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A low molecular weight component of serum inhibits biofilm formation in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus has a variety of genes that can influence the process of biofilm formation. The ability to establish a biofilm is an important virulence factor for this pathogen, and yet, the regulation of this process *in vivo* is not well understood. *S. aureus* can form biofilms on intravenous catheters and this process plays a key role in the pathogenesis of catheter infections. In order to investigate whether or not serum is conducive to the process of biofilm formation, we grew *S. aureus* in serum and analyzed biofilm thickness and expression of biofilm-related genes. Whereas serum supported planktonic bacterial growth, it was a potent inhibitor of biofilm formation. The inhibitory serum component had a molecular weight less than 3,000 kDa. The component was protease-resistant and heat stable. The serum component induced a significant increase in the transcription of the intercellular adhesin gene *icaA* and the fibronectin binding protein gene *fmbA*. Transcription of other biofilm-related genes were affected in a strain-dependent manner. These results reveal that serum inhibits biofilm formation despite the fact that biofilms form on intravenous catheters. This may suggest that *in vivo*, biofilm formation is “selected for” by the force of blood flow and/or immune pressure rather than “induced” by serum.

Keywords

Serum; Biofilm; *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is one of the leading causes of nosocomial infections. It is an opportunistic pathogen that can infect a variety of different human tissues leading to various clinical manifestations ranging in severity from minor purulent infections like impetigo to more severe, potentially fatal, conditions like endocarditis and osteomyelitis [1]. *S. aureus* has a large arsenal of virulence factors that allow the bacteria to evade the host immune system, adhere to host tissue, and acquire nutrients from the host [2]. In addition, *S. aureus* has the capacity to form biofilms, which is recognized as an important virulence factor particularly in the pathogenesis of medical device-related infections such as intravenous catheter infections.

A biofilm is a community of microorganisms that adheres to a surface and is encased within an extracellular polymeric matrix. The staphylococci account for more than half of prosthetic-

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device-related infections and biofilm formation is a key component to the pathogenesis of such infections [3]. Biofilm formation can be broadly divided into two stages: adherence of the bacteria to a surface followed by cell-cell adhesion (aggregation). In *S. aureus*, adherence is mediated by surface adhesins such as the *S. aureus* surface protein G (SasG) [4] and fibronectin binding proteins (FnbA and FnbB) [5] while the aggregation is brought about, under certain conditions, by the synthesis of β -1,6 poly-N-acetylglucosamine (PNAG) also referred to as polysaccharide intercellular adhesion (PIA). First identified in *S. epidermidis*, and now known to be functionally conserved within *S. aureus*, the polysaccharide is synthesized by proteins encoded by the *icaADBC* operon [6,7]. Transcription of *icaADBC* and subsequent polysaccharide production and biofilm formation, is negatively regulated by the product of the *icaR* gene, which is transcribed divergently from *icaADBC*. Conversely, biofilm formation is positively regulated by the global transcriptional regulator SarA and the carbon catabolite protein CcpA [8,9,10,11,12,13]. The alternative sigma factor σ^B is also a positive regulator of biofilm formation but it is not clear how it exerts this function [14]. Together these intracellular components and others coordinate to orchestrate the process of biofilm formation. In addition to these intracellular components, extracellular factors play a key role as well, as they cue the bacterial cell to conditions under which the biofilm mode of growth is preferable. External cues that inform bacteria that they are *in vivo* and affect expression of biofilm-related genes are poorly understood. Because *S. aureus* can form biofilms on intravenous catheters and other inserted medical devices that come into contact with blood, we hypothesized that serum would be an external cue to trigger biofilm formation. However, we found the opposite to be true; serum was a potent inhibitor of the biofilm phenotype, and this inhibition occurred at least in part, at the level of gene expression.

2. Results and Discussion

2.1 Effect of serum on biofilm formation

Biofilm formation plays a key role in the pathogenesis of medical device-related infections and in the case of intravenous catheter-related infections and other device-related infections, *S. aureus* is exposed to blood. Furthermore, pre-coating polystyrene with serum is frequently used as a method to augment biofilm formation [21]. We therefore hypothesized that biofilm formation would be induced in serum. To test this hypothesis, the strong biofilm-producing strain SA113 was grown in TSBG supplemented with human serum or PBS (as a control). While TSBG and TSBG containing 10% or 50% PBS fostered the elaboration of a thick biofilm, addition of 10% or 50% human serum inhibited biofilm formation (Fig. 1A). The effect was not limited to human serum as 50% fetal bovine serum (FBS) possessed similar inhibitory activity (Fig. 1A). Human plasma also prevented the formation of a biofilm (data not shown). In order to confirm that inhibition of biofilm formation was not due solely to growth inhibition, we measured growth under the same conditions. The optical densities of the cultures after 16 hr revealed that the presence of 50% serum supported planktonic growth (Fig. 1B). To confirm that this effect was not specific to strain SA113, we tested the unrelated strain Newman. We found that serum inhibited biofilm formation in strain Newman as well, and that 10% FBS had inhibitory activity in this strain (supplementary Fig. S1).

2.2 Characterization of the inhibitory component/s

To obtain a crude estimation of the molecular weight of the components responsible for the inhibitory activity in serum, we removed components with approximate molecular weights $>3,000$ kDa using a centrifugal concentrator. The low ($<3,000$ kDa) molecular weight fraction (LMWF) of human serum retained the ability to effectively inhibit biofilm formation compared to control TSBG alone or with supplemented PBS (Fig. 2A, B). It has been shown previously that serum proteins such as lactoferrin, apo-transferrin, and albumin inhibit aggregation and biofilm formation [15, 16, 17, 18], but a role for a small molecular weight component/s has

never been established. To further investigate the nature of the small molecular weight component in the flow-through we heated the <3,000 MW fraction to 100°C for 5 min. Heating did not inactivate the inhibitory activity (Fig. 2C). To eliminate the possibility that a heat stable protein was responsible for the biofilm inhibition, Proteinase K was used to degrade proteins in the FBS flow-through but this did not affect the ability of this low molecular weight fraction to inhibit biofilm formation (Fig. 2D). The results were similar when FBS was added instead of human serum (Fig. S2) and results were similar for strain Newman (Figs. S3 and S4). Together these results suggest that the compound in serum that inhibits *S. aureus* biofilm formation is not proteinaceous. We found in a previous study that basic conditions can inhibit biofilm formation [19]. Because the pH of serum is slightly basic, we buffered the <3,000 kDa fraction to pH 7.0 to determine whether the effect was due to high pH; however, this did not affect the biofilm-inhibiting activity (data not shown).

2.3 Serum components affect expression of biofilm-related genes

To determine whether inhibition of biofilm formation occurred at the level of gene expression, total RNA was isolated from cells grown in TSBG + 50% PBS or 50% of the LMWF of serum and expression levels of specific genes that have been previously implicated in biofilm formation or regulation were determined by realtime RT-PCR. The individual genes were normalized to the 16S rRNA and the percentage difference with respect to the TSBG is shown (Fig. 3). In comparison to TSBG, expression of the *icaA*, *ccpA*, and *fnbA* genes was significantly lower (by Student's *t*-test $p < 0.002$) within the serum fraction. IcaA (Intercellular Adhesin protein A), is a glucosyltransferase, necessary for the synthesis of PNAG/PIA, the primary component of *S. aureus* biofilms. Interestingly, there were strain-variable differences in the expression of other biofilm-related genes. The serum fraction lead to a drastic decrease in *sasG* transcript levels (0.0005) in strain SA113 but actually lead to a slight increase in Newman. SasG (*S. aureus* surface protein G), is a cell walled anchored protein that functions as an adhesin, facilitating the initial steps associated with binding to the substrate during biofilm formation [4,20]. Furthermore, the level of *sarA* transcript increased approximately 250% in SA113 ($p < 0.002$) but was repressed in strain Newman and levels of the *sigB* and *fnbB* transcripts were inhibited by the serum fraction in SA113 but remained unchanged in Newman ($p > 0.05$). These findings indicate that the response of different strains to serum varies. This is not surprising, as biofilm formation in different strains depends upon different proteins and polysaccharides, and can even depend on extracellular DNA. The findings suggest that inhibition of biofilm formation by FBS flow-through is due, at least in part, to inhibition of expression of biofilm-related genes. Therefore, biofilm formation in vivo may be "selected for" (possibly by immune pressure and sheer forces) rather than "induced" at the level of transcription. It is possible that *S. aureus* exhibits the planktonic mode of growth in vivo to maximize its growth potential in a nutrient-rich environment. The few bacteria that happen to adhere to the device would have a selective advantage over the planktonic population, which would be killed by immune defenses. Serum-mediated inhibition of biofilm formation may represent a previously uncharacterized aspect of host innate immunity that targets the expression of a key bacterial virulence factor: the ability to establish a resistant biofilm.

3. Material and Methods

3.1 Biofilm assay

SA113, also known as ATCC 35556, is a biofilm-positive strain of *S. aureus*. Newman is a strong-coagulase-producing isolate from a case of osteomyelitis. SA113. Planktonic cultures of *S. aureus* were grown in Luria Bertani (LB) broth media (EMD Chemicals, Gibbstown, NJ) at 37°C with shaking at 200rpm and maintained on Tryptic Soy Agar (TSA) plates (Becton Dickinson, Sparks, MD). Static biofilm assays were performed essentially as described by Christensen *et al.* [22]. Briefly, overnight planktonic cultures of *S. aureus* were diluted 1:200

within fresh media and 200 μ L of the culture was aliquoted into individual wells of two replicate, 96 well Corning Cell Bind Plates (Corning Glass Works, Corning, NY). The plates were incubated at 37°C for 16–18 hrs. For one of the replicate plates, the spent medium was removed, the wells were washed with 200 μ L 1X PBS to remove non-adherent bacteria, and the plates were dried and stained with safranin (PML Microbiologicals, Wilsonville, OR). After the biofilms were visually analyzed and imaged using a flatbed scanner, the safranin was dissolved with 200 μ L of 33% (v/v) acetic acid per well and the plates were analyzed at an optical density of 415nm using a 96-well plate spectrophotometer (BioTek 800 Plate reader, Winooski, VT). The second replicate plate was used to determine growth. Bacteria and biofilms within the wells were resuspended and the A_{595nm} was determined using the plate spectrophotometer. For each condition, three biologic replicates, each consisting of 5 technical replicates were performed. Conditions included Tryptic Soy Broth (TSB) supplemented with 1% Glucose (TSBG), TSBG + 50% or 10% Phosphate Buffer Solution (PBS) (GIBCO, Invitrogen, Carlsbad, CA), TSBG + 50% or 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, West Sacramento, CA) and TSBG + 50% or 10% Human Serum (HS) (MP Biomedicals, Solon, OH).

3.2 Characterization of serum components

Small molecular weight components were separated from FBS by adding 15mL FBS to Amicon Ultra filter units (Millipore, MA) with a 3,000 MWCO. The samples were centrifuged at 4,000 rpm for approximately 60 min at 4°C. For some experiments, heat labile components in the <3,000 kDa fraction were destroyed by heating at 100°C for 5 min. In others, proteinaceous serum components were destroyed by incubating with 0.4mg/mL proteinase K at 65°C for 1 hr followed by incubation at 100°C for 5 min to inactivate the protease. All fractions were filter sterilized.

3.3 Quantitative Real time RT-PCR analysis

Overnight cultures of SA113 and Newman grown in TSBG were diluted 1:10 in fresh TSBG or human serum and incubated at 37°C for 3 hours with aeration. To isolate RNA, approximately 5×10^8 bacteria were resuspended in 500 μ L of Qiagen Buffer RLT and transferred to Lysing Matrix B tubes (MP Biomedicals). 500 μ L cold Acid Phenol was added and the cells were lysed using FastPrep FP120 (Thermo, Pittsburgh, PA). The beads were removed by centrifugation and the supernatant was purified using the RNeasy® Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Contaminating DNA was removed with Turbo DNase (Ambion, Austin, TX) of 20 min at 37°C. 4 μ g of RNA was converted to cDNA using Accuscript reverse transcriptase (Agilent Technologies, La Jolla, CA) according to manufacturer's instructions. The primer mix consisted of the reverse primers listed in Table 1, at a final concentration of 10pmol each. Control reactions lacked reverse transcriptase enzyme. Realtime RT-PCR reactions contained 2 μ L cDNA or no-RT control (diluted 1:500 for 16S reactions and 1:5 for other genes), 1pmol forward and reverse primers, 8.5 μ L nuclease free deionized H₂O, and 12.5 μ L Sensimix plus SYBR Green mix (Quantace, Bioline, Randolph, MA). Realtime RT-PCR was performed using an iQ5 Multicolor Realtime PCR (BioRad, Hercules, CA) under the following conditions: 94°C for 2 min, 40 cycles of 94°C for 10 s, 54°C for 15 s, 72°C for 15 s. The normalized amount of transcript for each gene was expressed as the n-fold difference relative to the control gene ($2^{\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target gene and the 16s rRNA gene). Percentage change was calculated as the ratio of the normalized values in the serum fraction minus the values in TSBG divided by the values in TSBG. Experiments were performed in triplicate and the average is shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Serum inhibits biofilm formation

Overnight cultures of SA113 grown in LB were diluted 1:200 and inoculated in: TSBG, TSBG + 50% or 10% PBS, Human Serum (HS), TSBG + 50 or 10% HS, Fetal Bovine Serum (FBS), TSBG + 50% or 10% FBS. Biofilms were allowed to form in 96 well polystyrene plates at 37°C for 18 hours. Non-adherent cells were removed by washing with 1× PBS and the adherent bacteria were dried and stained with safranin. **A.** Scanned image of the bacteria grown within the wells. **B.** Quantitative representation of growth versus biofilm formation under the respective conditions. Growth (blue bars) was measured by resuspending the bacteria and measuring A_{595nm} . Biofilm formation (Red bars) was assessed by solubilizing the safranin in 33% (v/v) acetic acid and measuring A_{415nm} . Each condition was tested 5 times within each experiment and experiments were performed a minimum of three times on separate days with similar results and shown here is a representative image.

Figure 2. The low molecular weight fraction (LMWF) of human serum inhibits biofilm formation Serum components >3,000 kDa were removed by filtration. SA113 cultures were grown in 96 well plates in: A. TSBG, TSBG + 50% or 10% PBS, B. <3,000 kDa component of human serum (LMWF), TSBG + 50% or 10% LMWF, C. Boiled LMWF, TSBG + 50% or 10% heated LMWF, D. Proteinase K treated LMWF, TSBG + 50% or 10% Proteinase K-treated LMWF. Biofilms were imaged and thereafter read at A_{595nm} or A_{415nm} to determine growth versus biofilm formation under the respective conditions. Results represent the data from 3 independent experiments.

Figure 3. Transcript levels of biofilm associated genes under various media conditions

Total RNA was isolated from A. strain SA113 and B. strain Newman grown in TSBG or LMWF of serum during mid-log phase of growth. The transcript levels of specific genes that have been previously implicated in biofilm formation were analyzed by realtime RT-PCR. Individual genes were normalized to 16s rRNA and relative fold difference is shown with respect to TSBG. Each gene was assessed in triplicate and the experiment itself was performed in biologic duplicate. The data shown here is a representative graph of one experiment.

Table 1

Primers used for qRT-PCR

Primer Description	Sequence
16S RT Fwd	5'-TCCGGAATTATTGGGCGTAA-3'
16S RT Rev	5'-CCACTTTCCTCTTCTGCACTCA-3'
icaA RT Fwd	5'-AAACTTGGTGCGGTTACAGG-3'
icaA RT Rev	5'-GTAGCCAACGTCGACAACTG-3'
sarA RT Fwd	5'-TTGCTTTGAGTTGTTATCAATGG-3'
sarA RT Rev	5'-TTTCTCTTTGTTTTCGCTGAT-3'
sasG RT Fwd	5'-ACCACAGGGTGTAGAAGCTAAATC-3'
sasG RT Rev	5'-CGAGCTTTTCTAACCTTAGGTGTC-3'
sigB RT Fwd	5'-TTATGGGGCAACAAGATGAC-3'
sigB RT Rev	5'-TAAACCGATACGCTCACCTG-3'
ccpA RT Fwd	5'-AGTGTCGCGTGTGTTAATG-3'
ccpA RT Rev	5'-AAGTCCACGAGCAAGTTGTG-3'
fnbA RT Fwd	5'-ATCAGCAGATGTAGCGGAAG-3'
fnbA RT Rev	5'-TTTAGTACCGCTCGTTGTCC-3'
fnbB RT Fwd	5'-AAGAAGCACCGAAAAGTGTG-3'
fnbB RT Rev	5'-TCTCTGCAACTGCTGTAACG-3'