An Essential Role for Coagulase in *Staphylococcus aureus* Biofilm Development Reveals New Therapeutic Possibilities for Device-Related Infections

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High-level resistance to antimicrobial drugs is a major factor in the pathogenesis of chronic *Staphylococcus aureus* biofilm-associated, medical device-related infections. Antimicrobial susceptibility analysis revealed that biofilms grown for ≤24 hours on biomaterials conditioned with human plasma under venous shear in iron-free cell culture medium were significantly more susceptible to antistaphylococcal antibiotics. Biofilms formed under these physiologically relevant conditions were regulated by SaeRS and dependent on coagulase-catalyzed conversion of fibrinogen into fibrin. In contrast, SarA-regulated biofilms formed on uncoated polystyrene in nutrient-rich bacteriological medium were mediated by the previously characterized biofilm factors poly-N-acetyl glucosamine, fibronectin-binding proteins, or autolytic activity and were antibiotic resistant. Coagulase-mediated biofilms exhibited increased antimicrobial resistance over time (>48 hours) but were always susceptible to dispersal by the fibrinolytic enzymes plasmin or nattokinase. Biofilms recovered from infected central venous catheters in a rat model of device-related infection were dispersed by nattokinase, supporting the important role of the biofilm phenotype and identifying a potentially new therapeutic approach with antimicrobials and fibrinolytic drugs, particularly during the early stages of device-related infection.

**Keywords.** *Staphylococcus; biofilm; infection; coagulase; antimicrobial; susceptibility.*
A, SasG, SasC, ClfB, and the secreted proteins Eap and Emp [3]. The ability of a number of cell wall and extracellular proteins from *S. aureus* to recruit and deposit extracellular matrix proteins such as fibrinogen or fibrin on the cell surface has led to the idea that so-called fibrin shields play a role in resistance to opsonophagocytosis and perhaps vaccine failure [2]. Furthermore, a number of cell wall proteins involved in biofilm accumulation (FnBPA, FnBPB, and ClfB) are known to bind fibrinogen, suggesting a relationship between these 2 phenotypes. Consistent with this, Dastgheyb et al recently reported an important role for staphylothrombin-mediated fibrin deposition in the pathogenesis of catheter-related infections caused by *S. aureus* [10, 11]. Similarly, coagulase was significantly implicated in the formation of biofilm-like aggregates, which play an important role in the pathogenesis of joint infections [12, 13].

The potential contribution of fibrin deposition to biofilm formation under in vivo conditions highlights both the importance of the biofilm phenotype and the possible redundancy among biofilm mechanisms used by *S. aureus*, given that cells can be embedded in a matrix of bacterial proteins, host glycoproteins, polysaccharide, or extracellular nucleic acids [4, 14, 15]. However, it remains unclear which of these biofilm mechanisms is preferentially deployed under in vivo conditions, and hence the relative contribution of different staphylococcal biofilm matrixes to the antimicrobial resistance of biofilms has been largely unexplored. To date, in vitro analysis of staphylococcal biofilm has generally focused on measurements of bacterial cell attachment to and accumulation on abiotic surfaces by using nutrient-rich bacteriological growth medium [16] and, more recently, under physiologically relevant shear [17].

In this study, we describe the development of an in vitro biofilm model to more closely reflect the in vivo milieu. Attachment and biofilm accumulation by *S. aureus* strains grown in cell culture medium on surfaces conditioned with human plasma were measured under venous shear. Antimicrobial susceptibility of biofilms grown under traditional methods and under these more physiologically relevant conditions was compared. Using the Nebraska Transposon Mutant Library (NTML) [18], we studied the relative contribution of known biofilm factors in the community-associated MRSA strain USA300 JE2 and validated this finding in other *S. aureus* backgrounds. Our data reveal a new biofilm phenotype mediated by coagulase and ClfA and regulated by the SaeRS system. Furthermore, these biofilms exhibit a distinct antimicrobial susceptibility profile and, as demonstrated by in vitro and in vivo findings, can be dispersed by fibrinolysins.

**MATERIALS AND METHODS**

**Preparation of Platelet-Rich Plasma (PRP), Platelet-Poor Plasma (PPP), and Serum**

PRP was prepared by collecting human blood specimens in syringes containing heparin at 16 IE/mL. Whole blood was centrifuged at 150 × g for 10 minutes, and the top layer, consisting of PRP, was carefully removed. PPP was obtained by centrifugation of PRP or whole blood at 500 × g for 10 minutes. Serum was obtained by centrifugation of clotted whole blood at 2000 × g for 10 minutes at 4°C.

**Biofilm Analysis Under Static Conditions**

Strains used in the study are listed in Table 1. Biofilms were grown in hydrophilic (Nunclon) or hydrophobic (Cosvar) plates, as indicated. A total of 100 μL of 20% (v/v) plasma in 50 mM carbonate buffer, pH 9.6, was incubated in the wells for 2 hours at 37°C. A range of plasma concentrations (5–100% v/v) were tested and showed that a 20% PPP v/v supported optimal biofilm formation (data not shown). *S. aureus* overnight cultures in Roswell Park Memorial Institute (RPMI) 1640 medium were diluted at a ratio of 1:1 (v/v) in fresh medium and incubated in microtiter wells for 1 hour at 37°C. Unattached bacteria were removed, and the well was refreshed with sterile medium. To grow biofilms in rich medium, overnight cultures were diluted a ratio of 1:200 in either tryptic soy broth (TSB) or brain-heart infusion (BHI) broth, and biofilm assays were performed as described above. Biofilms were grown for 24 hours at 37°C, were stained with 0.5% crystal violet, and underwent absorbance measurement at OD_{490}. To quantify viable bacteria in biofilms, TrpLE (recombinant trypsin; Gibco) was used to disperse biofilms for 5 minutes at 37°C, and the number of colony-forming units (CFU) was determined.

**Biofilm Treatment With Antibiotics or Enzymes**

Antibiotics or enzymatic agents (proteinase K, plasmin, nattokinase, serrapeptase, and TrpLE) were added to biofilms and incubated for 24 hours unless otherwise indicated. Viability after treatment was measured using a resazurin-conversion assay, and biofilm thickness was determined by crystal violet staining.

**Resazurin-Conversion Assay**

Conversion of the nonfluorescent redox dye resazurin into the fluorescent resorufin was used to measure viability of bacteria in biofilms. A stock solution of resazurin (Sigma) at 440 μM was diluted to 10% (v/v) in RPMI 1640 medium and added at a ratio of 1:1 (v/v) to microtiter well biofilms before being incubated in the dark at 37°C for 1 hour. Fluorescence at 544-nm excitation and 590-nm emission was read using a Perkin Elmer 2030 Multilabeled Reader Victor X3.

**Biofilm Analysis Using a Cellix Microfluidic Flow Cell System**

Vena8 Fluoro+ flow chambers were primed 3 times with 20 μL of PPP at 37°C for 30 minutes. *S. aureus* overnight cultures were diluted 1:1 in RPMI 1640 medium or 1:200 in TSB. Bacterial suspensions were injected into flow chambers and incubated for 4 hours before the microfluidic pump was engaged to visualize biofilm growth at 37°C for 24 hours under a shear of 6.25 dynes/cm² (200 μL/min).
**Table 1. Staphylococcus aureus Strains Used in This Study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>BH1CC</td>
<td>MRSA; device-related infection isolate</td>
<td>[9]</td>
</tr>
<tr>
<td>BH48</td>
<td>MSSA; device-related infection isolate</td>
<td>[9]</td>
</tr>
<tr>
<td>USA300</td>
<td>CA-MRSA</td>
<td>[41]</td>
</tr>
<tr>
<td>USA300 ΔsaeRS USA300 derivative; ΔsaeRS</td>
<td>[42]</td>
<td></td>
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<tr>
<td>USA300 lux USA300 constitutively expressing luciferase (lux) from Photobacterium luminescens</td>
<td>[43]</td>
<td></td>
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<tr>
<td>USA300 JE2 USA300 lacking plasmids p01 and p03; NTML parent</td>
<td>[18]</td>
<td></td>
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<tr>
<td>JE2 srtA::Tn</td>
<td>NTML mutant</td>
<td>[18]</td>
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<td>JE2 srtB::Tn</td>
<td>NTML mutant</td>
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<tr>
<td>JE2 spa::Tn</td>
<td>NTML mutant</td>
<td>[18]</td>
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<tr>
<td>JE2 atl::Tn</td>
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<td>[18]</td>
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<tr>
<td>JE2 clfA::Tn</td>
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<tr>
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<tr>
<td>JE2 fnbA::Tn</td>
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<tr>
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<td>JE2 eap::Tn</td>
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<td>JE2 icaA::Tn</td>
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<td>JE2 icaC::Tn</td>
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<tr>
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<td>Strain 8325-4 with a repaired rsbU gene; SigB positive</td>
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<td>SH1000 protein A mutant</td>
<td>[45]</td>
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<tr>
<td>SH1000 clfA</td>
<td>SH1000 carrying frameshift mutation in clfA introduced using pJH plasmid</td>
<td>H. Miajlovic and T. J. Foster, unpublished data</td>
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**Biofilm Formation Using a BioFlux Microfluidic Flow Cell System**

The BioFlux 1000z microfluidic system (Fluxion Biosciences, California) was used to assess biofilm formation at 0.6 dynes/cm² for 18 hours as described previously with the following modification. Each channel of a 48-well plate was coated with 50 µL of undiluted platelet-poor human plasma for 30 minutes at 37°C before biofilm assays were set up.

**Coagulase Activity Assay**

*S. aureus* cultures were grown for 18 hours and adjusted to equal optical densities at 600 nm (OD₆₀₀). For RPMI 1640 medium, cultures were adjusted to an OD₆₀₀ of approximately 1, while for TSB, cultures were adjusted to an OD₆₀₀ approximately 10. Bacterial suspensions were centrifuged to separate culture supernatants, which were collected for coagulase assay. Microtiter wells were inoculated with 100 µL of human plasma. A total of 100 µL of culture supernatant, 1:1 diluted culture supernatant, or medium control was added to the wells. Microtiter plates containing the mixtures were incubated for 4 hours at 37°C following absorbance reading at 600 nm.

**Peptide Synthesis**

D- Fg wt γ (1–17) D16A (D-enantiomer of the γ chain of fibrinogen) peptide (NH₂-gegqqhhlggakqagac-CONH₂) was synthesized by automated solid-phase peptide synthesis on a 433A synthesizer (Applied Biosystems, United Kingdom) from 9-fluorenylmethoxy carbonyl (Fmoc)-protected D-amino acids (Merck, United Kingdom).

**Rat Jugular Vein Catheter Infection Model**

Sprague-Dawley rats with preimplanted jugular vein catheters were obtained from Charles River (United Kingdom). The 13.5-cm-long catheters (outside diameter, 1.1 mm; inside diameter, 0.6 mm) were inoculated with a 40-µL suspension of USA300lux containing 10⁶ CFU/mL. The catheters were flushed daily with 150 µL of sodium chloride (0.1% w/v), and the lumen was locked with 40 µL of sodium chloride (0.1% w/v). Intravenous vancomycin (50 mg/kg) was administered twice daily at 1-hour intervals. After 10 days, infected catheters were removed, and luminescence was imaged using a Perkin Elmer IVIS Spectrum instrument (exposure, 20 seconds; binning: 4, f1) before being treated three times with 100 µg/mL nattokinase (2000 FU) for 1 hour at 37°C.

**Ethics Approval**

Blood donations were obtained from healthy adult donors. Written, informed consent was obtained from participants at the time of collection. Ethics approval for collection and use of blood was granted by the Ethics Committee of the Royal College of Surgeons in Ireland (RCSI; REC820). Animal experiments were conducted under Irish Government Department of Health guidelines, with ethical approval from the RCSI Ethics Committee (REC931).

**Statistical Analysis**

The data presented by this study represent the means ± SD of three experiments unless otherwise stated. Statistical significance was assessed using one-way ANOVA and indicated as * for P < .05, ** for P < .001 and *** for P < .0001.
Figure 1. Susceptibility of *Staphylococcus aureus* biofilms to rifampicin and vancomycin. A, Biofilms of *S. aureus* USA300 (gray bars) and SH1000 (checkered bars) grown in plasma-coated wells in RPMI-1640 at 37°C for 1, 3, or 5 days and then treated with rifampicin or vancomycin at indicated concentrations. B, Biofilms of *S. aureus* USA300 (gray bars) and SH1000 (checkered bars) grown in wells coated with human plasma in brain-heart infusion broth at 37°C for 1 or 5 and subsequently treated with rifampicin or vancomycin at 50 mg/mL. Biofilm viability was determined using a resazurin conversion assay. Fluorescence intensity data are the means ± standard deviation of 3 independent experiments.
RESULTS

Biofilm Antimicrobial Susceptibility Is Increased on Human Plasma–Conditioned Surfaces

To more closely mimic in vivo conditions, PRP, PPP, and serum were used to condition microtiter wells and revealed that plasma proteins but not serum promoted biofilm formation (Supplementary Figure 1). Plasma conditioning contributed significantly to the thickness of biofilms involving of all *S. aureus* strains, including isolates from device-related infections, such as BH1CC and BH48 (Supplementary Figure 1). To reflect iron limitation encountered by bacteria in vivo, biofilms were also grown in RPMI 1640 medium. All wild-type *S. aureus* strains, including USA300, MW2, UAMS-1, SH1000, Newman, BH1CC, and BH48, formed biofilms when grown on surfaces conditioned with plasma in RPMI 1640 medium (data not shown). Viable counts revealed that USA300 and SH1000 biofilms grown for 24 hours in RPMI 1640 medium contained 1–2 log fewer cells than biofilms grown in TSB or BHI broth (Supplementary Figure 2A). Similar data were obtained with BH1CC and BH48 (data not shown). Notably the numbers of viable bacteria in RPMI 1640 medium–grown biofilms were similar to the previously reported numbers of bacteria in biofilms recovered from infected catheters in vivo [19].

USA300 and SH1000 biofilms grown for 24 hours on human plasma–coated surfaces in RPMI-1640 medium were susceptible to the antistaphylococcal antibiotics rifampicin and vancomycin at a concentration of <1 mg/mL (Figure 1A), whereas biofilms grown in BHI medium were resistant to 50 mg/mL of either antibiotic (Figure 1B). Resistance of RPMI-1640 medium–grown biofilms to both antibiotics increased significantly beginning at 3 days (Figure 1A), when cell densities were similar to those of biofilms grown in rich laboratory broths (Supplementary Figure 2B). These data suggest that the in vivo biofilm cell density may be an important indicator of susceptibility and identify a therapeutic window for biofilm eradication in the early stages of biofilm-associated infections.
The SaeRS System and Coagulase Are Required for Biofilm Formation in an Iron-Free Environment on Human Plasma–Coated Surfaces

*S. aureus* USA300 JE2 mutants from the NTML, which are deficient in known biofilm mediators and regulators, were grown statically in RPMI-1640 medium or TSB on plasma-coated surfaces (Figure 2A). In RPMI-1640 medium only, the *saeS*:Tn mutant exhibited a biofilm defect (Figure 2A), whereas in TSB medium, the *sara*:Tn, *srtA*:Tn, and *spa*:Tn mutants revealed a significant biofilm impairment (Figure 2A).

Extending this analysis to a physiologically relevant high venous shear (6.25 dynes/cm²), use of a Cellix microfluidic flow cell system further implicated SaeS in RPMI-1640 medium (Figure 2B) and defibrinated human blood (Figure 2C) and identified a role for ClfA, which was not evident under static conditions. Like the *clfA*:Tn mutant, the *srtA*:Tn mutant was also biofilm negative under venous shear in RPMI-1640 medium (Figure 2B). A *ΔsaeRS* mutation also impaired biofilm formation by UAMS-1, USA300 LAC, and MW2 under venous shear in RPMI-1640 medium but not TSB, and the MW2
supplemented with glucose, dose-dependently inhibited biofilm formation by USA300 and SH1000 (Supplementary Figure 4).

To elucidate the mechanism of SaeRS-dependent biofilm formation on human plasma-coated surfaces the role of coagulase (coa), which is part of the SaeRS regulon, was investigated. SaeRS positively regulates coagulase [21], which catalyses the conversion of fibrinogen, an abundant protein in human plasma, into fibrin [22]. A JE2 coa::Tn mutant was biofilm negative when grown in RPMI 1640 medium under flow on plasma (Figure 4A). Interestingly, a mutation in the gene encoding von Willebrand factor–binding protein, which produces a second coagulase in S. aureus [23], had no impact on biofilm (Figure 4A). Human plasmin, which degrades fibrin, dispersed USA300 biofilms (Figure 4B), as did proteinase K (Figure 4B). Dispersin B, which degrades PIA/PNAG and nuclease had no effect on biofilms grown on plasma in RPMI 1640 medium (Figure 4B), indicating no role for polysaccharide or eDNA in this biofilm phenotype. Plasmin also dispersed UAMS-1, MW2, and SH1000 biofilms grown on plasma in RPMI 1640 medium (Figure 4C). Consistent with these phenotypes, coagulation of human plasma under iron-limited conditions by MW2 culture supernatants was show to be SaeRS dependent (Supplementary Figure 5).

Taken together, these data support previous findings implicating sortase [7] and SarA [24] in the S. aureus biofilm phenotype in rich medium and identify new roles for SaeRS, ClfA, and coagulase in biofilm production on human plasma–coated surfaces under physiologically relevant growth conditions.

**Critical Role of ClfA in Biofilm Formation on Human Plasma–Coated Surfaces Is Shear Dependent**

Further investigation of SaeRS/coagulase-mediated biofilm at venous shear (6.25 dynes/cm²) in the Cellix system or 0.6 dynes/cm² in either Cellix or Bioflux system revealed that the clfA mutation in strains SH1000 and Newman had no effect at the lower shear rate but impaired biofilm at venous shear (Figure 5A). ClfA binds the C-terminal region of the Fg γ chain by using the so-called dock, lock, and latch mechanism [25]. A variant peptide that mimics the γ chain region of Fg and interacts with ClfA with higher affinity has been identified as Fg γ (1–17) D16A [26]. Biofilm formation by SH1000, which was incubated with this peptide prior to inoculation of the microfluidic flow cell chamber, was inhibited in a dose-dependent manner (Figure 5B), indicating that ClfA-dependent biofilm production on human plasma is related to its fibrinogen-binding activity, which is then likely to facilitate coagulase-mediated conversion of fibrinogen to fibrin.

**Dispersal of Biofilms Grown In Vitro and In Vivo With Fibrinolysins**

Consistent with the effect of plasmin, mature 3-day and 14-day coagulase-mediated US300 and SH1000 biofilms were also effectively dispersed with nattokinase and serrapeptase (Figure 6A). Furthermore, biofilms formed by USA300lux on
Central venous catheter in a rat model were also dispersed effectively with nattokinase (Figure 6B), supporting the importance of coagulase-mediated biofilm in vivo and the therapeutic potential of targeting this colonization mechanism for the treatment of device-related infections.

**DISCUSSION**

The challenge of managing chronic device-associated infections is primarily associated with the inherent drug resistance of biofilms colonizing biomaterial surfaces. Characterization of biofilm susceptibility has generally been performed under in vitro conditions on artificial surfaces. However, the rapid coating of implanted materials by plasma and extracellular matrix proteins and host sequestration of nutrients during infection suggest that in vitro characterization of biofilm antibiotic resistance may not fully reflect the in vivo milieu. To address this, *S. aureus* biofilms were grown on human plasma–conditioned surfaces under shear flow in chemically defined, iron-limited medium (RPMI 1640 medium). Early biofilms grown for ≤24 hours under these conditions were significantly more sensitive to the antistaphylococcal antibiotics rifampicin and vancomycin than biofilms grown on polystyrene in bacteriological medium. Increased drug susceptibility on plasma-coated surfaces was transient, and older (growth time, >48 hours), denser biofilms exhibited high levels of resistance. The 2-component system SaeRS, which is expressed by all *S. aureus* strains [27], was required for biofilm production under these conditions. SaeRS has been identified as an important regulator of global gene expression under in vivo conditions [28], including during device-related infections [29]. Furthermore mutation of *sae* blocks upregulation of proteases, toxins, and surface proteins such as α- and β-hemolysin, FnbA, coagulase, and protein A, which are important for the pathogenesis of *S. aureus* infection [30–34].

Under iron-limiting conditions, SaeRS was previously shown to increase biofilm production by strain Newman by activating...
Figure 6. Dispersal of in vitro–grown and in vivo–grown *Staphylococcus aureus* biofilms with nattokinase and serrapeptase. A, Biofilms of *S. aureus* SH1000 (gray bars), BH1CC (white bars), and USA300 (checker bars) grown in plasma-coated microtiter plate wells in RPMI-1640 medium at 37°C for 3 or 14 days before being treated with nattokinase or serrapeptase for 3 hours at 37°C. After treatment, the density of crystal violet–stained biofilms was measured at \( A_{490} \). Data presented are the means ± standard deviation of 3 independent experiments. B, Whole-animal imaging of luminescence from *lux*-expressing USA300 on a central venous catheter implanted into a rat, 2 days after infection. C, Luminescence on catheters recovered from 3 independent rats 10 days after infection before (left) and after (right) treatment with nattokinase for 3 hours.
expression of Emp and Eap [20, 35, 36]. However, use of the biofilm model described in this study biofilm production was not dependent on Emp or Eap. Similarly, mutations in the hla, hlb, fnbA, or spa genes, which are also regulated by the Sae system [21], had no significant effect on this biofilm phenotype. A subsequent screen of Sae-regulated genes identified a key role for staphylococcal coagulase (Coa) in biofilm production on human plasma–coated surfaces. Coagulase is a secreted enzyme that binds prothrombin to form staphylothrombin, which in turn converts fibrinogen to fibrin, resulting in blood clotting [22]. Our data showed that this process was induced by wild-type S. aureus supernatants but not by supernatants from sae mutant cultures, confirming that coagulase production is controlled by SaeRS in our experimental set up. Moreover, Sae- and Coa-dependent biofilms grown in vitro or in a rat model of central venous catheter infection were dispersed by human plasmin and nattokinase, which both degrade fibrin, further implicating coagulase-catalyzed production of fibrin in the S. aureus biofilm phenotype on human plasma–coated surfaces. Under venous shear in RPMI 1640 medium (and not under static conditions), ClfA, which binds fibrinogen and fibrin [25], was also important for coa-dependent biofilm formation on plasma-coated surfaces. A peptide that binds to ClfA with a higher affinity than the γ chain fragment of fibrinogen blocked biofilm formation, indicating that S. aureus uses this fibrinogen/fibrin receptor to promote attachment via a mechanism that is not critical at lower shear or during static biofilm growth.

Our data support a recent report by Vanassche et al demonstrating that an S. aureus mutant lacking both Coa and the second S. aureus coagulase, von Willebrand factor–binding protein, exhibited defective in vitro colonization of polyurethane catheters conditioned with fresh human plasma [10]. Furthermore, chemical inhibition of coagulase activity using dabigatran reduced S. aureus colonization of catheters in a murine model of jugular vein catheter infection [10, 11]. Interestingly, our data showed that mutation of the vwb gene had no significant impact on biofilm formation on plasma-coated surfaces.

Consistent with previous reports on the importance of SarA for biofilm formation by S. aureus under in vitro [24, 37–39] and in vivo conditions [40], our data showed that SarA-regulated biofilm production on polystyrene (either uncoated or coated with human plasma) in nutrient-rich bacteriological medium was dependent on the previously characterized biofilm mediators PIA/PNAG, on FnBPs or on autolytic activity [14]. The absence of a role for SarA in biofilm production in RPMI 1640 medium underlines the importance of the experimental conditions used for biofilm analysis and the importance of the SaeRS/Coa-mediated biofilm phenotype under physiologically relevant conditions.

Taken together, these data suggest that the adhesion and biofilm mechanisms used by staphylococci to colonize implanted biomaterials may, in part, be dependent on the degree of surface conditioning by plasma and extracellular matrix proteins. Insoluble fibrin, produced as a result of coagulase activity, can apparently function as a biofilm scaffold, allowing S. aureus to accumulate on human plasma–coated surfaces. In keeping with studies showing the therapeutic benefit of coagulase inhibitors or plasmin in S. aureus cardiovascular and joint infections [10–12], the data presented in this study reveal the enhanced therapeutic potential of fibrinolytic and antimicrobial drug combinations for S. aureus biofilm eradication, compared with currently used antibiotics, particularly during the early stages of device-related infection.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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