Aminoglycoside inhibition of *Staphylococcus aureus* biofilm formation is nutrient dependent

Michelle J. Henry-Stanley,¹ Donavon J. Hess²,³ and Carol L. Wells¹,²

¹Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA
²Department of Surgery, University of Minnesota, Minneapolis, MN, USA
³Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA

Biofilms represent microbial communities, encased in a self-produced matrix or extracellular polymeric substance. Microbial biofilms are likely responsible for a large proportion of clinically significant infections and the multicellular nature of biofilm existence has been repeatedly associated with antibiotic resistance. Classical *in vitro* antibiotic-susceptibility testing utilizes artificial growth media and planktonic microbes, but this method may not account for the variability inherent in environments subject to biofilm growth *in vivo*. Experiments were designed to test the hypothesis that nutrient concentration can modulate the antibiotic susceptibility of *Staphylococcus aureus* biofilms. Developing *S. aureus* biofilms initiated on surgical sutures, and in selected experiments planktonic cultures, were incubated for 16 h in 66% tryptic soy broth, 0.2% glucose (1× TSBg), supplemented with bactericidal concentrations of gentamicin, streptomycin, ampicillin or vancomycin. In parallel experiments, antibiotics were added to growth medium diluted one-third (1/3× TSBg) or concentrated threefold (3× TSBg). Following incubation, viable bacteria were enumerated from planktonic cultures or suture sonicates, and biofilm biomass was assayed using spectrophotometry. Interestingly, bactericidal concentrations of gentamicin (5 µg gentamicin ml⁻¹) and streptomycin (32 µg streptomycin ml⁻¹) inhibited biofilm formation in samples incubated in 1/3× or 1× TSBg, but not in samples incubated in 3× TSBg. The nutrient dependence of aminoglycoside susceptibility is not only associated with biofilm formation, as planktonic cultures incubated in 3× TSBg in the presence of gentamicin also showed antibiotic resistance. These findings appeared specific for aminoglycosides because biofilm formation was inhibited in all three growth media supplemented with bactericidal concentrations of the cell wall-active antibiotics, ampicillin and vancomycin. Additional experiments showed that the ability of 3× TSBg to overcome the antibacterial effects of gentamicin was associated with decreased uptake of gentamicin by *S. aureus*. Uptake is known to be decreased at low pH, and the kinetic change in pH of growth medium from biofilms incubated in 5 µg gentamicin ml⁻¹ in the presence of 3× TSBg was decreased when compared with pH determinations from biofilms formed in 1/3× or 1× TSBg. These studies underscore the importance of environmental factors, including nutrient concentration and pH, on the antibiotic susceptibility of *S. aureus* planktonic and biofilm bacteria.

**INTRODUCTION**

Biofilms can be found throughout nature, as well as in industrial and medical settings. Biofilms develop as microbial communities in liquid environments and can attach to biological or abiotic surfaces. Biofilm-associated microbes are phenotypically diverse and are often encased in a matrix containing varying amounts of polysaccharide, protein, glycolipid and extracellular DNA (Stewart & Franklin, 2008; Römling & Balsalobre, 2012). It has been estimated that biofilms may be involved in more than 65% of medically relevant infections, including ventilator and cystic fibrosis-related pneumonia, endocarditis, prostatitis, burn wound infections, colitis, vaginitis, urethritis, otitis and periodontitis, as well as infections associated with indwelling devices such as catheters, sutures, stents, orthopaedic implants and artificial heart valves (Hall-Stoodley *et al.*, 2004).

Antibiotic resistance represents a prominent feature of clinically relevant biofilm infections (Hall-Stoodley & Stoodley, 2009; Cos *et al.*, 2010) and there appear to be multiple mechanisms responsible for the decreased susceptibility of biofilms to antimicrobials. For example, antibiotics that affect rapidly dividing cells may not be effective against a...
subpopulation of biofilm cells thought to be relatively dormant (Lewis, 2010). It has also been suggested that the mere presence of biofilm matrix might hinder or delay antibiotic interactions with target bacteria (Suci et al., 1994; Tseng et al., 2013), but numerous studies using a variety of methodologies have shown that in many instances, antibiotics are able to penetrate the biofilm matrix (Dunne et al., 1993; Anderl et al., 2000; Walters et al., 2003).

Numerous strategies have been used to dissect differences in gene expression between biofilm and planktonic (free-living) cells. Although bacterial mutants have been isolated that are severely impaired in biofilm formation, and the genes responsible for this impairment identified, the impact of these mutations on the ability of an individual bacterial species to form biofilms varies, indicating that the diagnostic or therapeutic potential of these findings may be limited (Fux et al., 2005). Currently, there is no uniformly accepted method to assess the antimicrobial susceptibility of biofilm-associated bacteria.

Clinical microbiology laboratories routinely test antibiotic susceptibilities using low numbers of rapidly dividing planktonic cells (CLSI, 2006), but this scenario may have little relevance for their relatively slow-growing biofilm counterparts. Lewis (2001) explained that although antibiotic resistance is typically defined by clinical microbiologists as the ability of planktonic bacteria to grow in the presence of the MIC of a specific antibiotic, most biofilm-susceptibility studies assess antibiotic-mediated bacterial killing, which may be defined as minimum bactericidal concentration (MBC) rather than bacterial growth. Because biofilms are prevalent in clinical medicine, improved treatment modalities require that we clarify the factors that influence the antibiotic susceptibility of biofilm-associated bacteria.

Relatively little attention has been given to the role of the biofilm milieu in modulating the antibiotic susceptibility of infectious biofilm (Gilbert et al., 1997). Not only does the biofilm setting have importance in designing relevant in vitro testing methodologies, but elucidation of extra-bacterial factors may suggest antibiotic ‘co-therapies’ designed to augment the susceptibility of biofilm bacteria (Smith & Ronesberg, 2007). Therefore, we studied the effect of nutrient concentration on the susceptibility of developing Staphylococcus aureus biofilms to four antibiotics, namely gentamicin, vancomycin, ampicillin and streptomycin.

METHODS

S. aureus strains and biofilm development. S. aureus RN6390 and ATCC 25923 are wild-type strains that produce biofilms (Wells et al., 2011). Data from ATCC 25923 are presented in the online Supplementary Material primarily to show that the results presented in this manuscript are not unique to an individual strain. Bacterial inocula from overnight tryptic soy broth (TSB) cultures incubated at 37°C were washed twice in Hank’s balanced salt solution (HBSS) prior to cultivation with suture. TSB was obtained from Becton Dickinson and included 2.5 g D-glucose l−1. HBSS was obtained from Gibco-Life Technologies. Biofilm growth medium was 66 % TSB (0.37 % D-glucose) supplemented with 0.2 % D-glucose (TSBg), referred to as 1× TSBg (Shanks et al., 2005). Growth medium that was one-third or three times as concentrated is referred to as 1/3× TSBg or 3× TSBg, respectively. Unless otherwise stated, this formulation was used in all experiments; variations included adding glucose or NaCl to 1/3× and 1× TSBg to make the resulting glucose or NaCl concentration equivalent to that found in 3× TSBg.

In selected experiments, bacterial inocula from overnight cultures of S. aureus RN6390 were cultivated for 16 h in the above nutrient concentrations in the presence of gentamicin and viable bacteria enumerated. Suture-associated biofilms were cultivated as described previously (Wells et al., 2011), with minor modifications. Briefly, each well of a 24-well plastic plate (Becton Dickinson) contained a 1 cm segment of black braided 3-0 silk suture (Ethicon) suspended in 1 ml medium that was additionally supplemented with varying concentrations of gentamicin, vancomycin, ampicillin or streptomycin (Sigma-Aldrich). Control wells contained no antibiotic. Each well was inoculated with 106 S. aureus and incubated overnight at 37 °C with gentle rotation (50 r.p.m.). Concentrations of antibiotic included the MIC, as well as concentrations up to ten times the MIC, with the MIC defined as the lowest concentration that inhibits visible growth of planktonic cells after overnight incubation. For the S. aureus strains used herein, the MICs of planktonic cells were determined according to CLSI (2006) guidelines and were 2 μg gentamicin ml−1, 2 μg vancomycin ml−1, 0.25 μg ampicillin ml−1 and 32 μg streptomycin ml−1.

Suture-associated biofilms were analysed for both numbers of viable bacteria and biofilm biomass. In preparation for these studies, S. aureus suture-associated biofilms were sonicated in 2 ml sterile PBS for 0 (control), 5, 10, 30, 45 or 60 s at 50 J, 100 % amplitude and 20 kHz using a VCX 130 PB sonicator (Sonics and Materials), followed by dilution and plating of the sonicate overnight. The c.f.u. number was calculated the following day and decreased viability of the disrupted cells was noted at ≥ 45 s of sonication. Based on these results, data obtained from all experiments described herein represent S. aureus suture-associated biofilms disrupted by sonication for 5 s using the above parameters to assess bacterial numbers and viability. In selected experiments, the pH of spent growth media was measured using a Corning model 430 pH meter, accurate to 0.01 pH units.

Bacterial concentrations in sonicates were determined by standard microbiological methods, and the lower detection limit was 1.7 log10 c.f.u. per suture. Biofilm biomass was measured using the method of Peeters et al. (2008), with minor modifications. Briefly, biofilm-laden sutures were rinsed with PBS, fixed in 99 % methanol for 15 min, air-dried, incubated for 20 min with 0.5 % crystal violet (Fisher Chemical), washed, then incubated for 20 to 30 min in 33 % acetic acid to release biofilm-bound dye. Dye released from suture biofilms was placed in a 96-well plate (Sarstedt) and A590 measured using a Synergy HT multi-mode microplate reader (BioTek) spectrophotometer with a 1 cm path length correction. Crystal violet binds negatively charged surface molecules, including those on live and dead bacteria, as well as on matrix polysaccharides.

Statistical differences between two groups were analysed by Student’s t-test and differences among three groups were analysed by ANOVA followed by Fisher’s test for significant difference. The relationship between pH and c.f.u. or between pH and nutrient concentration was analysed by simple regression or ANOVA with Fisher’s post hoc Bacterial numbers were converted to log10 prior to statistical analysis and significance was set at P<0.05.

Uptake of gentamicin–Texas Red. Gentamicin–Texas Red was made according to the method of Dai et al. (2006), with modifications. Briefly, 440 μl 50 mg gentamicin sulfate ml−1 was mixed with 60 μl 20 mg amine-reactive Texas Red-X succinimidyl ester.
(Invitrogen) ml⁻¹ in anhydrous N,N-dimethylformamide, plus 500 µl 1M NaCl in 100 mM K₂CO₃, plus 4 ml 100 mM K₂CO₃, for a final salt concentration of ~100 mM. The mixture was rotated overnight at 4 °C to produce approximately a 30:1 molar ratio of gentamicin–Texas Red conjugate (~10 mM gentamicin and 0.3 mM Texas Red reagent) with excitation/emission maxima at ~595/615 nm. The mixture was then brought to room temperature, adjusted to pH 6.5–8.5 and excess free dye was removed using a fluorescent dye removal column (ThermoFisher Scientific) according to the manufacturer’s directions. The antibacterial activity (MIC/MBC) of the gentamicin–Texas Red conjugate was determined and the data compared to MIC/MBC data calculated for gentamicin alone. The MIC/MBC for the gentamicin–Texas Red conjugate is similar to that calculated for unconjugated gentamicin (2/4 µg ml⁻¹ and 1/2–4 µg/ ml⁻¹, respectively). Therefore, conjugation of Texas Red to gentamicin likely does not affect the antibacterial activity of gentamicin. S. aureus was cultivated for 16 h in 1/3 ×, 1 × or 3 × TSBg, washed, resuspended in gentamicin–Texas Red for 1 h at room temperature, centrifuged, resuspended in HBSS, placed on a microscopic slide for 15 min, rinsed, fixed with 4 % paraformaldehyde, rinsed, mounted in Vectashield hardset and observed using a Nikon E-800 microscope equipped with a spinning disc BD CARVII confocal image adaptor. Using a ×100, 1.45 NA objective lens, widefield z-stacks were acquired at 0.2 µm intervals with a Cascade 1k EMCCD camera (Photometrics). Maximum intensity projections were derived from deconvolved z-stacks (Huygens Pro; Scientific Volume Imaging) and overlaid onto differential interference contrast images.

RESULTS

Effect of nutrient concentration on the gentamicin susceptibility of developing biofilms

S. aureus RN6390 biofilms were incubated overnight in 1/3 ×, 1 × or 3 × TSBg supplemented with varying concentrations of gentamicin. At the bactericidal concentration of 5 µg gentamicin ml⁻¹, gentamicin inhibited biofilm development in samples incubated in 1/3 × or 1 × TSBg, but not in 3 × TSBg. Similar results were obtained after quantifying both viable c.f.u. numbers (Fig. 1a) and biofilm biomass (Fig. 1b). Compatible results were obtained with S. aureus ATCC 25923, showing that the effect of increased nutrients on the bactericidal effect of gentamicin was not unique to a single S. aureus strain (Fig. S1, available in the online Supplementary Material). It should be noted that in the absence of antibiotic there was significantly more biofilm growth in the presence of 3 × versus 1 × or 1/3 × media;

![Fig. 1. Effect of nutrient concentration on gentamicin susceptibility of developing S. aureus biofilms incubated for 16 h. (a) c.f.u. and (b) biofilm biomass following incubation in 3×, 1× and 1/3× TSBg; (c) c.f.u. following incubation in 3×, 1× and 1/3× TSB; (d) direct comparison of c.f.u. numbers after incubation in 3× TSBg versus 3× TSB at each gentamicin concentration. *, Bacteria in 3× medium increased at P<0.01 versus in 1× or 1/3× media; †, bacteria in 3× medium increased at P<0.01 versus in 1/3×; # and †, bacteria in TSB decreased at P<0.01 and P<0.05, respectively, compared to that in the corresponding TSBg. Each data point represents six biofilms.](http://jmm.sgmjournals.org)
nutrition, but this increase was relatively small compared with the differences (between no antibiotic and antibiotic supplementation) noted at the higher bactericidal concentrations of 5 and 10 μg gentamicin ml⁻¹ (Figs 1a–c and S1). In selected experiments, the effect of nutrition on the susceptibility of planktonic S. aureus cultures to gentamicin was studied. Data from these experiments indicate that increased nutrient concentration promotes increased survival and growth of gentamicin-treated planktonic as well as biofilm-associated S. aureus (Table S1).

To investigate whether glucose supplementation of the biofilm growth medium played a major role in the ability of 3×TSBg to overcome the bactericidal effect of gentamicin, the experiment was repeated using unsupplemented TSB. Here, 3×TSB supported significantly greater biofilm growth compared with 1/3× and 1×TSB (Fig. 1c). However, a direct comparison of the c.f.u. values obtained with 3×TSB and 3×TSBg showed that glucose supplementation increased biofilm growth (Fig. 1d). To further clarify whether the increased glucose or salt concentration in 3×TSBg was responsible for the ability of 3×TSB to overcome the bactericidal effect of gentamicin, glucose or NaCl was added to 1/3× and 1×TSB in sufficient quantities to make the glucose or salt concentrations in 1/3×TSBg or 1×TSB equivalent to those found in 3×TSB. Resulting data showed that equilibrating the glucose or salt concentration of 1/3× and 1×TSB to that found in 3×TSB did not markedly affect the antibiotic susceptibility of bacteria grown in 1/3×or 1×TSB to gentamicin (Table S2).

To assess the effect of increased nutrient concentration on the uptake of gentamicin by S. aureus cells, S. aureus RN6390 was incubated overnight in 1/3×, 1× or 3×TSB. Washed cells were then incubated for 1 h in gentamicin–Texas Red and analysed by confocal microscopy. Compared with bacteria incubated in 1/3× and 1×TSB, gentamicin–Texas Red uptake was decreased using S. aureus incubated in 3×TSB; this result was obtained by quantifying arbitrary fluorescent units and was confirmed by direct visualization of internalized gentamicin–Texas Red (Fig. 2). Similar results were obtained with S. aureus ATCC 25923, as noted by decreased uptake of gentamicin–Texas Red by cultures incubated in 3×TSBg compared with those incubated in 1/3× or 1×TSBg (Fig. S2). Aminoglycoside uptake (necessary for the antibacterial activity of gentamicin) requires that a proton motive force be generated across the bacterial cell membrane (Mates et al., 1982). The proton motive force represents an electrochemical gradient composed of an electrical potential (Δψ), as well as a transmembrane difference in H⁺ concentration (ΔpH) (Mates et al., 1982; Eisenberg et al., 1984). Specifically, it is the electrical component (Δψ) of this gradient that influences aminoglycoside uptake (Eisenberg et al., 1984). When S. aureus bacteria are subjected to pH ≤5.0, ΔpH will be increased and Δψ decreases (Eisenberg et al., 1984). If a Δψ threshold level of −155 MV is not obtained, gentamicin uptake may not be initiated (Eisenberg et al., 1984).

**Effect of nutrient concentration on the susceptibility of developing biofilms to streptomycin, vancomycin and ampicillin**

To determine whether the ability of increased nutrients to overcome the bactericidal effect of an antibiotic was specific to gentamicin, developing S. aureus RN6390 biofilms were incubated in 1/3×, 1× or 3×TSBg supplemented with varying concentrations of streptomycin, vancomycin or ampicillin. Again, the concentrations of antibiotic included those that flanked the MIC for planktonic cells, i.e. concentrations considered to be susceptible as well as within the bactericidal range (Fig. 3). Compatible with the results obtained with gentamicin, increased nutrient concentration was able to overcome the bactericidal effect of another aminoglycoside, namely streptomycin (Fig. 3a). With one exception, increased nutrition had a comparatively incremental effect on the bactericidal effect of vancomycin (Fig. 3b) and ampicillin, two cell wall-active agents (Figs 3c and S3). S. aureus RN6390 biofilms cultivated in 3×TSBg, in

![Fig. 2](https://www.microbiologyresearch.org/attachment/image.png)

**Fig. 2.** (a) The graph shows comparative quantitative uptake of gentamicin–Texas Red by S. aureus incubated for 16 h in 3×, 1× and 1/3×TSBg. *, Decreased at P<0.01 compared with 1× and 1/3×TSB. Each data point represents the mean of 15 random images. (b, c) Representative fluorescent images (overlaid onto their corresponding differential interference contrast images) showing comparative uptake of gentamicin–Texas Red after incubation for 1 h for S. aureus previously cultivated for 16 h in 3×TSBg (b) or 1/3×TSBg (c). Bars, 2.5 μm.
the presence of vancomycin, showed decreased susceptibility to vancomycin at concentrations greater or equal to the MBC for this antibiotic (2–4 µg vancomycin ml⁻¹). However, taken altogether these results verify that the effect of increased nutrients on ameliorated bactericidal activity may be associated with the aminoglycosides gentamicin and streptomycin, and not with the cell wall-active agents vancomycin and ampicillin.

**Association between pH and the ability of increased nutrients to overcome the bactericidal effect of gentamicin**

Because bacterial resistance to gentamicin is known to be correlated with low pH, the pH of spent medium from developing *S. aureus* biofilms cultivated in 1/3 ×, 1 × and 3 × TSBg was determined in the absence of gentamicin and in the presence of a high bactericidal concentration of 5 µg gentamicin ml⁻¹. Spent media from biofilms cultivated in all three nutrient concentrations with and without gentamicin revealed decreases in pH as a function of time, although the pH data obtained for spent media from biofilms cultivated in 1/3 × and 1 × TSBg showed greater variability and did not reach statistical significance when compared with data obtained from the spent media of biofilms cultivated in 3 × TSBg (Fig. 4).

To clarify the role of pH in the ability of increased nutrients to overcome the bactericidal effect of gentamicin, regression analysis was used to assess the association between pH and c.f.u., and between pH and nutrient concentration. Here, data were pooled for experiments performed with all concentrations of the aminoglycoside gentamicin and for experiments performed with all concentrations of the cell wall-active vancomycin. Our experiments showed that, for both gentamicin (R²=0.78, P<0.01) and vancomycin (R²=0.86, P<0.01), increasing numbers of viable cells were consistently correlated with decreased pH, verifying the expected result that some acid production was associated with bacterial growth (data not shown). Additional comparisons between pH and nutrient concentration revealed a significant correlation between low pH and nutrient concentration when experiments were performed in the presence of 3 × TSBg and varying concentrations of gentamicin (R²=0.26, P<0.01), but there appeared to be no correlation between pH and nutrient concentration when experiments were performed in the presence of varying concentrations of vancomycin (R²=0.006, P=0.65). Thus, with gentamicin, low pH (4.85 to 5.10) was associated with the ability of a high nutrient concentration (3 × TSBg) to overcome the bactericidal effect of gentamicin (data not shown).

Finally, to further demonstrate the relationship between pH and the antibiotic susceptibility of *S. aureus* biofilms to aminoglycoside antibiotics, selected suture-biofilm assays were performed in 1/3 ×, 1 × and 3 × TSBg media adjusted to the following pH values: 6.5, 6.0, 5.75, 5.50 and 5.00 (Tables 1, S3 and S4). For all three nutrient concentrations, suture biofilms cultivated at pH 5.00 showed significantly less growth than those formed at pH 7.0. *S. aureus* suture biofilms cultivated in 1/3 × TSBg showed at least partial susceptibility to 5 and 10 µg gentamicin ml⁻¹ when grown in media with a pH value above 5.00 (Table 1), and biofilms cultivated in 1 × TSBg showed susceptibility to 10 µg gentamicin ml⁻¹ at pH 7.0 (*S. aureus* RN6390) (Table S3). As expected, all *S. aureus* suture biofilms cultivated in 3 × TSBg from both strains appeared relatively resistant (when compared with data from biofilms cultivated in 1/3 × and 1 × TSBg) to 5 and 10 µg gentamicin ml⁻¹ at all pH values tested (Tables S3 and S4). Taken altogether, these results indicate that the growth of suture biofilms is impaired at low pH irrespective of nutrient concentration, and that pH likely has an impact on the susceptibility of *S. aureus* suture biofilms cultivated in 1/3 × and 3 × TSBg to gentamicin.

![Fig. 3. Effect of nutrient concentration on susceptibility of developing suture-associated S. aureus biofilms to streptomycin (a), vancomycin (b) and ampicillin (c) after incubation for 16 h in 3 ×, 1 × and 1/3 × TSBg. * Bacteria in 3 × medium increased at P<0.01 versus in 1 × or 1/3 × media; †, bacteria in 3 × medium increased at P<0.01 versus in 1/3 × medium; ‡, bacteria in 3 × medium increased at P<0.05 versus in 1 × and 1/3 ×. Each data point represents six biofilms.](http://jmm.sgmjournals.org)
Aminoglycosides are polycationic molecules that must traverse the bacterial cell membrane and bind rRNA to prevent protein synthesis (Taber et al., 1987; Kotra et al., 2000). Studies with S. aureus have shown that aminoglycoside uptake requires a transmembrane electrical potential threshold level of at least −155 mV and appears greatest (Eisenberg et al., 1984) in log versus stationary phase growth and at an extracellular pH between 7 and 8 (Mates et al., 1982; Eisenberg et al., 1984). Thus, aminoglycoside effectiveness relies heavily on S. aureus growth phase and extrabacterial factors, including the availability of oxygen and the pH in the surrounding milieu.

Resch et al. (2005) used comparative transcriptome analysis to show that S. aureus biofilms exhibit distinct metabolic patterns over time and grow microaerobically or anaerobically when compared with their planktonic counterparts. Specifically, genes involved in formate synthesis and metabolism, and mixed acid fermentation pathways, are expressed at high levels in 24 and 48 h S. aureus biofilms leading to acidification (decreased pH) of the biofilm milieu due to the build-up of formic, lactic and acetic acids.

In related studies, Zhu et al. (2007) reported that biofilms (as opposed to planktonic cultures) formed by two strains of S. aureus (one susceptible and one resistant to meticillin) specifically extracted glucose, serine, proline, arginine, glutamine, glycine and threonine, leading to accumulation of organic acids and alcohols, and decreased pH in the surrounding medium.

Our data revealed that, compared with 1/3 × and 1 × TSB or with 1/3 × and 1 × TSbg, S. aureus biofilms cultivated in 3 × TSbg had increased resistance to bactericidal concentrations of gentamicin and streptomycin, but not the cell wall-active antibiotics vancomycin and ampicillin (Fig. 1a, c, d). In addition, biofilm biomass was significantly increased in samples cultivated in 3 × TSbg compared with 1/3 × or 1 × TSbg (Figs 1b and 3a). Assessment of biofilm biomass using biofilms cultivated in plastic dishes relies on charge interactions between anionic biofilm constituents (bacterial cell wall and extracellular matrix) and the cationic dye, crystal violet. The extracellular matrix of S. aureus biofilms is known to contain high concentrations of extracellular DNA (Wu & Xi, 2009). In fact, binding between the polycationic aminoglycoside molecules and the anionic matrix-bound DNA does occur and may lead to sequesteration of antibiotic in the matrix, thereby preventing uptake by susceptible bacteria (Chiang et al., 2013). In the case of 3 × TSbg, increased biofilm biomass may result in decreased interactions between gentamicin and bacterial surfaces when compared with similar interactions between bacterial cells and gentamicin in biofilms cultivated in 1/3 × or 1 × TSbg. Also, in support of the notion that increased biomass might inhibit penetration of aminoglycosides, we have previously shown that sonication of gentamicin-resistant suture biofilms results in mechanically dispersed bacteria with gentamicin susceptibilities comparable to their planktonic counterparts (Hess et al., 2011).

**DISCUSSION**

Biofilm communities utilize multiple mechanisms of antibiotic resistance unavailable to free-living cells. Like their planktonic counterparts, individual biofilm bacteria may employ specific mechanisms of antibiotic resistance (efflux pumps and enzymes), as well as general antimicrobial-resistance strategies that may be attributed to the multicellular growth conditions and extracellular matrix production that define biofilms (Stewart & Costerton, 2001). The multicellular growth mode of bacterial biofilms promotes antibiotic resistance by inhibiting the penetration of antimicrobials and oxygen through multiple layers of matrix-associated bacteria. In addition, the effectiveness of many classes of antibiotic relies on rapidly dividing bacteria undergoing aerobic respiration and biofilms contain large numbers of stationary phase ‘persister’ cells thought to be dormant (Lewis, 2007).
We were surprised to learn that, as in the case with suture biofilms, the susceptibility of planktonic \textit{S. aureus} to gentamicin also appears to be nutrient dependent (and although not tested, possibly other aminoglycosides as well). Specifically, planktonic \textit{S. aureus} showed decreased susceptibility to gentamicin in the presence of nutrient-rich medium. Therefore, it seems reasonable to assume that in order for suture-biofilm formation to take place in the presence of bactericidal concentrations of gentamicin, a threshold number of gentamicin-resistant planktonic cells must be present in the initial biofilm inoculum.

As stated, an important limiting factor in the uptake of aminoglycoside antibiotics is environmental pH, and uptake of gentamicin by \textit{S. aureus} is decreased at low pH and largely inhibited at pH $\leq 5$ (Eisenbach \textit{et al.}, 1984). In our experiments, the pH of developing biofilms cultivated in $3 \times$ TSBg was lower than those cultivated in $1 \times$ or $1/3 \times$ TSBg overnight (Fig. 4). In related studies, regression analysis from \textit{S. aureus} biofilms cultivated in $1/3 \times$, $1 \times$ and $3 \times$ TSBg in the presence of gentamicin was performed. This analysis showed that decreasing c.f.u. numbers were directly correlated with increasing pH in developing biofilms.

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<tr>
<td></td>
<td>5.75</td>
<td>3.5$\pm$0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td>3.1$\pm$0.2</td>
<td></td>
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<tr>
<td></td>
<td>5.00</td>
<td>2.8$\pm$0.6</td>
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</tr>
</tbody>
</table>

* pH adjustments were made with 12M HCl with an accuracy of $\pm 0.01$ pH units, except in the case of biofilms cultivated in media at ambient pH (pH 7) where the pH of the non-adjusted media varied by $\pm 0.02$–0.16 pH units.
† For both strains, biofilms cultivated at pH 5.00 showed less growth than those grown at pH 7, $P \leq 0.01$. 

\textbf{Table 1.} Viability of \textit{S. aureus} RN6390 and ATCC 25923 in developing suture biofilms cultivated in $1/3 \times$ TSBg media adjusted to pH 6.5, 6.0, 5.75, 5.5 and 5.0 in the presence of gentamicin. Data represent the mean $\pm$ SEM from two biofilms.
S. aureus biofilms subjected to gentamicin treatment, thereby providing evidence for the link between environmental pH and aminoglycoside activity. To further document a relationship between gentamicin efficacy and pH, S. aureus biofilms were cultivated in 1/3 × 1 x and 3 × TSBg at pH 7.0, 6.50, 6.00, 5.75 and 5.00. In the case of all three nutrient concentrations, suture biofilms cultivated at pH 5.00 showed significantly less growth than those formed at pH 7.0. As previously stated, S. aureus suture biofilms cultivated in 3 × TSBg overnight appeared relatively resistant to 5 and 10 µg gentamicin ml⁻¹ at all pH values tested (Tables S3 and S4). These data indicate that increased nutrient concentration may alter the kinetics of biofilm growth and metabolism resulting in relatively rapid (16 h) decreases in pH of the biofilm milieu, thereby decreasing the susceptibility of S. aureus biofilms cultivated in 3 × TSBg to gentamicin.

Finally, using gentamicin conjugated to Texas Red, and confocal microscopy, we showed that uptake of gentamicin–Texas Red by S. aureus was greatest following cultivation of bacteria in media with a relatively high pH (1/3 × and 1 × TSBg) versus bacteria cultivated in media bearing a low pH (3 × TSBg) (Fig. 2). Although at first glance, differences in pH between the spent medium from S. aureus cultivated in 3 × TSBg versus those cultivated in 1/3 × and 1 × TSBg do not appear great, studies indicate that observed pH differences as small as 0.2 to 0.5 units at the bacterial surface may increase ATP levels in bacteria upon adherence to glass (Hong & Brown, 2009). Because aminoglycoside uptake may be driven by both ATP and pH, minor shifts in pH likely affect ATP levels and aminoglycoside susceptibility in developing S. aureus biofilms.

In summary, the in vitro studies described herein suggest that nutrient availability alone may exert a profound effect on the susceptibility of S. aureus to aminoglycoside antibiotics. These studies underscore the importance of in vivo, extrabacterial factors found in the biofilm milieu in the clinical situation where the choice of antibiotic may be critical to the outcome for patients with serious infections.

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REFERENCES


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