Paired methods to measure biofilm killing and removal: a case study with Penicillin G treatment of *Staphylococcus aureus* biofilm

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Biofilms are microbial aggregates that show high tolerance to antibiotic treatments in vitro and in vivo. Killing and removal are both important in biofilm control, therefore methods that measure these two mechanisms were evaluated in a parallel experimental design. Kill was measured using the single tube method (ASTM method E2871) and removal was determined by video microscopy and image analysis using a new treatment flow cell. The advantage of the parallel test design is that both methods used biofilm covered coupons harvested from a CDC biofilm reactor, a well-established and standardized biofilm growth method. The control Staphylococcus aureus biofilms treated with growth medium increased by 0□6 logs during a 3-h contact time. Efficacy testing showed biofilms exposed to 400 lmol l^{-1} penicillin G decreased by only 0□3 logs. Interestingly, time-lapse confocal scanning laser microscopy revealed that penicillin G treatment dispersed the biofilm despite being an ineffective killing agent. In addition, no biofilm removal was detected when assays were performed in 96-well plates. These results illustrate that biofilm behaviour and impact of treatments can vary substantially when assayed by different methods. Measuring both killing and removal with well-characterized methods will be crucial for the discovery of new anti-biofilm strategies.

Microbial biofilms exhibit increased tolerance to treat-ment with disinfectants and antibiotics, and often, only combinations of chemical and physical measures can reduce viable cell numbers and/or remove biofilm from surfaces as reviewed by Koo et al. (2017). Killing viable cells is the focus of traditional antibiotic therapy, although, removal of the matrix and/or prevention of initial attachment events are potentially important mechanisms in the battle to control biofilm (Høiby et al. 2015). Currently for a medical device colonized with bio-film, replacement is often the only option to re-establish proper functionality resulting in high socioeconomic bur-dens for patients and health care systems (Wilkins et al. 2014).

In vivo, biofilms grow in a diverse range of conditions and in vitro biofilms must therefore be studied using lab-oratory systems that model various conditions. Static sys-tems, such as well-plates, grow biofilm under batch

conditions (no replenishment of the nutrients) and minimal fluid shear. In a dynamic system, the nutrients are continuously replenished, and the fluid shear may vary from laminar to turbulent flow, depending upon the reactor system (Crusz *et al.* 2012). Flow cells are a useful tool for facilitating detailed investigations of initial attachment events and biofilm removal, both important aspects of biofilm control strategies. The preference for using dynamic assay systems is reflected by US FDA regulatory guidelines for testing medical devices containing antimicrobials (Food and Drug Administration, 2015).

ASTM method E2871-13, a biofilm efficacy test generally known as the single tube method, and ASTM method E2562-17 which describes how to grow a biofilm in the CDC biofilm reactor (ASTM International, 2013, 2017) were developed and statistically validated for measuring the efficacy of biocides against biofilm bacteria. The CDC biofilm reactor design allows for flexibility regarding biofilm growth conditions and sampling regimes due to the placement of three removable coupons in each of eight rods (Goeres *et al.* 2005; Buckingham-Meyer *et al.* 2007). By design, the single-tube method only measures the efficacy of biocides and antibiotics against biofilm. The single tube method is a static test system and does not provide information on biofilm removal. To address this limitation of the single tube method, the treatment-flow-cell (FC310; Biosurface Technologies Corp., Bozeman, MT) was designed as a complementary new tool (Fig. 1). The treatment-flow-cell can be used to assess biofilm removal in real time that results from treatment of a mature biofilm grown on coupons harvested from the CDC reactor. Using both the single tube method and the treatment flow cell in parallel allows a researcher to assess the kill and/or removal that results when a biofilm is exposed to an antibiotic or biocide, thereby providing key insights into the mechanism of action.

This paper highlights the advantages of combining kill and removal biofilm assays when finding new biofilm control strategies. Our treatment-flow-cell experiments, carried out in combination with the single tube method, allowed us to identify the effective biofilm removal properties of Penicillin G (Pen G) against a mature *Staphylococcus aureus* (*Staph. aureus*) biofilm. To the best of our knowledge, this antibiofilm property of Pen G has not been reported yet.

Results and discussion

Growth performance and Pen G susceptibility of planktonic *Staphylococcus aureus* AH2547

Planktonic *Staph. aureus* AH2547 had a generation time of 25 min, which lies in the normal bacterial proliferation range despite the metabolic burden of green fluorescent protein (GFP) expression (Domingue *et al.* 1996). A concentration of 0·15 μmol l^{-1} Pen G inhibited growth of the strain (Fig. 2a). We detected a difference in growth curve development for the treated *vs* control bacteria after 90 min of incubation (Fig. 2a). Due to the mode-of-action of Pen G no rapid bactericidal effect was detected, similar to previous experiments involving Pen G and the *Staph. aureus* strain ATCC 25923 (Ausbacher *et al.* 2014).

Treatment-flow-cell biofilm experiments and image analysis

Coupons containing *Staph. aureus* AH2547 biofilm grown in the CDC reactor were collected for either efficacy testing according to the single tube method or placement into the treatment-flow-cell to assess removal. This allowed for the parallel measurement of biofilm killing and biofilm removal eliminating any experiment-to-experiment variability that is

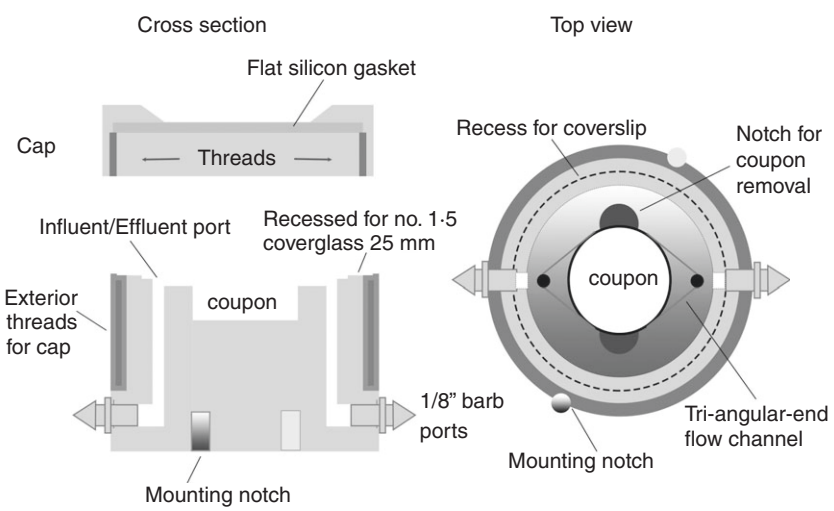

Fig. 1 Cross section and top view of the treatment flow cell illustrating coupon location, flow in- and outlet and mounting notches for attachment to the microscope stage. The treatment flow cell is sealed by a 25-mm cover slip, which is located under the silicon gasket, after the cap had been screwed on the bottom part of the cell. Barb ports accommodate 3 mm tubing for inlet and outlet (schematic adapted with permission from Biosurface Technologies Corp.).

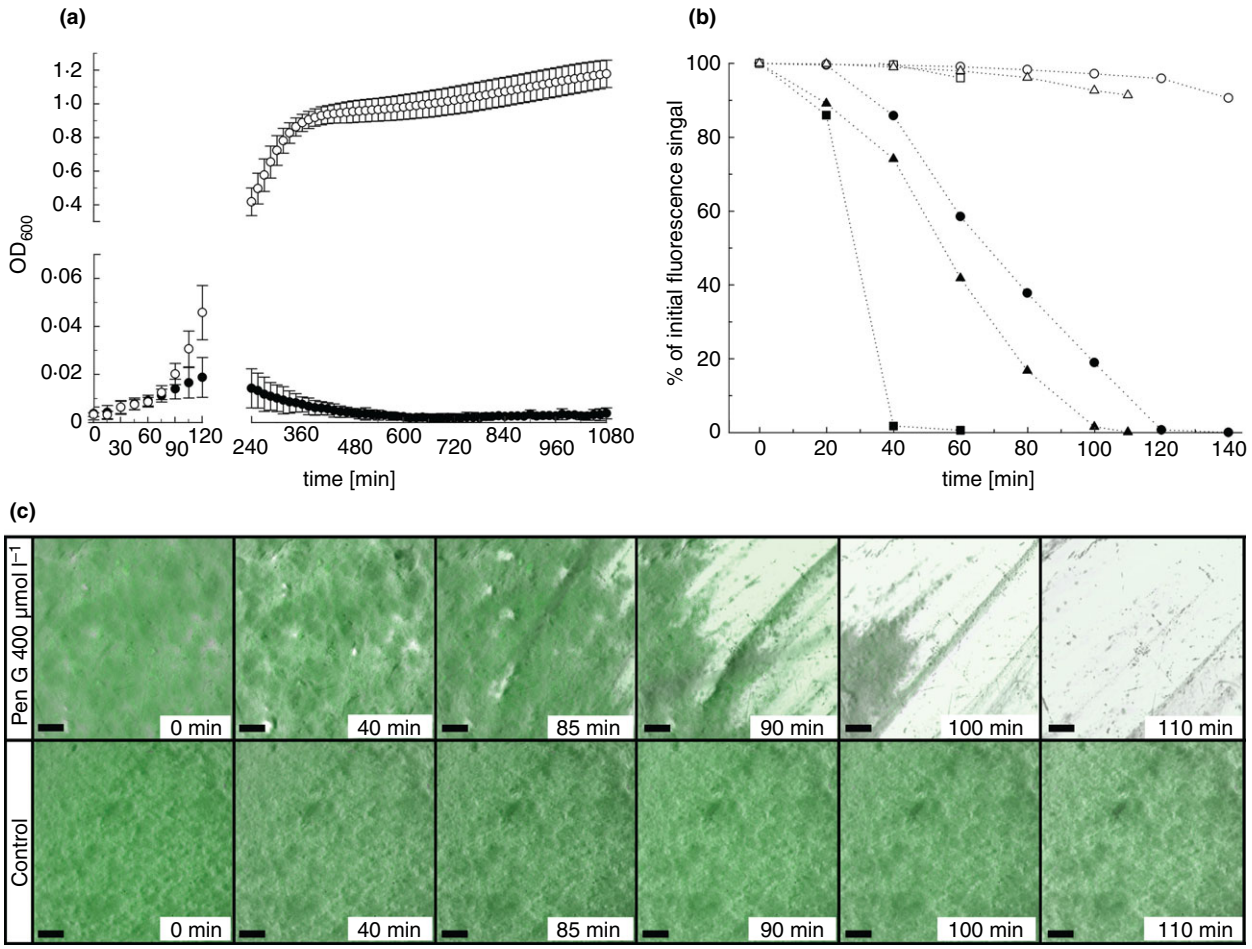

Fig. 2 Behaviour of planktonic *Staphylococcus aureus*, image analysis data and CLSM overlay images after real-time image acquisition of treated *Staph. aureus* biofilms. (a) Growth curves of Pen G (0·15 μmol l^{-1}) (●) treated and untreated (○) planktonic *Staph. aureus* over an incubation period of 18 h at 37°C. (b) Image analysis of untreated *Staph. aureus* biofilms and after treatment with 400 μmol l^{-1} of Pen G at 37°C in three independent experiments. Experiment 1-Pen G (●), experiment 1 – control (○), experiment 2-Pen G (▲), experiment 2-control (Δ), experiment 3-Pen G (■), experiment 3-control (□). For clarity, data points of each experiment were connected. (c) Green fluorescent protein-brightfield overlay images of control biofilms and Pen G treated biofilms at experiment start (0 min), start of erosion (40 min) and during dispersion phase (85–110 min). Scale bars represent 200 μm. Movies of Pen G treated and untreated biofilms are available as supplemental information in the online version of this article (videos S1 and S2). [Colour figure can be viewed at wileyonlinelibrary.com]

possible if the biofilm had been grown in different reactors on different days. Our microscopy studies showed that treatment with full-strength TSB in the treatment-flow-cell did not affect *Staph. aureus* biofilms. However, we observed a slight decrease in fluorescence intensity over time when performing image analysis. This can be attributed to bleaching from repeated laser light exposure (Fig. 2b, controls of experiments 1–3). Images of the untreated control coupons showed no removal events (Fig. 2c and videos S1 and S2). We used 400 μmol l^{-1} of Pen G during our biofilm experiments based upon data from previous studies where equally high concentrations had only a low to moderate impact on biofilm viability (Ausbacher *et al.* 2014; Manner *et al.* 2015). Exposing the biofilm to Pen G first caused erosion of the biofilm and finally resulted in complete removal of the biofilm after 40, 100 or 120 min (Fig. 2b,c and video S1). Image analysis showed a 60–100% biofilm removal within a 90-min time period. In contrast, growth curves of untreated controls and Pen G treated planktonic bacteria followed each other for 90 min due to the antibiotic's dependence on proliferating bacteria (Fig. 2a). Even though there is a discrepancy in Pen G concentration, it has been reported that increased penicillin dosing does not necessarily impact the effect of β-lactam antibiotics against planktonic bacteria (Van Herendael *et al.* 2012). We conducted our experiments in full strength TSB and the flow of nutrients facilitated hydrodynamic interactions. The increased mass transfer, higher shear

forces and the additional influence of Pen G, may therefore account for the substantial biofilm removal, which represents a cohesive material failure (Brindle *et al.* 2011). Physicochemical interaction of Pen G with the biofilm is plausible, considering that Pen G has surface active properties and is capable of forming micelles (Thakkar and Wilham 1971). Of note, Brindle *et al.* (2011) have made similar observations when testing an urea treatment, which in itself is not antimicrobial, against *Staphylococcus epidermidis* biofilms. In the study, urea removed biofilm within minutes when applied in conjunction with flow, whereas a static soak and subsequent fluid shear challenge did not result in biofilm removal. Besides urea, the anionic surfactant SDS, chloride and chlorine-releasing agents have also been reported as having good removal properties when *Pseudomonas aeruginosa* biofilms were treated (Chen and Stewart 2000). The dispersal of the *Staph. aureus* biofilm might, however, be the result of a synergistic combination of bacteria/matrix response to the presence of Pen G and demonstrates the benefits of testing in a system with hydrodynamics.

Single tube method

It was advantageous that we could investigate the effect of Pen G on biofilms collected from the same reactor as those used in the treatment-flow cell due to the 24 available coupons in the CDC biofilm reactor. Pen G had a bacteriostatic effect on the biofilm bacteria with a difference of 0·3 log units between 1 and 3 h of treatment (Fig. 3a). In contrast, bacteria in the untreated control biofilms proliferated under these conditions with a log increase of 0·6.

The viable plate count data collected during the single tube method experiments suggest that the observed biofilm removal cannot be explained by a decrease in bacterial viability. Data from our OD_{600} measurements (Fig. 2a) illustrate that Pen G, whose efficacy depends on dividing bacteria, does not have an instant effect on bacteria compared to what is generally known from rapidly acting biocides.

96-well plate biofilm assay

In order to check the treatment behaviour of AH2547 biofilms in other assay systems we chose a 96-well plate format. The 96-well plate is a favourable tool for drug screening and is commonly used by many laboratories for detecting potential anti-biofilm compounds. In this static assay system we tested if Pen G treatment led to fluorescence loss due to biofilm removal and/or cell lysis. The well-plate experiments did not result in substantial biofilm removal or loss in fluorescence after exchange of the planktonic phase (Fig. 3b). In contrast to the treatment flow cell, increased mass transfer and shear forces are absent in a 96 well-plate assay, similar to the single tube method. Lack of Pen G potency in equally high concentrations against biofilms of various *Staph. aureus* strains in well-plate based assays has been reported by others groups (Amorena *et al.* 1999; Pettit *et al.* 2009; Ausbacher *et al.* 2014; Manner *et al.* 2015). This suggests that the sole presence of 400 μmol l^{-1} of Pen G does not trigger biofilm removal. *Staphylococcus aureus* biofilms grown in a 96-well format in the presence of high concentrations of Pen G can provoke protein expression for the increased energy supply for strengthening of the proteoglycan (Savijoki *et al.* 2016). In addition to this defence strategy, Pen G is suspected to induce dormancy and thus support biofilm sustainability (Savijoki *et al.* 2016).

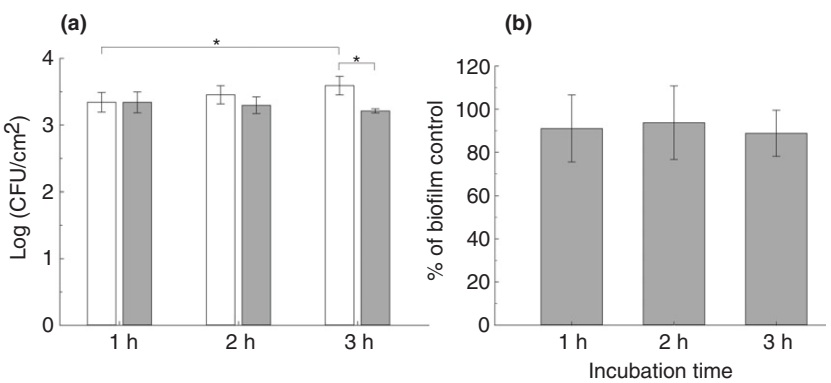

Fig. 3 Quantification of the Pen G impact on *Staphylococcus aureus* biofilms. (a) Quantification of viable cells of mature biofilms from a CDC biofilm reactor using the single-tube method. Biofilms of the untreated control (□) and biofilms treated with 400 μmol l^{-1} Pen G (■) were incubated at 37°C. Asterisk indicates significant difference, $P < 0{\cdot}05$ (Student's *t*-test). (b) Treatment and quantification of preformed 18 h biofilms (37°C) in 96-well plates (Nunclon™ Δ surface) utilizing green fluorescent protein fluorescence of *Staph. aureus*. Treatment of biofilms for 1–3 h (37°C) with 400 μmol l^{-1} of Pen G was followed by exchange of the planktonic phase, which did not lead to removal of biofilms. Results display the mean ± SD of three independent experiments.

Further studies are needed to fully elucidate the molecular bases of the biofilm dispersing mechanism of Pen G on *Staph. aureus* biofilms.

We demonstrated the usefulness of the treatment-flow-cell for visualizing biofilm removal in real-time. The ability to use coupons collected from the same CDC reactor for both the treatment flow cell and single tube method efficacy test allows for a more comprehensive evaluation of the mechanisms of action of potential antibiofilm treatments. Furthermore, our case study provides a good example of the importance of using multiple methods to reveal potent removal properties of Pen G, which has not been reported previously. The implementation of different test regimens can therefore be pivotal in identifying new biofilm control strategies.

Material and methods

Bacterial strain

We used the GFP expressing *Staph. aureus* strain AH2547 which and contains the GFP-expressing plasmid pCM29 (Pang *et al.* 2010) kindly provided by Dr. Alex Horswill.

Planktonic growth analysis and Pen G susceptibility

Overnight cultures of *Staph. aureus* AH2547 were prepared in TSB under aerobic conditions at 37°C, supplemented with chloramphenicol (10 μg ml^{-1}) for plasmid retention. Growth analyses of AH2547 were conducted in a 50 ml broth volume, supplemented with chloramphenicol as described above. The OD_{600} values of aliquots were measured every 30 min and plated on TSA agar for CFU ml^{-1} determination. Generation time was calculated from the log-phase of AH2547 proliferation. Growth curves of Pen G (Penicillin G sodium; Sigma-Aldrich, St. Louis, MO) treated and untreated bacteria were determined with a Biotek Synergy HT microplate reader (Biotek Instruments Inc., Winooski, VT) in a 96-well plate format as described by Ausbacher *et al.* (Ausbacher *et al.* 2014). In brief, overnight cultures were diluted 1000 times in fresh TSB and subsequently incubated under aerobic conditions at 37°C, 200 rev min^{-1} for 4 h. The planktonic cultures of *Staph. aureus* were diluted to 10^6 CFU ml^{-1} and incubated in TSB and under the presence of 0·15 μmol l^{-1} Pen G. Turbidity changes (OD_{600}) were recorded every 15 min at 37°C for a total of 18 h.

Formation of 48 h biofilms in CDC reactor

Biofilms were formed on glass coupons (diameter 1·27 cm) according to a modification of ASTM Method E2562-17 and Buckingham-Meyer *et al.* (Buckingham-Meyer *et al.* 2007; ASTM International, 2017). In brief, a CDC reactor containing 500 ml full strength TSB and chloramphenicol (10 μg ml^{-1}) was inoculated with 1 ml of a 10^9 CFU ml^{-1} overnight GFP *Staph. aureus* culture grown in full strength TSB supplemented with 10 mg ml^{-1} chloramphenicol for plasmid retention. The biofilm grew in batch conditions at 37°C, 125 rev min^{-1} for 24 h. Continuous flow of one-tenth TSB was applied subsequently for another 24 h at 37°C and 125 rev min^{-1} before coupons were sampled from the reactor.

Treatment-flow-cell and confocal microscopy

Coupons were transferred to the treatment-flow-cell (model FC310; Biosurface Technologies Corp., Bozeman, MT) with the low shear side up (side that faced the reactor wall). A flow of full strength TSB (2 ml min^{-1}, 37°C) was applied for three minutes to stabilize the system and for adjusting instrument settings. Untreated controls were treated with TSB. The penicillin G treatment (400 μmol l^{-1} Pen G in TSB) was applied after the system was stable. The pH of TSB was not affected by the presence of the antibiotic. Images were acquired of the bright field and GFP channel using a Leica SP5 confocal laser scanning microscope. The *z*-stack step size was set to 10 μm. Movie generation was carried out with IMARIS® (Bitplane Inc., South Windsor, CT) and image analysis with MetaMorph® (Molecular Devices, LLC, Sunnyvale, CA). The FIJI software bundle was used for generating overlay images (Schindelin *et al.* 2012).

Single tube method for treatment efficacy testing

ASTM Method E2871, generally known as the single tube method, was used to quantitatively measure the log reduction in viable biofilm cells exposed to a Pen G for 1, 2 and 3 h (ASTM International, 2013). Briefly, coupons containing *Staph. aureus* biofilm were removed from the CDC reactor, rinsed and then transferred to 50 ml conical tubes with tweezers. Subsequently, 4 ml of TSB or 400 μmol l^{-1} Pen G prepared in TSB were carefully added to the tubes. The tubes were incubated at 37°C under static conditions. At each specific time point, 36 ml D/E broth was added and the biofilm was disaggregated by sonication and vortexing according to ASTM E2871. All tubes were kept on wet ice and each sample was diluted immediately to neutralize the Pen G. The diluted samples were drop plated on TSA plates, incubated overnight at 37°C and enumerated.

Biofilm formation and treatment in 96-well plates

We performed a similar static assay in 96-well plates to investigate if a comparable effect was found in another

test system commonly used in biofilm research. Biofilms were formed and treated as described by Ausbacher *et al.* (2014). After treatment, the biofilm GFP fluorescence was measured using a BioTek Synergy H1 (Biotek Instruments Inc., Winooski, VT), multi-mode plate reader.

Statistics

The Students *t*-test was performed using the quantitative data from the single-tube method and 96-well plate assays using SigmaPlot 13.0 (Systat Software Inc., San Jose, CA).

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Conflict of Interest

No conflict of interest is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Staphylococcus aureus biofilm containing coupons, sampled from a CDC biofilm reactor, were transferred to the treatment-flow-cell with the low shear side up. TSB (2 ml min^{-1}, 37°C) was applied for three minutes to stabilize the system and continued for our untreated controls. Once stabilized, the treated coupons were exposed to 400 μmol l^{-1} Pen G in flowing TSB. Images were acquired with a Leica SP5 confocal laser scanning microscope using transmission and GFP channels. The *z*-stack step size was set to 10 μm and movie generation was carried out with IMARIS® (Bitplane, South Windsor, CT, USA).

Video S1. Video microscopy of experiment 2 – Pen G 400 μmol l^{-1} (GFP and brightfield channels)

Video S2. Video microscopy of experiment 2 – untreated control (GFP and brightfield channels)