

Growth limitation of *Staphylococcus epidermidis* in biofilms contributes to rifampin tolerance

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ABSTRACT

Tolerance of *Staphylococcus epidermidis* in biofilms to killing by rifampin was correlated with limitation of bacterial growth in the biofilm state. Intact biofilm experienced a 0.62 log reduction when treated with 0.1 µg rifampin/ml for 4 h whereas the same treatment of exponential-phase planktonic cells produced a log reduction of 4.48. Stationary-phase planktonic cells were nearly as tolerant as intact biofilm cells, experiencing a 1.11 log reduction. Biofilm bacteria grew at only 10% of the maximum rate at which they grew on the same medium in planktonic culture. Killing was localized near the surface of the biofilm adjacent to the nutrient source, as revealed by staining with a respiratory dye. Increased nutrient concentration during antibiotic treatment enhanced killing of biofilm cells. Changing the oxygen tension in the gas phase above the biofilm during antibiotic treatment barely affected killing. It was hypothesized that the biofilm harbors significant numbers of stationary-phase-like cells in the nutrient-limited depths of the biofilm, and that these inactive cells are the ones that survive antibiotic challenge.

INTRODUCTION

Staphylococci that adhere to implanted medical devices or damaged tissue can cause persistent infections such as those related to catheters, prosthetic heart valves, vascular grafts and osteomyelitis (Costerton *et al.*, 1999). These bacteria evade antimicrobial chemotherapy by growing in biofilms in which they are protected. While the reduced antibiotic susceptibility of bacteria in biofilms is widely recognized, the reasons for this protection remain unclear (Stewart & Costerton, 2001).

We have reported that rifampin penetrates biofilms formed by *Staphylococcus epidermidis* but fails to effectively kill the bacteria (Zheng & Stewart, 2002). This result corroborates other studies of antibiotic penetration in staphylococcal biofilms which concluded that poor antibiotic penetration was not an adequate explanation for reduced susceptibility in the biofilm state (Dunne *et al.*, 1993; Darouiche *et al.*, 1994; Yasuda *et al.*, 1994). The purpose of the work reported in this article was to test the hypothesis that slow killing of *S. epidermidis* biofilms by rifampin was due to nutrient limitation and slow or no growth of biofilm bacteria (Xu *et al.*, 2000).

The likely role of slow growth of biofilm cells in contributing to their protection from antimicrobial agents is a longstanding hypothesis (Brown *et al.*, 1988). Several investigations support this mechanism for staphylococcal biofilms in particular. Venables and coworkers showed that *Staphylococcus aureus* undergo physiological changes

in the early stages of biofilm formation (Williams *et al.*, 1999). These changes included smaller cell size, lower respiratory activity per cell and lower membrane potential in the attached population. This same group also reported that adherent *S. aureus* grew more slowly than free-floating bacteria (Williams *et al.*, 1997). Hodgson *et al.* (1995) measured slow growth of bacteria in *S. aureus* biofilms. Bacteria in the biofilm grew at an average specific growth rate of 0.06 h⁻¹ whereas free-floating cells grew at a rate of 0.7 h⁻¹ in the same medium. Duguid *et al.* (1992a,b) demonstrated growth rate-modulated sensitivity of *S. epidermidis* to tobramycin and ciprofloxacin; the more slowly the cells grew, the less susceptible they were to killing. Prior work therefore supports the ideas that staphylococcal biofilms harbor cells with reduced metabolic activity and that less active cells are less susceptible.

MATERIALS AND METHODS

Biofilm formation and antibiotic susceptibility

Biofilms of *S. epidermidis* ATCC 35984 were grown in continuous flow reactors by dripping one-tenth strength tryptic soy broth (TSB; Difco, Detroit, MI) over inclined stainless steel coupons in a covered chamber. Medium was applied dropwise at the top of a 7.8 cm × 1.2 cm coupon

and flowed down the long dimension of the coupon tilted at an angle of 10° to the horizontal. Medium drained from the reactor to a waste reservoir. The fluid residence time in the chamber was a few minutes or less. The chamber was covered, with ports on the lid that allowed for the introduction of gases other than air. The reactor system has been described in detail elsewhere (Stewart *et al.*, 2001). The medium flow rate was 50 ml/h. All work was carried out at 37°C .

The susceptibility of biofilm bacteria to antibiotics was measured using 48 h biofilms. The flow over the biofilm was switched from one-tenth strength TSB to one-tenth strength TSB containing either $0.1\ \mu\text{g}$ rifampin/ml or $2.5\ \mu\text{g}$ ciprofloxacin/ml. These concentrations of antibiotics were approximately 10 times the minimum inhibitory concentrations for planktonic cells. In some experiments, the strength of TSB during the antibiotic treatment period was varied or the gas phase above the biofilm was changed from air to oxygen or nitrogen. After exposure to the antibiotic for periods ranging from 4 to 48 h, biofilm was scraped from the sample slide into 50 ml of buffered water. Aggregates, dispersed by brief processing with a tissue homogenizer, were enumerated for viable cell numbers by serial dilution and plating on tryptic soy agar (TSA). The degree of killing was calculated as a log reduction using an untreated control as the reference cell count.

Planktonic antibiotic susceptibility

Planktonic bacteria were grown in shake flasks in one-tenth strength TSB at 37°C . The susceptibility of planktonic bacteria was measured by adding antibiotic to a subculture that was diluted in one-tenth strength TSB to an optical density 0.02 (1 cm path length) at 600 nm immediately prior to treatment. In some experiments the inocula were derived from exponential phase cultures (6 h) and in others the inocula came from stationary phase cultures (30 h). Rifampin was then added at $0.1\ \mu\text{g}/\text{ml}$ and the culture incubated with shaking at 37°C . Surviving bacteria were enumerated by serial dilution and plating on TSA.

Growth rates

The growth rate of bacteria in suspension cultures was determined by linear regression of natural log cell number versus time data; the slope of this line gave the growth rate. The average growth rate of biofilm bacteria was calculated from a material balance on cells. The average growth rate is equal to the effluent cell concentration multiplied by the volumetric flow rate of medium and divided by the total number of biofilm cells on the sample coupon.

Respiratory activity staining and microscopy

Biofilms were stained with 0.16% 5-cyano-2,3-ditolyltetrazolium chloride (CTC) for 2 h at 37°C (Huang *et al.*, 1995), cryoembedded (Yu *et al.*, 1994), frozen sectioned,

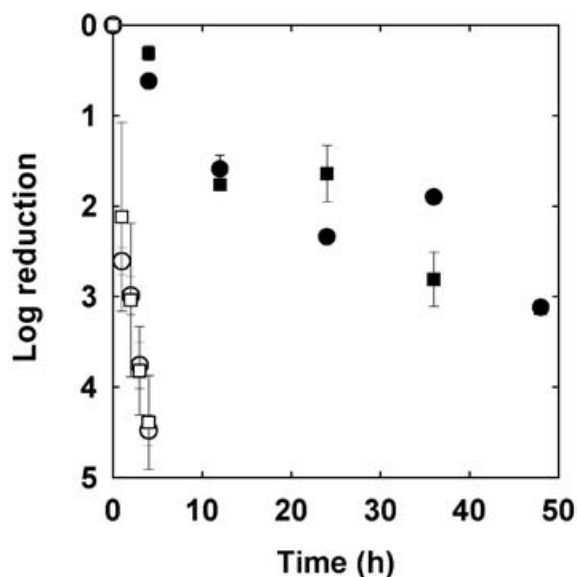


Fig. 1: Comparison of susceptibility of *S. epidermidis* exposed to $0.1\ \mu\text{g}$ rifampin/ml (circles) or $2.5\ \mu\text{g}$ ciprofloxacin/ml (squares) in the planktonic (open symbols) and biofilm (filled symbols) states. The treatment time was 4 h. Error bars indicate the standard error of the mean.

and counterstained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI), applied at $10\ \mu\text{g}/\text{ml}$ for 3 min. Specimens were examined with a Nikon E800 microscope using epifluorescence illumination.

RESULTS

Staphylococcus epidermidis biofilms are protected from killing by rifampin and ciprofloxacin

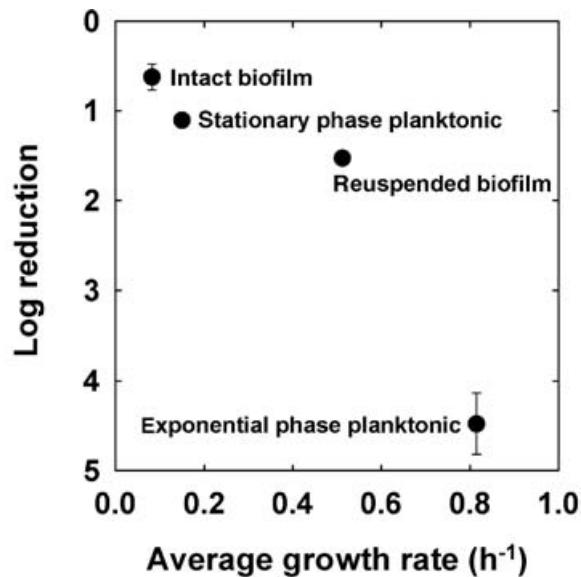
Free-floating bacteria from an exponential-phase growing culture were more susceptible than biofilm bacteria to killing by rifampin (Fig. 1). The untreated control biofilm at the time of initiation of treatment (48 h) was covered with a lush biofilm with a viable areal cell density of $8.94 \pm 0.12\ \log_{10}\ \text{cfu}/\text{cm}^2$. After 4 h of treatment, the log reduction in viable cell numbers for the treated biofilm was 0.62 ± 0.13 . The log reduction of growing planktonic bacteria in response to the same treatment was 4.48 ± 0.34 . This difference was statistically significant ($P = 0.002$). *Staphylococcus epidermidis* biofilms were also less susceptible to killing by $2.5\ \mu\text{g}$ ciprofloxacin/ml by comparison with growing planktonic bacteria (Fig. 1). With this antibiotic, the log reduction after 4 h treatment was only 0.32 ± 0.08 for biofilms as compared with 4.39 ± 0.52 for planktonic cells.

Stationary-phase planktonic cells are protected from killing by rifampin

Bacteria taken from a stationary-phase planktonic culture were less susceptible to killing by rifampin than were

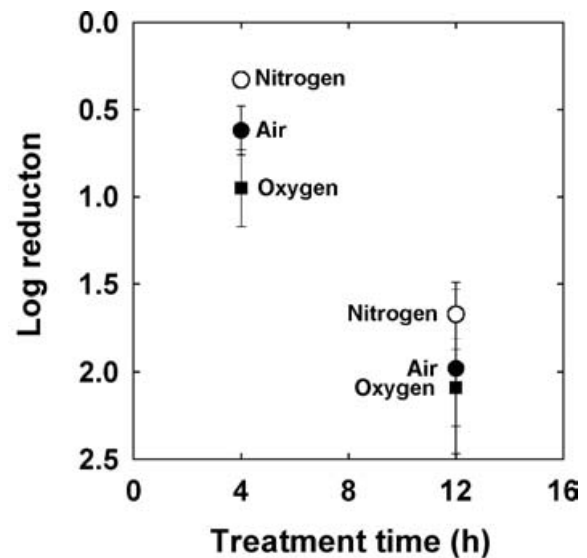
Table 1: Comparison of growth rates and susceptibilities of exponential-phase planktonic, stationary phase planktonic, resuspended biofilm, and intact biofilm bacteria. Killing was measured in response to 4 h rifampin treatment

Source of bacteria	Log reduction untreated control	Log reduction treated	Growth rate (h^{-1})
Exponential phase planktonic	-1.84 ± 0.54	4.48 ± 0.34	0.82 ± 0.34
Stationary phase planktonic	-0.29 ± 0.11	1.11 ± 0.05	0.15 ± 0.06
Resuspended biofilm	-0.93 ± 0.17	1.53 ± 0.06	0.51 ± 0.03
Intact biofilm	0	0.62 ± 0.13	0.08 ± 0.02

**Fig. 2:** *Staphylococcus epidermidis* killing by $0.1 \mu\text{g}$ rifampin/ml as a function of growth rate. The treatment time was 4 h. Error bars indicate the standard error of the mean.

bacteria taken from an actively growing culture. The log reduction of bacteria taken from a stationary-phase culture was 1.11 ± 0.05 , only about one-quarter of the kill measured when inocula were taken from growing cultures (Table 1). When bacteria were dispersed from biofilms by scraping and homogenization, resuspended in one-tenth strength TSB, and treated with rifampin, the degree of killing was similar to that measured for stationary phase planktonic cells. The log reduction after 4 h treatment in this case was 1.53 ± 0.06 . These results show that the growth status of the bacteria prior to, and at the time of, the antibiotic challenge is an important factor in determining susceptibility.

There was a rough correlation between average growth rate and rifampin sensitivity (Fig. 2). The more slowly bacteria grew, the less susceptible they were to killing by rifampin. Bacteria in the biofilm mode of growth were least susceptible, and also grew most slowly. The average specific growth rate of biofilm bacteria was only $0.08 \pm 0.02 \text{ h}^{-1}$. For comparison, the specific growth rate of bacteria in a rapidly growing planktonic culture was an order of magnitude larger, $0.82 \pm 0.34 \text{ h}^{-1}$. The trend of decreasing susceptibility with decreasing growth rate was statistically significant ($P=0.04$). Having established that the

**Fig. 3:** *S. epidermidis* biofilm susceptibility to $0.1 \mu\text{g}$ rifampin/ml as a function of oxygen availability. Error bars indicate the standard error of the mean.

growth status of the bacteria influences their antibiotic susceptibility, we hypothesized that biofilm susceptibility could be modulated by changing the level of oxygen or nutrients provided to the bacteria during antibiotic treatment. Experiments were therefore conducted in which biofilms were grown by the standard procedure and then challenged with $0.1 \mu\text{g}$ rifampin/ml under differing conditions of oxygen and nutrient provision. When the gas phase above the biofilm was changed from air to nitrogen or pure oxygen, little change in the extent of killing by rifampin was detected (Fig. 3). While these experiments supported a trend in increased killing as the oxygen tension increased, the differences were not statistically significantly different at the 95% confidence level in most cases. The statistical test used for this and other comparisons was a simple two-sided t-test. We conclude that oxygen availability was not a major factor in controlling the susceptibility of *S. epidermidis* biofilms under these conditions.

On the other hand, when the nutrient strength was changed during antibiotic treatment, a clear effect on the susceptibility of biofilm bacteria was evident (Fig. 4). Higher nutrient concentration during antibiotic treatment led to greater killing, while more dilute nutrients led to less killing. The trend of antibiotic killing increasing

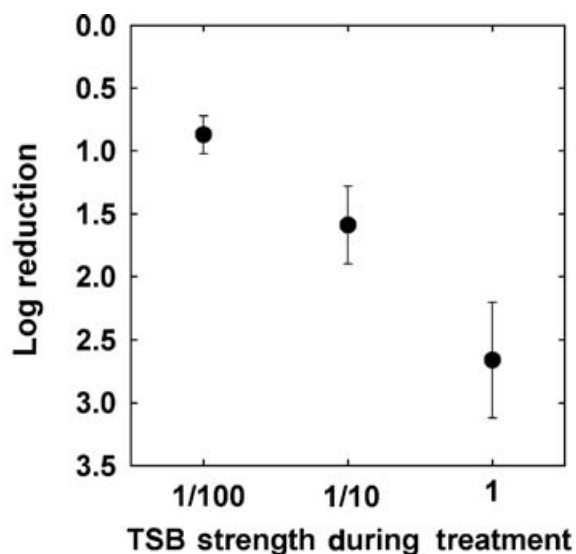


Fig. 4: *Staphylococcus epidermidis* biofilm susceptibility to 0.1 µg rifampin/ml as a function of nutrient concentration. The treatment time was 12 h. Error bars indicate the standard error of the mean.

with nutrient strength was statistically significant ($P = 0.01$). These experiments were conducted by growing the biofilms in the standard way, in one-tenth strength TSB. Only during the 12 h period of antibiotic treatment was the TSB strength changed. The log reduction was calculated in comparison to an untreated (no antibiotic) control that underwent the same upward shift in nutrient composition. These results suggest that nutrient availability may modulate rifampin susceptibility in these *S. epidermidis* biofilms.

If nutrient diffusion into the biofilm were important, then one would expect to see greater metabolic activity and greater antibiotic efficacy near the surface of the biofilm and reduced metabolic activity and diminished antibiotic action in the depths of the biofilm. As a test of this possibility, *S. epidermidis* biofilms were stained with the respiratory dye CTC. This stain deposits fluorescent red crystals at the site of respiratory activity. The use of the DNA intercalating dye DAPI stains all cells blue, independently of their metabolic state. Distinct patterns of respiratory activity, as indicated by CTC staining, were observed in control and antibiotic-treated biofilms (Fig. 5).

In the untreated control, an intense layer of red CTC-formazan formed at the biofilm–bulk fluid interface (Fig. 5A). The CTC staining was less intense near the substratum. DAPI counterstaining of the same section revealed a uniform blue intensity throughout the depth of the biofilm (Fig. 5A). From image analysis of DAPI-stained sections, the thickness of the untreated control biofilm was $636 \pm 64 \mu\text{m}$. In a biofilm treated with 0.1 µg rifampin/ml for 4 h, respiratory activity was suppressed near the biofilm–bulk fluid interface and was more intense throughout the remainder of the biofilm (Fig. 5B). DAPI staining again indicated a fairly uniform distribution of biomass (Fig. 5B). Image analysis of the treated biofilm

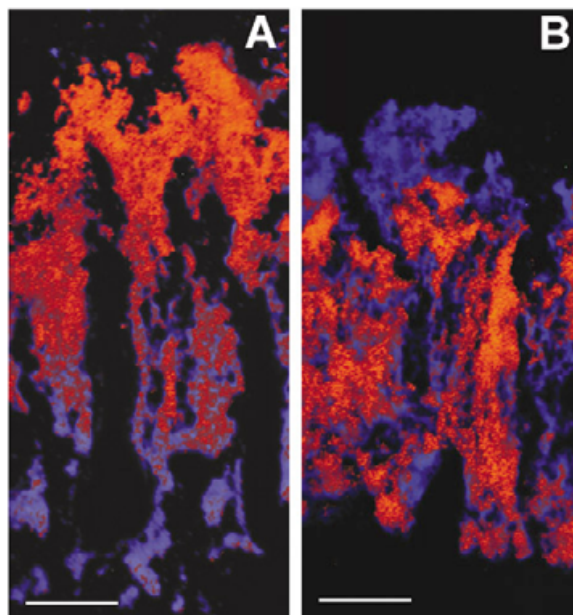


Fig. 5: Patterns of respiratory activity (red) and biomass distribution (blue) in untreated (A) and rifampin-treated (B) *S. epidermidis* biofilms. The treatment time was 12 h. Respiratory activity was visualized by CTC staining and biomass was revealed by staining DNA with DAPI. The substratum is at the bottom and the biofilm–nutrient interface at the top in both panels. The scale bar indicates 100 µm.

returned an average biofilm thickness of $466 \pm 13 \mu\text{m}$ and indicated that the zone of reduced respiratory activity near the biofilm surface was $91 \pm 13 \mu\text{m}$ in dimension. These results suggest that rifampin exerted its greatest activity near the surface of the biofilm, where nutrients are presumably most available.

DISCUSSION

The results of this study support the hypothesis that nutrient limitation resulting in slow or no growth of bacteria in *S. epidermidis* biofilm is responsible for tolerance to rifampin (Zimmerli *et al.*, 1994; Schierholz *et al.*, 1998). Evidence consistent with this mechanism includes: a general correlation, for both planktonic and biofilm bacteria, between growth rate and susceptibility; demonstration that the extent of killing depends on nutrient availability during antibiotic treatment; and the observation that antibiotic action is localized at the biofilm–nutrient interface. The lack of influence of oxygen tension on biofilm susceptibility measured in this study is consistent with the data of Norden & Shaffer (1983), who reported that minimum inhibitory concentrations and minimum bactericidal concentrations of rifampin challenging *S. aureus* were identical under aerobic and anaerobic conditions.

Because most antibiotics target macromolecular synthesis, it should not be surprising that slowly growing or non-growing cells are less susceptible to killing. Rifampin binds to DNA-dependent RNA polymerase and inhibits initiation of RNA synthesis. Ciprofloxacin binds

to gyrase, interferes with proper supercoiling of DNA and eventually halts replication. In a bacterial cell in which there is neither DNA replication nor transcription occurring, these antibiotics cannot be expected to have much effect. Antibiotics might still kill slow-growing cells over time, but against dormant or stationary-phase cells these agents might have little efficacy even during prolonged periods of exposure.

It is important to recognize that the growth of bacteria in a biofilm is likely to vary with spatial location in the biofilm (Xu *et al.*, 2000). Consider, by way of illustration, a biofilm in which cells grow, *on average*, at half the rate of their planktonic counterparts. This population might actually comprise two halves: one that is growing rapidly, at the full planktonic rate; and the other that is in stationary phase and not growing at all. Characterization of biofilm populations by average growth rates, to which we have also resorted in this article, may not be an adequate way of characterizing the growth status of a biofilm population. We suggest that it is those cells that are not growing at all, which are of the greatest importance in understanding antibiotic tolerance in biofilms. More work is needed to prove that biofilms harbor significant numbers of non-growing, yet viable, cells.

Given the likelihood that biofilms contain stationary-phase-like cells, batch cultures are arguably more appropriate than continuous culture for planktonic comparisons with biofilms. A cell in a biofilm probably experiences a history that is more like that in a batch culture. When the biofilm is thin, cells may be in exponential phase. As the biofilm gets thicker, those cells near the surface of the biofilm may continue to grow rapidly. But those cells that become more deeply embedded in the biofilm will experience diminishing nutrients and eventual complete depletion of nutrients. This progression is similar to a batch culture that enters stationary phase. Biofilm cells are unlikely to have the chemostat experience of a steady, precise nutrient level that maintains a certain growth rate over a long period.

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