Hyaluronan Modulation Impacts *Staphylococcus aureus* Biofilm Infection

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Staphylococcus aureus is a leading cause of chronic biofilm infections. Hyaluronic acid (HA) is a large glycosaminoglycan abundant in mammalian tissues that has been shown to enhance biofilm formation in multiple Gram-positive pathogens. We observed that HA accumulated in an *S. aureus* biofilm infection using a murine implant-associated infection model and that HA levels increased in a mutant strain lacking hyaluronidase (HysA). *S. aureus* secretes HysA in order to cleave HA during infection. Through *in vitro* biofilm studies with HA, the *hysA* mutant was found to accumulate increased biofilm biomass compared to the wild type, and confocal microscopy showed that HA is incorporated into the biofilm matrix. Exogenous addition of purified HysA enzyme dispersed HA-containing biofilms, while catalytically inactive enzyme had no impact. Additionally, induction of *hysA* expression prevented biofilm formation and also dispersed an established biofilm in the presence of HA. These observations were corroborated in the implant model, where there was decreased dissemination from an *hysA* mutant biofilm infection compared to the *S. aureus* wild type. Histopathology demonstrated that infection with an *hysA* mutant caused significantly reduced distribution of tissue inflammation compared to wild-type infection. To extend these studies, the impact of HA and *S. aureus* HysA on biofilm-like aggregates found in joint infections was examined. We found that HA contributes to the formation of synovial fluid aggregates, and HysA can disrupt aggregate formation. Taken together, these studies demonstrate that HA is a relevant component of the *S. aureus* biofilm matrix and HysA is important for dissemination from a biofilm infection.

Staphylococcus aureus is a Gram-positive pathogen and a leading cause of bacterial infection in both health care and community settings. Many of the complications caused by *S. aureus* are chronic biofilm-like infections, including those associated with implants and conditions such as osteomyelitis (1–3). Approximately one million nosocomial indwelling device-associated infections occur annually in the United States (4). Staphylococci are the most common isolates, accounting for nearly two-thirds of this type of infection (5). Treatment of these infections requires long courses of antibiotics and additional surgeries, which often leads to removal of the infected device (4, 6). The recalcitrance of biofilm infections to treatment is thought to be in part due to reduced penetration of antimicrobial agents through the extracellular biofilm matrix (7, 8).

The main components of the biofilm matrix *in vitro* are exopolysaccharides, proteins, and extracellular DNA (eDNA) (3, 9). However, little work has been done to determine if *S. aureus* can incorporate host matrix materials into the biofilm matrix during infection. *S. aureus* has been shown to bind host matrix molecules such as fibronectin, fibrin, and fibrinogen (10–12), and there are increasing reports of bacteria incorporating host glycosaminoglycans into the extracellular biofilm matrix. For example, heparin has been shown to enhance *S. aureus* biofilm formation and to be incorporated into the biofilm matrix *in vitro* (13). Additionally, hyaluronic acid (also known as hyaluronan [HA]) has been shown to promote biofilm formation in Gram-positive pathogens, including *Streptococcus pneumoniae* (14) and *Streptococcus intermedius* (15).

Hyaluronic acid (HA) is a large glycosaminoglycan with an average molecular mass of 7,000 kDa (16), composed of repeating disaccharide units of *N*-acetylglucosamine and glucuronic acid linked by alternating β -1,3 and β -1,4 glycosidic bonds. HA is a main component of the mammalian extracellular matrix (ECM) and is abundant in the skin, synovial fluid, skeletal tissues, heart

valves, lungs, kidneys, and brain (16). HA has been shown to have a number of host functions, which largely depend on the molecular mass of the polymer (16–18). High-molecular-mass HA (>450 kDa) is anti-inflammatory and immunosuppressive and there is increased production of high-molecular-mass HA at inflamed sites. In contrast, low- and mid-molecular-mass HAs (20 to 450 kDa) strongly promote production of inflammatory cytokines, adhesins, and cell proliferation of chondrocytes, endothelial cells, and fibroblasts (18–20). HA also acts as a physical barrier to prevent the spread of pathogens (16–18).

To penetrate the HA barrier, many pathogenic Gram-positive bacteria secrete hyaluronidases that cleave HA at the β -1,4 glycosidic bond. This cleavage yields unsaturated disaccharides as a final product of complete digestion. *S. aureus* produces a hyaluronidase, encoded by the gene *hysA*, that is conserved across all *S. aureus* lineages (21). Our group and others have shown that HysA is a virulence factor in a variety of infection models (22, 23), which is thought to be due to its role in facilitating local spread of the infection. However, it has become increasingly clear that hyaluronidases have additional functions that benefit pathogen survival in the host. A number of bacteria, including *S. pneumoniae* and *Streptococcus pyogenes*, have been found to catabolize HA in a

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
BW25141	Cloning strain	63
AH2856	HysA protein expression strain ER2566 containing pCR03	22
AH3053	Y306A HysA protein expression strain ER2566 containing pCR07	This work
<i>S. aureus</i>		
RN4220	Restriction-deficient 8325-4	64
AH1263	USA300 CA-MRSA, Erm ^s (LAC*)	65
AH2525	AH1263 Δ <i>hysA</i>	22
AH1712	AH1263 <i>ica::tet</i>	66
AH3741	AH1263 Δ <i>hysA ica::tet</i>	This work
AH3743	AH1263 <i>ica::tet</i> (pCM29)	This work
AH3744	AH1263 Δ <i>hysA ica::tet</i> (pCM29)	This work
AH3849	AH1263(pHC48)	This work
AH3908	AH1263 Δ <i>hysA</i> (pHC48)	This work
Other staphylococci		
AH2490	<i>S. epidermidis</i> strain 1457	67
AH2160	<i>S. lugdunensis</i> strain N920 143	68
AH3604	<i>S. saprophyticus</i> strain ATCC 15305	ATCC
AH3603	<i>S. xylosus</i> strain ATCC 29971	Naomi Ward
AH2452	<i>S. capitis</i>	Patrick Schlievert
AH3568	<i>S. caprae</i> DSM20608	DSMZ
AH2329	<i>S. carnosus</i> strain TM300	69
Plasmids		
pCM29	<i>S. aureus</i> constitutive sGFP expression vector, Cam ^r	28
pHC48	<i>S. aureus</i> constitutive DsRed expression vector, Cam ^r	This work
pCR03	<i>hysA</i> 6×His-tagged <i>E. coli</i> protein expression vector, Kan ^r	22
pCR07	Y306A <i>hysA</i> 6×His-tagged <i>E. coli</i> protein expression vector, Kan ^r	This work
pRMC2	Tetracycline-inducible expression vector, Cam ^r	70
pRMC2- <i>hysA</i>	Tetracycline-inducible <i>hysA</i> expression vector, Cam ^r	This work

hyaluronidase-dependent manner (24, 25). Additionally, hyaluronidase added exogenously to HA-containing *S. intermedius* biofilms led to dispersal of the cells, leading the authors to propose that hyaluronidase may be an important biofilm detaching agent (15).

Due to the abundance of HA in mammalian tissues, we hypothesized that HA is an important host polymer that will impact *S. aureus* biofilm development during infection. Here we show that *S. aureus* incorporates HA into the biofilm matrix *in vitro* and during implant infection. Additionally, we find that HysA can disperse established biofilms and disseminate *S. aureus* from a murine implant-associated infection model. This work provides strong evidence that HA is an important and overlooked component of the biofilm infection matrix and that HysA enzyme modulates HA levels, thereby facilitating *S. aureus* dissemination in the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. All *S. aureus* strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) with the appropriate antibiotics for plasmid maintenance or selection (chloramphenicol [Cam], 10 µg/ml) at 37°C with shaking at 220 rpm, unless otherwise specified. Hyaluronic acid sodium salt purified from *Streptococcus equi* was purchased from Sigma-Aldrich. Plasmid DNA was

purified from *Escherichia coli* and electroporated into *S. aureus* RN4220 as previously described (26). Plasmids were moved from RN4220 with bacteriophage ϕ11 to other *S. aureus* strains by transduction.

To make DsRed plasmid pHC48, DsRed.T3(DNT) was amplified from pRFP-F (27) with primers HC286 (5'-GTTTGGTACCTGATTAAC TTTATAAGGAGGAAAAACATATGGA) and HC287 (5'-CAACGAATT CTTATAAAAAACAAATGATGACGACCTTCTGTAC-3'), and the product was digested with KpnI and EcoRI. The green fluorescent protein (GFP) gene was excised from pCM29 (28), using the same enzymes, and DsRed was ligated in its place. This plasmid expresses DsRed under the control of the *S. aureus sarA* P1 promoter.

To create a tetracycline-inducible *hysA* plasmid (pRMC2-HysA), the *hysA* gene was amplified from AH1263 genomic DNA using primers CBR63 (5'-GTTGTTGAGCTCTTAATGATGATGATGATGATGT TTAGTTAATTCAAAGTG-3') and CBR64 (5'-GTTGTTGGTACCTT AGGAGGATGATTATTTATGACATATAGAATAAAGAAATGG-3'), which contain SacI and KpnI sites at their 5' ends, respectively. The resulting PCR product was ligated into pRMC2 linearized with KpnI and SacI, and the plasmid was verified.

The HysA catalytically inactive (Y306A) overexpression construct pET28a-Y306A HysA was generated from pCR03 using the QuikChange (Stratagene) mutagenesis method with primers CBR49 (5'-CCATACAC TGGTGCTGCGGGCGTTGTACTC-3') and CBR50 (5'-GAGTACAAC GCCCGCAGCACCAGTGTATGG-3') and transformed into *E. coli* strain ER2566 for protein expression. The resulting plasmid was confirmed by sequencing.

Murine catheter infection model. All animal procedures were performed in accordance with a protocol approved by the University of Iowa Institutional Animal Care and Use Committee. All animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (29).

C57BL/6 mice were purchased from Charles River (Wilmington, MA) and allowed to acclimate to the animal housing facility for at least 1 week prior to their inclusion into this study. For infections, the flanks of age- and sex-matched mice were shaved with a microtome blade (Sakura Finetek, Torrance, CA). Segments of 18-gauge intravenous (i.v.) catheters (BD, Franklin Lakes, NJ) were cut to 1 cm, UV sterilized, and incubated in C57BL/6 serum (Innovative Research, Novi, MI) for 1 h at 37°C. The *S. aureus* wild type (WT) or *hysA* mutant was grown overnight at 37°C with shaking at 225 rpm in TSB, subcultured 1:1,000 into fresh medium, and grown to an optical density at 600 nm (OD_{600}) equal to 1.0. One milliliter of culture from each strain was pelleted and resuspended into 1 ml of 0.9% saline (Baxter, Deerfield, IL). The catheters were removed from the serum and added to the washed *S. aureus* cells or as a negative control into saline alone and incubated at 37°C for 1 h. After precolonization, catheters were resheathed onto 18-gauge needles. While under isoflurane anesthesia, catheters were inserted subcutaneously into the flank after precleaning the skin with an alcohol prep pad (Covidian, Dublin, Ireland), and the insertion site was sealed with Dermabond (Ethicon, West Somerville, NJ). After infection, the overall health of the mice was monitored daily for 6 days, when mice were sacrificed and catheters were harvested for analysis of bacterial burdens and histology.

HA ELISA of infected catheters. Catheters were colonized and implanted subcutaneously in the right flank of male C57BL/6 mice as described above. After 6 days, catheters were harvested into 1 ml phosphate-buffered saline (PBS) to perform an HA detection assay using a hyaluronan Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Catheters were sonicated for 10 min in a water bath sonicator and then vortexed for 30 s to release HA. Samples were diluted 1:100 with the supplied calibrator buffer, and the assay was performed per the manufacturer's instructions. Concentrations were determined by calculation from a standard curve of HA.

Histological analysis of catheter biofilm infection. At necropsy, skin tissues were immediately prosected with a wide margin, pinned to a cork board, and fixed in 10% neutral buffered formalin for 3 to 5 days. Tissues were routinely processed, embedded, sectioned ($\sim 4 \mu\text{m}$), and stained with hematoxylin and eosin (HE). Tissues were examined by a postexamination method of masking by a boarded veterinary pathologist (30). For histopathological scoring of necropurulent debris, the following grades were assessed: 0, none; 1, scant necropurulent debris along the rim of the catheter; 2, small localized pools of necropurulent debris surrounding the catheter ($<33\%$ circumference); 3, moderate pools of necropurulent debris surrounding the catheter (33 to 66% circumference); and 4, extensive pools of necropurulent debris surrounding the catheter ($>66\%$ circumference). To evaluate the extent of necropurulent lesions, the total necropurulent area (square millimeters) surrounding the catheter was determined. We also measured the largest distance (millimeters) that the necropurulent lesion had expanded from the catheter site. These morphometric measurements were made using a high-resolution microscope/digital camera (BX52 and DP73 camera; Olympus) and specialized software (CellSens Software; Olympus). Statistical evaluation between groups was made with the Mann-Whitney test.

Synovial fluid aggregates. The synovial fluid aggregates were prepared by adapting a previously published method (31). Synovial fluid was isolated from WT newborn pigs and centrifuged to remove any host cells. *S. aureus* strains were grown overnight in TSB at 37°C with shaking at 220 rpm. Cultures were normalized by optical density, 1 ml was pelleted, and cells were resuspended into 1 ml of phosphate-buffered saline (PBS). Two hundred microliters of PBS or 10% synovial fluid diluted with PBS was

FIG 1 HA is present at high concentrations in a biofilm infection. (A) SEM of a biofilm formed in the lumen of a catheter infected with the LAC *ΔhysA* mutant in a murine implant infection model and removed for imaging after 6 days. (B) Close-up SEM images indicate a large biofilm in the catheter lumen encased in matrix material. Arrows are shown to highlight *S. aureus* cells in the biofilm. (C) There is increased HA in the biofilm formed on the catheter after 6 days, as demonstrated by HA-specific ELISA. Statistical analysis was performed using a Student's *t* test (**, $P < 0.01$).

added to each well of a 48-well microtiter plate. Twenty microliters of *S. aureus* washed cells was added to each well. Plates were incubated for 1 h at 37°C with shaking at 220 rpm. To image aggregates, ethidium bromide (2.5 $\mu\text{g/ml}$) was added to each well, and the plate was incubated for 30 min at 37°C with shaking at 220 rpm. Cells were visualized by UV light and imaged with a Bio-Rad GelDoc 2000.

Preparation of biofilms for SEM. Catheters were colonized with the *S. aureus* wild-type strain LAC and implanted subcutaneously in the right flank of male C57BL/6 mice as described above. After 6 days, catheters were harvested, transferred to a 48-well microtiter plate, and fixed with 2.5% glutaraldehyde overnight at 4°C. The synovial fluid biofilms were formed in a 48-well polystyrene microtiter plate with strain LAC as described above. After 1 h of incubation in 10% porcine synovial fluid,

FIG 2 HA enhances staphylococcal biofilm formation. (A) HA enhances biofilm formation of LAC $\Delta hysA$ *in vitro*. (B) Both LAC and LAC $\Delta hysA$ have enhanced biofilm formation in the presence of heparin. (C) HA must be larger than 50 kDa to enhance biofilm formation of LAC $\Delta hysA$. (D) HA enhances biofilm formation of many staphylococcal species *in vitro*, but only the *S. aureus* WT strain produces a hyaluronidase, as shown on HA agarose (underneath the graph). Statistical analysis was performed using a Student's *t* test. Asterisks denote statistical significance compared to biomass with brain heart infusion plus 0.4% glucose (BHlg) alone: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

biofilms were transferred to a fresh well and fixed overnight at 4°C with 2.5% glutaraldehyde. All biofilms were then prepared for scanning electron microscopy (SEM) in the same manner. In brief, biofilms were washed three times for 15 min with 0.1 M sodium cacodylate buffer to remove fixative and treated with 1% osmium tetroxide for 1.5 h. Samples were washed three times with buffer, followed by one wash with double-distilled water. Increasing concentrations of ethanol were then added to dehydrate the samples for 15 min each. Biofilms were incubated with 95% ethanol for 30 min and 100% ethanol twice for 30 min each. Samples were cross-linked with a 1:1 mixture of hexamethyldisilazane (HMDS) and 100% ethanol for 15 min, followed by HMDS alone twice for 15 min each. HMDS was removed, and samples were allowed to air dry in a tissue culture hood overnight. Coverslips were mounted on stubs and coated with gold particles using an Emitech K550 sputter coater. Images were captured with a Hitachi S-4800 scanning electron microscope with Super ExB filter technology.

Microtiter biofilms. Microtiter biofilms were generated by a variation of previously used method (32). Bacterial strains were grown overnight in TSB and subcultured 1:500 into biofilm medium (brain heart infusion [BHI] plus 0.4% glucose) or biofilm medium containing HA as indicated. Wells of a 48-well microtiter plate (Corning Costar 3548) were inoculated, and plates were incubated at 37°C with shaking at 500 rpm in a Stuart microtiter plate incubator for 18 h in quadruplicate. The medium was aspirated, and wells were washed once with water and stained with 0.1% crystal violet. The wells were washed again with water, and stained biomass was solubilized with 1 ml of isopropanol for 20 min. The absorbance

was measured using a Tecan Infinite M200 plate reader at between 595 and 615 nm. The 615-nm absorbance wavelength was used to avoid dilution when measurements were above the limit of detection.

The following modifications were used for those biofilms formed with the tetracycline-inducible plasmids (pRMC2 and pRMC2-*hysA*). For induction at time zero, strains were grown overnight in TSB in the presence of Cam (10 μ g/ml) to maintain the plasmid. The next day, strains were subcultured 1:500 into biofilm medium (BHI plus 0.4% glucose) that contained 2 mg/ml HA, Cam, and anhydrotetracycline (aTet) at the concentrations indicated in the legend to Fig. 2. Biofilms were grown in a 48-well microtiter plate for 18 h at 37°C with shaking at 500 rpm in a Stuart microtiter plate incubator, when biomass was determined with the microtiter biofilm assay as described above. For the biofilms that were induced at 18 h, strains were also grown overnight in TSB in the presence of Cam. The next day, strains were subcultured 1:500 into biofilm medium containing HA and Cam as indicated above. Strains were inoculated into 48-well microtiter plates and incubated at 37°C with shaking at 500 rpm in a Stuart microtiter plate incubator. After 18 h, aTet was added as indicated in the legend to Fig. 2, and the microtiter plate was incubated for an additional 4 h. Biomass was then determined with the microtiter biofilm assay as described above.

Confocal microscopy of HA biofilms. *S. aureus* strains LAC and LAC $\Delta hysA$, containing a plasmid that constitutively expresses DsRed (pHC48), were grown overnight in TSB, and then subcultured 1:500 into BHI–0.4% glucose that contained 2 mg/ml HA (1,500 kDa), 1% labeled with fluorescein isothiocyanate (FITC) (Creative PEGworks, Chapel Hill,

FIG 3 HA is incorporated into the *S. aureus* biofilm matrix. The LAC WT and LAC Δ *hysA* strains constitutively expressing DsRed (red) were grown in biofilm medium. Biofilms were prepared with or without 2 mg/ml of 1% FITC-labeled HA (green). Images were obtained at $\times 100$ magnification by confocal laser scanning microscopy, and red and green channel images were captured at 45 by 45 μ m; a merged image is also shown. (A) LAC WT with HA. (B) LAC Δ *hysA* without HA. (C) LAC Δ *hysA* with HA. (D) Enlarged inset of LAC Δ *hysA* with HA.

NC). Three hundred microliters was added to each well of the 8-well μ -slide (ibiTreat surface; ibidi, Martinsried, Germany), and the slide was incubated statically for 18 h at 37°C and afterwards fixed for 15 min at room temperature with 2% paraformaldehyde. Wells were washed with PBS to remove the fixative and imaged with a Zeiss LSM510 confocal microscope.

Confocal microscopy of synovial fluid aggregates. Synovial fluid aggregates were formed as described using the *S. aureus* WT and *hysA* mutant strains (AH3743 and AH3744) that lack the *ica* locus and express superfolder GFP (sGFP) constitutively from a plasmid (pCM29). Aggregates were fixed with 4% paraformaldehyde for 10 min at room temperature and washed 3 times with PBS to remove excess fixative. Hyaluronic acid was stained with wheat germ agglutinin (WGA) at room temperature for 15 min, and aggregates were washed with PBS to remove excess stain. Aggregates were transferred to a microscope slide and imaged by confocal laser scanning microscopy using a Leica SP3 microscope. Images were assembled using FIJI Image J software.

Purification of HysA and the HysA catalytically inactive mutant. AH2856 and AH3053 were grown overnight at 37°C in LB medium containing kanamycin (50 μ g/ml) and subcultured into 1 liter of fresh LB

medium at a ratio of 1:250. Cultures were grown at 30°C to the mid-logarithmic phase, IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a 1 mM final concentration, and the culture was allowed to grow for an additional 4 h. Cells were harvested by centrifugation, washed with water, pelleted by centrifugation, and frozen at -20°C . Cells were mechanically lysed with two passes through a Microfluidics microfluidizer (model LV1; Microfluidics, Newton, MA) at 25,000 lb/in². Cell lysate was clarified by centrifugation at 15,000 $\times g$ for 20 min at 4°C. Proteins were purified using Fractogel His-bind resin (Novagen) per the manufacturer's instructions, dialyzed into phosphate-buffered saline (PBS), concentrated using Millipore centrifugal filter units (molecular-weight cutoff of 30,000), and brought to 3 mg/ml in 1 $\times$ PBS. The protein suspensions were frozen at -20° in 10% glycerol until use.

Statistical analysis. Statistical significance of all data was determined using a Student *t* test with GraphPad Prism (version 6) software (GraphPad, La Jolla, CA) unless otherwise noted in the figure legends.

RESULTS

HA is present during *S. aureus* biofilm infections. We hypothesized that HA is present at the site of a *S. aureus* biofilm infection.

To test this question, we used a murine implant-associated infection model and challenged the mice with a community-associated methicillin-resistant *S. aureus* (CA-MRSA) USA300 wild-type strain, LAC, or an isogenic hyaluronidase deletion mutant (LAC $\Delta hysA$). After 6 days, mice were sacrificed, and catheters were prepared for scanning electron microscopy (SEM). In the lumen of the catheter during infection, a large accumulation of host- and pathogen-derived biomass developed that was encased in abundant extracellular matrix material (Fig. 1A and B). We next measured the HA concentration of the biofilm formed in the catheter lumen by ELISA. The presence of *S. aureus* led to an increase in HA levels over those in uninfected control catheters, while those infected with the *hysA* mutant had a dramatic increase in HA over the WT and control (Fig. 1C). These initial findings indicate that HA is present at the site of *S. aureus* biofilm infection and the production of HysA limits HA accumulation.

HA enhances staphylococcal biofilm accumulation. HA has been shown to enhance biofilm formation in multiple low-GC Gram-positive bacteria (14, 15). Therefore, we assessed *S. aureus* biofilm formation using a microtiter plate assay in the presence and absence of HA. Under the conditions tested, the presence of different levels of HA did not alter LAC WT strain biofilm formation (Fig. 2A). However, there was a significant increase in biofilm formation in the *hysA* mutant in the presence of HA at 1 and 3 mg/ml, which are relevant levels in body sites such as synovial fluid (16). This finding led us to predict that the HysA enzyme may cleave HA in the biofilm matrix and limit the accumulation of biomass. To assess this possibility, we tested if the LAC and LAC $\Delta hysA$ mutant strains could incorporate an alternative, uncleavable polymer into the biofilm matrix. Heparin is another glycosaminoglycan and is similar in composition to HA, but it is not a substrate for HysA (33). In the presence of heparin, both LAC and the LAC $\Delta hysA$ mutant had enhanced biofilm formation, but only the *hysA* mutant was enhanced by HA (Fig. 2B). From this finding, we speculated that HA breakdown was limiting its utility as a biofilm matrix component in the wild type, and the size of the HA must need to be larger than a certain threshold to promote biofilm formation. We assessed multiple sizes of HA, ranging from 10 kDa to 1,500 kDa, for their ability to enhance biofilm formation in the *hysA* mutant. We found that HA needed to be larger than 50 kDa to promote biofilm enhancement (Fig. 2C).

Based on the differential responses of LAC and the LAC $\Delta hysA$ mutant to the HA biofilm enhancement, we wondered how other *Staphylococcus* species would behave in the presence of HA. Only *S. aureus* secretes a hyaluronidase enzyme (Fig. 2D), suggesting other staphylococci might act similarly to the *S. aureus hysA* mutant in biofilm development. In the presence of HA, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, *Staphylococcus xylosus*, *Staphylococcus capitis*, and *Staphylococcus caprae* all formed significantly larger biofilms than under standard conditions (Fig. 2D). Interestingly, there was no difference in biofilm formation between the two conditions for *Staphylococcus carnosus* (Fig. 2D). This may indicate that *S. carnosus* lacks a factor necessary to incorporate HA into the biofilm matrix.

HA is incorporated into the *S. aureus* biofilm matrix. After observing the HA-induced biofilm enhancement, we performed confocal microscopy studies to visualize the localization of HA in the biofilm matrix. For this, we used *S. aureus* constitutively expressing the red fluorescent protein DsRed and FITC-labeled

FIG 4 HysA can disperse HA-containing biofilms. (A) Biofilms of LAC $\Delta hysA$ were performed in microtiter plates with HA. Purified HysA at 1 or 3 μ g/ml can disperse these established biofilms, but a catalytically inactive site-directed mutant HysA(Y306A) protein has no impact on the biofilm. (B) Induction of *hysA* expression (pRMC2-*hysA*) prevented HA-containing biofilm formation in a dose-dependent manner (shown by the aTet inducer in nanograms per milliliter) compared to the empty-vector control. (C) Induction of *hysA* expression dispersed established biofilms that contain HA in the matrix. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

high-molecular-mass HA, and we compared LAC and the LAC $\Delta hysA$ mutant. In the presence of exogenously added HA, the LAC WT strain had almost no discernible HA in the extracellular matrix, and the amount of the cell-surface-bound HA was low (Fig. 3A). The WT biofilms were not stable and in some areas completely detached from the bottom of the well. When the LAC $\Delta hysA$ mutant was tested in the absence of HA, the microscopy revealed biofilms composed of tightly packed cells (Fig. 3B). The addition of HA to the mutant restored the space between bacterial

FIG 5 The *hysA* mutant has reduced dissemination in an implant-associated infection model. (A) Mice infected with the LAC WT lose significantly more weight than those infected with the LAC $\Delta hysA$ mutant or the PBS negative control. (B) The bacterial burdens on the catheters are similar at 6 days of infection between the WT and *hysA* mutant. (C) There is a significant reduction in bacterial burden for the *hysA* mutant compared to the WT in the tissue surrounding the catheters at 6 days. (D) There is reduced dissemination of the *hysA* mutant to the kidneys compared to the WT. *, $P < 0.05$; **, $P < 0.01$.

aggregates, similar to the WT biofilm. However, the LAC $\Delta hysA$ mutant matrix was filled with HA, visible as a diffuse green staining (Fig. 3C). A large proportion of HA was bound to the bacterial surfaces, as indicated by a green ring-like staining of cell walls visible on individual cells (Fig. 3D). Taken together, these observations indicate that HA is incorporated into *S. aureus* biofilms, both as a loose component of the extracellular matrix and as a cell-surface-bound form. In the case of wild-type *S. aureus*, the majority of the matrix-localized HA is degraded by the HysA enzyme.

HysA disperses biofilms that contain HA. Based on our biofilm assay results, we hypothesized that secreted HysA enzyme may disperse *S. aureus* from HA-containing biofilms. To test this hypothesis, biofilms were preformed in the presence of HA with the LAC $\Delta hysA$ mutant. These biofilms were treated exogenously with purified HysA (at 1 and 3 $\mu\text{g/ml}$) or a purified catalytically inactive version of HysA (Y306A), for 3 h before measurement of the remaining biomass. Active HysA enzyme significantly reduced biomass compared to the no-treatment control (Fig. 4A), and the catalytically inactive HysA had no impact on the biofilm biomass. Our observations indicated that HysA enzyme activity could be involved in dispersal from HA-containing biofilms. To further assess the role of HysA in biofilm dispersal, we used strains engi-

neered to express *hysA* under the control of a tetracycline-inducible promoter to determine if induction of HysA at time zero could prevent biofilm formation. We found that with increasing concentrations of inducer (anhydrotetracycline [aTet]), the expression of HysA prevented HA-containing biofilm formation compared to the empty-vector control (Fig. 4B). To evaluate the ability of HysA to disperse an established biofilm, a biofilm was formed for 18 h, at which point, the expression of *hysA* was induced with increasing concentrations of aTet for 4 h, prior to assessment of biofilm biomass. We found that induction of *hysA* in an established HA-containing biofilm resulted in biofilm dispersal (Fig. 4C) compared to the uninduced control, confirming our finding that exogenous HysA could disperse HA-containing biofilms.

***S. aureus* uses HysA to escape from a biofilm infection.** Based on our observed HysA impact on *S. aureus* biofilms, we assessed the contribution of HysA to an *S. aureus* biofilm infection. Mice were challenged with the LAC WT or LAC $\Delta hysA$ mutant using a murine implant-associated infection model. Catheters were coated with murine serum, and *S. aureus* cells were allowed to adhere prior to subcutaneous implantation in the flank of the mouse. During the course of the infection, mice that were inoculated with the WT lost significantly more weight than those inoc-

FIG 6 Histopathological evaluation and scoring of skin catheters with *S. aureus* infection. (A) Catheters (asterisks) infected with WT ($n = 8$ mice) and LAC $\Delta hysA$ mutant ($n = 6$ mice) bacteria had peripheral zones (arrows) of purulent inflammation, necrosis, and debris (insets), but $\Delta hysA$ mutant groups had a reduction in lesion distribution and severity. HE stain was used. (B to D) Similarly, semiquantitative histopathological scoring (B) along with quantitative morphometry of the lesion area (C) and maximum lesion length from the catheter (D) demonstrated that $\Delta hysA$ mutants had reduced lesion extent and severity compared to WT bacteria ($P < 0.05$, Mann-Whitney test).

ulated with the LAC $\Delta hysA$ mutant or the PBS control (Fig. 5A). After 6 days, mice were sacrificed and bacterial burdens were determined for the catheters, surrounding tissue, and kidneys. There was no difference in bacterial burdens between the WT and LAC $\Delta hysA$ mutant on the catheters themselves (Fig. 5B). However, there was a significant reduction in bacterial load in the tissue surrounding the catheters for the mice infected with the LAC $\Delta hysA$ mutant compared to the WT (Fig. 5C). This difference was further amplified in a distal organ site, the kidney (Fig. 5D), indicating that the $\Delta hysA$ mutant was defective in dissemination in the host.

Histopathological examination and analysis of infected catheters. Histopathologic examination of the tissues with catheters showed that LAC WT infection had a more profound and overt tissue reaction than infection with the LAC $\Delta hysA$ mutant (Fig. 6A). Within the lesion, purulent and cellular necrotic (necropurulent) debris was morphologically well delineated (Fig. 6A), and so this was targeted for histopathological scoring and morphometry. Using semiquantitative morphometric approaches, the extent of necropurulent debris around the catheter was significantly decreased in the $\Delta hysA$ group (Fig. 6B and C). Spread of *S. aureus* from the infected catheter to adjacent tissues is an important consideration in this model. We measured the maximum distance that the necropurulent lesion had traversed from the infected catheter site and found that the LAC $\Delta hysA$ mutant had a significantly reduced lesion distance compared to the WT (Fig. 6D).

HA is involved in the formation of synovial fluid aggregates. To assess the generality of the presence of HA in infectious biofilms, we investigated the contribution of HA to other types of host-dependent biofilm structures. *S. aureus* was recently found to form macroscopic biofilm-like aggregates in synovial fluid of joint

infections (31). The highest concentrations of HA in any tissues are in synovial fluid, with concentrations of 1 to 4 mg/ml in humans (34). We hypothesized that HA might be involved in the formation of these synovial fluid aggregates. To test this, we isolated synovial fluid from newborn pigs and replicated the formation of biofilm-like aggregates with the porcine synovial fluid (Fig. 7A). Scanning electron microscopy (SEM) was performed to further examine the architecture of the aggregates (Fig. 7B), and we observed large clusters of cells tightly encased in a polymeric rope-like substance. As anticipated, the levels of HA were present at high concentrations in porcine synovial fluid—between 2 and 4 mg/ml (Fig. 7C)—similar to concentrations found in humans.

We predicted that HA might be incorporated into the synovial fluid aggregates, similar to our other observations with HA-containing biofilms (Fig. 3). Aggregates were formed using LAC WT and LAC $\Delta hysA$ mutant strains, expressing GFP and containing deletions of the polysaccharide intracellular adhesion (PIA) locus. These strains formed macroscopic aggregates in microtiter plates (data not shown), and the aggregates were exposed to wheat germ agglutinin (WGA). WGA binds β -1,4-linked *N*-acetylglucosamine, and we used this lectin to stain for HA in the synovial fluid aggregates. Using confocal microscopy, a high degree of staining with WGA was observed within the aggregates of the WT strain, and compared to the GFP-producing cells, the majority were colocalized with WGA (Fig. 8). The LAC $\Delta hysA$ mutant also stained at a similar high level with WGA. Knowing that HA is abundant in synovial fluid, we pretreated the fluid with purified HysA enzyme to fully digest the HA present. The LAC $\Delta hysA$ mutant strain formed significantly smaller aggregates with the HysA-treated fluid (Fig. 8), providing evidence for the important role of HA as a structural component of these synovial fluid aggregates.

FIG 7 *S. aureus* forms large biofilm aggregates in porcine synovial fluid. (A) *S. aureus* forms biofilm-like aggregates in porcine synovial fluid (10% SF). (B) SEM images of the cells at different magnification show aggregates encased in a matrix composed of long polymers. (C) Quantification of HA in the porcine fluid reveals that it is present at high concentrations. Dotted lines indicate the upper and lower ranges of HA concentrations found in human synovial fluid.

DISCUSSION

In this report, we investigated whether the mammalian extracellular glycosaminoglycan hyaluronic acid (HA) has an impact on *S. aureus* biofilm development. *S. aureus* biofilm studies to date have mostly been performed using commercially available rich or chemically defined media, which has provided a wealth of information about important bacterial contributors to the biofilm process, but these defined growth conditions have limited application to the host environment. In some studies, human matrix proteins have been used to aid *S. aureus* attachment and enhance biofilm accumulation (35–38), providing a biotic substratum that better imitates a realistic host surface. Although these approaches are an improvement and likely enhance the relevance of biofilm observations, the abundance of host glycosaminoglycans in many tissues and their contribution to *S. aureus* biofilm formation remain mostly overlooked.

To assess the specific involvement of HA in *S. aureus* biofilm formation, we first determined whether the polymer was present in the vicinity of a biofilm infection. Using an HA-specific ELISA

(Fig. 1), we detected increased levels of HA in the catheter lumen of a mouse implant infection, and HA levels were further increased when the hyaluronidase gene (*hysA*) was deleted. Going further, we demonstrated that HA was incorporated into the biofilm structural matrix, encasing bacterial cells (Fig. 3), and that the presence of HA enhanced the ability of staphylococci to form a biofilm (Fig. 2D). Similarly, we demonstrated that HA was abundant in synovial fluid aggregates (Fig. 7), a biofilm-like clump that mimics an *S. aureus* joint infection (31), and we also observed that HA had a structural role in these aggregates (Fig. 8). Taken together, using different approaches to represent an actual biofilm implant infection, we present convincing evidence that HA is present at the infection site, incorporated into the extracellular matrix encasing the bacterial cells, and serves a structural role in the biofilm.

The challenge of HA as an effective matrix component is that *S. aureus* secretes a potent hyaluronidase (HysA) enzyme that can rapidly degrade this substrate. Using *in vitro* biofilm assays, we did not observe *S. aureus* biofilm enhancement until the *hysA* gene was deleted (Fig. 2A). Presumably under the conditions tested, adequate HysA is secreted during biofilm development to reduce levels of high-molecular-mass HA until the pool of this polymer is under the 50-kDa threshold found to be critical for use as a functional matrix component (Fig. 2C). In support of this statement, confocal microscopy failed to identify HA between cells of a WT biofilm (Fig. 3A). In the host, the HysA activity is likely further suppressed, as a number of reports have suggested inhibitors of bacterial hyaluronidases are present in serum (39–41). Interestingly, the normal amount of HysA secreted from the *S. aureus* WT strain did not prevent synovial fluid aggregates from forming (Fig. 7A), perhaps due to the presence of these enzyme inhibitors. Thus, there seems to be a delicate balance where HA can serve a structural role in the biofilm—likely when the activity of hyaluronidase is kept limited. Although this seems unexpected, it is not unusual for enzyme levels to impact *S. aureus* biofilm development, as other extracellular matrix-degrading enzymes must be repressed or surface proteins and extracellular DNA (eDNA) will be destroyed (42, 43). Additionally, such a dichotomy is not unusual among bacterial pathogens, as *Streptococcus pyogenes* secretes a hyaluronidase while simultaneously producing a HA capsule, resulting in a biological state that appears mutually exclusive (44).

How does HA function in the *S. aureus* biofilm matrix? This pathogen might contain surface proteins that bind HA and facilitate its incorporation into the biofilm structure. Heparin and eDNA binding proteins have been identified in a number of bacteria, including *S. aureus* (45–50), lending support to this idea. Due to the high degree of HA association with the individual cell surfaces in *S. aureus* biofilm (Fig. 3), we predict that *S. aureus* may also have HA binding proteins. HA was able to enhance biofilm formation in other staphylococci (Fig. 2B), which suggests that these proteins might be conserved across the genus. In addition, *S. aureus* and other staphylococci produce the PIA polysaccharide (3), which is composed of β -1,6-linked *N*-acetylglucosamine and has some structural similarity to HA. It is possible that negatively charged polymers, including eDNA, heparin, HA, and PIA, all play a similar structural role in the biofilm matrix, and the staphylococci may have conserved mechanisms to bind and incorporate these polymers. The ability of *S. aureus* to repurpose abundant ECM components such as HA as a biofilm building material

FIG 8 HA is an important component of the synovial fluid aggregate matrix. Synovial fluid (SF) aggregates were formed over a 1-h period with *S. aureus* LAC (WT) or the LAC $\Delta hysA$ mutant that constitutively expresses GFP and lacks PIA production. Biofilms were stained with WGA to label HA and imaged by confocal laser scanning microscopy. In the right panels, synovial fluid was predigested with purified HysA and then added to *S. aureus* cells.

would greatly improve the likelihood of constructing a protective niche within a hostile host environment.

The success of biofilm formation as an immune evasion strategy is reflected by the characteristic chronic nature of biofilm-associated infections within immunocompetent hosts. Given that even under favorable conditions, biofilms are heterogeneous with nutrient-limiting regions (51), these communities must be highly responsive to environmental perturbations that further challenge the metabolic status. Within the context of intense homeostatic stress such as nutrient deprivation, HysA might play a multifunctional role as a mediator of biofilm dispersal and tissue invasion, which are both vital to a recolonization effort. Coupling the proposed HysA behaviors with nutritional distress, we recently showed that the global regulator CodY represses *hysA* expression (22) in response to nutrient levels in the cell. Thus, CodY could control biofilm dissemination in a nutrient-dependent manner and facilitate *S. aureus* survival when facing a resource-depleted environment (i.e., nutritional immunity [52]). In addition, multiple hyaluronidase-producing pathogens, such as *S. pneumoniae*, *S. pyogenes*, *Streptococcus agalactiae*, and *Mycobacterium tuberculosis*, have been shown to utilize HA as the sole carbon source (24, 25, 53, 54). Although the ability of *S. aureus* to catabolize HA has

not been demonstrated definitively (55), it is possible that HysA-mediated liberation of *N*-acetylglucosamine-glucuronate disaccharides during biofilm dispersal serves as a carbon source available to planktonic cells invading host tissue. Future studies will be necessary to understand *S. aureus* HA catabolism and the contribution of CodY to regulating this pathway.

As the leading cause of infectious mortality in the United States, *S. aureus* has an alarming propensity to spread from sites of incipient infection and cause life-threatening invasive diseases (56). Given that *S. aureus* systemic infections can readily arise from skin or mucosal sites, it is reasonable to infer that the active invasion of host tissue is a key facet of *S. aureus* pathogenicity. Consistent with this view, *S. aureus hysA* mutants exhibit a hypovirulent phenotype in models of acute pulmonary and cutaneous infection (22, 23). In addition, hyaluronidases from numerous Gram-positive pathogens have been shown to promote tissue penetrance of bacteria, toxins, and large-molecular-mass dyes in the vicinity of the challenge site (25, 57). In the present study, we employed a medical device-associated infection model to show that *S. aureus*-derived HysA is an important virulence factor within the context of chronic biofilm infection. In support of this,

catheter preparations from HysA mutants showed a reduction in overall morbidity and a corresponding decrease in both local and systemic dissemination from the implant site (Fig. 5). Additionally, cross-sectional comparisons of catheter-associated tissue following mutant and WT infections revealed profound differences in the histopathological manifestations of disease between groups. Whereas infections with HysA mutants resulted in minimal injury, analogous infections with their WT counterparts caused expansive tissue lesions typified by neutrophilic infiltration and necropurulent debris (Fig. 6). Altogether, the results presented herein show that HysA plays an important role in virulence with broad functional activities that encompass biofilm assembly, dispersal, tissue invasion, and host injury.

While the present study strengthens the case for HysA as a mediator of *S. aureus* virulence, its function as a “spreading factor” would appear to be challenged by the recognition of low-molecular-mass fragments of HA as a danger-associated molecular pattern (DAMP) via host Toll-like receptor 2 (TLR2) and/or TLR4 (17, 19, 20, 58, 59). Numerous reports have shown that the low-molecular-mass HA fragments generated by host mechanisms (e.g., endogenous hyaluronidases and reactive oxygen species-mediated cleavage) serve as TLR agonists (17). Thus, while HysA seems to improve *S. aureus* motility through HA-rich connective tissue, this behavior may appear tactically neutral as it would come at the cost of alerting host immune surveillance via DAMP production. It should be noted, however, that the preponderance of evidence demonstrating the immunogenic effects of low-molecular-mass HA fragments have studied HA fragments generated by mammalian hyaluronidases or catabolically equivalent microbial enzymes (i.e., *Streptomyces* HysA) that yield polymeric products (tetrasaccharides and oligosaccharides) of a similar size range (4- to 16-mers) (17, 58–61). In contrast, HysA from *S. aureus* and other Gram-positive pathogens (e.g., group A streptococci and *S. agalactiae*) possess greater enzymatic processivity and break down HA into disaccharide units (62). Inactivation of hyaluronidase in *S. agalactiae* elicits enhanced cytokine responses from both macrophage cultures and splenic homogenates following infectious challenge (54), suggesting that such catabolic end products may actually promote immune evasion. Given that within the context of *S. aureus* infection, polymeric HA is subject to both host- and pathogen-mediated degradation, further investigation will be needed to precisely define the immunoregulatory properties of HysA-derived disaccharides and their significance to the infective process.

Collectively, the results from this study demonstrate that HA makes an underappreciated contribution to *S. aureus* biofilm infections. As the bacterial biofilm field advances, it will be important to consider the impact host factors such as glycosaminoglycans have on biofilm development in order to appropriately translate these findings to understanding infection progression and developing treatment approaches. HA and heparin are two examples of relevant glycosaminoglycans that should be considered in assay development going forward, as both now have been shown to impact *S. aureus* biofilm formation. Considering that many Gram-positive pathogens secrete hyaluronidases, the broad significance of this enzyme's contribution to modulation of biofilm infections, dissemination in the host, and immune evasion is only beginning to be appreciated.

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