Evidence for persisters in *Staphylococcus epidermidis* RP62a planktonic cultures and biofilms

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The pathogenesis of *Staphylococcus epidermidis* in foreign device-related infections is attributed primarily to its ability to form biofilms on a polymer surface. One mechanism proposed for the survival of organisms in a biofilm is the presence of persister cells. Persister cells survive antibiotic treatment without acquiring heritable antibiotic resistance. This study was conducted to determine if *S. epidermidis* RP62a growing in planktonic cultures and biofilms could survive as persister cells following treatment with levofloxacin and vancomycin. *S. epidermidis* RP62a produced a small percentage of persisters (levofloxacin, 3.09×10^{-7} %; vancomycin, 8.21×10^{-5} %) when grown to exponential phase, whereas biofilms contained 28 and 94 % persisters, following exposure to levofloxacin and vancomycin, respectively. The highest percentages of persisters were obtained during stationary phase in planktonic cultures and the lowest percentages of persisters were obtained during mid-exponential phase. An increase in persister number was not due to activation of quorum-sensing regulons. Confocal laser scanning microscopy images of biofilms exposed to levofloxacin demonstrated that the antibiotic was able to kill bacteria throughout the biofilm. Our results suggest that antibiotic tolerance in biofilms and in planktonic cultures of *S. epidermidis* RP62a is due in part to the presence of persister cells.

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INTRODUCTION

Staphylococcus epidermidis is a commensal bacterium of the human skin and mucous membranes (Otto, 2008). It is a frequent cause of health-care-associated infections and of infections on implanted medical devices (Mack *et al.*, 2000; O’Gara & Humphreys, 2001). The pathogenesis of *S. epidermidis* in foreign device-related infections is attributed primarily to its biofilm formation on the surfaces of implanted medical devices (Qin *et al.*, 2007).

A biofilm is an assemblage of surface-attached microbial cells that are embedded in a matrix of exopolysaccharide (EPS) material (Xu *et al.*, 2006). Biofilms can form on living tissues and indwelling medical devices (Donlan, 2002). The growth of a biofilm on medical devices plays a significant role in health-care-associated infections as they are responsible for the majority of recalcitrant infections (Keren *et al.*, 2004a). Despite prolonged antibiotic therapy, pathogens linger within these biofilms for long periods of time. The long-term survival of organisms in biofilms appears to be due, in part, to the presence of persister cells (Lewis, 2005, 2007).

Unlike antibiotic-resistant bacteria, which grow in the presence of an antibiotic, bacterial persisters consist of a small subpopulation of bacteria that survive exposure to

lethal concentrations of antibiotics (Lewis, 2005, 2007). Persister cells are phenotypic variants that lack genetic traits for antibiotic resistance, resulting in progeny that are just as sensitive to antibiotic treatment as their parent cells (Lewis, 2005; Shah *et al.*, 2006). They are responsible in part for high levels of multi-drug tolerance and exhibit tolerance in that they do not multiply, nor do they die in the presence of antibiotics (Keren *et al.*, 2004a; Lewis, 2007).

The persistence phenomenon and the identification of genes responsible for the generation of persisters have been studied extensively in *Escherichia coli*, and to a lesser extent in *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Bigger, 1944; Jöers *et al.*, 2010; Keren *et al.*, 2004a, b; Möker *et al.*, 2010; Singh *et al.*, 2009). However, little has been done thus far to demonstrate the production of persisters by *S. epidermidis*. We proposed that in a subpopulation of a biofilm-producing methicillin-resistant *S. epidermidis* (MRSE), *S. epidermidis* RP62a, persisters would be detected after treatment with clinically effective doses of antibiotic.

METHODS

Bacterial strains and standard culture conditions. *S. epidermidis* RP62a was purchased from the American Type Culture Collection (ATCC 35984), and suspended in trypticase soy broth (TSB) containing 10 % glycerol (Fisher Scientific) for long-term storage

Abbreviations: CLSM, confocal laser scanning microscopy; DFR, drip flow reactor; EPS, exopolysaccharide; MRSE, methicillin-resistant *Staphylococcus epidermidis*; PI, propidium iodide.

and kept at -70°C (Christensen *et al.*, 1990). Frozen cultures were thawed at room temperature and streaked for isolation on trypticase soy agar plates (TSA). Plates were stored at 4°C following incubation. Overnight cultures used to produce biofilms of *S. epidermidis* RP62a were prepared by inoculating an isolated colony from the TSA plates in 5 ml TSB and incubating overnight at 37°C (16–18 h) with rotation (60 r.p.m.). When overnight cultures were needed for planktonic cultures, the frozen cultures were thawed at room temperature, then 50 μl *S. epidermidis* RP62a was inoculated in 5 ml TSB and incubated overnight at 37°C . The overnight cultures (16 h) were diluted 1:1000 into 100 ml TSB in a 500 ml flask. In all planktonic culture experiments, cultures were incubated at 37°C with shaking at 100 r.p.m..

Determination of optimal antimicrobial agent concentration and time for maximal killing. Standard culture conditions as described above were used to prepare a *S. epidermidis* RP62a culture. During exponential phase ($\text{OD}_{600} = 1.25 \pm 0.05$, or about 5×10^8 c.f.u. ml^{-1}), 1 ml culture was removed for plating assays (Keren *et al.*, 2004a). The remaining culture was divided into six aliquots and received various concentrations (0, 10, 25, 50, 75 and 100 $\mu\text{g ml}^{-1}$) of the antimicrobial agents levofloxacin (Sigma) or vancomycin (Sigma) dissolved in sterile water, and shaken at 37°C for 3 or 16 h, respectively. The samples treated with antimicrobial agents were washed twice in 1 ml cold TSB, centrifuged for 2 min at 14 000 g, pelleted, resuspended in 1 ml cold TSB for serial 10-fold dilutions and then plated on TSA plates. The optimal concentrations of the antimicrobial agents were considered to be the lowest concentration that yielded the fewest c.f.u. ml^{-1} (25 $\mu\text{g ml}^{-1}$ for levofloxacin and 50 $\mu\text{g ml}^{-1}$ for vancomycin). The optimal concentrations of the antimicrobial agents were then used to treat *S. epidermidis* RP62a and at various times (0, 16, 24, 48, 72 and 96 h) samples were removed for plating assays (c.f.u. ml^{-1}). All experiments were completed in triplicate.

Determination of the number of persister cells present at each phase of the growth cycle. Standard culture conditions as described above were used to prepare a 100 ml culture of *S. epidermidis* RP62a in TSB. At various times during the growth cycle (0, 0.5, 1, 1.5, 2–12 h), one sample was removed to determine the number of c.f.u. ml^{-1} and two other samples were treated with 25 $\mu\text{g ml}^{-1}$ levofloxacin or 50 $\mu\text{g ml}^{-1}$ vancomycin. After 24 h of exposure to levofloxacin and 48 h exposure to vancomycin, samples from each culture were washed twice in 1 ml cold TSB, centrifuged for 2 min at 14 000 g, pelleted, and then resuspended in 1 ml cold TSB for serial dilutions to be plated on TSA. All experiments were completed in triplicate.

Preparation of extracellular sterile supernatant. Standard culture conditions as described above were used to prepare a 16 h culture of *S. epidermidis* RP62a. The 16 h culture was diluted 1:1000 into 500 ml warm TSB and grown to stationary phase (8 h). The bacterial cells in the stationary phase culture of *S. epidermidis* RP62a were pelleted by centrifugation at 16 270 g for 15 min at 4°C and the supernatant was filter-sterilized (0.2 μm filter). The sterile supernatant was frozen at -70°C and then lyophilized. The supernatant was then suspended in distilled water and filter-sterilized (0.2 μm filter) to yield a $10 \times$ final concentration.

Effect of extracellular factors on persister cell number. A 16 h *S. epidermidis* RP62a culture was diluted 1:1000 in 90 ml warm TSB and 10 ml $10 \times$ TSB (control) or in 90 ml warm TSB and 10 ml $10 \times$ extracellular sterile supernatant. At various times (4, 6 and 8 h) samples were removed from both cultures. Samples were diluted for plating assays to determine c.f.u. ml^{-1} at each time period for both cultures. Samples of both cultures (10 ml) were removed at all time periods and treated with 25 $\mu\text{g ml}^{-1}$ levofloxacin for 24 h and 50 μg

vancomycin ml^{-1} for 48 h. At the end of the incubation period, samples were removed from the antimicrobial-treated cultures, washed (see below), diluted and plated to determine the number of c.f.u. ml^{-1} . All the samples treated with antimicrobial agents were washed twice in 1 ml cold TSB, centrifuged for 2 min at 14 000 g, pelleted, and then resuspended in 1 ml cold TSB for the plating of serial dilutions. All experiments were completed in triplicate.

Preparation of *S. epidermidis* biofilms utilizing a modified drip flow reactor (DFR). Biofilms were grown using a modified DFR (model 110; Biosurface Technologies Corporation) on glass microscope slides (25×75 mm) as described by Goeres *et al.* (2009). Each slide was washed with detergent, rinsed with distilled water, dried and placed in the DFR.

To produce a biofilm, the bacterial cells were allowed to attach to the surface of the glass slide in the DFR as it lay flat (batch mode) on an incubator shelf. Overnight cultures of *S. epidermidis* RP62a were prepared using the methods described above. A sample of the overnight culture (3 ml) was inoculated into 45 ml TSB in a 50 ml conical tube. Sixteen millilitres of the inoculum was placed in each of two DFR chambers at a time and incubated for various times (2, 4, 6 and 8 h) at 37°C in batch mode. Following the batch mode incubation time, four adjustable metal legs were inserted into the bottom of the DFR, to produce a 10° incline. A biofilm developed on the glass slides in the DFR device while TSB was supplied drop-wise over the glass slides for 48 h, providing a continuous flow of nutrients (flow mode). TSB was supplied through the influent port with a peristaltic pump (model 3385; Fisher Scientific) with the flow rate set at 50 ml h^{-1} . Once the optimal attachment time was determined, the slides were incubated in the batch mode at 37°C and then incubated in the flow mode for various times (12, 24, 48 and 72 h) at 37°C to determine the optimal maturation time. The biofilms were then removed from the glass slides and the number of viable adherent bacterial cells was determined as described below.

In each experiment, the viable adherent bacterial cells were expressed as the number of c.f.u. ml^{-1} . The glass slides containing the attached biofilm were removed from the DFR and rinsed with sterile distilled water to remove planktonic and loosely adherent cells. Biofilms were scraped from the glass surface into a 50 ml conical bottom tube containing 40 ml sterile ice-cold TSB per biofilm using a sterile wooden applicator stick. All tubes were placed on ice to inhibit cell growth. The suspensions of the biofilms were placed on ice and subjected to sonication (sonic dismembrator model 500; Fisher Scientific) for 2 min at 30 s intervals and 40 % amplitude to disperse cell aggregates. The dispersed micro-organisms were then diluted and plated on TSA plates. All plates were incubated for 48 h at 37°C . The set of conditions that produced the greatest number of viable adherent bacterial cells was considered the optimal time for cell attachment and maturation of the biofilm. Data from these experiments are represented as means $\pm$ SEM from quadruplicate experiments and differences in the means were compared using a Student's *t*-test with a *P* value ≤ 0.05 .

Antibiotic treatment of biofilms. Stock solutions of levofloxacin (10 mg ml^{-1}) and vancomycin (5 mg ml^{-1}) were diluted to a final concentration of 25 and 50 $\mu\text{g ml}^{-1}$, respectively, in sterile Petri dishes containing 10 ml TSB and mixed. A volume of sterile dH_2O equivalent to the volume used for the antibiotics was added to 10 ml TSB in a sterile Petri dish and mixed to serve as a control. Biofilms formed in the DFR were rinsed as above, removed and placed into the Petri dish containing the antibiotic-treated TSB or TSB, one slide per Petri dish. The Petri dishes were then incubated at 37°C for 24 h. The glass slides were then rinsed in sterile dH_2O and scraped into 50 ml conical tubes containing 40 ml sterile ice-cold TSB as above. All tubes were placed on ice to prevent further cell growth. The

suspensions of the biofilms were subjected to sonication as described above. Bacterial cells recovered from the biofilms were diluted and plated on TSA plates in triplicate. All plates were incubated for 48 h at 37 °C. The colonies were counted and the means of the c.f.u. ml⁻¹ were calculated for the control and antibiotic-treated biofilms. The surviving fraction was calculated by dividing the number of viable organisms subsequent to antibiotic treatment per biofilm by the number of control viable organisms per biofilm. Data for the percentage survival in biofilms are represented as means $\pm$ SEM from quadruplicate experiments and analysed using Student's *t*-test with a *P* value $\leq$ 0.05.

Confocal laser scanning microscopy (CLSM) analysis of biofilms. To observe the heterogeneity of *S. epidermidis* biofilms, CLSM and fluorescent dyes were utilized. Biofilms were grown using the DFR at the optimal attachment and maturation times. Standard glass microscope slides were too thick for the visualization of the biofilm; therefore, biofilms were grown on glass coverslips in the following manner. Glass microscope slides (25 $\times$ 75 mm) were placed into each chamber of the DFR. Three square glass coverslips (22 $\times$ 22 mm) were placed on top of the glass slide to form a single layer covering the slide. The slides and coverslips were then sterilized in the DFR. Bacterial cells were then placed on the glass slides in the DFR and treated as described above to produce a biofilm (6 h for attachment, 48 h for maturation). Control biofilms and those biofilms that had been exposed to antibiotic as described above were washed with sterile dH₂O to remove non-adherent bacteria. A LIVE/DEAD BacLight bacterial viability staining kit (Invitrogen Molecular Probes) was used to monitor the viability of bacterial cells as a function of the membrane integrity (Moscoco *et al.*, 2006). Biofilms were treated with 100 μ l of a freshly prepared solution of 6 μ M SYTO 9 stain and 30 μ M propidium iodide (PI). SYTO 9 stains all bacteria in a population, including those with intact and damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the green fluorescence of the SYTO 9 stain (dead cells appear yellow, live cells appear green). Biofilms were incubated for 15 min in the dark at room temperature. Excess staining was removed by washing the biofilms once with PBS (0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 0.14 M NaCl, pH 7.2). Stained biofilms were visualized using a Leica SP500 confocal laser scanning microscope. Green fluorescence emitted from SYTO 9 (excitation 488 nm and emission 500 nm) and red fluorescence emitted from the PI (excitation 543 nm and emission 565 nm) was recorded using a magnification of $\times$ 63. Biofilm structure was analysed as a series of horizontal optodigital sections, each 1 μ m thick, over the entire height (*z*-axis) of the biofilm.

Antibiotic susceptibility testing. The MICs for isolates from the antibiotic-treated and control *S. epidermidis* RP62a cultures were determined using a broth microdilution assay in 96-well microtitre plates as described by Darouiche *et al.* (2008). Ten isolated colonies from the surviving cells in the biofilms or planktonic cultures following antibiotic treatment and five isolated colonies from the control biofilms or planktonic cultures were randomly selected for overnight growth at 37 °C in TSB and diluted to $\sim$ 10⁵ c.f.u. ml⁻¹. Twofold dilutions of levofloxacin or vancomycin were prepared in the wells of a 96-well microtitre plate. A 100 μ l aliquot of bacterial suspension was added to each well. Plates were incubated at 37 °C for 24 h, and the lowest concentration of antibiotic with no visible growth was considered to be the MIC. MICs were obtained for cultures that survived antibiotic treatment and compared to MICs of the original culture without antibiotic treatment. Data are given as means $\pm$ SEM from quadruplicate experiments. A Shapiro-Wilk test showed MIC values for bacterial cultures were not normally distributed. Mean MIC values for persister-derived and original cultures were compared using a non-parametric Mann-Whitney U test; MICs were considered statistically different if the *P* value was $\leq$ 0.05. MICs were also compared to MICs of *S.*

epidermidis RP62a determined by the bioMérieux VITEK system at the North-East Regional Medical Center Clinical Laboratory, Kirksville, MO, USA. The VITEK system identified the micro-organism as *S. epidermidis* and determined the MICs for several different antibiotics (Spanu *et al.*, 2003). The VITEK system is an automated system that determines MIC values in a manner similar to the 96-well microtitre plate method described above.

Concentration-dependent kill curves of control and levofloxacin-treated biofilms. Control and levofloxacin-treated (25 μ g ml⁻¹, 24 h, 37 °C) biofilms were suspended by sonication, diluted 1:1000 in TSB and incubated overnight at 37 °C (16–18 h) with rotation (60 r.p.m.). The overnight cultures were diluted (1:200) and incubated at 37 °C with shaking until mid-exponential phase (OD₆₀₀ 1.25 $\pm$ 0.05). Levofloxacin was added at final concentrations of 5, 10, 25, 50 or 75 μ g ml⁻¹ to the mid-exponential phase cultures and incubated at 37 °C with shaking for 3 h. Samples were obtained after 3 h of antimicrobial treatment, diluted and plated to determine the number of c.f.u. ml⁻¹. Data are given as means $\pm$ SEM from triplicate experiments.

RESULTS AND DISCUSSION

Determination of optimal levofloxacin and vancomycin concentration for maximal killing of *S. epidermidis* RP62a

S. epidermidis RP62a is a MRSE and is resistant to several different antimicrobial agents (Sieradzki *et al.*, 1999). As a result, antimicrobial sensitivities were assessed to determine which antimicrobial agents could be used in this study. Sensitivity testing completed at the North-East Regional Medical Center Clinical Laboratory revealed that *S. epidermidis* RP62a was sensitive to levofloxacin (MIC 0.0625 μ g ml⁻¹) and vancomycin (MIC 2 μ g ml⁻¹; Table 1).

To determine the concentration of levofloxacin or vancomycin that resulted in the highest amount of killing, various concentrations (0, 10, 25, 50, 75 and 100 μ g ml⁻¹) of levofloxacin or vancomycin were added to cultures of *S. epidermidis* RP62a at early exponential phase (3 h) and the number of c.f.u. ml⁻¹ was determined at each concentration after 3 h of levofloxacin treatment or 16 h of vancomycin treatment at 37 °C (Fig. 1a).

The concentration of antimicrobial agent that killed the maximal number of bacterial cells was 25 μ g ml⁻¹ for levofloxacin and 50 μ g ml⁻¹ for vancomycin (Fig. 1a). Addition of higher concentrations of antimicrobial agent did not result in a significant increase in killing. The maximal serum concentration of levofloxacin is approximately 4.5 μ g ml⁻¹ (Brunton *et al.*, 2006a). Survivors were still observed even though the concentration of levofloxacin used was fivefold higher than can be attained in the serum of patients. It was from 400 to 384 times the MIC in Table 1 and Table 2, respectively. *S. aureus* has been reported to be resistant to levofloxacin with MICs greater than or equal to 32 μ g ml⁻¹ (Evans & Titlow, 1998).

Survivors were still observed even though the concentration of vancomycin was over twofold higher than the peak serum

Table 1. Antimicrobial sensitivities for *S. epidermidis* RP62a

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$) *	Interpretation according to testing laboratory
Levofloxacin	0.0625	Sensitive
Vancomycin	2	Sensitive
Ampicillin/sulbactam	>32	Resistant
Cefazolin	>32	Resistant
Clindamycin	>8	Resistant
Erythromycin	>8	Resistant
Gentamicin	>16	Resistant
Oxacillin	>8	Resistant
Penicillin G	>16	Resistant
Trimethoprim/sulfamethoxazole	160	Resistant

*MIC data were generated by the North-East Regional Medical Center Clinical Laboratory using the bioMérieux Vitek system.

level for vancomycin administered intravenously to humans ($18.5 \mu\text{g ml}^{-1}$; Brunton *et al.*, 2006a). The concentration of vancomycin used in this study was around 25 times higher than the MICs in this study (Tables 1 and 2).

Determination of the optimal time for maximal killing of *S. epidermidis* RP62a by levofloxacin and vancomycin

The amount of time a culture is exposed to an antimicrobial agent has an effect on the amount of killing observed; therefore, $25 \mu\text{g levofloxacin ml}^{-1}$ or $50 \mu\text{g vancomycin ml}^{-1}$ was used to treat a mid-exponential phase broth culture of *S. epidermidis* RP62a at 37°C for various times (0, 16, 24, 48, 72 and 96 h; Fig. 1b). The rate of killing was quite rapid up until 24 h of exposure to levofloxacin or 16 h of exposure to vancomycin. Increasing the time of incubation beyond 24 h did not significantly decrease the number of c.f.u. ml^{-1} . As a result, all of the following experiments were performed by incubating the bacteria for 24 h with $25 \mu\text{g levofloxacin ml}^{-1}$ or $50 \mu\text{g vancomycin ml}^{-1}$.

Both antimicrobial agents suppress growth of the surviving micro-organisms for up to 96 h. It appears from this experiment that the survivors of high concentration antimicrobial exposure can live at 37°C for significant periods of time.

Mean MIC of levofloxacin and vancomycin for *S. epidermidis* RP62a original and persister-derived cultures

The original *S. epidermidis* RP62a culture and persister-derived isolates' MICs were compared to determine if levofloxacin- or vancomycin-treated cultures were resistant or survived as persister cells. If treatment with these

Fig. 1. Determination of optimal concentrations and exposure times for levofloxacin and vancomycin for maximal killing of *S. epidermidis* RP62a. (a) Levofloxacin and vancomycin concentrations of 0, 10, 25, 50, 75 or $100 \mu\text{g ml}^{-1}$ were added to a mid-exponential phase culture ($\text{OD}_{600} 1.25 \pm 0.05$) of *S. epidermidis* RP62a and incubation was continued at 37°C with shaking for 3 and 16 h, respectively. Samples were obtained after 3 h of antimicrobial treatment, washed, diluted and plated to determine the number of c.f.u. ml^{-1} . (b) Levofloxacin ($25 \mu\text{g ml}^{-1}$) or vancomycin ($50 \mu\text{g ml}^{-1}$) was added to a mid-exponential phase culture ($\text{OD}_{600} 1.25 \pm 0.05$) of *S. epidermidis* RP62a and incubation was continued at 37°C with shaking. Samples were obtained at various times (0, 16, 24, 48, 72 and 96 h), washed, diluted and plated to determine the number of c.f.u. ml^{-1} . Data in (a) and (b) are means $\pm$ SEM from triplicate experiments. ●, Levofloxacin treated; ○, vancomycin treated.

antimicrobial agents selected for resistance, then the MICs of the antibiotic-treated isolates should be higher than the MIC of the original *S. epidermidis* RP62a culture. However, if the antibiotic-treated isolates developed from persisters, then no difference in MIC should be observed when compared to the MIC of the original *S. epidermidis* RP62a culture. In this experiment, when the original *S. epidermidis* RP62a culture was compared with the isolates exposed to $25 \mu\text{g levofloxacin ml}^{-1}$ for 24 h or $50 \mu\text{g vancomycin ml}^{-1}$ for 24 and 48 h there was no statistically significant difference between their MIC values (Table 2).

Table 2. Mean MICs of levofloxacin and vancomycin for *S. epidermidis* RP62a original cultures and cultures treated with antimicrobial agents

MICs were performed as twofold serial dilutions of the antimicrobial agents, levofloxacin and vancomycin. Data are shown as means $\pm$ SD from triplicate experiments.

Treatment	Original <i>S. epidermidis</i> cultures [mean $\pm$ SD ($\mu\text{g ml}^{-1}$)]	Treated <i>S. epidermidis</i> cultures [mean $\pm$ SD ($\mu\text{g ml}^{-1}$)]
25 μg levofloxacin ml^{-1} (24 h)	0.0651 $\pm$ 0.0282	0.0651 $\pm$ 0.0282*
50 μg vancomycin ml^{-1} (24 h)	2.734 $\pm$ 0.781	3.906 $\pm$ 1.563*
50 μg vancomycin ml^{-1} (48 h)	2.604 $\pm$ 0.902	5.208 $\pm$ 1.804*

*There was no statistically significant difference ($P > 0.05$) from *S. epidermidis* RP62a (paired *t*-test).

It appears from these data (Table 2) that the survivors from antimicrobial-treated isolates are just as sensitive to these antimicrobial agents as the original culture. The original culture of *S. epidermidis* RP62a had a MIC for levofloxacin of 0.0625 $\mu\text{g ml}^{-1}$ and a MIC of 2 $\mu\text{g ml}^{-1}$ for vancomycin (Table 1). In this study, levofloxacin-treated survivors had a MIC for levofloxacin of 0.0651 $\mu\text{g ml}^{-1}$ and vancomycin-treated survivors had MICs for vancomycin ranging from 2–5 $\mu\text{g ml}^{-1}$ (Table 2). The statistical analysis showed there was no difference between the original strain and the cultures derived from the survivors of antibiotic treatment. This demonstrated that the survivors were likely to be persisters.

Determination of persister number during growth of *S. epidermidis* RP62a treated with levofloxacin and vancomycin

Previous investigators demonstrated with *E. coli* that persister cells were present sometime after early exponential phase and during stationary phase (Keren *et al.*, 2004a). Similar experiments were performed in this study with *S. epidermidis* RP62a. *S. epidermidis* RP62a was incubated at 37 °C with shaking for 12 h and at various times (0, 0.5, 1, 1.5, 2–12 h) three samples were removed. One sample was plated immediately to determine the total number of c.f.u. ml^{-1} . The second sample was treated with 25 μg levofloxacin ml^{-1} for 24 h and then plated to determine the number of c.f.u. ml^{-1} . The third sample was treated with 50 μg vancomycin ml^{-1} for 48 h and then plated to determine the number of c.f.u. ml^{-1} . At each time point, the number of persisters following levofloxacin or vancomycin treatment was obtained and compared with each other to determine the times that resulted in the highest and lowest number of persisters (Fig. 2). The lowest numbers of persisters occurred in exponential phase cultures for both antibiotics. Stationary phase cultures produced the highest numbers of persisters. At various times in stationary phase, no killing was observed (9 h for levofloxacin, and 8, 9 and 10 h for vancomycin). Lag phase cultures contained more persisters than exponential phase but fewer persisters than stationary phase. Analysis of the data by percentage survival resulted in similar findings (data not shown).

A previous study using *E. coli*, *P. aeruginosa* and *S. aureus* also found few persisters in the lag and exponential phases of growth, and the highest number of persisters in the stationary phase (Keren *et al.*, 2004a; Lewis, 2007). Antimicrobial agents from the fluoroquinolone and β -lactam (cell-wall inhibitor) groups were used in that study. Both antimicrobial agents obtained the highest and lowest number of persisters in the stationary phase and mid-exponential phase, respectively. Similar findings were obtained in this study using biofilm-producing *S. epidermidis* RP62a treated with a fluoroquinolone and a cell-wall inhibitor from the glycopeptide group.

Although both antimicrobial agents produced persister cells in *S. epidermidis* cultures, more killing was obtained with levofloxacin than vancomycin. This is similar to what was observed when *S. aureus* was treated with a fluoroquinolone (ciprofloxacin) and a cell-wall inhibitor

Fig. 2. Determination of persister number during growth of *Staphylococcus epidermidis* RP62a treated with levofloxacin or vancomycin. An overnight culture was diluted 1 : 1000 in TSB and shaken at 37 °C. Samples were taken at various times, exposed to levofloxacin at 25 $\mu\text{g ml}^{-1}$ for 24 h or vancomycin at 50 $\mu\text{g ml}^{-1}$ for 48 h, washed, diluted and plated to determine the number of c.f.u. ml^{-1} . Data are shown as means $\pm$ SEM from triplicate experiments. ●, Untreated; ▼, treated with 25 μg levofloxacin ml^{-1} ; ■, treated with 50 μg vancomycin ml^{-1} .

(ampicillin; Keren *et al.*, 2004a; Lewis, 2007). Fluoroquinolones target DNA replication and can kill rapidly growing as well as slow-growing cells, resulting in the ability to kill more bacterial cells (O'Donnell & Gelone, 2000). Vancomycin and many other cell-wall inhibitors only kill rapidly dividing cells and so kill fewer cells (Brunton *et al.*, 2006b). Slow-growing cells and persister cells that remained dormant had little or no cell-wall synthesis occurring during antimicrobial treatment and therefore a cell-wall synthesis inhibitor like vancomycin was not able to kill as many cells as levofloxacin (Lewis, 2007). Interestingly, even though these two antimicrobial agents target different metabolic processes and cause different amounts of killing, the increases in persister cell numbers occurred at similar times (Fig. 2).

Effect of an extracellular factor on persister cell number

Quorum-sensing regulators can be induced by small extracellular factors produced during cell growth in *S. epidermidis* (Fitzpatrick *et al.*, 2005). If persister formation is controlled by a quorum-sensing regulator, then an increase in persister number might be observed earlier in the culture if an extracellular peptide is added during lag phase. A filter-sterilized, 10 × extracellular concentrate of a stationary phase culture from *S. epidermidis* RP62a was tested for its ability to induce persister formation earlier in exponential phase by adding it to a culture in lag phase. Based on the results from these experiments, there were no increases in persister number when a filterable extracellular substance was added to *S. epidermidis* RP62a cultures (Table 3).

Lewis observed that quorum-sensing signalling molecules did not increase persister number in experiments performed with *E. coli* (Lewis, 2007). No increase in persister numbers was shown in early exponential phase cultures

that received growth medium from stationary phase cultures to induce persister production (Lewis, 2007). However, other investigators demonstrated that *P. aeruginosa* did produce more persisters when induced by the quorum-sensing signalling molecules phenazine pyocyanin and acylhomoserine lactone (Möker *et al.*, 2010). The observations from this study demonstrated there was no increase or decrease in the number of persisters in cultures of *S. epidermidis* containing a 10 × stationary culture concentrate.

In vitro model of biofilm development on glass slides

Experiments were performed to obtain reproducible *S. epidermidis* RP62a biofilms on glass slides with the DFR system. The optimal incubation time for adherence of the bacterial cells to the glass slides and the optimal time for biofilm aggregation and maturation were determined. The number of *S. epidermidis* viable cells adherent to the surface was quantified by direct plating and calculation of the mean c.f.u. ml⁻¹. After incubation in batch mode all samples were then subjected to continuous flow of TSB for 48 h to obtain a biofilm. The mean number of c.f.u. ml⁻¹ of *S. epidermidis* in the biofilm was greatest after an incubation time of 6 h for cell attachment (Fig. 3a). The numbers of c.f.u. ml⁻¹ following 4 and 8 h of attachment were significantly decreased when compared to biofilms obtained following a 6 h incubation time for cell attachment.

Using 6 h for cell attachment, experiments were performed to determine optimal times for biofilm formation. At 12 h and 24 h of incubation with a continuous flow of TSB, the mean viable counts of bacteria in the biofilms were $(6.15 \pm 1.81) \times 10^8$ c.f.u. ml⁻¹ and $(1.31 \pm 0.72) \times 10^9$ c.f.u. ml⁻¹, respectively. There was no statistically significant difference between the two groups ($P=0.107$) (Fig. 3b).

Table 3. Effect of extracellular factor on persister cell number

An overnight culture was diluted in 90 ml TSB and 10 ml 10 × TSB (control) or 10 ml 10 × extracellular concentrate. At 4, 6 and 8 h, samples were removed for plating assays to determine c.f.u. ml⁻¹. Samples (10 ml) were removed at various times and treated with 25 µg levofloxacin ml⁻¹ for 24 h or 50 µg vancomycin ml⁻¹ for 48 h. Treated samples were washed, diluted and plated to determine the number of c.f.u. ml⁻¹. Data are shown as means ± SD from triplicate experiments.

Treatment	Time		
	4 h [mean ± SD (c.f.u. ml ⁻¹)]	6 h [mean ± SD (c.f.u. ml ⁻¹)]	8 h [mean ± SD (c.f.u. ml ⁻¹)]
Untreated control	$2.62 \pm 2.84 \times 10^7$	$3.61 \pm 4.45 \times 10^8$	$9.86 \pm 1.36 \times 10^9$
Untreated 10 × concn control*	$3.50 \pm 3.53 \times 10^7$	$5.98 \pm 1.87 \times 10^8$	$1.42 \pm 0.10 \times 10^9$
Levofloxacin control	$3.36 \pm 4.31 \times 10^4$	$4.85 \pm 0.21 \times 10^2$	$4.43 \pm 6.23 \times 10^7$
Levofloxacin 10 × concn*	$4.49 \pm 6.34 \times 10^3$	$6.65 \pm 7.56 \times 10^2$	$1.55 \pm 2.19 \times 10^7$
Vancomycin control	$2.42 \pm 3.04 \times 10^5$	$6.00 \pm 8.10 \times 10^9$	$4.82 \pm 0.92 \times 10^9$
Vancomycin 10 × concn*	$1.35 \pm 0.61 \times 10^5$	$1.11 \pm 0.81 \times 10^9$	$4.24 \pm 0.33 \times 10^9$

*10 × concentrate culture supernatant.

The mean c.f.u. ml⁻¹ of biofilms incubated for 48 h increased significantly compared to 12 and 24 h of incubation [$(9.00 \pm 0.62) \times 10^9$ c.f.u. ml⁻¹, $P \leq 0.001$]. There was no statistically significant difference between 48 and 72 h of incubation ($P=0.708$) (Fig. 3b). Therefore, the optimal time for biofilm formation was determined as 54 h (6 h cell attachment, 48 h biofilm formation) and this was used in all subsequent experiments.

One obstacle in quantification of biofilm studies is that independent rounds of biofilm experiments are highly variable (Heydorn *et al.*, 2000). In this study we were able to establish reproducibility between biofilms of *S. epidermidis* RP62a, a biofilm-positive strain, on glass slides using a DFR. Optimized cell attachment and biofilm maturation times were those that produced the greatest number of

Fig. 3. Optimal time for *S. epidermidis* RP62a cell attachment and biofilm formation. (a) *S. epidermidis* RP62a inoculum (16 ml) was placed in each chamber of the DFR and incubated for various times (2, 4, 6 and 8 h) at 37 °C in batch mode. After incubation in batch mode all samples were then subjected to continuous flow of TSB for 48 h to obtain a biofilm. (b) Using 6 h for cell attachment, a mature biofilm was developed while TSB was supplied drop-wise over the glass slides, providing a continuous flow of nutrients for various times (12, 24, 48 and 72 h) at 37 °C. Samples in (a) and (b) were washed, sonicated, diluted and plated to determine the number of c.f.u. ml⁻¹. Data in (a) and (b) are means $\pm$ SEM from quadruplicate experiments.

c.f.u. ml⁻¹, and were obtained following 6 h of incubation at 37 °C for cell attachment and 48 h of incubation at 37 °C for biofilm formation (54 h total).

Survival of *S. epidermidis* RP62a in a biofilm following levofloxacin and vancomycin treatment

S. epidermidis biofilms incubated at various times (0, 12, 24 and 48 h) following the 6 h attachment phase were treated with 25 µg levofloxacin ml⁻¹ for 24 h (Fig. 5). The highest percentage survival was observed at 24 and 48 h of biofilm maturation. The percentage survival of biofilm at time zero after 6 h of attachment was significantly lower (8.1×10^{-5} %) than the survival of bacterial cells at later times (12 h=9.7 %, 24 h=20.5 %, 48 h=28 %). Control and levofloxacin-treated 48 h biofilms were suspended and then treated with various concentrations of levofloxacin to determine if the survivors of levofloxacin treatment from biofilms were persistent or resistant to the antibiotic. No significant differences were observed between the biphasic curve obtained from the control and levofloxacin-treated biofilm cultures (Fig. 4). In addition, mean MICs were obtained for isolates from control and levofloxacin-treated 48 h biofilms. Both the control and levofloxacin-treated cultures had MICs of 0.098 µg ml⁻¹.

Biofilms incubated at 48 h following the 6 h attachment phase were treated with 50 µg vancomycin ml⁻¹ for 24 h. The survival was 94 %. The mean MICs for control and vancomycin-treated biofilm isolates were 1.99 ± 0.78 µg

Fig. 4. Concentration-dependent kill curves of control and levofloxacin-treated biofilms. Control and levofloxacin-treated (25 µg ml⁻¹) biofilms were suspended, diluted and incubated for 16 h at 37 °C. The cultures were then diluted (1:200) and incubated at 37 °C with shaking until mid-exponential phase (OD₆₀₀ 1.25 $\pm$ 0.05). Levofloxacin concentrations of 5, 10, 25, 50 or 75 µg ml⁻¹ were added to the mid-exponential phase cultures and incubated at 37 °C with shaking for 3 h. Samples were obtained after 3 h of antimicrobial treatment, washed, diluted and plated to determine the number of c.f.u. ml⁻¹. Data are shown as means $\pm$ SEM from triplicate experiments. ●, Control biofilm; ○, levofloxacin-treated biofilm.

ml^{-1} and $2.17 \pm 0.80 \mu\text{g ml}^{-1}$, respectively, with no statistically significant difference between MIC values of the two groups ($P=0.416$).

This study revealed that *S. epidermidis* RP62a produces persisters in biofilms as well as in planktonic culture. The numbers of persisters in biofilms increased for at least 24 h but then did not increase after 24 h of maturation. As was observed in planktonic cultures of *S. epidermidis*, the cells within the biofilms required some time to mature to produce maximal numbers of persisters. The number of persisters in the biofilm (Fig. 5) was higher as others have observed when comparing the 54 h biofilm (6 h attachment; 48 h maturation) with lag and exponential phase planktonic cultures (Fig. 2) following vancomycin and levofloxacin treatment (Singh *et al.*, 2009).

Almost all antibiotics are more effective against rapidly growing cells; however, fluoroquinolones, such as levofloxacin, have bactericidal activity against even slow-growing cells. Vancomycin, a narrow spectrum antibiotic, is a glycopeptide that acts by inhibiting cell-wall peptidoglycan synthesis (Hammes & Neuhaus, 1974). It is active against rapidly dividing cells, and in general, has a slower rate of killing than the fluoroquinolones (Kang-Birken, 2000).

Treatment of foreign device-related infections is difficult with the emergence of MRSE. Vancomycin remains the major antibiotic used to treat MRSE infections (Polonio

et al., 2001). However, it has been reported that vancomycin efficacy is reduced when *S. epidermidis* exists within a biofilm on the surfaces of indwelling medical devices (Polonio *et al.*, 2001). Zameer & Gopal (2010) reported that vancomycin penetration through *S. epidermidis* biofilms was delayed by about 4 h in comparison to planktonic cultures. This suggests that recalcitrant infections associated with *S. epidermidis* biofilms could be due to relatively poor access of the antibiotic to bacterial cells adhering to the surface of the foreign device, resulting in reduced susceptibility to vancomycin.

In this study, vancomycin was incubated with the biofilm for 24 h and its concentration was 25 times the MIC ($50 \mu\text{g ml}^{-1}$; MIC $2 \mu\text{g ml}^{-1}$) and yet it had little effect on the killing of biofilm cells (94 % survival). When compared with planktonically grown cells, the majority of rapidly growing mid-exponential phase cells (5 h) were killed by vancomycin, resulting in the lowest number of survivors (Fig. 2). These findings indicated that *S. epidermidis* RP62a biofilm cells were similar to slow-growing stationary phase cells, which may explain the high amounts of tolerance in the biofilm to vancomycin. In this study the survival of the bacteria may have been due more to the presence of slow growth and persisters than to poor access of the antibiotic to biofilm cells. The findings in this study were in agreement with other reports (Otto, 2008; Spoering & Lewis, 2001).

Planktonic stationary phase cultures produced the highest number of survivors when compared with biofilm cultures, and at various times no killing was observed (8, 9 and 10 h; Fig. 2). It is generally believed that biofilms are more tolerant to killing than are stationary-phase planktonic cells; however, these experiments demonstrated similarities between stationary-phase and biofilm cultures of *S. epidermidis* in their production of high levels of survivors and overall tolerance to vancomycin. Keren *et al.* (2004a) reported similar results in fluoroquinolone-treated *P. aeruginosa*, *E. coli* and *S. aureus*, which suggests that biofilms are not unique in producing large persister populations.

Levofloxacin, the fluoroquinolone used in this study, killed significantly more biofilm cells compared with those treated with vancomycin ($P=0.001$). The percentage survival of *S. epidermidis* biofilm cells was compared with that of planktonic cultures; the levels of survivors were statistically higher in the dense planktonic stationary phase and biofilm cultures than in the exponential phase cells (5 h) ($P \leq 0.001$). However, there was no significant difference in surviving cells between planktonic stationary phase cells at 9 and 12 h compared with biofilm cultures ($P=0.229$ and $P=0.057$, respectively).

Visualizing biofilms with CLSM

The first direct light and electron microscopic observation of microbial biofilms demonstrated that the adherent

Fig. 5. Biofilm maturation time versus the percentage survival of *S. epidermidis* RP62a following treatment with levofloxacin. After an attachment time of 6 h the biofilms were incubated for various times in flow mode at 37 °C. The biofilms were then washed and incubated in $25 \mu\text{g levofloxacin ml}^{-1}$ for 24 h at 37 °C, washed, scraped from the glass slide, sonicated, diluted and plated on TSA. Following incubation of the plates for 48 h at 37 °C the colonies on the plates were counted and the mean number of c.f.u. ml^{-1} was calculated. The percentage survival was calculated by dividing the number of c.f.u. ml^{-1} from the biofilm treated with levofloxacin by the number of c.f.u. ml^{-1} from the control biofilm. Data are shown as means $\pm$ SEM from quadruplicate experiments.

population contained many more cells than their planktonic counterparts (Geesey *et al.*, 1977). Biofilm bacteria were enveloped in an extremely hydrated and dense EPS matrix, with a species-specific chemical composition. Limited resolution of light microscopy and dehydration associated with electron microscopy created the perception that biofilm bacteria were distributed in a homogeneous manner throughout the EPS matrix of the biofilm (Costerton *et al.*, 1994). Recently, however, the application of CLSM shed a new light on the study of biofilms, providing a more accurate depiction of the heterogeneous architecture of these bacterial populations (Donlan, 2002). CLSM allows for non-destructive, *in situ*, three-dimensional investigation of biofilms in their naturally hydrated state and for detecting fluorescence from specific constituents such as live/dead cells or extracellular polymeric substances (Merod *et al.*, 2007).

Viable bacteria with intact cell membranes were stained with SYTO 9 (green fluorescence), whereas dead bacteria with damaged cell membranes were stained with PI (yellow fluorescence; Flemming *et al.*, 2009). Biofilms formed after a total of 54 h (6 h cell attachment; 48 h biofilm formation) exhibited a three-dimensional multicellular structure typical of a biofilm (Fig. 6a). The biofilms were approximately 9–11 μm thick and were composed of mostly live cells. In this study the cell density of 54 h biofilms was approximately 1.92×10^{13} c.f.u. cm^{-3} assuming the biofilm was 10 cells thick and the cells in the biofilm were 1 μm in diameter. Planktonic stationary cell cultures in this study contained around 5×10^9 c.f.u. cm^{-3} . The cell density of the biofilm was significantly higher than that obtained in stationary planktonic cultures.

CLSM images of biofilms exposed to levofloxacin demonstrated that the antibiotic was able to diffuse through the biofilm and kill cells throughout the biofilm (Fig. 6b).

One significant virulence factor identified in *S. epidermidis* is its ability to produce biofilm (Qin *et al.*, 2007). *S. epidermidis* is a common cause of recalcitrant infections on indwelling medical devices (Mack *et al.*, 2000; O’Gara & Humphreys, 2001). In some cases the infection requires removal of the indwelling medical device and with removal significant effects on patient quality of life and functioning are experienced. Understanding how the organisms in these biofilms survive antimicrobial treatment is essential to patients with indwelling medical devices. If the mechanisms of survival could be understood, then better means of treating these infections may be developed. This study was conducted to better understand the mechanisms a well characterized biofilm-producing MRSE utilizes to survive following antibiotic treatment.

This study demonstrated the presence of persister cells at high concentrations in stationary planktonic cultures and in biofilms of *S. epidermidis* RP62a. Persister cell numbers in *S. epidermidis* biofilms are quite low at first but within 24 h they become high. The persister number increases as the cell density increases in planktonic cultures and biofilms. Killing of *S. epidermidis* cells occurred uniformly throughout the biofilm when exposed to levofloxacin. Therefore, survival of the organisms did not appear to be due to inactivation of the antibiotic or to the lack of diffusion throughout the biofilm. Differences in the number of survivors treated with vancomycin and levofloxacin indicate that many of the survivors of vancomycin

Fig. 6. CLSM of *S. epidermidis* RP62a biofilm formation on surfaces of glass slides for 54 h (a) and following levofloxacin treatment for 24 h (b). (a) The biofilms were stained with LIVE/DEAD stain, which allowed for observation of channel-like spaces and the heterogeneity characteristic of a mature biofilm. (b) Fifty-four hour biofilms were treated with levofloxacin for 24 h and then stained with LIVE/DEAD viability stain. Cells that appeared green were considered viable, whereas nonviable bacteria with compromised cell membranes appeared yellow. The CLSM image is of the bottom layer of a biofilm exposed to antibiotic and demonstrates that the antibiotic was able to diffuse through the thick layer of cells and kill cells throughout the biofilm. Images in (a) and (b) were taken with a $\times 63$ oil immersion objective.

treatment are slow-growing cells and that the high levels of survival in vancomycin-treated biofilms (94 %) are due in part to reductions in growth rate. However, when an antibiotic capable of killing slow-growing cells, levofloxacin, was used to treat the biofilm, significant numbers of survivors still remained (28 %). It appears that another mechanism of survival following antibiotic treatment for *S. epidermidis* is the production of persisters. The factors involved in triggering increases in persister numbers are not fully understood and such increases do not appear from this study to be due to an extracellular factor produced during stationary phase in planktonic culture. Future studies will be conducted to better understand the mechanisms *S. epidermidis* utilizes to increase persister numbers in biofilms.

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