy agar (TSA) plate and then cultured overnight at 37 °C. The minimum concentration at which bacteria did not completely grow in the TSA medium was defined as the MBC.

Antibiotics susceptibility test on biofilm *S. aureus*

To measure the MIC in biofilm bacteria, a modification of the existing Bio-Timer assay (BTA) was performed as previously described (Pantanella et al. 2008). In brief, CYL1135 and CYL1106 strains were inoculated into tryptic soy broth (TSB) medium (Becton Dickinson, NJ, USA) containing 0.25% glucose and cultured overnight at 37 °C in a shaking incubator. After measuring the optical density at 600 nm (OD$_{600}$), the absorbance of the cultured strains was adjusted to 2.5, diluted 5000-fold in TSB medium containing 0.5% glucose, and then incubated overnight at 37 °C in a 6 cm dish (Nunc, Roskilde, Denmark) containing sterilized glass beads (Marienfeld, Lauda-Königshofen, Germany). The culture medium was rinsed twice with sterilized phosphate-buffered saline (PBS) at 6.5 rpm for 5 min. A glass bead containing biofilm was placed in each well of a 24-well plate containing 1 ml of diluted antibiotics at a maximum of 1024 μg ml^{-1} in the BioTimer-phenol red (BT-PR) medium. As a positive control, a glass bead containing biofilm was placed in a BT-PR medium without antibiotics. Sterilized glass beads in BT-PR medium were used as a negative control. The number of biofilm-forming strains was approximately 5×10^5 CFU ml^{-1}. The concentration of antibiotics in the last well (red color) was defined as the MIC of biofilm bacteria. To measure MBC in the biofilm state, beads were taken from each well of the MIC plate where bacteria did not grow, transferred to a new 24-well plate containing 1 ml of BT-PR medium, and incubated overnight at 37 °C. The concentration of antibiotics in the last well showing red color was defined as the MBC in biofilm *S. aureus.*

Biofilm matrix detachment assay

Overnight cultures with CYL1106 strain were adjusted to an OD$_{600}$ of 3 and diluted 500-fold in TSB medium supplemented with glucose. Then, a 200 μl aliquot of the cell suspension was seeded into each well of a 96-well plate (Costar; Corning Inc., NY, USA). After overnight incubation at 37 °C, each well was gently washed twice with PBS. Antibiotics (200 μl) were serially diluted two-fold with TSB medium and incubated for 6 h at 37 °C. After incubation, the supernatant was discarded, and the cells were rinsed twice with PBS. The plates were dried at room temperature for 1 h and stained with 0.25% crystal violet. Absorbance at 590 nm was measured using a microplate reader (Sunrise™; Tecan Co., Grödig, Austria).

Results and discussion

MICs of several classes of antibiotics against planktonic and biofilm *S. aureus*

We first constructed a *rbf*-inactivated strain (CYL1135) and a *rbf*-complemented strain (CYL1106) of *S. aureus*, as described in the Materials and Methods. The ability of CYL1135 and CYL1106 strains to form biofilms was studied using crystal violet staining (Fig. 2A). The CYL1106 strain showed a more than fourfold higher ability to form biofilms than the CYL1135 strain (Fig. 2B). These results demonstrate that the *rbf* gene is involved in biofilm formation *by S. aureus.* Therefore, the CYL1135 and CYL1106 strains were used for low and high degrees of biofilm formation by *S. aureus*, respectively. Figure 2C illustrates the planktonic, low biofilm formation, and high biofilm-forming strains. We then measured the antimicrobial activities of several classes of antibiotics against planktonic and biofilm bacteria. Table 1 shows the MICs of antibiotics in planktonic *S. aureus*, low biofilm-forming CYL1135, and high biofilm-forming CYL1106 strains. Most antibiotics were more effective against planktonic *S. aureus* than biofilm *S. aureus*, suggesting that biofilm-forming ability influences antimicrobial resistance (Castano-Arriba et al. 2020; Qi et al. 2016). However, there was no significant difference in MICs between the CYL1135 and CYL1106 strains. The MICs of penicillin-type and quinolone-type antibiotics against planktonic bacteria were 1–4 times lower than those for biofilm bacteria. In contrast, the biofilm-forming strains were 2–16 times more resistant to aminoglycoside-type and glycopeptide antibiotics than planktonic *S. aureus.* Interestingly, moxifloxacin, ceftriaxone, erythromycin,
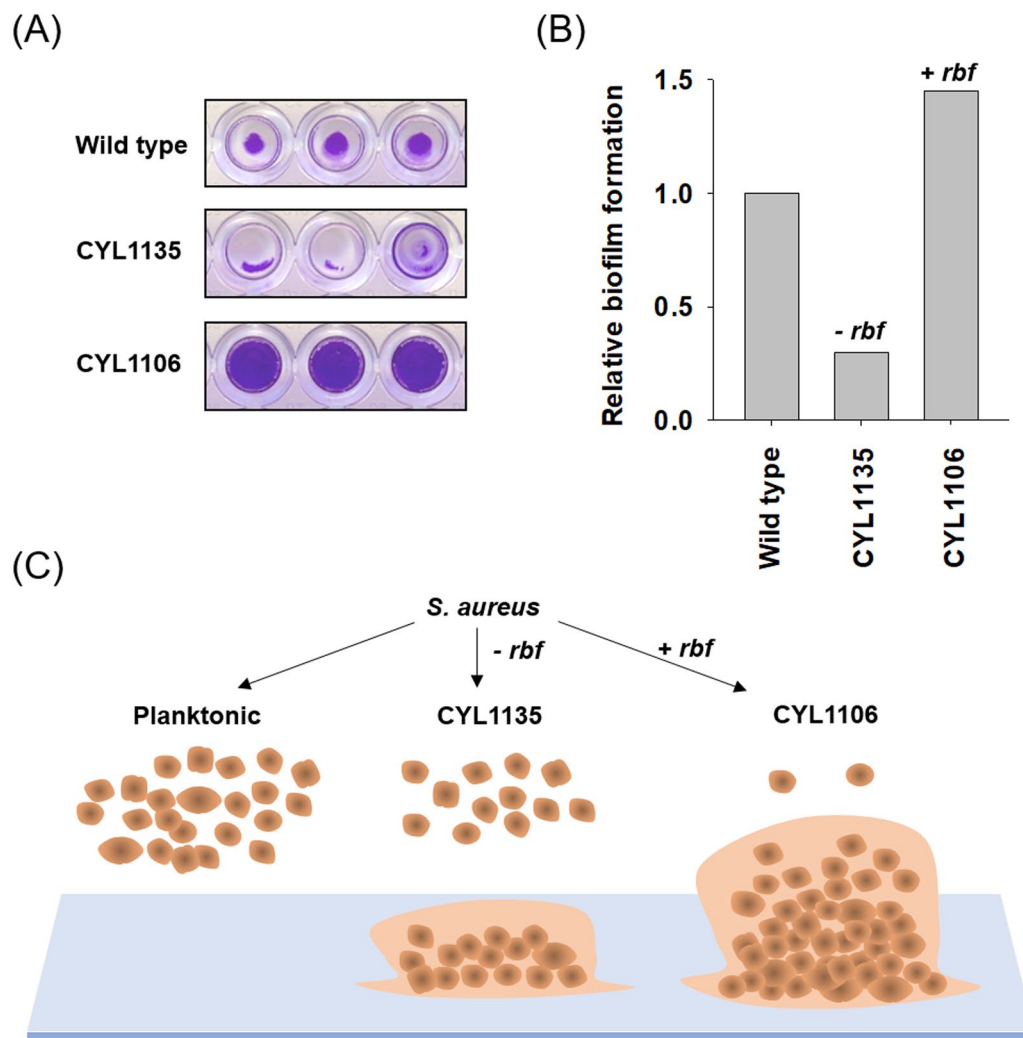
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Fig. 2 Effect of the *rbf* gene on *S. aureus* biofilm formation.**A** Wild type, CYL1135, and CYL1106 strains were tested for polystyrene binding in wells of a microtiter plate. The biofilm formed was stained with crystal violet. **B** Biofilm formation of wild type, CYL1135, and CYL1106. The absorbance was determined at 590 nm. The results represent the averages of three independent experiments. **C** Schematic representation of planktonic *S. aureus*, low biofilm-forming CYL1135, and high biofilm-forming CYL1106

and quinupristin–dalfopristin showed the same MIC values in planktonic and biofilm states, regardless of the degree of biofilm formation.

MBCs of several classes of antibiotics against planktonic and biofilm *S. aureus*
Table 2 shows the MBCs of antibiotics in planktonic and biofilm *S. aureus* strains. The antibiotics used in this study exhibited 2–512 times higher MBC in the CYL1135 and CYL1106 strains than in planktonic bacteria (Table 2), whereas the MIC difference between planktonic and biofilm *S. aureus* was 1–64 times (Table 1). The greater decrease in bactericidal than bacteriostatic capacity is probably due to the different growth modes

between planktonic and biofilm *S. aureus*. Among the antibiotics used in the experiment, moxifloxacin, amikacin, tobramycin, and quinupristin–dalfopristin showed the lowest increase rate in MBC in biofilm cells compared to planktonic cells, indicating that they are effective bactericidal antibiotics under biofilm conditions. Some antibiotics in the same class showed different antimicrobial activity against *S. aureus*. For example, among the fluoroquinolones, moxifloxacin was more effective in killing *S. aureus* than ciprofloxacin, supporting the view that moxifloxacin has enhanced activity against gram-positive bacteria compared to ciprofloxacin (Zhanel et al. 2002). Interestingly, given the difference in MBC between planktonic and biofilm *S. aureus*, there was no significant

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Table 1 Minimum inhibitory concentrations (MICs) of antibiotics in planktonic *S. aureus*, low biofilm-forming CYL1135, and high biofilm-forming CYL1106 strains

Antimicrobial agents	MIC (μg ml^{-1})		
	Planktonic	CYL1135	CYL1106
Penicillins			
Bacampicillin	0.25	0.5	0.5
Amoxicillin-clavulanate	0.5	1	1
Ampicillin-sulbactam	1	2	2
Quinolones			
Ciprofloxacin	0.125	0.5	0.5
Norfloxacin	0.5	1	1
Moxifloxacin	0.06	0.06	0.06
Cephalosporins			
Cefaclor	1	4	8
Cefotaxime	0.5	1	2
Ceftriaxone	2	2	2
Aminoglycosides			
Amikacin	2	8	16
Kanamycin	1	16	16
Tobramycin	0.5	2	2
Glycopeptides			
Teicoplanin	0.125	0.25	1
Vancomycin	0.5	1	2
Others			
Erythromycin	0.25	0.25	0.25
Fosfomycin	2	64	128
Quinupristin–dalfopristin	0.125	0.125	0.125

Table 2 Minimum bactericidal concentrations (MBCs) of antibiotics in planktonic *S. aureus*, low biofilm-forming CYL1135, and high biofilm-forming CYL1106 strains

Antimicrobial agents	MBC (μg ml^{-1})		
	Planktonic	CYL1135	CYL1106
Penicillins			
Bacampicillin	0.25	8	16
Amoxicillin-clavulanate	0.5	16	64
Ampicillin-sulbactam	1	64	64
Quinolones			
Ciprofloxacin	0.125	4	4
Norfloxacin	0.5	1	16
Moxifloxacin	0.06	0.125	0.5
Cephalosporins			
Cefaclor	1	128	512
Cefotaxime	0.5	32	64
Ceftriaxone	2	128	128
Aminoglycosides			
Amikacin	2	16	16
Kanamycin	1	16	64
Tobramycin	0.5	4	4
Glycopeptides			
Teicoplanin	0.125	2	4
Vancomycin	0.5	4	16
Others			
Erythromycin	0.25	4	8
Fosfomycin	16	128	1024
Quinupristin–dalfopristin	0.125	0.5	1

difference in MBC between the CYL1135 and CYL1106 strains. These results suggest that once the biofilm is formed, it is difficult to kill *S. aureus*, regardless of the degree of biofilm formation.

Effect of antibiotics on biofilm detachment of CYL1135 cells

Next, we measured the detachment effect of antibiotics on the biofilm matrix of *S. aureus* (Fig. 3). Biofilms grown with the high biofilm-forming CYL1106 strain were incubated with antibiotics. Among the antibiotics of each class used in the experiment, those with the lowest difference in MBC between planktonic and biofilm cells were selected: (A) bacampicillin, (B) moxifloxacin, (C) cefotaxime, (D) tobramycin, (E) teicoplanin, and (F) erythromycin. Moxifloxacin, tobramycin, and teicoplanin showed little biofilm degradation at concentrations below 8 μg ml^{-1}. However, cefotaxime, bacampicillin, and erythromycin at concentrations above 2 μg ml^{-1} led to a 40–50% removal of the biofilm. Considering that there is a close correlation between drug resistance and biofilm development, the high

biofilm-forming CYL1135 strain could be very useful for understanding biofilm mechanisms or screening novel antibiotics to eradicate biofilms.

Conclusions

Pathogenic biofilm formation is recognized as a major challenge in treating many persistent infections (Gebreyohannes et al. 2019). Susceptibility testing of planktonic bacteria can be an impediment to the successful treatment of chronic infections caused by biofilm-forming pathogens. In this study, we generated *S. aureus* with different degrees of biofilm formation and measured the MICs and MBCs for low and high biofilm-forming strains. Most importantly, we found a large difference in MBC between the planktonic and biofilm states, but the difference in MBC between low and high levels of biofilm formation was insignificant. We propose that once the biofilm is formed, the bactericidal activity of antibiotics is significantly reduced, regardless of the degree of biofilm formation by *S. aureus*.
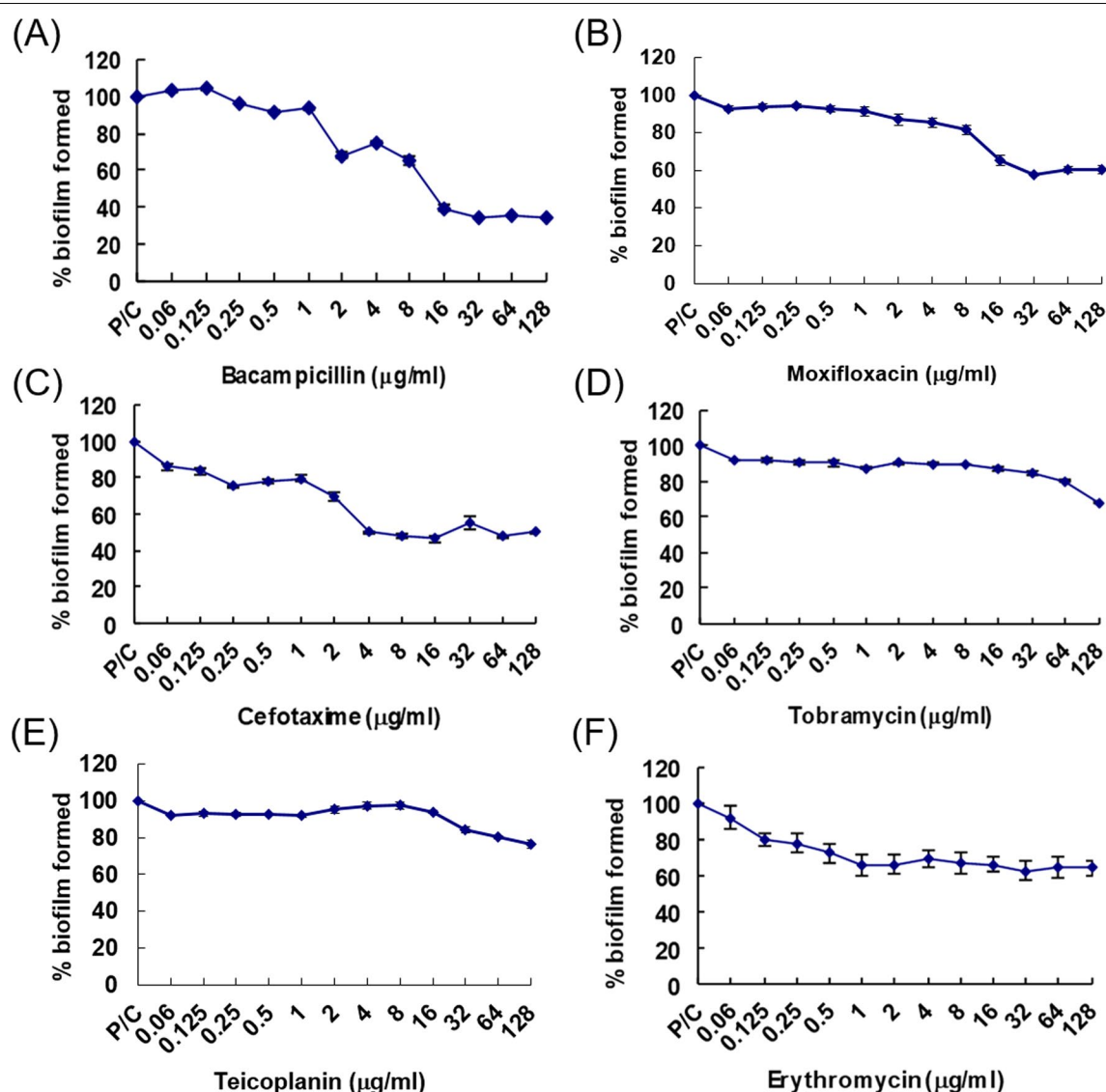
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Fig. 3 Biofilm detachment for CYL1106 strains in the presence of the antibiotics. Antibiotic with the lowest difference in MBCs between planktonic and CYL1106 strains was selected: **A** bacampicillin, **B** moxifloxacin, **C** cefotaxime, **D** tobramycin, **E** teicoplanin, and **F** erythromycin

Abbreviations
MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; *rbf*: Regulator of biofilm formation; PCR: Polymerase chain reaction; BTA: BioTimer assay; BT-PR: BioTimer-phenol red; TSA: Tryptic soy agar; TSB: Tryptic soy broth; PBS: Phosphate-buffered saline; MHB: Mueller Hinton broth; CFU: Colony forming unit.

Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no competing interests.

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