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Staphylokinase Control of *Staphylococcus aureus* Biofilm Formation and Detachment Through Host Plasminogen Activation

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Staphylococcus aureus biofilms, a leading cause of persistent infections, are highly resistant to immune defenses and antimicrobial therapies. In the present study, we investigated the contribution of fibrin and staphylokinase (Sak) to biofilm formation. In both clinical *S. aureus* isolates and laboratory strains, high Sak-producing strains formed less biofilm than strains that lacked Sak, suggesting that Sak prevents biofilm formation. In addition, Sak induced detachment of mature biofilms. This effect depended on plasminogen activation by Sak. Host-derived fibrin, the main substrate cleaved by Sak-activated plasminogen, was a major component of biofilm matrix, and dissolution of this fibrin scaffold greatly increased susceptibility of biofilms to antibiotics and neutrophil phagocytosis. Sak also attenuated biofilm-associated catheter infections in mouse models. In conclusion, our results reveal a novel role for Sak-induced plasminogen activation that prevents *S. aureus* biofilm formation and induces detachment of existing biofilms through proteolytic cleavage of biofilm matrix components.

Keywords. *Staphylococcus aureus*; biofilm; fibrin; plasminogen; staphylokinase.

Biofilms are involved in the majority of infections and cause an enormous burden to healthcare systems [1]. One of the leading pathogens in biofilm-associated infections is *Staphylococcus aureus* [2]. Although this pathogen is a major cause of skin, soft-tissue, respiratory, bone, joint, and endovascular infections [3], the staphylococcal biofilm-related infections are among the most difficult to manage. Biofilms are resistant to antibiotics and host immune defenses [1, 4, 5], so their treatment is challenging and costly.

Biofilms are multilayered communities of bacteria proliferating on surfaces, for example on biomedical implants. First, bacteria attach by binding directly to the biomaterial surface or to host proteins conditioning the surface after implantation. Biofilm growth and maturation form a complex 3-dimensional structure, with bacterial cells embedded in an extracellular matrix. Finally, some bacteria detach from the mature biofilm leading to dispersal and spread to new sites of infection [6–9]. Numerous global regulators (*agr*, *sarA*, *sigB*, and *sae*) are involved in control of *S. aureus* biofilm formation and dispersal, and their exact roles are probably strain specific [4, 7]. Furthermore, the main bacterial components of the staphylococcal biofilm matrix (either polysaccharides or proteins) are strain dependent [4, 7]. Much less is known about the role of host-derived factors in biofilm formation and regulation.

Staphylokinase (Sak) is a plasminogen activator secreted by the majority of *S. aureus* strains [10]. It forms complexes with trace amounts of plasmin present in host plasma. Those complexes subsequently cleave plasminogen to form active plasmin, a potent broad spectrum protease targeting host proteins, including fibrin clots. Plasminogen activation by Sak promotes bacterial entry and further spread in the skin [11, 12], but it decreases the severity of invasive bloodstream infections [13, 14]. The role of Sak in *S. aureus* biofilm infections has not been studied, but plasmin could potentially cleave proteins present in the biofilm matrix. We hypothesize that this activity has a profound impact on biofilm development in vivo. Indeed, our findings suggest that interplay between Sak and host plasminogen and fibrin plays a crucial role in controlling biofilm formation, both in vitro and in vivo.

METHODS

Bacterial Strains and Growth Conditions

Congenic *S. aureus* strains differing in the level of Sak secretion—LS-1*EP*, LS-1*sak*, and LS-1*spa-sak*—were described elsewhere [13]. The Newman strain and its congenic *coa/vwb* mutant

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lacking both coagulases were described elsewhere [15]. Growth curves of congenic strains did not differ [13].

We used a collection of *S. aureus* clinical isolates from invasive infections, described elsewhere [11, 16]. This included 28 isolates from biofilm-associated infections on foreign materials (line-associated infections and prosthetic joint infections) and 131 isolates from non–biofilm-associated invasive infections, including soft-tissue infections and bacteremia without focus.

Bacteria were grown with shaking at 37°C in trypticase soy broth (TSB). For biofilm experiments, if not indicated otherwise, a mixture of 50% TSB and 50% human plasma was used, supplemented with 0.5% glucose. In some experiments, various reagents were added (see Supplementary Methods).

Measurement of Sak

The level of Sak produced by bacteria was measured using a chromogenic assay [11, 17] in 20-hour-old cultures initiated by adding equal inoculum of each strain to a fresh broth (all strains reached stationary phase by the 20-hour time point). The "high Sak" production in clinical isolates was comparable to that of LS-1*spa-sak*.

Biofilm Microplate Assay

A classic microplate method for biofilm measurement was used [18], with minor modifications (see Supplementary Methods).

Biofilm Microscopy

Methods for visualization of biofilms and neutrophil-biofilm interactions in confocal and electron microscopy, along with details of immunostainings, are described in the Supplementary Methods.

Primary Adhesion Under Shear Stress

Measurement of bacterial adhesion under flow conditions is described in the Supplementary Methods.

Fibrinolytic and Proteolytic Markers

Plasmin activity in biofilm supernatants was measured using chromogenic assay, as described elsewhere [19]. Fibrin D-dimers in supernatants were measured with Asserachrom D-DI enzyme-linked immunosorbent assay (ELISA; Diagnostica Stago). Biofilm matrix was extracted [20], and protein concentrations were measured with the Bradford assay. Sak protein concentration was measured with ELISA [12] (details in Supplementary Methods).

Sak Expression in a 7-Day Experiment

To measure Sak expression on different days, we used biofilms of *S. aureus* LS-1*sak* or of 3 randomly chosen clinical isolates from biofilm-associated infections, secreting similar amounts of Sak as the LS-1*sak*. Growth medium was removed daily, biofilms were washed, and fresh medium was added. On selected days, biofilms were scraped from the bottom of the wells, and expression of *sak* mRNA in biofilm and in planktonic cells was measured by means of reverse-transcription polymerase chain reaction [11].

Response of Neutrophils to Biofilms

Human neutrophils were separated from buffy coats from healthy donors by dextran sedimentation and Ficoll-Paque centrifugation [21] and stored on ice until use.

Production of intracellular superoxide was measured by chemiluminescence in a 96-well plate in a Mithras LB 940 reader (Berthold Technologies) [22]. Neutrophils (2×10^5 cells per well) were added to preformed biofilm in a Krebs-Ringer phosphate buffer containing the cell permeable substrate luminol (10 μg/mL; Sigma) in the presence of the extracellular scavengers superoxide dismutase (50 U/mL, Worthington Biochemical Corporation) and catalase (2000 U/mL; Worthington).

Determination of Minimum Inhibitory Concentration for Antibiotic

The minimum inhibitory concentration (MIC) of vancomycin against planktonic bacteria was measured with a microdilution assay according to the ISO 20 776 standard, and the MIC against biofilms was determined with a thiazolyl blue tetrazolium bromide (MTT) chromogenic assay to detect biofilm viability [23]. Duplicate determinations were performed.

Animals

C57BL/6 mice (Charles River) aged 6–9 weeks were used, of both sexes (subcutaneous catheter infections) or male (jugular vein catheter infections). All experiments were approved by the Animal Research Ethical Committee of Gothenburg and the Ethical Committee of the University of Leuven.

Subcutaneous Catheter Biofilm Model

Catheter segments (1.5 cm) (BD Venflon; 16 gauge; Becton Dickinson) were coated overnight with 20% mouse plasma, washed, filled with 10 μL of 1% overnight bacterial suspension in TSB with 0.5% glucose and 0.05 mg/mL human plasminogen (or phosphate-buffered saline [PBS]), and implanted subcutaneously on the back of mice. An implant infected with *S. aureus* strain LS-1*EP* was inserted on one flank, and an implant with LS-1*spa-sak* on the other. To provide human plasminogen, 50 μL of 200 μg/mL plasminogen (or PBS) was injected daily into the lumen of the catheter. On day 3 after implantation, catheters were excised, rinsed, placed in 1 mL of PBS, and sonicated for 5 minutes to dislodge attached biofilm, and colony-forming units were then counted.

Jugular Vein Catheter Biofilm Model

The intravascular jugular vein catheter model was performed as described elsewhere [24], with minor modifications (described in Supplementary Methods, along with details of adenovirus-mediated human plasminogen expression in mice).

Statistical Analysis

Statistical calculations were done with Prism software, version 6.03 (GraphPad Software) and differences were considered significant at $P < .05$ (2 tailed). Test details are described in the Supplementary Methods.

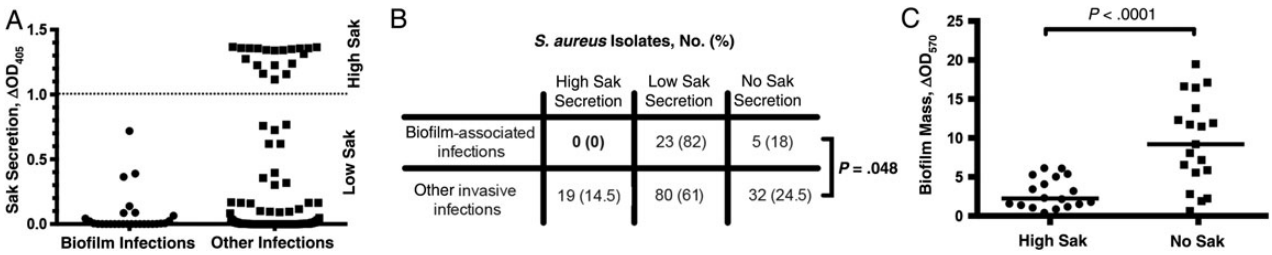

Figure 1. High staphylokinase (Sak) production inhibits biofilm formation in clinical *Staphylococcus aureus* isolates. *A, B,* Levels of Sak secretion by clinical isolates from biofilm-associated infections (n = 28) and non–biofilm-associated invasive infections (n = 131). *C,* Biofilm formation in vitro in medium supplemented with human plasma by clinical isolates with high Sak secretion and by randomly chosen isolates with no Sak secretion (n = 19). Horizontal bar represents median. Abbreviation: ΔOD_{405}, change in optical density at 405 nm.

RESULTS

Secretion of Sak in *S. aureus* Isolates From Biofilm Infections

Clinical *S. aureus* invasive infection isolates produced varying levels of Sak, ranging from undetectable (no optical density increase in the chromogenic assay) to very high levels (optical density increase of >1). Strikingly, none of the isolates from biofilm-associated infections secreted high levels of Sak (Figure 1*A* and 1*B*). To examine whether there is a negative association between high Sak production and *S. aureus* biofilm, we measured biofilm formation by selected strains in broth supplemented with 50% human plasma. High Sak-secreting clinical strains formed significantly less biofilm than Sak-negative strains (Figure 1*C*). This suggests that a high level of Sak is not compatible with biofilm formation when host plasma is present.

Effect of Sak on Biofilm Formation and Dispersal in Plasma

We used a panel of congenic *S. aureus* strains differing in the Sak secretion level [13]. Strain LS-1*EP* does not secrete Sak, LS-1*sak* secretes Sak moderately, and LS-1*spa-sak* has high Sak secretion (comparable to the high-secreting clinical isolates). In the biofilm assay incorporating plasma, striking differences were seen between the strains (Figure 2*A*). LS-1*EP* formed dense biofilm, which grew further between 24 hours and 48 hours of incubation. In contrast, LS-1*spasak* was unable to form biofilm. For LS-1*sak* strain, biofilm formed at 24 hours was similar to that produced by the Sak-negative control strain. However, the biofilm partially disintegrated after 48 hours, and the biofilm mass was significantly lower than at 24 hours. Viable bacteria counts in biofilm followed the same pattern (Figure 2*B*). Differences between strains disappeared when pure TSB without host plasma was used (Figure 5*D*).

Confocal microscopy of the biofilms confirmed that both LS-1*EP* and LS-1*sak* initially formed thick, 3-dimensional biofilm structures, whereas LS-1*spa-sak* formed only small clusters of cells and even after 48 hours was unable to form a complete biofilm (Figure 2*C*). Biofilms of LS-1*sak* disintegrated after 48 hours, but LS-1*EP* biofilm retained its thick structure. The same pattern was observed with scanning electron microscopy (Figure 2*D*). Thick biofilms of LS-1*EP* covered the entire surface and were composed of cell clusters embedded in an extracellular matrix. Biofilms of LS-1*sak* had a similar appearance after 24 hours, although after 48 hours they appeared thinner and less compact, and in many places the underlying surface became exposed. With LS-1*spa-sak,* barely any matrix was visible, with only a few "unembedded" bacteria attached to the surface.

When initial adhesion to plasma-coated surface was investigated under physiological shear stress, the LS-1*spa-sak* failed to adhere, whereas LS-1*EP* did adhere (Figure 3*A*), showing that Sak not only prevented accumulation but also inhibited initial adhesion to the surface—the first step of biofilm formation. The addition of recombinant Sak to an established biofilm formed by LS-1*EP* caused detachment of biofilm (Figure 3*B*), showing directly that Sak causes disintegration and dispersal of biofilm. Overall, the in vitro experiments showed that a high level of Sak inhibited biofilm formation in the presence of plasma. The moderate production of Sak by *S. aureus* did not interfere with initial biofilm formation but promoted subsequent detachment of already formed biofilms.

To investigate the expression of the *sak* gene, *sak* mRNA levels were measured in cells from biofilms grown in microwells for up to 7 days. A characteristic pattern was observed in both LS-1*sak* (Supplementary Figure 1*A*) and 3 clinical isolates from biofilm-associated infections (Supplementary Figure 1*B*). The expression of Sak by cells within the biofilm was low and decreased over time. In contrast, the expression of Sak by planktonic cells was higher and markedly increased with time. This suggests that Sak expression might be associated with planktonic bacteria detaching from the existing biofilm rather than with bacteria persisting inside the biofilm and/or that Sak expression is radically decreased inside biofilms. Such a hypothesis is strengthened by a direct ELISA measurement, showing reduced levels of Sak protein secreted from biofilms, compared with planktonic cultures (Supplementary Figure 1*C*), in line with observations published elsewhere [25].

Mediation of Sak Effect by Plasminogen Activation

A plausible mechanism for the observed effects is the activation of host plasminogen by Sak into plasmin and subsequent

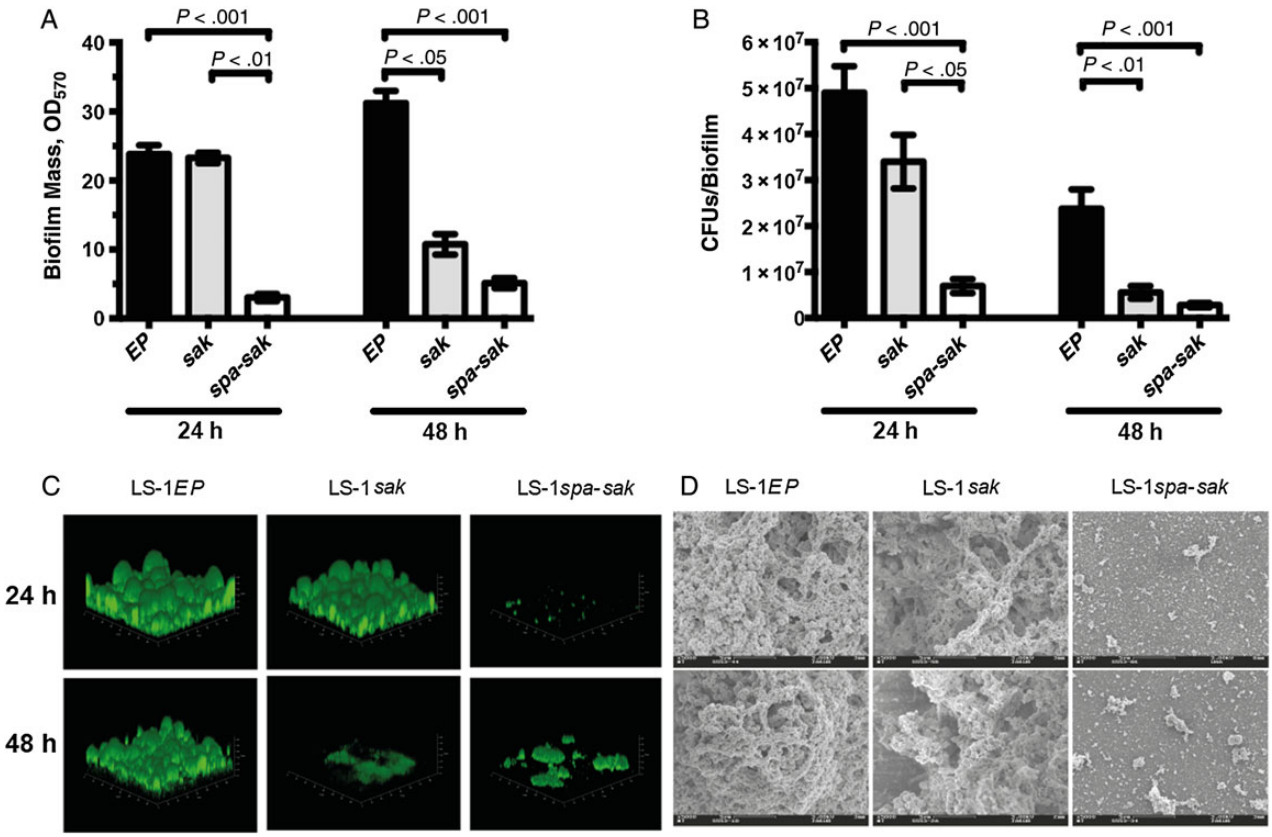

Figure 2. Staphylokinase (Sak) modulates biofilm formation and detachment by *S. aureus*. Biofilm formation by congenic *S. aureus* strains differing in the level of secreted Sak (*EP,* no secretion; *sak,* moderate secretion; *spa-sak,* high secretion) assessed in medium supplemented with human plasma after 24 and 48 hours. *A,* Biofilm mass (n = 9). *B,* Colony-forming unit (CFU) counts (n = 7–10). *C,* Three-dimensional appearance with confocal microscopy (diameter, 160 × 160 × 65 µm). *D,* Scanning electron microscopy (image width, 23 µm). Means and standard errors of the mean are shown. Abbreviation: OD_{570}, optical density at 570 nm.

cleavage of fibrin and other biofilm proteins. To test this hypothesis, we measured the plasmin activity in biofilm supernatants was measured and found that it correlated inversely with biofilm formation and Sak secretion (Figure 4*A*). The level of the D-dimer (a fibrinolysis marker) was also higher in supernatants of strains producing Sak (Supplementary Figure 2*A*). Furthermore, the overall protein content of the biofilms negatively correlated with the plasmin activity (Supplementary Figure 2*B*).

Blockade of either plasmin activity by aprotinin- or Sak-induced plasminogen activation by tranexamic acid restored

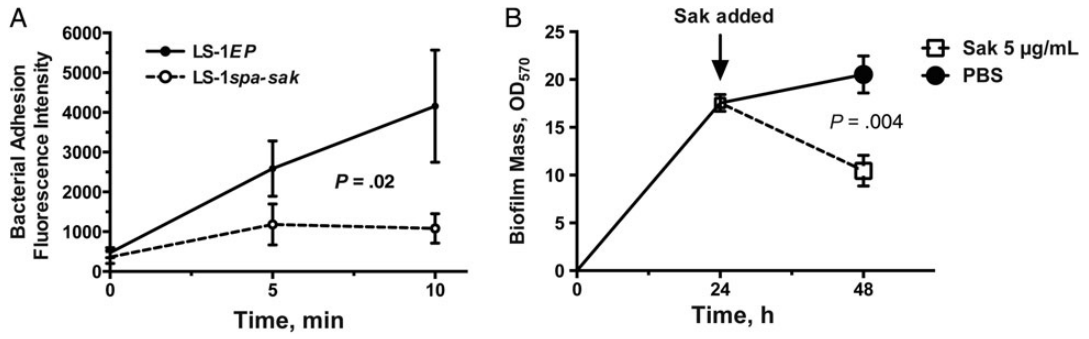

Figure 3. Staphylokinase (Sak) affects different phases of *Staphylococcus aureus* biofilm development. *A,* Effect of high Sak secretion on primary adhesion under shear stress in flow system (n = 7). *B,* Detachment of biofilm mass after addition of exogenous Sak (5 µg/mL) (n = 6). Means and standard errors of the mean are shown. Abbreviations: OD_{570}, optical density at 570 nm; PBS, phosphate-buffered saline.

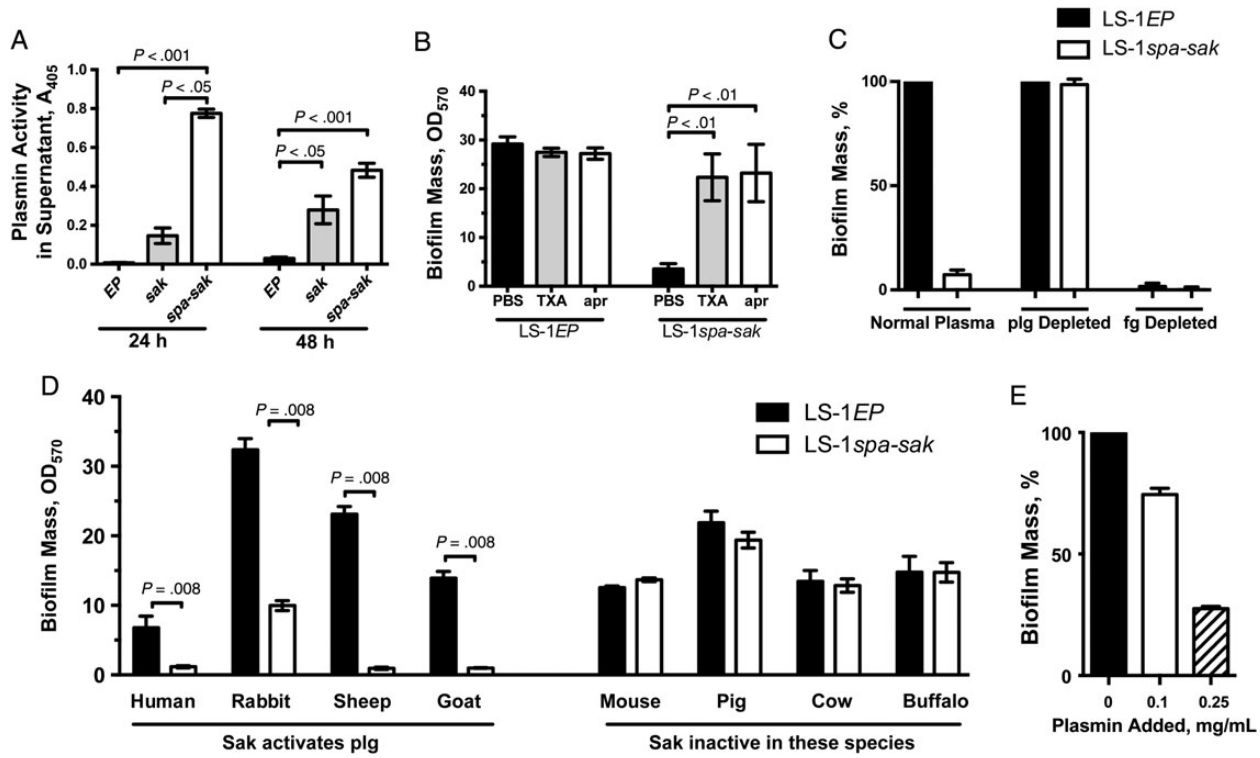

Figure 4. Staphylokinase (Sak) mediates its effect on *Staphylococcus aureus* biofilm by activating plasminogen. *A*, Plasmin activity in the supernatants of biofilm cultures of *S. aureus* strains differing in Sak secretion (n = 9). *B*, Effect of plasmin inhibitors tranexamic acid (TXA) and aprotinin (apr) on biofilm formation by strain secreting no Sak or high level of Sak (n = 6–9). *C*, Biofilm formation by strain secreting no Sak or high level of Sak in human plasma depleted of plasminogen (plg) or fibrinogen (fg) (n = 3). *D*, Biofilm formation by strain secreting no or high level of Sak in plasma from various animal species, where Sak is either active or unable to activate plasminogen (n = 5). *E*, Effect of plasmin addition on biofilm formation by strain LS1*EP* (n = 3). Means and standard errors of the mean are shown. Abbreviations: A_{405}, absorbance at 405 nm; OD_{570}, optical density at 570 nm.

biofilm formation by LS-1*spa-sak* to the control level (Figure 4*B* and Supplementary Figure 2*C*). Depletion of plasminogen from plasma also restored normal biofilm formation in LS-1*spa-sak* (Figure 4C). Sak is active only toward plasminogen from human and certain animals (Supplementary Figure 2*D*); therefore, LS-1*spa-sak* formed biofilms only when bacteria grew in the presence of plasma from animal species where Sak was unable to activate plasminogen (Figure 4*D*). Adding active plasmin to medium inhibited biofilm development in a dose-dependent fashion (Figure 4*E*), confirming plasminogen activation as the mechanism of observed differences.

Fibrin as a Scaffold for the Biofilm Matrix

Because fibrin is the main target of active plasmin and biofilm formation was negligible in fibrinogen-depleted plasma (Figure 4*C*), our data point to a crucial role for fibrin in biofilm formation. On examination of the biofilm structure, it seemed that *S. aureus,* when grown in the presence of plasma, uses host proteins to construct the biofilm matrix. Immunostaining revealed that, in addition to bacterial cells, biofilms incorporated human serum proteins, including fibrin(ogen) (Supplementary Figure 3*A*). Fibrin clumps were present in the biofilm matrix and were a part of the biofilm's scaffold. They were also present on the bacterial surface, tethering individual cells to the biofilm fibrin matrix (Figure 5*A*). This suggests an important structural role for fibrin within staphylococcal biofilms, especially because *S. aureus* secretes its own coagulases inducing fibrin deposition [26]. Indeed, biofilm formation was inhibited and the fibrin scaffold surrounding bacteria disappeared with the addition of a direct thrombin inhibitor (dabigatran), which also inhibits *S. aureus*–induced fibrin formation [26]. No biofilm was formed when *S. aureus* mutant lacking coagulases was used or when the experiment was performed in pure medium without host plasma (Figure 5*B*).

Pure broth was supplemented with components needed for coagulation and fibrinolysis, that is, prothrombin (to be activated by staphylococcal coagulases and mediate fibrin deposition), fibrinogen (to form a fibrin mesh in the biofilm matrix), and plasminogen (to lyse fibrin after its SAK-mediated activation). Under these conditions, the same pattern of biofilm formation by LS-1*EP*, LS-1*sak* and LS-1*spa-sak* was seen as in broth with plasma (Figure 5*C*). No difference between the strains was visible in unsupplemented broth (Figure 5*D*). In accordance with the proposed model, omission of plasminogen from the broth

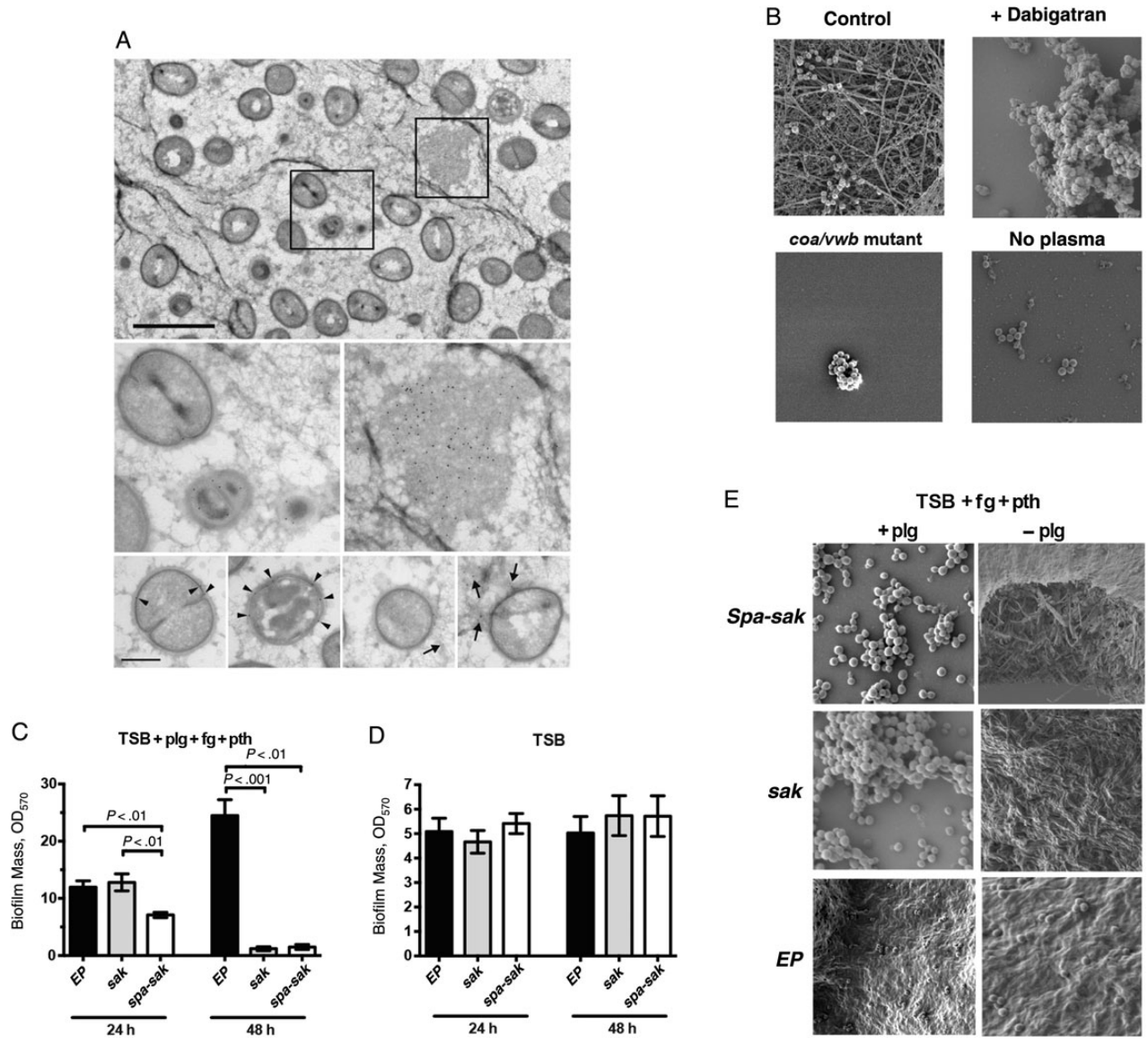

Figure 5. Fibrin is an essential component of biofilm matrix and is destroyed by staphylokinase (Sak)–induced fibrinolysis. *A*, Section of *Staphylococcus aureus* LS-1*EP* biofilm in transmission electron microscopy, stained with 10 nm immunogold technique (black dots) against fibrin(ogen). Upper panel shows even distribution of bacteria in the matrix containing flocculent material and irregular strands. The "darkest" lines are sectioning artifacts. Middle panels show magnified insets from the upper row. Left panel shows positive staining of fibrin on bacterial surface. Central and rightmost bacterial cells were "topped" by tangential sectioning, thus including more cell surface in section and showing more intensive staining than cells sectioned equatorially. Right panel shows intensive labeling of a dense structure in the matrix, identifying it as a fibrin mesh. Lower panels show various patterns of immunostaining. Staining on the staphylococcal surface (*arrowheads*) is shown in the left panels. Staining of filamentous material radiating from bacterial surface (*arrows*), linking individual staphylococci to the intracellular fibrin scaffold in matrix are shown in the right panels (scale bars, 2 and 0.5 μm). No staining was seen in isotype controls (not shown). *B*, Scanning electron microscopy images of *S. aureus* Newman biofilms formed in medium with plasma in different conditions: control, with dabigatran (coagulation inhibitor) added, with congenic *S. aureus coa/vwb* mutant lacking both coagulases or when no plasma was present (×3000 magnification). *C*, Effect of Sak on biofilm formation in trypticase soy broth (TSB) supplemented with plasminogen (plg), fibrinogen (fg), and prothrombin (pth) (n = 9). *D*, No effect of Sak on biofilm formation in pure TSB, n = 6–9. Means and standard errors of the mean are shown. *E*, Scanning electron microscopic images of biofilms of congenic *S. aureus* strains differing in the level of secreted Sak in TSB supplemented with fibrinogen and prothrombin, with or without plasminogen added (×3000 magnification). Abbreviation: OD_{570}, optical density at 570 nm.

completely inhibited biofilm dispersal and allowed all 3 strains to form a thick, fibrin-rich matrix (Figure 5*E*).

Effect of Sak on Biofilm Susceptibility to Antibiotics and Host Defenses

When neutrophils engulf a microbe, there is a robust production of reactive oxygen species (ROS) within the phagosome of these cells [27, 28]. Intracellular ROS production from neutrophils was very weak on interaction with biofilms formed by LS-1*EP* or LS-1*sak*. In contrast, neutrophils interacting with the bacterial clumps formed by the LS-1*spa-sak* strain rapidly responded with high-level ROS production (Figure 6*A*). This difference was not due to direct effects of Sak on neutrophils,

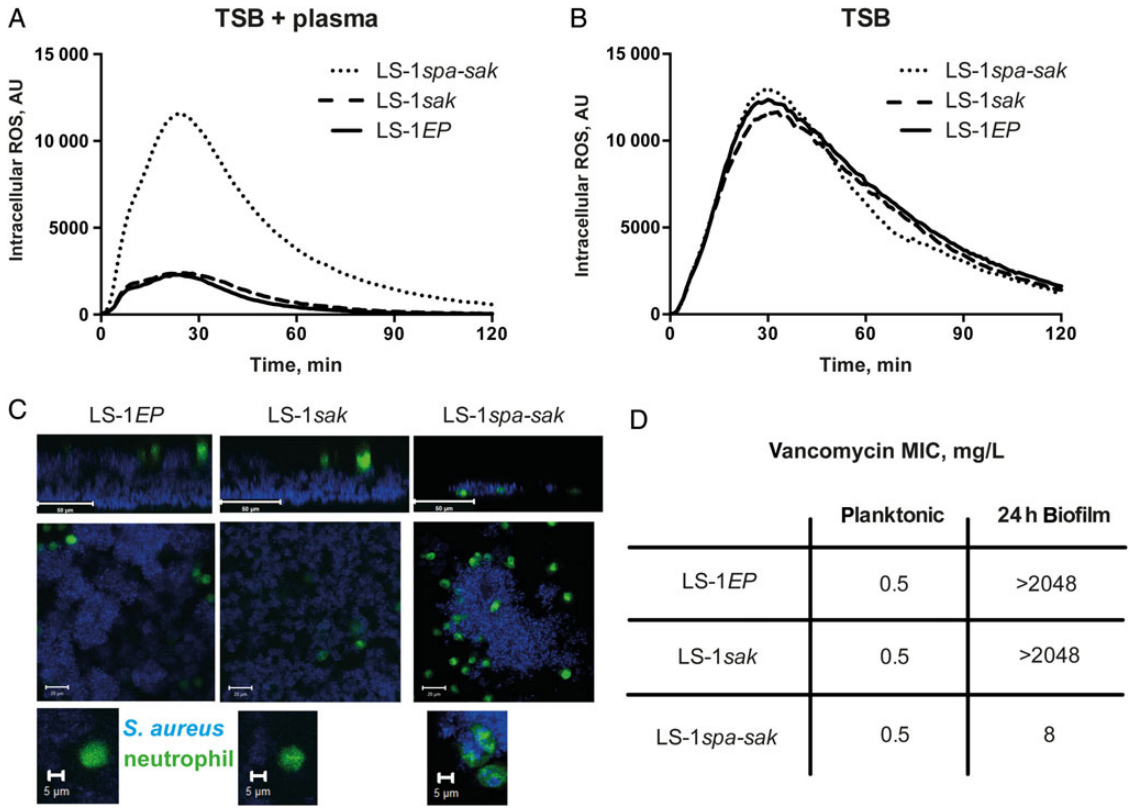

Figure 6. Staphylokinase (Sak) reduces the resistance of biofilm resistance to immune system and antibiotics. *A*, Intracellular reactive oxygen species (ROS) production in neutrophils encountering biofilms of the congenic strains differing in Sak secretion grown in presence of human plasma or *B*, in pure trypticase soy broth (TSB) medium (n = 3). *C*, Representative images of neutrophils interacting with biofilms differing in Sak secretion, including vertical (*upper panel*) and horizontal (*middle panel*) sections through biofilm obtained from confocal imaging (scale bar, 50 µm) and: magnification of neutrophils next to biofilm pieces (scale bar, 5 µm) (*lower panel*). *D*, Minimum inhibitory concentration (MIC) of vancomycin against *Staphylococcus aureus* strains in planktonic form or in biofilm. Abbreviation: AU, arbitrary units.

because Sak alone had no impact on ROS production (data not shown). Moreover, no difference in ROS production was found among the strains when biofilms were grown in broth without plasma (Figure 6*B*). This indicates that the thick biofilm matrix of fibrin and host proteins protects LS-1*EP* and LS-1*sak* from immune recognition and attack, whereas the biofilm residues formed by LS-1*spa-sak*, lacking the shielding matrix of host proteins, are exposed to neutrophil phagocytosis. This was also confirmed by microscopic observation: many neutrophils phagocytosing bacterial clumps were seen in LS-1*spa-sak* samples compared with LS-1*EP* and LS-1*sak* (Supplementary Figure 4).

When fluorescently labeled neutrophils and staphylococci in biofilm were visualized with confocal microscopy, the same pattern emerged. Neutrophils attached to and penetrated into the clumps of LS-1*spa-sak*; many of them contained ingested bacteria. In contrast, neutrophils failed to enter into the LS-1*EP* and LS-1*sak* biofilms and remained on the top of the biofilm matrix. Despite their proximity to bacteria, very few neutrophils contained phagocytosed staphylococci (Figure 6*C*).

All 3 strains exhibited the same vancomycin susceptibility when tested as planktonic cells (MIC, 0.5 mg/L). In the case of biofilm-embedded bacteria LS-1*EP* and LS-1*sak*, resistance increased dramatically, and even >4000 times higher concentrations of vancomycin failed to kill bacteria (MIC, >2048 mg/L). This was in sharp contrast to LS-1*spa-sak*, where the adherent clumps of cells had only a slightly elevated vancomycin MIC (8 mg/L) (Figure 6*D*). Overall, the biofilm matrix was essential both for antibiotic resistance and for immune evasion. Dissolution of this matrix induced by Sak rendered bacteria vulnerable.

Effect of Sak on Biofilm Formation in Mouse Infection Models

Both the in vitro data and the observations with clinical isolates strongly suggested an inhibitory effect of high Sak secretion on biofilm formation. To confirm this in vivo, we used mouse subcutaneous and intravascular catheter infection models. Because Sak is specific for human plasminogen and has very low activity toward murine plasminogen (Supplementary Figure 2*D*), we provided human plasminogen either locally, by injection into the catheter lumen, or systemically, using an adenoviral vector system that expressed human plasminogen in mice.

In subcutaneous catheters inoculated with staphylococci, injections of human plasminogen resulted in significantly smaller

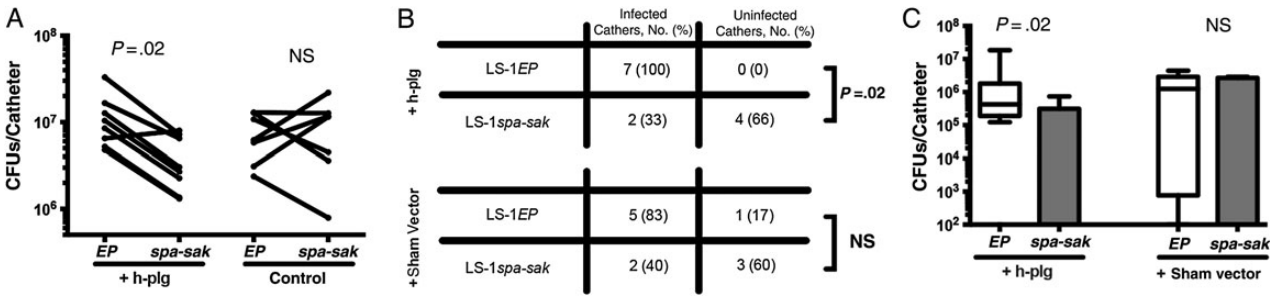

Figure 7. High staphylokinase (Sak) secretion inhibits biofilm formation in mouse catheter infection models. *A,* Colony-forming units (CFUs) in biofilms formed by *Staphylococcus aureus* strains either producing no (LS-1*EP*) or high amounts of Sak (LS-1*spa-sak*) within subcutaneous catheters. The implanted catheters were injected into the lumen by either human plasminogen (h-plg; n = 8) or buffer (n = 7). Lines connect paired samples from a single mouse. *B,* The percentage of infected catheters. *C,* CFUs in biofilms formed in catheters implanted in the jugular vein after inoculation with the *S. aureus* LS-1*EP* or LS-1*spa-sak* in mice treated with an adenovirus gene therapy vector expressing human plasminogen (n = 6–7) or in control mice treated with vector carrying a sham irrelevant sequence (n = 5–6). Median value (*horizontal line*), area between 25th and 75th percentiles (*box*), and minimal and maximal values (*whiskers*) are shown. Abbreviation: NS, not significant.

levels of LS-1*spa-sak* attached to the biomaterial compared with LS-1*EP* (Figure 7*A*). In contrast, no such effect occurred in control animals injected with saline, which indicates that the inhibitory effect of Sak on catheter infection was due to interaction of Sak and human plasminogen.

The same pattern was observed in the model of intravascular jugular vein catheter infection in mice expressing human plasminogen from an adenoviral vector. In the LS-1*EP*–infected animals, biofilms developed on all catheters (7/7), compared with 2 of 6 catheters in the LS-1*spa-sak*–infected mice (Figure 7*B*). Furthermore, significantly fewer LS-1*spa-sak* cells were found on implanted catheters compared with strain LS-1*EP* (Figure 7*C*). This effect did not occur in control mice without expression of human plasminogen, confirming that the capacity of Sak to activate plasminogen is essential for reducing *S. aureus* intravascular catheter infection.

DISCUSSION

Our study demonstrates that Sak controls development of *S. aureus* biofilm by activating plasmin(ogen)-dependent proteolysis and fibrinolysis, which in turn prevents bacterial adhesion to surfaces and destroys biofilm matrices composed of fibrin, leading to breakdown of the biofilm architecture and bacterial detachment.

Host proteins deposited on implant surfaces mediate the primary adhesion of staphylococci [2, 7]. Bacteria-induced fibrin formation plays a role in this attachment [24], but as we noted, the importance of fibrin is not limited to the attachment phase. Abundant fibrin(ogen) was found incorporated into the biofilm matrix, confirming other observations of structures resembling fibrin meshes inside biofilms [24, 29–31]. Electron microscopy showed a fibrin scaffold inside the biofilm matrix, with individual bacteria attached to it. Fibrin molecules on the *S. aureus* cell surface cross-linked bacteria with the interstitial deposited fibrin, which is likely to be mediated by *S. aureus* fibrin (ogen)-binding surface proteins [32]. Biofilms formed in clinical situations are therefore most likely composed not only of bacteria-derived but also of host-derived constituents. Unlike bacteria-derived matrix components, proteins and polysaccharides, present in some strains only [4, 6, 7], those host-derived constituents are present in vivo in biofilms of all strains. Interestingly, this biofilm matrix protects bacteria from neutrophil phagocytosis, because neutrophils were unable to penetrate into the biofilm. The presence of host proteins such as fibrin in this matrix hampered the immune recognition of the pathogen. This is in line with other reports suggesting inhibition of leukocyte activation and bacterial killing by fibrin [33, 34]. Therefore, both the inhibition of fibrin formation and the fibrinolysis induced by Sak enhance the clearance of *S. aureus* by neutrophils.

Production of high Sak levels, as observed with the LS-1*spa-sak* strain and a subset of clinical isolates, blocks bacterial attachment and subsequent biofilm formation by activating local fibrinolysis and proteolysis. Even if some cells manage to attach to the surface, the absence of a fibrin scaffold prevents successful biofilm formation. Without the fibrin-rich matrix, bacteria remain vulnerable to attack by phagocytes and antibiotics.

In contrast to the strains secreting high amount of Sak, the strains with lower Sak levels managed to adhere and form a biofilm matrix. Expression of Sak within those biofilms was reduced in comparison with planktonic cells. Under these circumstances, Sak might play a role in biofilm dispersal during the detachment phase. Biofilm dispersal happens as biofilms mature and can be mediated by phenol-soluble modulins, bacterial proteases, and nucleases [6, 7, 35–38]. Because fibrin scaffold forms a significant part of biofilm matrix, cleavage of fibrin matrix might be necessary for successful bacterial detachment. Indeed, addition of exogenous Sak to a preformed biofilm induced detachment. It is noteworthy that Sak, proteases, and phenol-soluble modulins are all regulated by the *agr* quorum-

sensing system [35, 37], so they are likely to be coexpressed and form a powerful, synergistic mechanism of biofilm detachment.

Our observation of LS-1*EP* and LS-1*sak* biofilms interacting with immune cells and antibiotics demonstrated that at early time points (at 24 hours, when assays were performed), before Sak-induced dispersal occurs, biofilms of Sak-moderate strains would be as protected as those of Sak-deficient strains.

S. aureus captures plasminogen directly on its surface through numerous receptors [39–42] and either activates it through Sak secretion or relies on host plasminogen activators. The latter method, however, is less efficient than the pronounced Sak-induced activation, and probably has a negligible role in control of biofilm structuring and dispersal, because no induction of plasmin activity was observed in supernatants of Sak-deficient biofilms. This suggests that Sak is the main regulator of fibrinolysis in biofilms.

In summary, our study demonstrates the importance of host-derived factors in *S. aureus* biofilm formation and draws attention to the central role of Sak in regulation of different phases of biofilm development (Supplementary Figure 5). By inducing cleavage of host-derived fibrin, Sak prevents bacterial attachment to surfaces and the subsequent formation of a biofilm matrix, whereas in mature biofilms it induces detachment of bacteria by degrading the fibrin scaffold holding biofilms together. Staphylococci exploit both host coagulation and fibrinolysis, manipulating them to control the biofilm formation and dispersal. Importantly, many other bacterial species can induce fibrinolysis in a similar fashion to *S. aureus*, either by secreting a plasminogen activator or by attracting host plasmin to their surfaces [43]. Therefore, the mechanism described in our study is relevant to other pathogens, and fibrinolysis might become a universal target for antibiofilm therapies.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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