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Densely adherent growth mode, rather than extracellular polymer substance matrix build-up ability, contributes to high resistance of *Staphylococcus epidermidis* biofilms to antibiotics

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Objectives: (i) To evaluate the role of the adherent growth mode and extracellular polymer substance build-up in biofilm resistance to antibiotics. (ii) To re-assess various mechanisms leading to biofilm resistance to antibiotics.

Methods: We compared the biofilm MICs, biofilm MBCs using the viable count method, biofilm MBCs based on broth recovery methods and minimum biofilm eradication concentrations (MBECs) of antistaphylococcal antibiotics for multilayer biofilms formed by 'biofilm-positive' *S. epidermidis* strains and monolayer biofilms formed by their 'biofilm-negative' mutants/variants. Bacterial densities and the quantity of persister cells in both multilayer and monolayer biofilms were assessed to evaluate their roles in biofilm resistance.

Results: Monolayer and multilayer biofilms presented similar susceptibilities to multiple antibiotics, based on biofilm MIC, broth recovery-based biofilm MBC and MBEC results. Multilayer biofilms demonstrated higher viable count-based MBCs than monolayer biofilms. Both monolayer and multilayer biofilms had very high bacterial densities of $\sim 10^{11-12}$ cfu/mL. Persister cells were found in both monolayer and multilayer biofilms, but not in planktonic cultures at log phase. The presence of persister cells in monolayer and multilayer biofilms appeared to be strain and antibiotic dependent.

Conclusions: The adherent growth mode, rather than the ability to build up a typical multilayer biofilm structure, contributes to the high resistance of biofilms to antibiotics, and therefore might be the main virulence factor of coagulase-negative staphylococci (CoNS) with respect to antibiotic resistance. The presence of persister cells in CoNS biofilms plays an important role in antibiotic resistance. Growth at high bacterial densities is another significant factor in biofilm resistance.

Keywords: coagulase-negative staphylococci, multilayer biofilms, monolayer biofilms, antibiotic resistance, persister cells

Introduction

Coagulase-negative staphylococci (CoNS), in particular *Staphylococcus epidermidis*, are among the most common opportunistic pathogens of low virulence causing nosocomial infections.[1] Human infections associated with CoNS include various device-associated infections, neonatal necrotizing enterocolitis, endophthalmitis and urinary tract infections.[2] Biofilm formation has been recognized as the main virulence factor of CoNS.[3,4] The ability of CoNS to adhere to a substratum and establish biofilms renders the bacteria highly tolerant to external stresses, such as the presence of antibodies, antimicrobials and other adverse environmental conditions.[4,5] High resistance to multiple antibiotics has been accepted as the main characteristic of biofilms responsible for the frequent failures of treatment of biofilm-related infections. Antibiotic resistance of CoNS biofilms is likely to have multiple causes, including failure of antibiotics to reach the extracellular polymer substance (EPS)-embedded biofilm cells, slow bacterial growth, a microenvironment unfavourable to antimicrobial activity, activation of stress response within biofilms, phenotypically resistant persister cells and genotypically resistant cells selected by antibiotic exposure in biofilms.[6,7]

CoNS have been previously categorized by Christensen *et al.*[8] and Deighton *et al.*[9] into three groups (biofilm positive, biofilm weak and biofilm negative) based on the optical density measurement of the adherent growth stained with Hucker Crystal Violet. It should be noted that even the widely accepted

biofilm-negative CoNS reference strain, *Staphylococcus hominis* SP2 (ATCC 35982), forms an adherent monolayer on the substratum.[8] Though not specifically stated, it is generally believed that the thicker the EPS matrix the biofilm cells build up, the higher the resistance they gain to multiple antibiotics. New categories have been recently proposed for bacterial biofilms. Karatan and Watnick[10] used a specific term 'multilayer biofilms' to represent the conventional biofilms, in which the bacteria attach to both the substratum and the neighbouring bacteria. Biofilm-forming bacteria in this category are characterized by their capability for previously defined 'cell to cell adherence'. The adherent monolayer of Christensen *et al.*[8] was then defined as 'monolayer biofilm'. Bacteria in this category are only able to perform 'initial attachment', the first stage of conventional CoNS biofilm formation.[10] In contrast to multilayer biofilms, the monolayer biofilms represent a homogenous collection of surface-attached cells.[10]

To date, most biofilm research concerning antibiotic resistance has focused on the 'biofilm-positive' phenotype or the multilayer biofilms.[11,12] There is a lack of studies on the antibiotic susceptibility of the 'biofilm-negative' phenotype or monolayer biofilms.

Different concentrations have been used to guide the treatment of biofilm-associated infections with antimicrobial agents. The biofilm MIC represents a concentration that inhibits biofilms from immediately establishing a planktonic bacterial population. This concentration targets the early acute phase of biofilm-related infections.[13] The biofilm MBC represents a concentration that reduces the bacterial load by 3 logs (99.9% kill) in the biofilm population[14] or kills most of the biofilm cells.[15] This concentration was proposed to be used to cure biofilm-related infections at a chronic stage. However, vulnerable patients with CoNS biofilm infections often have a weakened immune system, which can be further compromised by the biofilm EPS. Any survivors from antibiotic treatment at a biofilm MBC level might fail to be eradicated by the immune system and would eventually re-build the biofilm structure.[16,17] Therefore, the minimum biofilm eradication concentration (MBEC), which defines the concentration to definitively eradicate biofilm cells (100% kill), is necessary for the successful treatment of biofilm-related infections.[13]

The aims of this study were to: (i) evaluate the role of the adherent growth mode and biofilm EPS build-up in biofilm resistance to antibiotics; and (ii) re-assess various mechanisms leading to biofilm resistance to antibiotics.

Materials and methods

Bacterial isolates and growth conditions

Four *S. epidermidis* strains were used in this study; RP62a (ATCC 35984), RP62a^{CRV}, M187 sp11 and M187 sn3. *S. epidermidis* M187 mutants sp11 and sn3 were kindly donated by Dr Gene Muller (previously in the Channing Laboratory, Brigham and Women's Hospital, Boston, MA, USA) and were produced by an insertion of Tn*917*-LTV1 into a 'biofilm-positive' *S. epidermidis* clinical strain M187.[18] M187 sp11 is phenotypically identical to M187 and M187 sn3 is a biofilm-deficient mutant. *S. epidermidis* RP62a^{CRV} was also donated by Dr Gene Muller. It was previously selected from RP62a and has a 'biofilm-negative' phenotype.

Antibiotic susceptibilities of *S. epidermidis* in a planktonic mode

Four antistaphylococcal antibiotics were chosen for this study; oxacillin (β-lactam), vancomycin (glycopeptide), ciprofloxacin (fluoroquinolone) and rifampicin (rifamycin) (Sigma–Aldrich, Sydney, Australia). Antibiotic susceptibilities of the four strains were evaluated by determining the MICs and MBCs, according to CLSI (formerly NCCLS) guidelines.[19]

MICs for monolayer/multilayer biofilms

The method to determine MICs for biofilm-grown bacteria was adapted from that of Ceri *et al.*[13] Biofilms were established in 96-well microplates and exposed to 100 μL of 2-fold serial dilutions of antibiotics ranging from 2560 or 640 mg/L (for rifampicin) to 0.001 mg/L, in Mueller–Hinton broth (MHB; Oxoid, Hampshire, UK) or MHB plus 1% NaCl (only for oxacillin). Microplates were then incubated at 35°C for 18 h (for ciprofloxacin and rifampicin) or 24 h (for oxacillin and vancomycin). After overnight challenge, the supernatants from each well were carefully transferred to a new 96-well microplate without disturbing the biofilms, and the turbidity of the contents was visually assessed. The biofilm MIC was defined as the lowest concentration of antibiotics at which no visible growth was observed.

MBCs for monolayer/multilayer biofilms

Three different methodologies were used to define MBCs of antibiotics for *S. epidermidis* monolayer/multilayer biofilms. The first methodology was referred to as the viable count-based biofilm MBC.[20] To determine the number of bacteria in established monolayer/multilayer biofilms before antibiotic exposure, 100 μL of MHB was added to a microwell containing an established biofilm. The biofilm matrix was immediately scraped with sterile pipette tips and sonicated (Branson 450 sonifier with a microtip) for 8 s (2 s×4) at 10% of the maximum amplitude. Any undetached cells were then collected with a sterile cotton-tipped swab by carefully swabbing the well bottom. The bacterial suspensions, together with the cotton tip of the swab, were placed in a sterile tube containing 900 μL of PBS. The tube was vortexed at a high speed to dislodge the bacteria from the swab and to break up the bacterial clumps (30 s×4). Viable counts were performed for the suspensions containing single biofilm cells. The minimum concentration of antibiotics that reduced bacterial numbers by at least 3 logs (99.9%) was taken as the viable count-based biofilm MBC.

The second and third MBC methodologies were both based on a published broth recovery method, with or without a modification.[15] After treatment, antibiotics were removed and the microwells were washed three times with sterile PBS. The microwells were then processed with: (i) a broth recovery-based method [200 μL of tryptone soya broth (TSB; Oxoid, Hampshire, UK) was added into each well and the microplates were then incubated for a further 48 h with shaking (100 rpm)]; and (ii) a modified broth recovery-based method [200 μL of TSB and 0.2 g of antibiotic-removal resin beads (Amberlite XAD-16) were added into each well and the microplates were then incubated for a further 24 h with shaking (100 rpm)]. The supernatants were carefully transferred to a new 96-well microplate, and the turbidity of the contents was visually assessed. The minimum concentrations of antibiotics that resulted in no visible bacterial growth in the suspensions were defined as the broth recovery-based biofilm MBC and the modified broth recovery-based biofilm MBC, respectively.

MBECs for biofilms

After the broth recovery-based biofilm MBCs were determined, the remaining biofilms were then swabbed with a cotton-tipped swab, cultured on a nutrient agar plate and incubated for 3 days at 35°C.[21] The MBEC was the minimum concentration resulting in no bacterial growth.

Confocal laser scanning microscopy (CLSM)

One of the following treatments was applied to the *S. epidermidis* monolayer/multilayer biofilms for 24 h: (i) MHB, as an untreated biofilm control;

(ii) 2560 mg/L oxacillin; (iii) 1280 mg/L vancomycin; (iv) 128 mg/L ciprofloxacin; or (v) 32 mg/L rifampicin. The treated monolayer/multilayer biofilms were stained with the LIVE/DEAD BacLight bacterial viability kit (3.35 μM Syto-9 and 20 μM propidium iodide) at 22°C for 15 min in the dark. The viability of biofilm cells was examined immediately with a Nikon A1R confocal laser scanning microscope equipped with a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., USA).

Bacterial densities of monolayer/multilayer biofilms

The calculation of bacterial densities of monolayer/multilayer biofilms was based on the formula:

$$\text{biofilm bacterial density} = \frac{\text{viable count of biofilms}}{\text{volume of biofilms}}$$

Bacterial numbers of biofilms were determined by performing scraping, sonication, swabbing and viable count, as described earlier. The volume of biofilms was calculated by the following formula:

$$\text{biofilm volume} = \text{average biofilm thickness} \times \text{average biofilm area}$$

Biofilm thickness can be measured after constructing a three-dimensional biofilm structure using CLSM. The mean of nine thickness values from three different biofilms was used to represent the overall biofilm thickness. The area covered by attached cells was quantified by using the area of the microwell bottom and the percentage of bacterial coverage of the microwell bottom (Image J software, NIH, USA). The mean of nine individual values from three different biofilms was used as the overall biofilm area.

Production and quantification of persister cells in monolayer/multilayer biofilms and planktonic cultures at log phase

The prerequisite of the biofilm persister cell study was to obtain a culture of single biofilm cells, excluding biofilm-related factors that might affect the performance of antibiotics, such as the presence of EPS matrix and highly concentrated hydrolytic enzymes.[7,22] To collect single biofilm cells, 2 mL of MHB was added to an established biofilm in 12-well microplates (Corning, NY, USA) and the wells were scraped thoroughly with sterile tips. The microwells were then treated with sonication, swabbing and vortex, as described earlier. The suspensions were filtered with a 1.2 μm Acrodisc syringe filter (Pall, MI, USA) to remove any bacterial clumps.

Bacterial suspensions of biofilm cells and planktonic cells at log phase were then adjusted to a turbidity equivalent to that of a 0.5 McFarland standard. The choices of antibiotic concentrations and exposure times to select the persister cells were based on our preliminary bacterial killing curve of *S. epidermidis* RP62a. Volumes of 0.9 mL of the bacterial suspensions were transferred to sterile Eppendorf tubes (Sarsted, Nombrecht, Germany) containing 0.1 mL of the designated antibiotics to reach a final concentration of broth recovery-based biofilm MBC. The tubes were then incubated statically at 35°C for 24 h (for oxacillin, ciprofloxacin and rifampicin) or 48 h (for vancomycin). After antibiotic exposure, 1 g of the antibiotic-removal resin beads (Amberlite XAD-16) was added into the tube, followed by vortexing, incubation for 15 min and then viable counts.[23,24] The ratio of bacterial survival (bacterial density after antibiotic treatment) in the initial culture (bacterial density before antibiotic treatment) (×100) was estimated as the percentage of persister cells in the corresponding bacterial populations.

Data analysis

Antibiotic susceptibility experiments were repeated at least three times. A fourth replicate was performed if the results were inconsistent. The geometric mean ($\log_2$) was calculated and used for comparison. Based on the acceptable range of MICs for antibiotic quality control for *S. aureus* ATCC 25913, an increase of $>2\times\log_2$ in MIC, MBC or MBEC of oxacillin, vancomycin, ciprofloxacin and rifampicin was considered significant.[19] Persister cell experiments were performed twice in triplicate. The paired *t*-test was used to compare the percentage of persister cells in different populations, and a P value <0.05 was considered to indicate significance.

Results

Antibiotic susceptibilities of *S. epidermidis* isolates

Both *S. epidermidis* RP62a and its biofilm-negative variant RP62a^{CRV} and *S. epidermidis* M 187 sp11 and its biofilm-negative mutant sn3 had the same susceptibilities to oxacillin, vancomycin, ciprofloxacin and rifampicin, respectively (Table 1).

Antibiotic susceptibilities of monolayer/multilayer biofilms

In general, there were no significant differences in biofilm MICs, broth recovery-based biofilm MBCs, modified broth recovery-based biofilm MBCs or MBECs of antibiotics between multilayer biofilms of *S. epidermidis* 'biofilm-positive' strains and monolayer biofilms of their 'biofilm-negative' mutant/variant (Table 1). The biofilm MICs of rifampicin and ciprofloxacin were generally similar to the CLSI MICs for planktonic cultures; however, biofilm MICs of oxacillin and vancomycin were significantly greater than CLSI MICs in the cases of the two *S. epidermidis* M187 strains. Modified broth recovery-based biofilm MBCs and MBECs were much higher than CLSI MBCs as well as broth recovery-based biofilm MBCs, with most values above the detection limit (640 mg/L for rifampicin and 2560 mg/L for the other antibiotics). Although there were generally no significant differences between broth recovery-based MBCs of any antibiotics for multilayer and monolayer biofilms, there were significant differences in viable count-based biofilm MBCs. Multilayer biofilms showed much higher resistance to all four antibiotics than monolayer biofilms, when the analysis was based on viable count-based biofilm MBCs (Table 1).

Bacterial densities of monolayer and multilayer biofilms

Though multilayer biofilms after overnight incubation in TSB in a 96-well microplate contain more bacterial cells in total number than the corresponding monolayer biofilms ($\sim10^8$ cfu versus $\sim10^7$ cfu), they had similar bacterial densities of 10^{11-12} cfu/mL ($10^{11.9\pm0.1}$ cfu/mL for *S. epidermidis* RP62a, $10^{11.7\pm0.1}$ cfu/mL for *S. epidermidis* M187 sp11, $10^{11.5\pm0.1}$ cfu/mL for *S. epidermidis* RP62a^{CRV} and $10^{11.1\pm0.1}$ cfu/mL for *S. epidermidis* M187 sn3, respectively). The bacterial densities of both multilayer and monolayer biofilms were much higher than those of planktonic cultures at either log phase ($\sim10^{6-8}$ cfu/mL) or stationary phase ($\sim10^{9-10}$ cfu/mL).

Table 1. Antibiotic susceptibilities of monolayer biofilms and multilayer biofilms (mg/L)[a]

	Antibiotics and bacteria															
	oxacillin				vancomycin				ciprofloxacin				rifampicin			
	RP62a	RP62a^{CRV}	M187 sp11	M187 sn3	RP62a	RP62a^{CRV}	M187 sp11	M187 sn3	RP62a	RP62a^{CRV}	M187 sp11	M187 sn3	RP62a	RP62a^{CRV}	M187 sp11	M187 sn3
CLSI MIC	64	64	0.5	0.5	1	1	1	1	0.12	0.12	0.12	0.12	0.004	0.004	0.004	0.004
CLSI MBC	64	64	1	1	2	2	1	2	0.12	0.12	0.12	0.12	0.008	0.008	0.004	0.004
Biofilm MIC	128	320	64	128	4	4	8	4	0.25	0.25	0.5	0.5	0.002	0.002	0.004	0.004
Viable count-based biofilm MBC	>2560	640	>2560	128	>2560	64	>2560	32	128	0.5	1280	1	0.03	0.015	320	0.25
Broth recovery-based biofilm MBC	>2560	>2560	>2560	>2560	640	1280	64	128	128	64	128	64	64	16	32	32
Modified broth recovery-based biofilm MBC	>2560	>2560	>2560	>2560	>2560	>2560	2560	>2560	1280	640	>2560	2560	128	64	>640	>640
MBEC	>2560	>2560	>2560	>2560	>2560	>2560	>2560	2560	>2560	1280	>2560	>2560	>640	>640	>640	>640

[a]All MIC, MBC and MBEC values are geometric means of the results of at least three independent measurements, taken to the closest doubling dilution.

CLSM

The CLSM study showed that even when exposed to a very high concentration of antibiotics, a small fraction of bacteria in both monolayer biofilms and multilayer biofilms still survived antibiotic killing (Figure 1).

Quantification of persister cells in monolayer/multiplayer biofilms and planktonic cultures at log phase

After exposure to antibiotics at concentrations equivalent to the broth recovery-based biofilm MBC for a pre-determined period, there were no surviving persister cells in planktonic cultures at log phase. In contrast, ∼1%–0.01% of persister cells remained viable in the biofilm populations (Figure 2). The percentage of persister cells in biofilms appeared to be strain and antibiotic dependent. There was no significant difference in the percentage of oxacillin persister cells present in monolayer biofilms and multilayer biofilms. When selected with vancomycin or ciprofloxacin, multilayer biofilm formed by *S. epidermidis* M187 sp11 had significantly more persister cells than monolayer biofilm of M187 sn3 (P values of 0.005 and <0.001); however, fewer persister cells were found in multilayer biofilms of RP62a than the corresponding monolayer biofilms of RP62a^{CRV} (P values of 0.014 and 0.007). Selection of persister cells by rifampicin was not successful due to the emergence of mutant strains after overnight exposure (data not shown).

Discussion

In this study, we used pairs of 'biofilm-positive' *S. epidermidis* strains and their 'biofilm-negative' mutants (or variants) to show that at least for *S. epidermidis*, once grown in an adherent mode, the bacteria become highly resistant to antibiotics, regardless of their abilities to build up an EPS matrix. There was no significant difference in antibiotic resistance defined by the biofilm MIC, broth recovery-based biofilm MBC, modified broth recovery-based biofilm MBC and MBEC between the monolayer biofilms produced by those so-called 'biofilm-negative' strains and multilayer biofilms established by 'biofilm-positive' strains. The CLSM study confirmed that, after exposure to antimicrobial agents at a high concentration (usually ≥1000× the MIC), viable cells still remained in the monolayer biofilms. Results from this study are supported by a recent study that showed that strains of *Pseudomonas syringae* pv. *theae* with different biofilm production abilities had similar susceptibilities to bactericides.[25] In addition, it has been reported that no correlation exists between the antibiotic susceptibility of *S. epidermidis* biofilms and the amount of slime produced.[26,27] We have also found similar responses of monolayer/multilayer biofilms of different CoNS isolates to antibiotic lock solutions, regardless of the amount of biofilm they produced.[28] Taken together, it should be questioned whether the conventionally defined 'biofilm formation' is a true virulence factor of CoNS with respect to high resistance to antibiotics, as the biofilm EPS build-up ability is not directly related to antibiotic resistance. We propose that the adherent growth mode of high density, rather than the ability of bacterial strains to produce EPS matrix or to form a typically 'conventional' biofilm, is responsible for CoNS biofilm resistance to various antibiotics and should be considered as a major

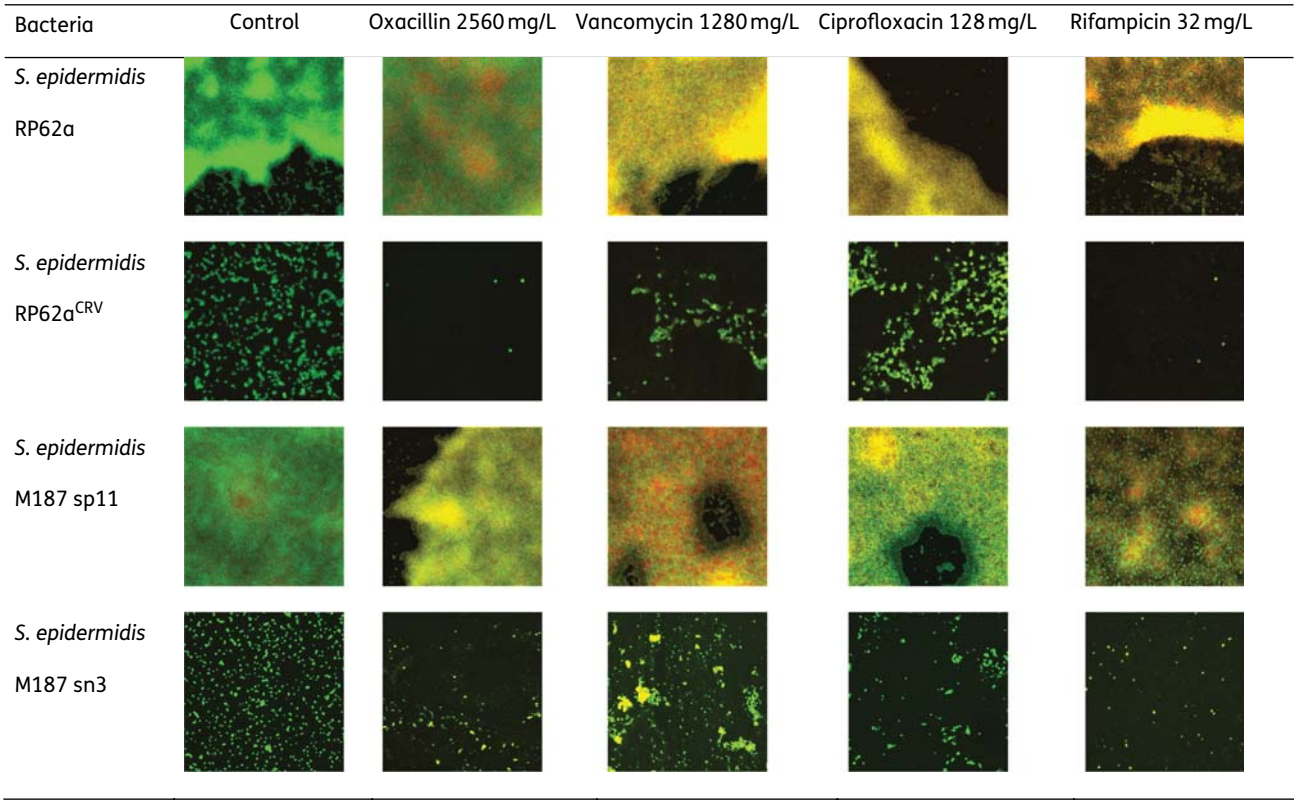

Figure 1. Survival of biofilm cells from antibiotic exposure. Biofilms of *S. epidermidis* RP62a, RP62a^{CRV}, M187 sp11 and M187 sn3 were treated with antibiotics at usually 1000× the MICs or even higher, then stained with Syto-9 (bright green for live cells) and propidium iodide (red for dead cells). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

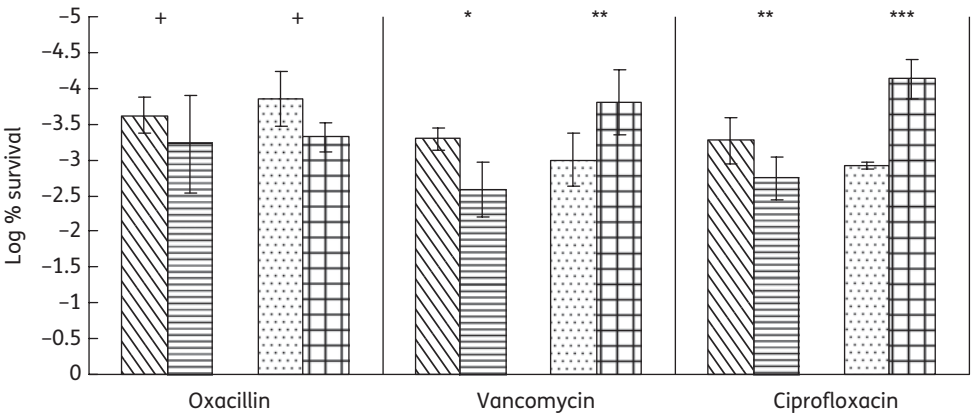

Figure 2. Survival of the persister cells in *S. epidermidis* multilayer biofilms [RP62a (diagonal lines) and M187 sp11 (dots)] and monolayer biofilms [RP62a^{CRV} (horizontal lines) and M187 sn3 (check pattern)]. Log % survival is a logarithm format of the percentage of persister cells for statistical analysis. For example, log % survival of −2 stands for ∼1% (10^{-2}) of the original population persisting the antibiotic exposure. The lower the log % survival is, the fewer persister cells the corresponding cell population can produce. $+P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

virulence factor of CoNS with regard to antibiotic resistance. EPS build-up ability of CoNS mainly contributes to their tolerance to innate immune defences of the human body.[29]

In contrast to similar levels of resistance defined by biofilm MICs, broth recovery-based biofilm MBCs, modified broth recovery-based biofilm MBCs and MBECs, multilayer biofilms demonstrated significantly higher viable count-based MBCs than monolayer biofilms. This could be explained by the different stresses that monolayer and multilayer biofilm cells encounter. We propose that there are three subpopulations in a bacterial biofilm: a small fraction of normal cells; a large fraction of stressed cells with variable tolerance to antibiotics; and a small

fraction of persister cells. Our preliminary bacterial killing curves of *S. epidermidis* RP62a biofilm supported this hypothesis (data not shown). The tri-phasic curve consisted of an initially small but sharp drop in viable count, followed by a gradual drop, and then a level plateau of viable cells, corresponding to the normal cells, stressed cells and persister cells, respectively. CLSI MBCs, modified broth recovery-based biofilm MBCs and MBECs target these three populations, respectively. In a multilayer biofilm, cells are stressed by a series of factors, including a high cellular density, a lack of oxygen and nutrients, and accumulated waste products from cellular metabolism. These stressed cells have an overall high resistance to antibiotics. In contrast, cells in a monolayer biofilm, mostly stressed by the high-density growth, have a comparatively low tolerance to antibiotics. Thus, it is not surprising that viable count-based biofilm MBCs, which mainly target most but not all of the stressed cells (99.9% of the total population), would be higher for multilayer biofilms than for monolayer biofilms.

Broth recovery-based biofilm MBCs obtained with the previously published methods were generally lower than those by our modified method that used resin beads to bind antibiotics, suggesting the presence of antibiotic carryover effects in biofilms after antibiotic exposure. This difference could be explained by the slow diffusion of antibiotics such as vancomycin and rifampicin through biofilms.[30,31] Any trapped antibiotic would continue to destroy biofilm bacteria unless bound with resin beads.

The percentages of persister cells in different bacterial populations were compared in this study. The presence of persister cells in biofilms, rather than differences in their numbers, seemed to influence their antibiotic resistance. Persister cells are defined as a small fraction of cells recognized as 'drug-tolerant' cells that neither grow nor die in the presence of bactericidal antibiotics.[32] Our study found that both monolayer and multilayer biofilms of *S. epidermidis* contain persister cells that were not present in planktonic log-phase cultures, suggesting an important role for persister cells in biofilm resistance. Our finding is in agreement with recently published studies;[32,33] however, other studies reported the presence of persister cells in planktonic culture at log phase.[34,35] This contradiction could be explained by the different methods used to eradicate the non-persister cells in the different studies. We specifically chose pre-determined concentrations and exposure times based on a bacterial killing curve to select the persister cells and to ensure complete eradication of all normal cells and stressed cells. However, other studies used antibiotics at 8–10× the MICs, or even a random high concentration, and exposure time of 3 or 24 h to isolate the persister cells.[34] It is possible that exposure to antibiotics at 8–10× the MICs for even 24 h would select a mixture of persister cells and stressed cells from biofilms, rather than a pure culture of persister cells. In addition, the percentage of persister cells isolated from monolayer and multilayer biofilms appeared to be antibiotic and strain dependent. No unique difference in persister cell production was found between monolayer and multilayer biofilms. It seems that adherent growth mode, rather than formation of complex biofilm architecture, plays an important role in persister formation. Similar findings of persister cells in *Candida albicans* biofilms were presented by LaFleur *et al.*[36]

Antibiotic resistance of CoNS biofilms has been attributed to multiple causes.[6,7] Based on the present study, neither the diffusion of antibiotic into the biofilm matrix nor the unfavourable microenvironment appeared to play a major role in the antibiotic resistance of biofilms, as the monolayer biofilms and multilayer biofilms showed similar antibiotic susceptibilities. In our study, despite the different number of persister cells, monolayer and multilayer biofilms showed a generally similar response to antibiotics. We therefore propose that the presence of persister cells, rather than the quantity of persister cells in biofilm bacterial populations, is likely to be the key to biofilm resistance. Both monolayer and multilayer biofilms were found to be densely populated with bacteria. Thus activation of high density-related stress responses is probably another important cause of biofilm resistance. In addition, as the monolayer biofilms presented high resistance to antibiotics, expression of resistance genes induced by contact with a surface (adherent growth mode) cannot be excluded from factors causing biofilm resistance.[37,38] The genotypic resistance of biofilm cells was not considered as a major factor leading to biofilm resistance to antibiotics as in our study planktonic culture, monolayer and multilayer biofilms all produced resistant mutants after exposure to rifampicin, but not to other antibiotics.

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Transparency declarations

None to declare.

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