SURGICAL INFECTIONS
Volume 16, Number 5, 2015

DOI: 10.1089/sur.2014.162

The Natural Surfactant Glycerol Monolaurate Significantly Reduces Development of *Staphylococcus aureus* and *Enterococcus faecalis* Biofilms

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Abstract

Background: Bacterial biofilms are involved in a large proportion of clinical infections, including device-related infections. Unfortunately, biofilm-associated bacteria are typically less susceptible to antibiotics, and infected devices must often be removed. On the basis of a recent observation that lipid-rich biofilm matrix material is present in early biofilm formation and may protect a population of bacteria from interacting with ordinarily diffusible small molecules, we hypothesized that surfactants may be useful in preventing biofilm development.
Methods: Experimental *Staphylococcus aureus* or *Enterococcus faecalis* biofilms were cultivated on surgical suture suspended in a growth medium supplemented with the natural surfactant glycerol monolaurate (GML) or with a component molecule, lauric acid. After 16 h incubation, the numbers of viable biofilm-associated bacteria were measured by standard microbiologic techniques and biofilm biomass was measured using the colorimetric crystal violet assay.
Results: Both GML and lauric acid were effective in inhibiting biofilm development as measured by decreased numbers of viable biofilm-associated bacteria as well as decreased biofilm biomass. Compared with lauric acid on a molar basis, GML represented a more effective inhibitor of biofilms formed by either *S. aureus* or *E. faecalis*.
Conclusions: Because the natural surfactant GML inhibited biofilm development, resulting data were consistent with the hypothesis that lipids may play an important role in biofilm growth, implying that interfering with lipid formation may help control development of clinically relevant biofilms.

Microbial biofilms develop in a variety of clinical situations and it is now recognized that biofilms are involved in more than 60% of infections [1]. Biofilms can be defined as surface-associated microbial communities that develop in liquid environments. Microbes within biofilms are often embedded in a hydrated matrix composed of an extracellular polymeric substance containing proteins, glycoproteins, glycolipids, polysaccharides, and extracellular DNA [2–5]. Biofilm-related infections encompass a variety of clinical processes and include periodontitis, otitis media, ventilator- and cystic fibrosis-related pneumonias, endocarditis, biliary tract infections, prostatitis, osteomyelitis, burn wound infections, other surgical site infections, and device-related infections such as those associated with catheters, sutures, and stents [1,6]. Device-related infections complicate treatment and may require removal of the infected device.

Biofilm-associated bacteria are generally less susceptible to antibiotic therapy compared with free-living planktonic bacteria [1,2,6] and the mechanisms responsible for this resistance are unclear. One explanation for the decreased antibiotic susceptibility of biofilm bacteria may be that antibiotic molecules are unable to interact directly with bacteria because of the proximity of impermeable matrix substance, or that charge characteristics of the matrix may interfere with binding between the antibiotic and its target microbe. For example, positively charged aminoglycosides are inhibited by negatively charged matrix material [6]. Although a variety of studies have reported unrestricted antibiotic diffusion through the biofilm [1,7,8], none of these studies had the resolution required to observe whether antibiotic was able to diffuse to each cell within the biofilm, i.e., none of these studies was able to verify that antibiotics were uniformly accessible to individual cells within a biofilm. We have used cytochemistry and fluorescent microscopy to observe and characterize the biofilm matrix material of in vitro and in vivo biofilms [9]. Our studies revealed the presence of occasional areas of lipid-containing matrix encasing some bacteria within the biofilm. This lipid matrix prevented comparatively

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small, ordinarily diffusible molecules from coming into contact with the encased bacterial cells. In light of these findings, it is conceivable that antibiotics may be able to diffuse through the biofilm but not come in contact with all bacterial cells throughout the biofilm. There may be areas in the biofilm that are shielded by a lipid hydrophobic barrier that prevents diffusion of antibiotics into these areas. Because we observed that lipid material may be identified early in biofilm development and appears to prevent penetration of small molecules into a portion of the bacterial cells [9], we now hypothesize that surfactants (surface-acting agents capable of disrupting lipid-containing structures) may interfere with biofilm development.

As an initial challenge to this hypothesis, the present study was designed to determine whether a natural surfactant, namely glycerol monolaurate (GML), could prevent development of experimental *Staphylococcus aureus* or *Enterococcus faecalis* biofilms. GML is a monoester composed of glycerol and lauric acid and is used as a surfactant in cosmetics and as an emulsifier in foods. In human beings, lauric acid is converted into GML and can be found in human breast milk. Although the clinical usefulness of GML has not been established firmly, GML has potent antimicrobial activity against enveloped viruses [10] as well as a variety of planktonic (free-living) bacteria including some gram-negative bacteria and some gram-positive bacteria such as *S. aureus* and *Streptococcus* species [11]. Resulting data from our study indicated that both GML and lauric acid interfered with development of experimental *S. aureus* or *E. faecalis* biofilms cultivated on surgical suture.

Materials and Methods

Bacterial strains and surfactants

Staphylococcus aureus RN6390 and ATCC 25923 are wild-type strains known to produce biofilms [12–15]. *Enterococcus faecalis* OG1RF is a plasmid-free strain, often used as a parent strain for genetic manipulations of this species [16] and *E. faecalis* VA1128 is a clinical isolate; both *E. faecalis* strains are also known to produce biofilms [15]. Bacterial inocula were washed cells from overnight cultures incubated at 35°C in tryptic soy broth, with bacterial concentrations confirmed by standard microbiologic methods. Surfactants (Sigma-Aldrich, St. Louis, MO) included GML (also known as glyceryl laurate or 1-Lauroyl-glycerol) and lauric acid, and the original powders were stored at −20°C. A 182 mM stock solution of GML was diluted in chloroform and stored at room temperature in the dark, and two stock solutions of 500 and 50 mM lauric acid were diluted in 100% ethanol and stored at −20°C. For experiments, working dilutions of GML and lauric acid were further diluted in biofilm growth medium (described below). Preliminary experiments showed that the residual chloroform in working dilutions of GML (<2.5 mcL/mL) and the residual ethanol in working dilutions of lauric acid (<25 mcL/mL) did not affect bacterial viability.

Development and analysis of suture-associated biofilms

Suture-associated biofilms were cultivated as described [12,13,15] with minor modifications. Briefly, each well of a 24-well microtiter plate contained a 1-cm segment of black braided 3-0 silk suture (Ethicon, Inc., Somerville, NJ) suspended in 1 mL of biofilm growth medium, namely 66% tryptic soy broth supplemented with 0.2% glucose [14], and the medium was supplemented additionally with varying concentrations of GML or lauric acid. Control wells contained no surfactant. Each well was inoculated with 10^7 *S. aureus* or *E. faecalis* and incubated 16 h at 37°C with gentle rotation (50 rpm). Suture-associated biofilms were photographed under phase contrast microscopy with an Olympus IMT-2 inverted microscope (Lake Success, NY). Suture-associated biofilms were analyzed for numbers of viable bacteria and for biofilm biomass as described below.

To assess the numbers of viable suture-associated biofilm bacteria, each suture was gently rinsed, transferred to 3 mL of sterile Hank's balanced salt solution, sonicated at approximately 50 joules at 100% amplitude for 5 sec using a sonicator at 20 kHz (Sonics and Materials, Newtown, CT). Sonication had no noticeable effect on bacterial viability [17], and microscopy confirmed that sonicated bacteria were single-cell suspensions. Bacterial concentrations in sonicates were determined by standard microbiologic methods, and the lower detection limit was 1.7 $\log_{10}$ colony forming units per suture. Biofilm biomass was measured with the basic dye crystal violet as described [18] with minor modifications. Crystal violet binds negatively charged surface molecules, including those on live and dead bacteria, as well as on polysaccharides in the biofilm extracellular matrix. Biofilm-laden sutures were gently rinsed with Hank's balanced salt solution, fixed in 99% methanol for 15 min, air-dried, incubated for 20 min with 0.5% crystal violet (Fisher Chemical, Pittsburgh, PA), washed, then incubated 20 to 30 min in 33% acetic acid to release the crystal violet, with absorbance read at 590 nm.

Statistical analysis

Comparisons of two treatment groups were analyzed by unpaired Student *t*-test and more than two groups were analyzed by one-way analysis of variance with Fisher post hoc. Significance was set at $p < 0.05$.

Results

Effect of GML and lauric acid on S. aureus *biofilm development*

Figure 1 shows that GML inhibited biofilm development with both strains of *S. aureus* used in this study. In the absence of GML, each suture held a biofilm containing approximately 10^7 *S. aureus*. As little as 0.35 mM GML inhibited biofilm development by at least 100,000-fold, reflected in the decreased numbers of viable bacteria from 10^7 bacteria per control suture-associated biofilm to $<10^{1.7}$ bacteria per suture, the lower limit of assay detection (Fig. 1A). Similarly, the biomass associated with both *S. aureus* strains was effectively inhibited at a concentration of 0.35 mM GML (Fig. 1B), and this is presented in Figure 2. This inhibition of biomass was noted with both *S. aureus* strains, although the biomass of samples treated at the lower concentrations of 0 and 0.1 mM GML were greater with the 6390 strain compared with the 25923 strain.

Figure 3 presents evidence that lauric acid alone also inhibited biofilm development of both strains of *S. aureus* used

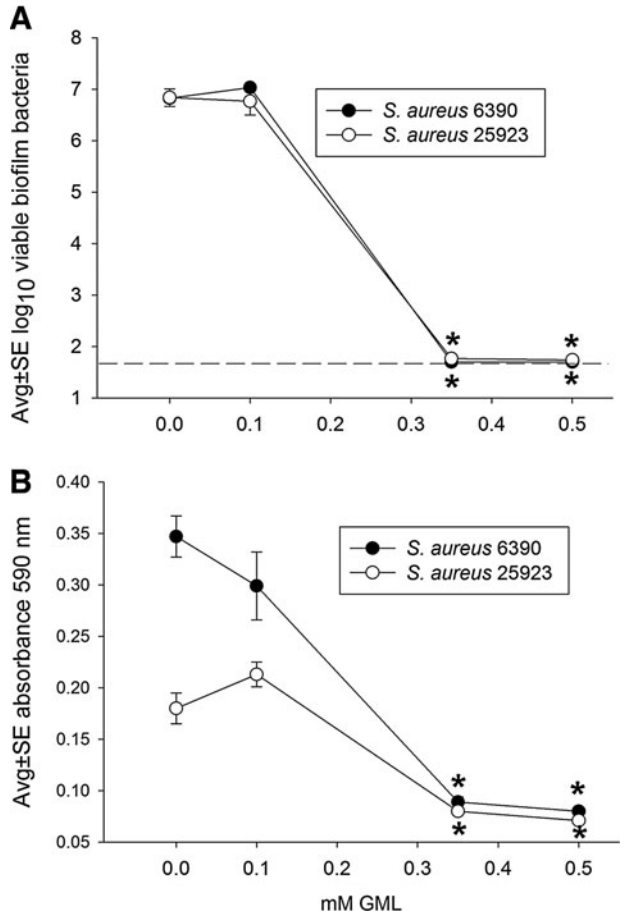

FIG. 1. Effect of glycerol monolaurate (GML) on development of *Staphylococcus aureus* RN 6390 and ATCC 25923 biofilms incubated 16 h on silk suture, as measured by the numbers of viable biofilm bacteria (**A**) and biofilm biomass (**B**). Each data point represents 12 biofilms. Dashed line represents the lower limit of assay detection. *, decreased at $p < 0.01$ compared with corresponding 0 mM GML.

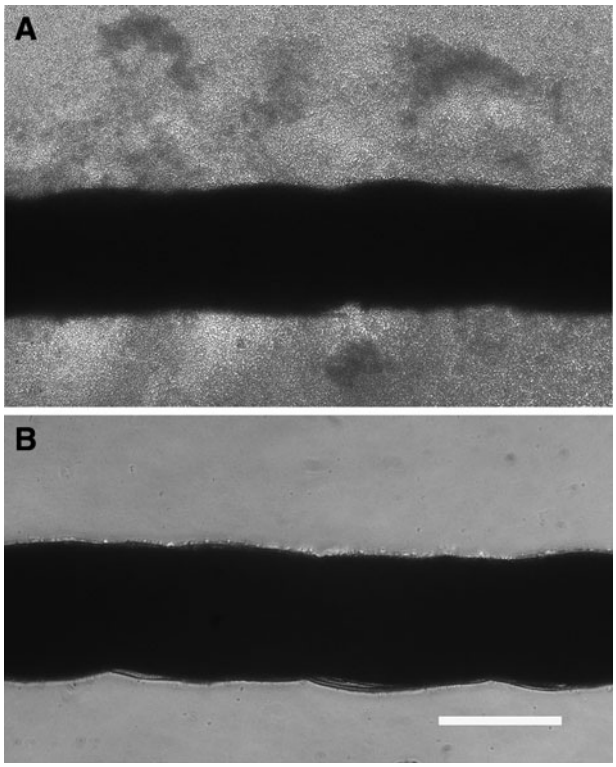

FIG. 2. Phase contrast micrographs of silk suture incubated 16 h with *Staphlococcus aureus* RN 6390 cultivated in growth medium alone (**A**) or in growth medium supplemented with 0.35 mM GML (**B**). Scale bar is 200 μm.

in this study. As expected, in the absence of lauric acid, each suture again held a biofilm containing approximately 10^7 *S. aureus* (Fig. 3A). In contrast to the results with GML in which only 0.35 mM of GML (Fig. 1A) was needed to inhibit bacterial viability down to the lower detection limit ($<10^{1.7}$ bacteria per suture-associated biofilm), 2.5 to 5 mM lauric acid was needed to achieve a similar result (Fig. 3A). However, similar to the results with GML (Fig. 1B), only 0.35 mM lauric acid was required to decrease the biomass of *S. aureus* RN6390 biofilms (Fig. 3B). In contrast to GML, lauric acid was not associated with a decrease in the biomass of the 25923 strain at all concentrations of lauric acid tested, a result likely because of the comparatively low biomass in control samples treated with 0 mM lauric acid (Fig. 3B).

Effect of GML and lauric acid on E. faecalis *biofilm development*

Figure 4A shows that GML also inhibited biofilm development with both strains of *E. faecalis* used in this study. In the absence of GML, each suture held a biofilm containing approximately $10^{6.4}$ *E. faecalis*. At a concentration of 0.4 mM, GML was bactericidal for *E. faecalis* biofilms, resulting in a reduction in the numbers of viable enterococci, i.e., approximately a 100- to 1,000-fold decrease from control values. Thus, similar concentrations of GML (0.3 to 0.4 mM) were bactericidal for both *S. aureus* and *E. faecalis*, and approximately 0.5 mM inhibited enterococcal viability to values near the lower limit of assay detection ($1.7 \log_{10}$ or 50 bacteria). Interestingly, similar to experiments with *S. aureus*, lauric acid alone effectively limited development of *E. faecalis* biofilms (Fig. 4B), where 1.25 mM lauric acid resulted in a decrease in the numbers of viable bacteria and 5 mM lauric acid decreased these numbers down to values near the lower limit of assay detection. In addition, similar to the results with *S. aureus*, developing *E. faecalis* biofilms were approximately 10-fold more susceptible to GML compared with lauric acid, where 0.5 mM GML and 5 mM lauric acid were associated with bacterial numbers that were at the lower limit of assay detection. (The effects of GML and lauric acid on *E. faecalis* biomass were not determined because preliminary experiments revealed a comparatively low biomass associated with enterococcal suture biofilms, making it difficult to observe a biomass decrease with this assay [data not shown]).

Discussion

The goal of this study was to assess the effectiveness of GML in preventing development of *S. aureus* and *E. faecalis* biofilms cultivated on surgical suture. This investigation was based on substantial evidence that GML has an antibacterial

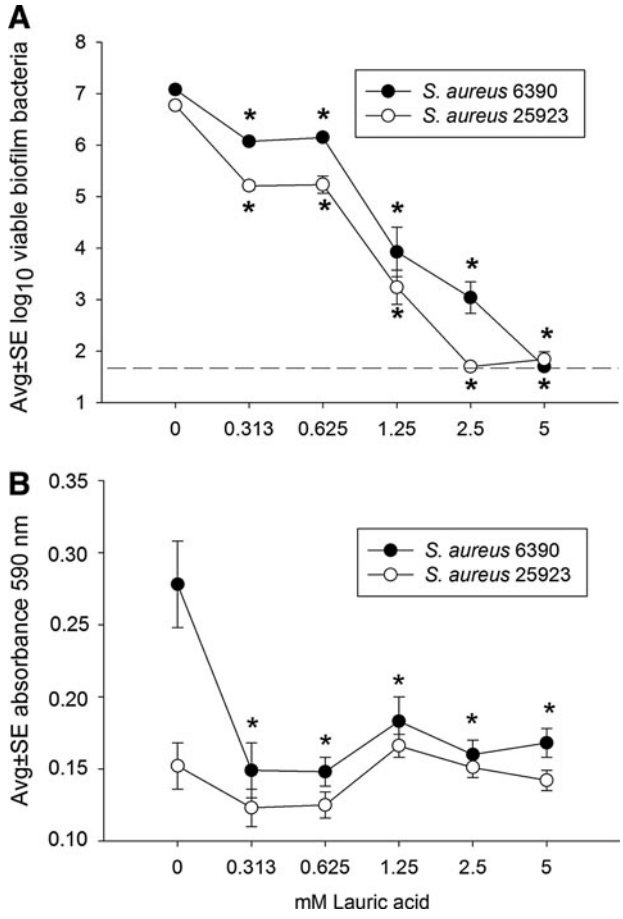

FIG. 3. Effect of lauric acid on development of *Staphylococcus aureus* RN 6390 and ATCC 25923 biofilms incubated 16 h on silk suture, as measured by the numbers of viable biofilm bacteria (**A**) and biofilm biomass (**B**). Each data point represents 12 biofilms. Dashed line represents the lower limit of assay detection. *, decreased at $p < 0.01$ compared with corresponding 0 mM lauric acid.

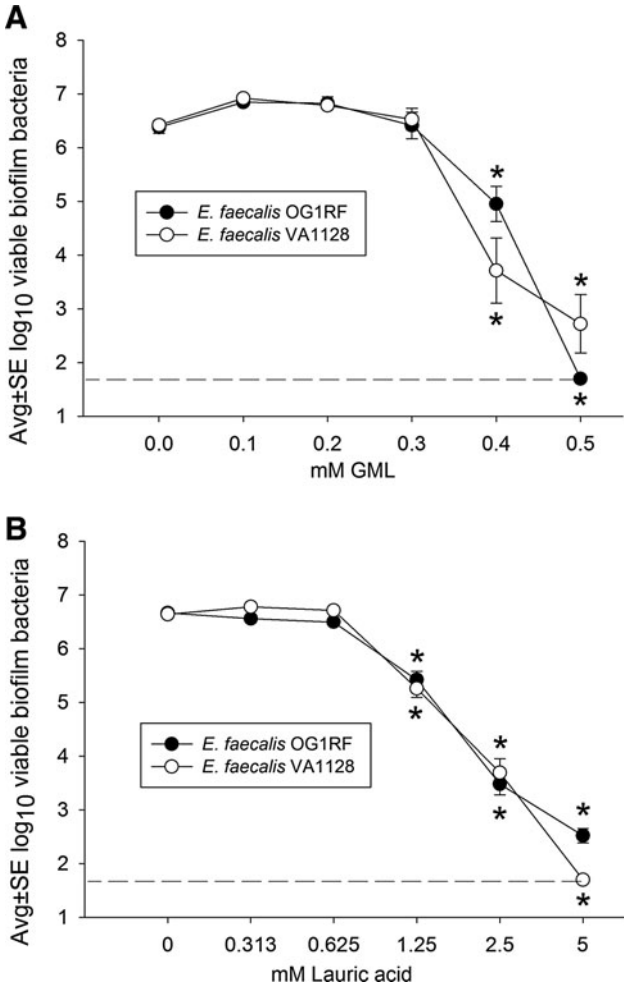

FIG. 4. Effect of glycerol monolaurate (GML) (**A**) and lauric acid (**B**) on development of *Enterococcus faecalis* OG1RF and VA1128 biofilms incubated 16 h on silk suture, as measured by the numbers of viable biofilm bacteria. Each data point represents 8 biofilms. Dashed line represents the lower limit of assay detection. *, decreased at $p < 0.01$ compared with corresponding 0 mM GML or lauric acid.

effect on a wide variety of clinically relevant microbes cultivated as planktonic cultures. For example, Schlievert et al. [11] reported that GML was bactericidal for a wide variety of aerobic and anaerobic gram-positive bacteria, including *S. aureus* and *Streptococcus* species, but gram-negative bacteria in the family Enterobacteriaceae (such as *Escherichia coli*) and *Pseudomonas aeruginosa* were resistant. Preuss et al. [19] also noted that GML was bactericidal for *S. aureus*, but not *E. coli* or *Klebsiella pneumoniae*, another member of Enterobacteriaceae. There is evidence that GML inhibited production of *S. aureus* virulence factors, such as β-lactamase, α-hemolysin, and toxic shock syndrome toxin-1, presumably by inhibiting signal transduction [20,21]. Glycerol monolaurate also inhibited induction of vancomycin resistance in *E. faecalis*, and this mechanism also appeared to involve signal transduction [22]. Strandberg et al. [23] reported that GML inhibited effectively *Candida* species and *Gardnerella vaginalis*, two potential vaginal pathogens. In addition, GML inhibited biomass formation in *S. aureus* biofilms cultivated in plastic dishes, and GML also inhibited production of toxic shock syndrome toxin-1 in a model of biofilms cultivated on tampon fibers [11]. Thus, there is substantial evidence that GML is bactericidal for a wide variety of microbes but the effect of GML on biofilm development has received relatively little attention.

Data from the present study indicated that GML inhibited the development of detectable viable *S. aureus* and *E. faecalis* biofilms, two gram-positive bacteria. Because others have shown that many gram-negative bacteria may not be susceptible to GML, it will be interesting to test the effect of GML on development of biofilms initiated with gram-negative bacteria such as *P. aeruginosa* or *E. coli*. With the gram-positive species in this study, GML-associated inhibition of biofilm development was noted with 0.35 mM GML (Figs. 1 and 4A), which corresponds to approximately 100 mcg/mL GML. On a molar basis, compared with lauric acid, GML was approximately 10-fold more effective in inhibiting development of viable *S. aureus* or *E. faecalis* biofilms because 2.5 to 5.0 mM lauric acid was needed to prevent recovery of viable bacteria in this model (Figs. 3 and 4B). Thus, although lauric acid was antibacterial on its own, its

ability to inhibit biofilm development appeared to be enhanced by the presence of the glycerol molecule in GML. This may be important clinically because, in liquid culture, *S. aureus* can rapidly hydrolyze GML to glycerol and lauric acid with a half-life of approximately 5 min, but lauric acid persists for at least 2 h [20].

Using *S. aureus* planktonic cells, others have similarly noted that GML has greater bactericidal activity than lauric acid, and that GML is more effective in inhibiting production of toxic shock syndrome toxin 1 [11]. However, Ruzin and Novick [20] reported that both lauric acid and GML have similar effects on production of staphylococcal exoproteins, including beta-lactamase and toxic shock syndrome toxin 1. Because GML inhibited suture biofilm formation more effectively than lauric acid and because the antimicrobial effects of GML and lauric acid on planktonic cultures vary between studies, our data indicated that the mechanisms responsible for the antimicrobial action of GML and lauric acid may be different in the case of biofilm-associated versus planktonic bacteria.

Our original hypothesis was that if lipids are important in development of *S. aureus* biofilms, then the addition of a surfactant should interfere with biofilm formation. Because the surfactant GML inhibited biofilm development, the resulting data were consistent with that hypothesis, and data from the present study provided additional evidence that the natural surfactant GML might be a viable candidate for controlling clinically relevant biofilms.

Acknowledgments

This work was supported in part by U.S. National Institutes of Health Grant R01 GM095553 and in part by funds from the Department of Surgery, University of Minnesota, Minneapolis, Minnesota.

Author Disclosure Statement

None of the authors has any commercial associations that might create a conflict of interest in connection with this article.

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