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Staphylococcus aureus Hyaluronidase Is a CodY-Regulated Virulence Factor

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Staphylococcus aureus is a Gram-positive pathogen that causes a diverse range of bacterial infections. Invasive *S. aureus* strains secrete an extensive arsenal of hemolysins, immunomodulators, and exoenzymes to cause disease. Our studies have focused on the secreted enzyme hyaluronidase (HysA), which cleaves the hyaluronic acid polymer at the β -1,4 glycosidic bond. In the study described in this report, we have investigated the regulation and contribution of this enzyme to *S. aureus* pathogenesis. Using the Nebraska Transposon Mutant Library (NTML), we identified eight insertions that modulate extracellular levels of HysA activity. Insertions in the *sigB* operon, as well as in genes encoding the global regulators SarA and CodY, significantly increased HysA protein levels and activity. By altering the availability of branched-chain amino acids, we further demonstrated CodY-dependent repression of HysA activity. Additionally, through mutation of the CodY binding box upstream of *hysA*, the repression of HysA production was lost, suggesting that CodY is a direct repressor of *hysA* expression. To determine whether HysA is a virulence factor, a Δ *hysA* mutant of a community-associated methicillin-resistant *S. aureus* (CA-MRSA) USA300 strain was constructed and found to be attenuated in a neutropenic, murine model of pulmonary infection. Mice infected with this mutant strain exhibited a 4-log-unit reduction in bacterial burden in their lungs, as well as reduced lung pathology and increased levels of pulmonary hyaluronic acid, compared to mice infected with the wild-type, parent strain. Taken together, these results indicate that *S. aureus* hyaluronidase is a CodY-regulated virulence factor.

Staphylococcus aureus is a leading cause of both hospital and community-associated infections in the developed world, accounting for \$14.5 billion in health care costs annually in the United States alone (1). These infections range from mild skin and soft tissue infections to potentially fatal infections, such as pneumonia, endocarditis, osteomyelitis, sepsis, and toxic shock syndrome (2). Additionally, the increasing prevalence of methicillin-resistant *S. aureus* (MRSA) strains in both the hospital and community has exacerbated the problem (3–5). *S. aureus* is a successful pathogen due to its extensive arsenal of virulence factors that consist of both surface-associated proteins, such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and secreted proteins, including hemolysins, immunomodulators, and a number of exoenzymes (2, 3). Many emerging MRSA isolates are known for causing tissue-destructive infections (5, 6), and it has been proposed that the excessive damage is due, in part, to the large quantities of toxins and secreted enzymes produced by these new isolates (7).

Our studies have focused on the exoenzyme hyaluronidase (also called hyaluronate lyase), encoded by the gene *hysA* (8). Hyaluronidases are bacterial enzymes that cleave the β -1,4 glycosidic bond of hyaluronic acid (HA), a high-molecular-weight polymer composed of repeating disaccharide units of *N*-acetylglucosamine and D-glucuronic acid (9, 10). Hyaluronic acid is synthesized and secreted from the plasma membrane of mammalian cells, and it is abundant in the skin, skeletal tissue, umbilical cord, lungs, heart valves, brain, and a number of other tissues (9–11). Hyaluronic acid also performs a multitude of functions for the host, including providing structure to tissues and water homeostasis, assisting with cell proliferation, and acting as an immune regulator (10, 11). Many of these tissues with high HA concentrations are frequently infected with *S. aureus* (2).

Cleavage of HA by bacterial hyaluronidases occurs by β elimination of the β -1,4 glycosidic bond and results in unsaturated disaccharides as the product of complete digestion (9). In contrast, the eukaryotic hyaluronidases hydrolyze the β -1,4 glycosidic bond, producing mostly tetra- and hexasaccharides as end products. For a number of Gram-positive organisms, hyaluronidases have been shown to be essential virulence factors because of their ability to disseminate cells and virulence factors through tissue (9). For example, μ -toxin, a hyaluronidase of *Clostridium perfringens*, was found to significantly increase the ability of α -toxin to penetrate tissue (12). Similarly, a mutant with a mutation in the hyaluronidase of *Streptococcus pyogenes* showed reduced tissue penetration compared to the wild type in a murine skin abscess model (13). Additionally, there have been a number of structural studies on the hyaluronidases from *Streptococcus pneumoniae* and *Streptococcus agalactiae* (14–16), and the *S. aureus* enzyme is reported to have a structure similar to the structures of these streptococcal enzymes (9).

Reports of *S. aureus* hyaluronidase date back to pioneering 1933 studies performed by Duran-Reynals (17), who identified a spreading factor found in the spent medium of invasive *S. aureus* strains. This spreading factor increased the lesion size in a rabbit

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skin infection model, as measured by the spread of India ink (17), and it could increase the size of lesions caused by other bacterial pathogens. Subsequent reports concluded that the enzyme hyaluronidase was responsible for the spreading factor activity (18–21), but Hobby et al. suggested that other agents may contribute to the phenomenon (22). A number of follow-up studies investigated the enzymatic properties and expression of *S. aureus* hyaluronidase (23–25), but in the intervening years, progress stagnated until the *hysA* gene was identified and sequenced (26). More recently, the HysA enzyme was detected in proteomic studies (27) and shown to be required for increased wound size in a murine skin infection model (28). Little is known about *hysA* regulation, although SarA is thought to repress *hysA* gene expression, and there is some evidence that the *agr* quorum-sensing system induces expression (28, 29).

To expand our knowledge of HysA in *S. aureus*, we proceeded to investigate the regulation and the contribution of this enzyme to pathogenicity. We identified genes involved in the regulation of *hysA* utilizing a transposon mutant library, and the most prominent of these was the global regulator CodY. We characterized the role of HysA in the pathogenesis of *S. aureus* using a murine pulmonary infection model, and we present evidence that HysA promotes virulence through the breakdown of hyaluronic acid during infection.

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 (ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; erythromycin, 10 µg/ml; tetracycline, 0.625 µg/ml) at 37°C with shaking at 220 rpm with a flask-to-volume ratio of approximately 5:1, 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 (30). Plasmids and transposon insertions were moved from *S. aureus* RN4220 or *S. aureus* JE2 with bacteriophage Φ11 to other *S. aureus* strains by transduction. All of the oligonucleotides used for this study can be found in Table 2.

The *ΔhysA* mutant was constructed using the pJB38-*hysA* modified pKOR1 system as described previously (31). Briefly, 600-bp flanking regions upstream and downstream of *hysA* were amplified and joined by overlap PCR extension using genomic DNA from strain AH1263 as the template and oligonucleotides CBR12 and CBR13 to generate the upstream fragment and oligonucleotides CBR14 and CBR15 to generate the downstream fragment. The outermost primers (CBR12 and CBR15) were engineered to include SbfI and XmaI sites at their 5' ends, respectively, and the inner primers (CBR13 and CBR14) were similarly engineered with XhoI and MluI sites at their 5' ends to aid with the overlap extension. PCR products were ligated with T4 DNA ligase (Invitrogen) into pJB38 at the SbfI and XmaI sites and electroporated into *E. coli* strain BW25141. Plasmid DNA was purified from this strain, isolated using a high-speed plasmid minikit (IBI Scientific), and electroporated into RN4220. The plasmid construct was isolated from *S. aureus* RN4220 and transduced into AH1263 using bacteriophage Φ11. The *hysA* mutation was constructed on the chromosome as previously outlined in the pJB38 method (31). The final colonies were screened for plasmid loss by determination of antibiotic susceptibility, and loss of the plasmid was confirmed by PCR.

The complementing plasmid pHysA (pCR01) was created by amplifying the *hysA* promoter region (~600 bp upstream of the putative translational start site) through the stop codon with the flanking primers CBR35 and CBR29. Amplified DNA containing the *hysA* coding sequence

TABLE 1 Strains and plasmids

Strain or plasmid	Description	Reference(s) or source
Strains		
<i>E. coli</i>		
BW25141	Cloning strain	58
ER2566	Protein expression strain	New England Biolabs
DH5α	Cloning/protein expression strain	Gibco
AH2856	HysA protein expression strain, ER2566 containing pCR03	This work
<i>S. aureus</i>		
RN4220	Restriction-deficient strain 8325-4	59
AH1263	USA300 CA-MRSA Erm ^s (LAC)	39
AH2525	AH1263 <i>ΔhysA</i>	This work
AH2839	AH2525 containing pHysA	This work
JE2	CA-MRSA USA300 Erm ^s , plasmid-cured LAC derivative	40
AH2914	JE2 <i>hysA</i> ::ΦNΣ Erm ^r	40
AH2936	JE2 <i>sarA</i> ::ΦNΣ Erm ^r	40
AH2937	JE2 <i>sigB</i> ::ΦNΣ Erm ^r	40
AH2938	JE2 <i>rsbW</i> ::ΦNΣ Erm ^r	40
AH2939	JE2 <i>rsbU</i> ::ΦNΣ Erm ^r	40
AH2942	JE2 SAUSA300_0543::ΦNΣ Erm ^r	40
AH2945	JE2 <i>hslU</i> ::ΦNΣ Erm ^r	40
AH2946	JE2 <i>codY</i> ::ΦNΣ Erm ^r	40
AH3052	AH1263 <i>Δspa</i>	This work
AH3134	AH1263 <i>Δspa codY</i> ::ΦNΣ Erm ^r	This work
AH3207	AH1263 <i>Δspa codY</i> ::ΦNΣ Tet ^r <i>hysA</i> ::ΦNΣ Erm ^r	This work
AH1292	AH1263 <i>Δagr::tetM</i>	60
AH2032	AH1263 <i>Δagr::tetM ΔsigB</i>	60, 61
AH1483	AH1263 <i>ΔsigB</i>	62
AH3453	AH1263 <i>Δagr::tetM codY</i> ::ΦNΣ Erm ^r	This work
AH3172	AH1263 <i>ΔhysA codY</i> ::ΦNΣ Erm ^r	This work
Plasmids		
pET28a	<i>E. coli</i> protein expression vector	Novagen
pJB38	<i>S. aureus</i> gene knockout vector, Cam ^r	31
pTet	Antibiotic cassette replacement vector, Cam ^r Amp ^r	32
pSKerm-MCS	<i>E. coli</i> - <i>S. aureus</i> shuttle vector, Erm ^r Cam ^r	63
pALC2073	<i>E. coli</i> - <i>S. aureus</i> shuttle vector, Cam ^r	64
pALC2073-RNAIII	Tetracycline-inducible RNAIII expression vector, Cam ^r	60
pCR02	pJB38 <i>hysA</i> knockout vector, Cam ^r	This work
pHysA (pCR01)	<i>hysA</i> complementation plasmid, Cam ^r Erm ^r	This work
pCR03	<i>hysA</i> 6× His-tagged <i>E. coli</i> protein expression vector, Kan ^r	This work
pCR04	pCR01 mutated CodY box, Cam ^r Erm ^r	This work
pCR05	pCR01 mutated CodY box, Cam ^r Erm ^r	This work
pCR06	pCR01 mutated CodY box, Cam ^r Erm ^r	This work

and the promoter region was restriction digested with BamHI-HF and NheI-HF (New England BioLabs) and ligated into linearized pSKerm-MCS plasmid DNA at the BamHI and NheI sites. Ligated DNA was electroporated into *E. coli* strain BW25141 using standard molecular techniques.

To create the HysA overexpression construct pCR03, the *hysA* gene without the encoded signal sequence was amplified from AH1263 genomic DNA using primers CBR41 and CBR42, which contain NheI and

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence
CBR12	GTTGTTCCCTGCAGGGATAAGCGATTCAATACTGACG
CBR13	GTTGTTCTCGAGTGTGTTGTTACGCGTCATGTCTCCTTTTTGTGTTGCG
CBR14	GACGCGTAACAACAACACTCGAGGCAAGAAAATCTCGACACTATAC
CBR15	GTTGTTCCCGGGCAGTGCCGACAACAGATTGC
CBR29	GTTGTTGCTAGCGTATAGTGTGAGATTCTTCTGTC
CBR35	GTTGTTGGATCCCGATTCAATACTGACGCGCTG
CBR41	GTTGTTGCTAGCGATACGAATGTTCAAACGCCAG
CBR42	GTTGTTCTCGAGGTGTCGAGATTCTTCTGCATT
CBR82	AGATTGATAATATTTCCCTCCCTGAAAATTATTTTTAG
CBR83	CTAAAATAATTTTTCAGGGAGGGAAAATTATATCAATCT
CBR84	CTAAGATTGATAATATTTTCATCCCCGAAAATTATTTTTAGT
CBR85	ACTAAAATAATTTTTCGGGGGATGAAATAATATCAATCTTAG
CBR86	AATAATTAATTTTTTCCACCCCATTTTAGTTGCAATT
CBR87	AATTGCAACTAAAATGGGGTGGAAAAAATTAATATTT
spa_delA_EcoRI	ATGGAAATTCCAATCCACCATAAAATACCCTCAA
spa_delB	ACGGTGGTACCCTGAGCCTTTTTCAAATTAATACCCCTGTATG
spa_delC	GCTAGCGGTACCACGCGTCCGAAATATAAAAAACAAACAATACAC
spa_delD_SalI	GATGTCGACGCTAAAGCGGGGCAATTTTC
spa_upstream	ATAGCGTGATTTGCGGTTT
spa_downstream	GCAACAAAAGATGTTGCTCGT

XhoI sites at their 5' ends, respectively. The resulting PCR product was ligated into pET28a linearized with NheI and XhoI and electroporated into *E. coli* strain ER2566 to generate strain AH2856.

To mutate the CodY binding box upstream of *hysA*, plasmids pCR04, pCR05, and pCR06 were constructed using overlap PCR to engineer the six nucleotide changes. A 5' region introducing the mutations into the CodY box upstream of *hysA* was amplified using CBR35 and either CBR83, CBR85, or CBR87. Using homologous primers to introduce the same mutations and amplify the remainder of the *hysA* promoter region and *hysA*, a corresponding 3' region was generated using CBR29 and either CBR82, CBR84, or CBR86. The 5' and 3' fragments were mixed at a ratio of 1:1 in combinations, as follows: CBR35-CBR83-CBR82-CBR29, CBR35-CBR85-CBR84-CBR29, and CBR35-CBR87-CBR86-CBR29. Overlap PCR was done using the mixed fragments as a template and CBR29 and CBR35 to amplify *hysA* and the promoter region containing the mutated CodY boxes. pSKermMCS and the PCR product inserts were restriction digested with BamHI-HF and NheI-HF and ligated using T4 DNA ligase. The ligations were transformed into *E. coli* strain DH5 α as previously described. The resulting plasmids were confirmed by sequencing.

The *spa* mutant was generated using the same protocol for deleting *hysA* described above. Flanking regions of *spa* (*SAUSA300_0113*) were amplified with primer pair spa_delA_EcoRI and spa_delB and primer pair spa_delC and spa_delD_SalI. The products were fused using overlap extension PCR with primers spa_delA_EcoRI and spa_delD_SalI. The resulting fragment was digested with EcoRI and SalI and ligated into pJB38. The *spa* deletion was confirmed by PCR with primers spa_upstream and spa_downstream. The construct with the *spa* and *codY* double mutation was generated by transducing the *codY:: Φ N Σ* cassette into *S. aureus* strain LAC Δ *spa* with bacteriophage Φ 11. To generate strain AH3207, the pTet marker switching plasmid was used to replace the erythromycin resistance cassette of the *codY:: Φ N Σ* transposon insertion in *S. aureus* strain AH2946 with a tetracycline resistance cassette, as described by Bose et al. (32). This construct was then transduced by bacteriophage Φ 11 into the *spa* deletion strain that also harbored the *hysA:: Φ N Σ* transposon insertion.

Protein purification. AH2856 was 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. The culture was grown at 30°C to 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 once with water, pelleted by centrifugation, and frozen at -20°C. Cells were mechanically lysed using a Microfluidics microfluidizer (model LV1; Newton, MA) at 25,000 lb/in² and running the samples through the machine twice. Cell lysate was clarified by centrifugation for 20 min at 15,000 \times g and 4°C. Protein was purified using Fractogel His-Bind resin (Novagen) per the manufacturer's instructions, dialyzed into phosphate-buffered saline (PBS), concentrated using a Millipore centrifugal filter unit (30,000-molecular-weight cutoff), and brought to 10 mg/ml in 1 \times PBS. The protein suspension was frozen in 10% glycerol at -20°C until used.

Transposon library screen. The Nebraska Transposon Mutant Library (NTML) was grown overnight in TSB in 96-well microtiter plates. Cultures were stamped onto hyaluronic acid (HA) plates (TSB, 1% agarose, 0.4 mg/ml HA, 1% [wt/vol] bovine serum albumin [BSA]), prepared as described previously (8), and incubated at 37°C for 4 h. Acetic acid (10%) was added to the plates to visualize zones of clearing. Plates were scored for their hyaluronidase activity, and mutations that increased or decreased activity were rescreened in the same manner. Mutations that affected activity in both screens were quantitatively assessed for hyaluronidase activity by growing each strain overnight in TSB, subculturing to an optical density at 600 nm (OD₆₀₀) of 1.0, and dispensing the diluted culture as five aliquots (4 μ l) onto HA plates. After 6 h of incubation, the plates were flooded with 10% acetic acid, and zones resulting from HA cleavage were enumerated with a caliper.

Hyaluronidase activity assay. The hyaluronidase activity assay was performed as previously described (28), with some modifications. Briefly, *S. aureus* strains were grown overnight in TSB at 37°C with shaking, subcultured 1:1,000, and grown at 37°C for the time specified below. Spent culture medium was isolated with Spin-X filters (pore size, 0.22 μ m) and frozen at -20°C until assayed. Hyaluronic acid (100 μ l at 1 mg/ml) was mixed with 50 μ l of spent medium and allowed to react at 37°C for 15 min. The reaction was stopped by adding 25 μ l of potassium tetraborate solution (0.8 M, pH 9.1), and the reaction mixture was vortexed and boiled for 3 min. In parallel, 12.5 μ l of spent medium was added to 31.25 μ l of stop solution (0.8 mg/ml hyaluronic acid, 0.8 M potassium tetraborate, pH 9.1) at time zero, vortexed, and boiled for 3 min. The samples were dispensed into a 96-well microtiter plate in quadruplicate, and freshly prepared DMAB solution (10% [wt/vol] *p*-dimethylaminobenzaldehyde, 12.5% [vol/vol] 10 M HCl [Sigma-Aldrich], and 87.5% [vol/vol] glacial

acetic acid [Fisher Scientific]) was added to each well. The plate was incubated at 37°C for 20 min to allow the color reaction to take place. A Tecan Infinite M200 plate reader was used to measure the absorbance at 590 nm. Hyaluronidase specific activity is expressed as $10^3 \times$ the number of μmol *N*-acetylglucosamine (NAG; Sigma-Aldrich) released $\text{ml}^{-1} \text{min}^{-1}$ per OD_{600} unit and is calculated by the equation $10^3 \times 3 \times (1/15) \times (1/m) \times \Delta A_{590} \times (1/\text{OD}_{600})$, where ΔA_{590} represents the difference in absorbance between the readings at 15 min and time zero for each sample, m represents the slope of the standard curve of NAG, OD_{600} is the optical density of the culture when the sample was taken, 3 is the dilution factor of the sample tested in substrate, and 1/15 is the reciprocal of the reaction time allowed at 37°C. The assay was performed in triplicate for each condition tested.

To determine the impact of RNAIII on hyaluronidase activity, strains with pALC2073 or pALC2073-RNAIII were grown overnight in TSB at 37°C and were subcultured 1:1,000 into fresh TSB with anhydrotetracycline (aTc) at various concentrations. Cultures were grown for 8 h, cells were pelleted, and spent medium was filtered and assayed for activity.

Assays with limiting to rich medium. Chemically defined medium (CDM) was made as described by Pohl et al. (33), except that the group 6 vitamin solution was replaced with nicotinamide (500 $\mu\text{g/liter}$), thiamine (500 $\mu\text{g/liter}$), pantothenate (500 $\mu\text{g/liter}$), and biotin (500 $\mu\text{g/liter}$) (final concentrations). Single amino acids were omitted or added in excess, as indicated in the figure legends. The medium was buffered to pH 7.0. *S. aureus* strains were grown overnight in TSB and subcultured 1:1,000 into CDM or CDM lacking isoleucine. Bacteria were grown for 5 h, at which point cells were pelleted, washed once with $1 \times$ PBS, and suspended into fresh CDM. For those cells grown in CDM lacking isoleucine, they were then suspended in fresh CDM containing $5 \times$ branched-chain amino acids (BCAAs; isoleucine, valine, leucine). Cells were grown in the new medium for an additional 2 h. Growth was monitored spectrophotometrically at 600 nm throughout by measuring the optical density of 100 μl of culture in a 96-well microtiter plate and a Tecan infinite M200 plate reader. Additionally, 200 μl of each culture was removed at the designated time points indicated in the figures, and spent medium was isolated using 0.22- μm -pore-size Spin-X centrifuge tube filters (Costar). Spent media were used to determine hyaluronidase activity and the presence or absence of Pantone-Valentine leukocidin (PVL) (see below).

Protein immunoblot for PVL. Spent culture medium isolated from cultures of cells grown in CDM, CDM lacking isoleucine, or CDM with $5 \times$ BCAAs as described above (5 μl for 4 h or 1 μl for the other time points) was brought to a total volume of 10 μl in SDS loading buffer containing 1% β -mercaptoethanol. Proteins were resolved by PAGE using Tricine-SDS gels containing 12% polyacrylamide and 170 kV. Proteins were transferred (160 mA for 1 h) onto nitrocellulose membranes and blocked overnight at 4°C in 0.1% Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% skim milk. The membrane was probed for PVL using Luk-S polyclonal antibody (IBT Bioservices, Gaithersburg, MD) at 1:10,000 in TBST containing 5% skim milk for 2 h at room temperature and then washed three times for 10 min each time with TBST. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied at 1:20,000 in blocking buffer at room temperature for 1 h. Membranes were washed three times for 10 min each time with 0.1% TBST, and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was added to detect proteins, followed by exposure to Classic X-ray film (Research Products International Corporation). Bands were quantified using ImageJ software.

Protein immunoblot for HysA. *S. aureus* strains AH3052, AH3134, and AH3207 were grown overnight in TSB at 37°C with shaking at 220 rpm. The strains were subcultured at a ratio of 1:100 into fresh TSB and grown for 8 h at 37°C with shaking at 220 rpm. Cultures were normalized to an OD_{600} of 3.0 with TSB, spent media were isolated by filtration using a 0.22- μm -pore-size Millex-GS syringe filter (Millipore), concentrated using Amicon Ultra 10,000-molecular-weight-cutoff centrifugal filters

(Millipore), and frozen at -20°C . The 10- μl samples were brought to a total volume of 15 μl in SDS loading buffer containing 1% β -mercaptoethanol, and proteins were resolved by PAGE using Tricine-SDS gels containing 9% polyacrylamide and 170 kV. Proteins were transferred (160 mA for 1 h) to nitrocellulose membranes and blocked overnight at 4°C in blocking buffer (0.1% TBST containing 5% skim milk). A rabbit polyclonal antibody to HysA was generated by Pacific Immunology, and the antibody was purified as described above. HysA was probed using a dilution of 1:40,000 with the anti-HysA rabbit serum for 1 h at room temperature in blocking buffer. The membrane was then washed three times for 10 min each time in 0.1% TBST. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG was applied at a 1:20,000 dilution in blocking buffer at room temperature for 1 h. Membranes were washed three times for 10 min each time with 0.1% TBST, and SuperSignal West Pico chemiluminescent substrate was added to detect proteins, followed by exposure to Classic X-ray film.

Neutropenic murine pneumonia model. Female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). All animals were housed under specific-pathogen-free conditions in filter-top cages at the Walter Reed Army Institute of Research (WRAIR) Animal Facility and provided sterile food and water *ad libitum*. Six- to 8-week-old mice were injected intraperitoneally with 150 mg/kg of body weight of cyclophosphamide (Baxter) 4 days prior to infection and 100 mg/kg of cyclophosphamide 1 day prior to infection, as previously described (34). Mice were anesthetized with isoflurane and infected intranasally with 1.0×10^7 CFU of *S. aureus* suspended in 25 μl of PBS. At 48 h postinfection, the mice were euthanized and lungs were extracted, homogenized, and serially diluted before suspensions of lung tissue were plated onto TSA. The CFU were enumerated to assess the bacterial burden. For survival studies, infected mice were monitored twice a day for signs of morbidity. Following sacrifice, paraffin-embedded lung tissue samples from infected and uninfected mice were sectioned and stained with hematoxylin-eosin (H&E). All procedures were performed in accordance with a protocol approved by the WRAIR Institutional Animal Care and Use Committee (protocol number IB02-10). 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 adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (35).

Immunohistochemistry. The lungs of PBS-treated and infected mice were harvested at 48 h postinfection, fixed in 4.0% paraformaldehyde, and cut into 5- μm sections. As a control, uninfected lung tissue samples were pretreated with 100 turbidity reducing units (TRU) of *Streptomyces hyalurolyticus* hyaluronidase (Sigma) for 2 h at 60°C (36). Hyaluronic acid was labeled as previously described (37). Briefly, samples were incubated in 0.2% BSA for 2 h at 4°C to block nonspecific binding. Following incubation, samples were treated with 2 $\mu\text{g/ml}$ of biotinylated hyaluronic acid binding protein (EMD Biosciences) for 16 h at 4°C. Fluorescein isothiocyanate-conjugated streptavidin (Invitrogen) was used to visualize hyaluronic acid. The presence of hyaluronic acid in lung sections was quantified by measuring the mean fluorescent intensity of 40 fields per section. Images were acquired with an Olympus AX80 microscope and analyzed with Image Pro (version 7.0) software.

Statistical analysis. The statistical significance of all data was analyzed using the Student *t* test with GraphPad Prism (version 6) software (GraphPad, La Jolla, CA), except for data from survival studies. Data from the survival studies were analyzed by the Mantel-Cox test ($P = 0.0022$).

RESULTS

The gene encoding hyaluronidase (*hysA*) is conserved across all *S. aureus* clonal lineages (8), and only one hyaluronidase is carried by the genome of a community-associated MRSA (CA-MRSA) USA300 strain (38). The *hysA* gene (*SAUSA300_2161*) is located near a number of genes of unknown function and is transcribed by itself in the opposite direction of neighboring genes (Fig. 1A). To

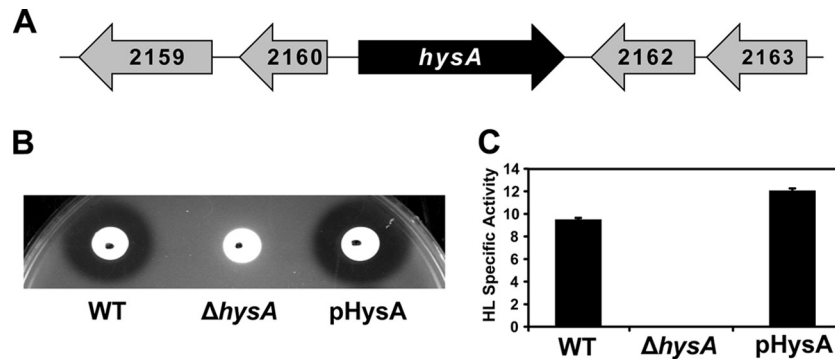


FIG 1 Generation and initial characterization of the CA-MRSA USA300 $\Delta hysA$ mutant and complemented strains. (A) Location of *hysA* on the CA-MRSA USA300 LAC chromosome. Numbers indicate *SAUSA300* locus numbers. The *hysA* gene is surrounded by genes with unknown function and is transcribed by itself on the chromosome. (B and C) HA plate and hyaluronidase (HL) specific activity assays of LAC-WT, LAC $\Delta hysA$, and complemented (pHysA) strains. As shown, HysA activity is abolished for the $\Delta hysA$ mutant and restored to WT levels when it is complemented in both assays.

begin our studies on *hysA*, we created a markerless deletion construct in strain AH1263 (39), an erythromycin-sensitive version of the USA300 isolate LAC (here called LAC wild type [LAC-WT]). The $\Delta hysA$ knockout mutant was confirmed by PCR and a lack of enzymatic cleavage of HA by an agar plate assay (Fig. 1B). We also verified the absence of hyaluronidase activity in the knockout mutant using a quantitative, spectrophotometric assay (Fig. 1C). The HysA activity was complemented to LAC-WT levels by providing *hysA* on a plasmid (Fig. 1), demonstrating that the phenotype was due to the mutant construction and not secondary or polar effects.

The regulation of *hysA* has received only limited attention, although there are reports for the potential involvement of global regulators *sarA* and *agr* (28, 29). To identify genetic loci that contribute to *hysA* regulation, we screened 1,952 mutants from the Nebraska Transposon Mutant Library (NTML) (40) for hyaluronidase activity using an HA plate assay. Screening of the NTML resulted in the identification of eight *bursa aurealis* transposon insertions that significantly altered hyaluronidase activity. To determine the relative extent to which each mutation affected activity, five replicates of each mutant were spotted on HA plates and the zone size was enumerated with a caliper (Fig. 2A). NTML WT strain JE2 had an average zone size of 1.01 ± 0.02 cm, while a mutation in *hysA* resulted in no detectable zone of clearing. Multiple strains with insertions in the sigma factor B operon (*sigB*, *rsbU*, and *rsbW*) and strains with insertions in *sarA*, *hslU*, and *codY* had elevated hyaluronidase activity. Interestingly, insertion into the putative deaminase, *SAUSA300_0543*, gave a reduced zone of clearing of HA (0.76 ± 0.03 cm) and was the only mutation in the library besides *hysA* to cause the strain to have a zone size smaller than the WT zone size.

To quantitatively measure the effect of the identified transposon insertions on hyaluronidase activity, we performed a spectrophotometric specific activity assay on spent medium of 8-h cultures (Fig. 2B). In support of the results of the HA plate assay, the *codY* transposon mutant was found to have activity that was increased 12-fold compared to that of the WT. Additionally, multiple insertions in the *sigB* operon, as well as one in *hslU*, increased the activity approximately 3-fold, and an insertion in *sarA* resulted in a 10-fold increase. The mutant with the transposon insertion in *SAUSA300_0543* had one-third of the activity of the WT (Fig. 2B), similar to the reduction in activity observed in the HA plate assay.

As expected, no activity by the *hysA* transposon mutant was observed in this assay.

Despite previous reports of *agr* quorum-sensing involvement in *hysA* regulation (28), we did not find any insertions in the *agr* locus that significantly altered hyaluronidase activity in our screen. We hypothesized that this was perhaps due to the fact that *S. aureus* strain 8325-4, which has a known mutation in *rsbU* that results in a defect in SigB activity, was used in the previous study. As shown in Fig. 2, we found multiple mutations in the *sigB* operon, including a mutation in *rsbU*, that increase hyaluronidase activity. Thus, a role for *agr* could be masked in the USA300 background that has an intact *rsbU* gene. To investigate this question, we shifted our studies to the USA300 LAC (LAC-WT) background, and we quantified HysA activity in LAC-WT as well as the *agr* and *sigB* single mutant and *agr sigB* double mutant strains. We found that HysA activity was unchanged in an *agr* mutant and increased significantly in a *sigB* mutant. Comparing the *sigB agr* double mutant to the *sigB* mutant (Fig. 3A), there was a significant decrease in activity when *agr* was deactivated. This observation is in agreement with published reports (28), and we also made similar observations in the USA400 MW2 background (data not shown), indicating that this effect occurs across multiple *S. aureus* strain types.

CodY is reported to regulate the *agr* system (41, 42), and we hypothesized that some *hysA* regulation might be occurring indirectly through the known CodY-*agr* interactions. To address this question, we initially confirmed that the effect of CodY was consistent in the LAC background by crossing the transposon insertion into LAC-WT, and we observed that HysA activity increased 16-fold in the *codY* mutant over that in LAC-WT (data not shown). Next, we quantified HysA activity in LAC-WT, as well as in the *agr* and *codY* single mutant and *agr codY* double mutant strains (Fig. 3B). As anticipated, HysA activity was unchanged in an *agr* mutant and increased in a *codY* mutant. However, HysA activity was significantly decreased in the *agr codY* double mutant compared to that in the *codY* mutant (Fig. 3B). We speculated that the unnaturally high levels of RNAPIII in the *codY* mutant could be distorting HysA production. To test this question, we transformed USA300 WT with a plasmid that expresses RNAPIII under the control of a tetracycline-inducible promoter. In the absence of the inducer, there was essentially no difference in HysA activity be-

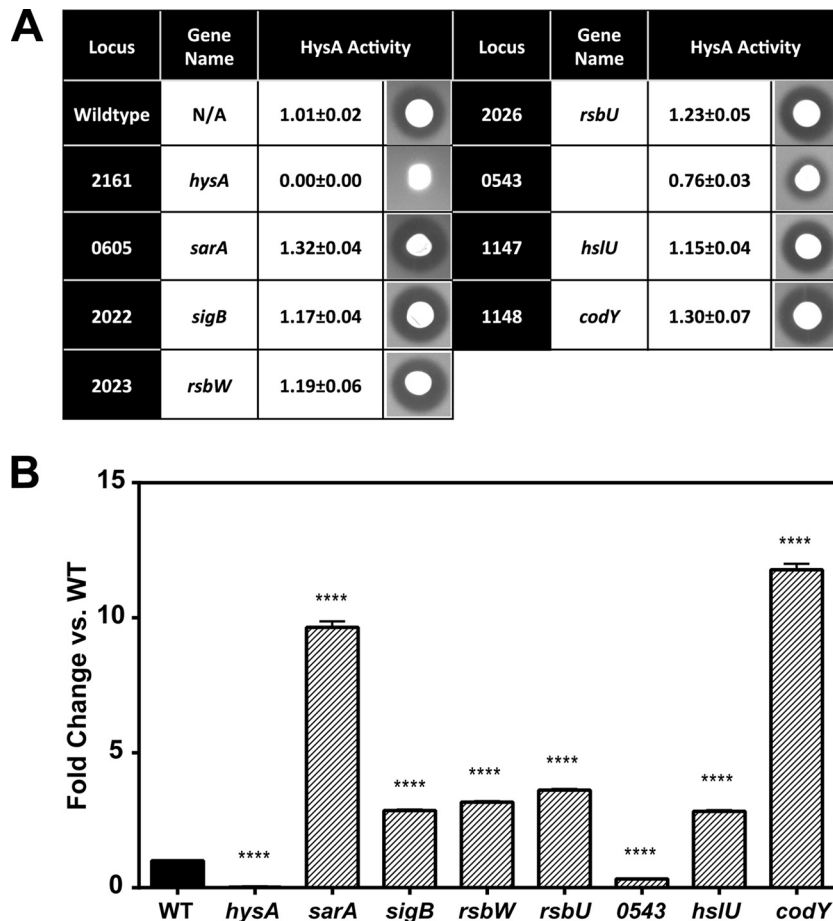


FIG 2 Transposon mutagenesis library screen for altered HysA activity. (A) Summary of results of screen for HysA activity on HA agarose plates. Values of HysA activity are reported as diameters (cm) of zones of clearing, measured with a caliper, and represent the means \pm standard deviations of five technical replicates for each strain plated on HA agarose. Pictures are representative images of each strain. (B) Hyaluronidase specific activity assay of strains containing mutations that significantly altered HysA activity on HA agarose plates. The data are presented as the fold changes compared to the activity of USA300 WT strain JE2. Values represent the means and standard deviations of four technical replicates for three independent biological determinations. Statistical significance (****, $P < 0.0001$) was determined by Student's t test.

tween this strain and an empty vector control (Fig. 3C). However, HysA activity increased with the induction of RNAIII expression compared to the activity of the control (Fig. 3C). Collectively, these data lead us to suggest the model depicted in Fig. 3D for CodY and *agr*-mediated regulation of HysA.

In considering the regulatory model (Fig. 3D), it still remained possible that CodY directly regulated *hysA* gene expression. The CodY regulon has been investigated previously (41–43), and *hysA* was not identified as a target in any of these studies. We inspected the sequence upstream of *hysA* for the presence of the previously published CodY consensus binding box (41), and we found a well-conserved CodY binding box 202 bp upstream of the putative translational start site (Fig. 4A), with one nucleic acid change from a cytosine to a thymine being found in the region between the inverted repeats. The presence of this box led us to predict that CodY may interact with the *hysA* putative promoter region. To confirm that we were not being misled by the results of the hyaluronidase activity assays, we tested to see if the HysA protein levels increased accordingly in a *codY* mutant. We used an antibody to HysA that was developed and found by immunoblotting that enzyme levels were increased in a *codY* mutant compared to those in

the WT strain (Fig. 4B). To eliminate background binding in the immunoblot, protein A was inactivated in each of these strains. The band corresponding to HysA was not detected in a *codY hysA* double mutant, demonstrating that the antibody was specific and suggesting that the increase in hyaluronidase activity is due to elevated HysA levels. Also of note, HysA was not stable in spent medium, and we frequently observed lower-molecular-weight breakdown products by immunoblotting (Fig. 4B).

To assess whether CodY was acting to directly regulate *hysA*, we constructed plasmids with CodY binding box mutations in the *hysA* promoter region (Fig. 4C). As our testing system, we used the LAC $\Delta hysA$ mutant with plasmid pHysA, which complements hyaluronidase activity to nearly WT levels (Fig. 1C). Using pHysA as a backbone, we introduced five to six nucleotide substitutions from the consensus sequence into the CodY box, and these were based on previous studies in *Bacillus subtilis*, where this number of changes was sufficient to disrupt CodY-based repression (44). These constructs were tested by transforming them into the LAC $\Delta hysA$ mutant or $\Delta hysA codY$ double mutant and measuring HysA activity from plasmid expression. We first introduced A-to-C and T-to-C changes into the first half site of the box in constructs

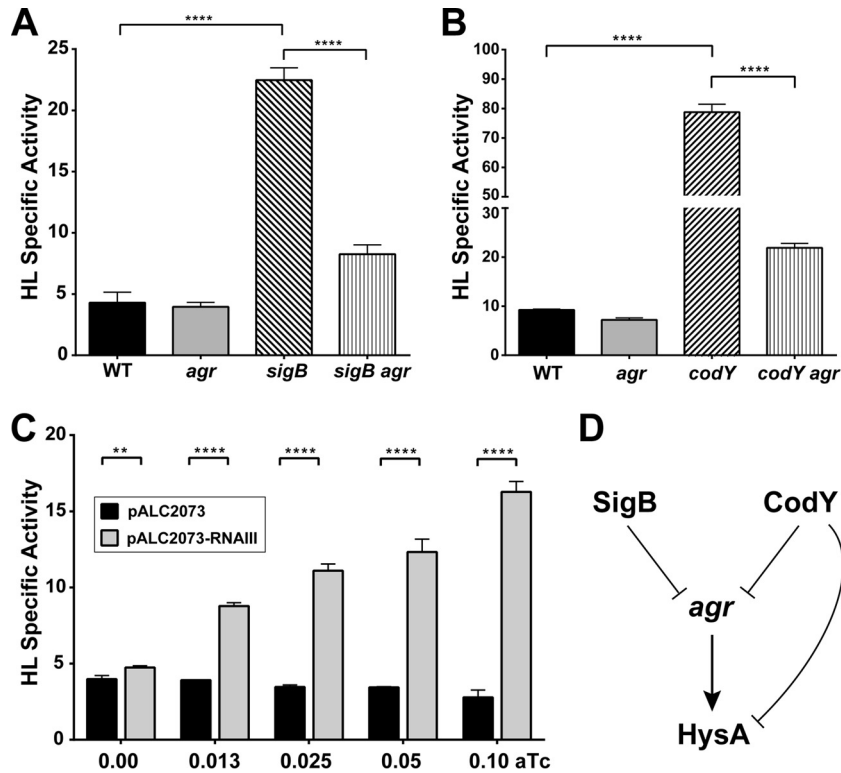


FIG 3 Role of the *agr* system in *hysA* gene regulation. Cultures of various LAC strains were grown for 8 h, spent medium was prepared, and the hyaluronidase specific activity in each sample was measured. (A) To assess the *agr* and SigB interaction, activity was measured in the WT, *agr* and *sigB* single mutant, and *agr sigB* double mutant strains and plotted. (B) To assess the *agr* and CodY interaction, activity was measured in the WT, *agr* and *codY* single mutant, and *codY agr* double mutant strains. For panels A and B, statistical significance (****, $P < 0.0001$) was determined by Student's *t* test. (C) Hyaluronidase specific activity for the WT containing the empty vector (black bars) or RNAlII under the control of a tetracycline-inducible promoter (gray bars). Student's *t* test was used to determine statistical significance (**, $P < 0.01$; ****, $P < 0.0001$). (D) Model for SigB and CodY regulation of HysA through the *agr* system.

pCR04 and pCR05 (Fig. 4C). In testing these constructs, the CodY-based repression of HysA activity was reduced markedly to approximately 2-fold (Fig. 4D). We made additional, independent nucleotide changes in the second box half site in construct pCR06 (Fig. 4C), and CodY-based repression of HysA activity was completely eliminated (Fig. 4D). These results provide strong support for the suggestion that CodY directly regulates *hysA* expression through binding to the box identified in the promoter region.

Finally, we took advantage of the CodY response to the availability of branched-chain amino acids (BCAAs) to test the possibility that CodY represses HysA production in response to environmental changes. CodY gains a high affinity for target promoters when BCAAs are available, leading to repression of its targets. When BCAAs are limiting, such as when cells are in stationary phase or under starvation, CodY loses this affinity, resulting in relieved repression of CodY targets (45, 46). We grew LAC-WT in chemically defined medium (CDM) lacking isoleucine or in CDM with standard levels of BCAAs until the cells began to enter stationary phase (Fig. 5A). If *hysA* is under CodY control, we would anticipate increased HysA activity in the cells grown without isoleucine compared to that in cells grown in CDM with standard levels of BCAAs. In support of this hypothesis, there was a more than 2.5-fold increase in hyaluronidase activity when BCAAs were limiting compared to that when bacteria were grown in CDM at both 4 and 5 h (Fig. 5B).

We also tested the impact of high levels of BCAAs on CodY

repression. The cells grown in medium lacking isoleucine were switched to medium that contained excess BCAAs. If *hysA* is under CodY control, we would anticipate that the excess BCAAs would bind to CodY and repress expression. As predicted, when BCAAs were supplied in excess, HysA production dropped over 5-fold compared to that in cells grown in CDM (Fig. 5B). These activity changes were not due to differences in growth (Fig. 5A). As a control, samples were taken from each time point and immunoblot assays for Pantone-Valentine leukocidin (PVL) (Fig. 5C and D), which is known to be regulated by CodY in USA300 lineages, were performed (47). The trends in PVL production mirrored those of HysA production under all the conditions tested, suggesting that they are regulated in a similar manner.

To determine whether HysA is required for virulence, neutropenic BALB/c mice were infected intranasally with 1×10^7 CFU of either LAC-WT or the $\Delta hysA$ mutant. After 4 days of infection, ~80% of mice infected with LAC-WT succumbed to infection, which is in striking contrast to the death of only ~20% of mice infected with the $\Delta hysA$ mutant (Fig. 6A). At 48 h postinfection, a 4-log-unit reduction in the bacterial burden was observed in the lungs of mice infected with the $\Delta hysA$ mutant compared to the bacterial burden in the lungs of mice infected with LAC-WT (Fig. 6B), suggesting that the virulence defect is likely caused by an inability of the mutant to replicate in the lungs of infected mice.

The hyaluronidase activity of multiple Gram-positive pathogens has been shown to cause severe tissue damage *in vivo* (12, 13,

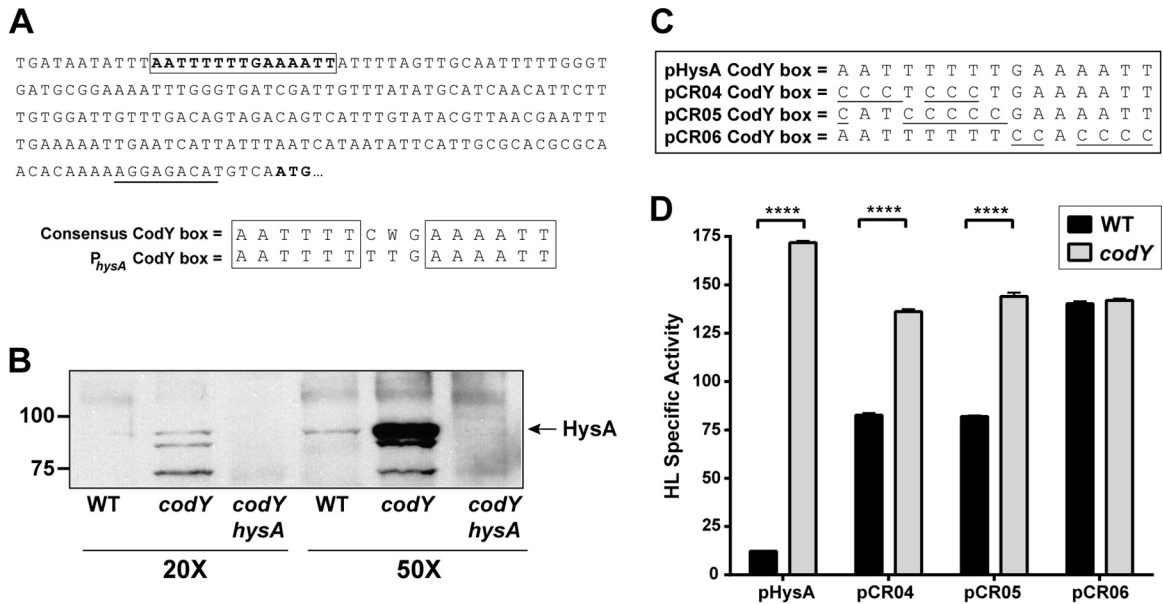


FIG 4 The CodY binding box is required for *hysA* regulation. (A) Location of the putative CodY binding box in the *hysA* promoter region. An alignment of the consensus CodY binding box and the proposed *hysA* box is shown. (B) Immunoblotting for HysA performed with a LAC-WT strain. Spent medium was concentrated 20- or 50-fold from the WT and the *codY* and *codY hysA* mutant strains, as indicated, and immunoblotted for HysA. In each strain, a Δspa deletion was engineered to remove background antibody binding. (C) Mutations generated in the CodY box in plasmids pCR04, pCR05, and pCR06. (D) Hyaluronidase specific activity assay at 8 h for the WT (LAC $\Delta hysA$ with one of the indicated plasmid constructs) or a *codY* mutant (the LAC $\Delta hysA codY$ double mutant with one of the indicated plasmid constructs) containing the pHysA, pCR04, pCR05, or pCR06 construct. Statistical analysis was performed using a Student's *t* test (****, $P < 0.0001$).

28). Therefore, we investigated whether the loss of virulence of the $\Delta hysA$ mutant strain was associated with reduced tissue damage in the lung. Histological analysis showed that the lungs of mice infected with the $\Delta hysA$ mutant exhibited less pathology and influx of alveolar macrophages than the lungs of LAC-WT-infected mice (Fig. 6C). To determine whether hyaluronidase activity contrib-

uted to the virulence of *S. aureus in vivo*, HA was fluorescently labeled and quantified in the lungs of infected mice. There was a significant increase in the amount of HA ($P < 0.0001$) present in the lungs of $\Delta hysA$ mutant-infected mice compared with the amount present in the lungs of LAC-WT-infected mice (Fig. 7A and B). In fact, the level of HA present in the lungs of wild-type-

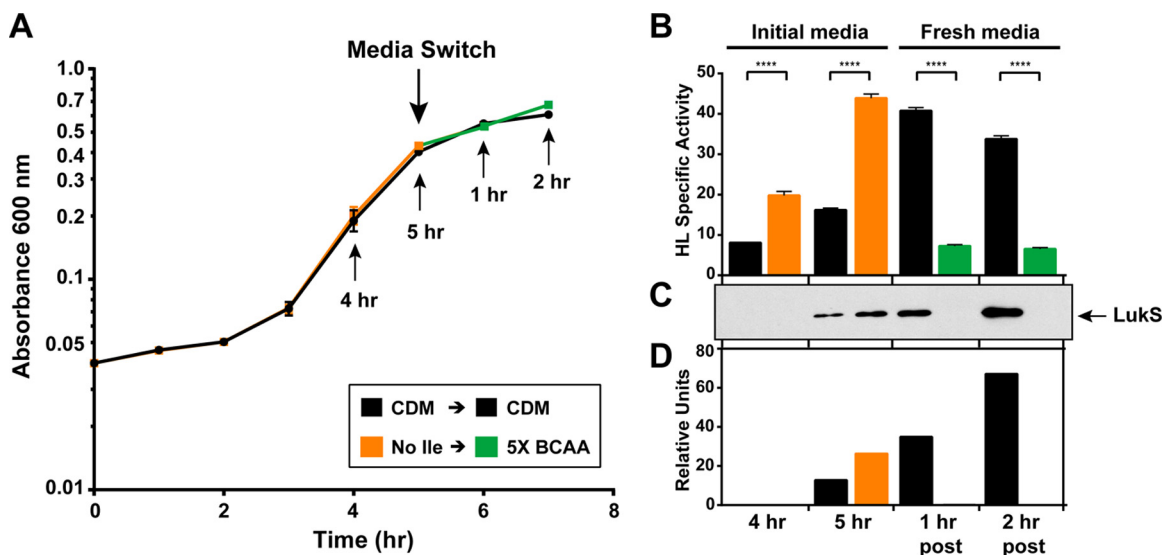


FIG 5 Effect of nutrient availability on *hysA* regulation. (A) Growth curve of LAC-WT grown in CDM or in CDM lacking isoleucine for the first 5 h. Cells were harvested by centrifugation, washed 1 time in PBS, and suspended in either fresh CDM or CDM containing 5 \times BCAAs. Arrows, times when samples were taken or where medium was switched, as indicated. (B) Hyaluronidase specific activity of samples taken at the time points indicated in panel A. Statistical analysis was performed using Student's *t* test (****, $P < 0.0001$). (C) Immunoblot for the LukS subunit of PVL at the time points indicated in panel A. (D) Quantification of LukS immunoblot using ImageJ software. post, samples taken at time points after the medium switch.

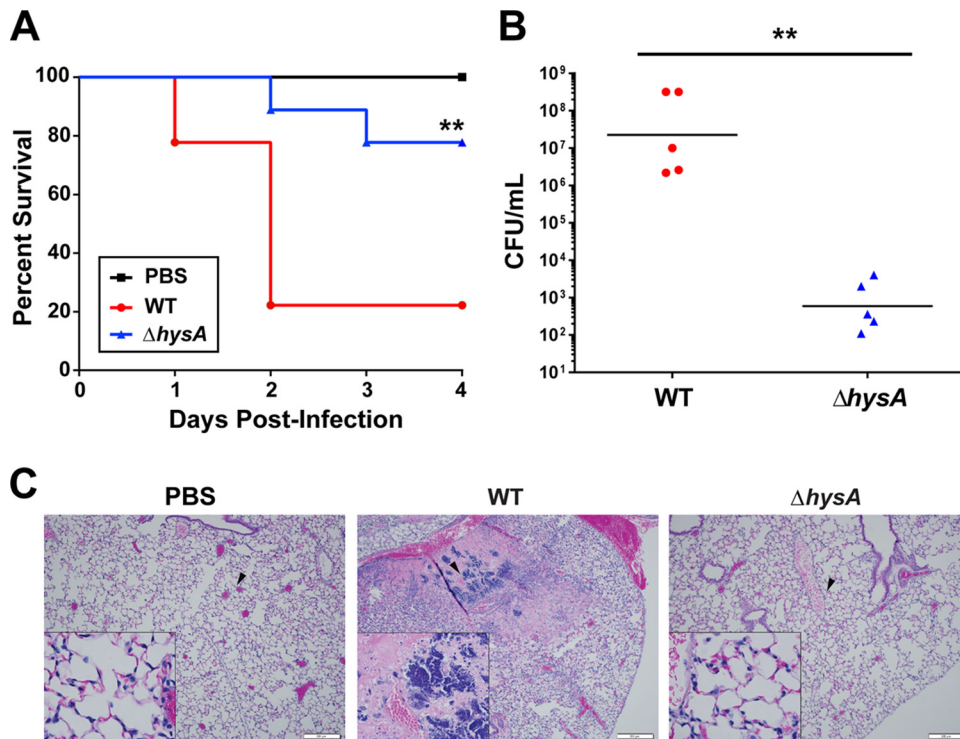


FIG 6 HysA is required for USA300 virulence. Mice were intranasally inoculated with 1.0×10^7 CFU of either LAC-WT, its $\Delta hysA$ mutant strain, or PBS. (A) Mice ($n = 27$) were monitored daily for signs of morbidity and mortality. **, $P = 0.0022$, Mantel-Cox test. (B) Groups of mice ($n = 21$) were euthanized after 48 h, and the bacterial burden in the lungs was quantified by CFU enumeration. **, $P = 0.0095$. (C) Histopathology of the lungs from mice treated with PBS or infected with either LAC-WT or the $\Delta hysA$ mutant. Black arrowheads, areas shown in the enlarged images in the insets. Data are representative of those from three independent experiments.

infected mice was similar to that found in the lungs of mice treated with purified hyaluronidase from *Streptomyces hyalurolyticus* (Fig. 7C). Taken together, these findings show that *hysA* is an important determinant as a cause of *S. aureus* infection.

DISCUSSION

Hyaluronidases are thought to act as spreading factors, allowing dissemination of pathogens throughout mammalian tissue during infection (9). In fact, the earliest studies on spreading factors described them as agents that increase lesion size when combined with various bacterial and viral pathogens (17, 21). In this report, we investigated the regulation and *in vivo* function of HysA during *S. aureus* infection using a CA-MRSA USA300 strain, and we found that HysA is indeed an important factor for causing an infection.

Through our screen of the NTML, we identified eight mutations that significantly altered HysA activity, including a mutation in *sarA*, multiple mutations in the *sigB* operon, and a mutation in an uncharacterized nucleoside deaminase encoded by the *SAUSA300_0543* gene. The deaminase was the only target identified that is required for the full expression of HysA. Surprisingly, we did not identify mutations in the *agr* locus during the screen, although it was reported that *agr* is required for HysA production (28). Considering that the previous studies were done with a strain with a natural mutation in *rsbU*, which is known to mimic a *sigB* mutation and elevate *agr* function (48), we investigated this question. When an *agr* deletion was introduced into a *sigB* mutant, we observed a drop in HysA activity (Fig. 3A), supporting the find-

ings of the previous studies. Taken together, when the *agr* system is hyperactivated, HysA levels increase, and by removing the *agr* locus, these levels drop, which supports the previously identified *agr*-dependent regulation. We speculated that this *agr* role occurred only at unnaturally high levels of RNAIII, and we confirmed this by expressing RNAIII from a plasmid and boosting HysA output. Thus, *agr* has a role in HysA production, but it seems to be important only in situations where RNAIII is elevated beyond normal WT levels.

Interestingly, our screen of the NTML revealed that a mutation in the global regulator CodY resulted in increased HysA activity. CodY responds to intracellular concentrations of branched-chain amino acids in *S. aureus* to control target gene expression (33, 41, 42, 45). We initially speculated that the CodY regulation might be indirect through the *agr* system, and indeed, the introduction of an *agr* deletion reduced the level of HysA production in a *codY* mutant (Fig. 3B). However, we observed that the *hysA* promoter region had a nearly perfect CodY binding box (Fig. 4A), and importantly, nucleotide changes to the conserved positions of the box eliminated CodY-based repression (Fig. 4C and D). Thus, there are indirect and direct mechanisms of CodY-dependent regulation occurring to coordinate HysA production in *S. aureus*.

CodY-dependent regulation suggests that HysA has a potential nutrient-scavenging function. HA is a major component of the viscous substance produced by connective tissues, and it acts as a host defense mechanism by creating a protective barrier that impedes the penetration of bacteria and bacterial products into deeper tissues (49). Sequestration of essential nutrients, such as

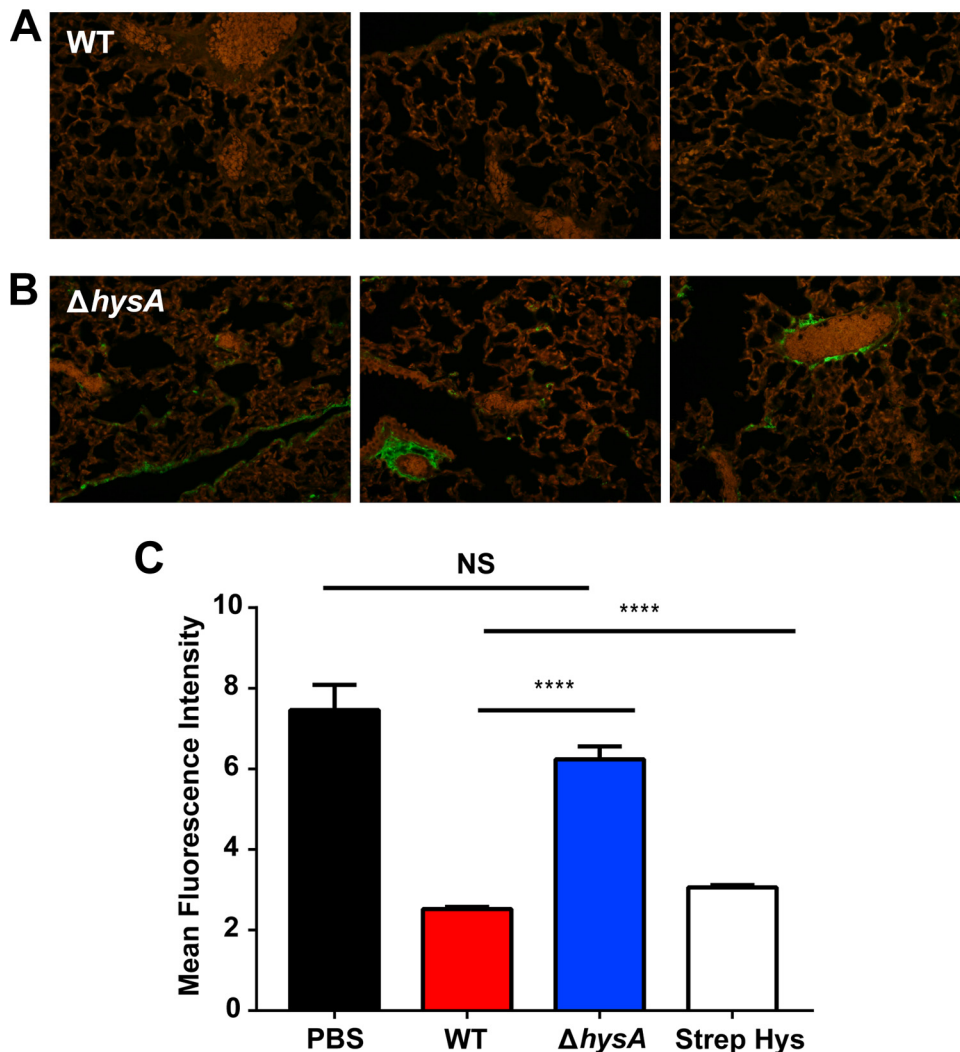


FIG 7 HysA is required for the breakdown of hyaluronic acid *in vivo*. (A and B) Micrographs of immunofluorescently labeled HA in the lungs of mice inoculated with 1.0×10^7 CFU of either strain LAC-WT or its $\Delta hysA$ mutant strain. (C) The mean fluorescence intensity was quantified for each sample (40 fields per section). A sample treated with hyaluronidase from *Streptomyces hyalurolyticus* (Strep Hys) was also included as a control. NS, not significant; ****, $P < 0.0001$.

iron (50) and tryptophan (51), is another mechanism of bacterial infection prevention that works through the generation of nutrient-limited environments. Bacterial pathogens can rapidly adapt to these hostile situations by regulating the expression of genes important for virulence and metabolism (52), and our findings suggest that *S. aureus* uses CodY to regulate HysA in this manner. HA can be broken down by hyaluronidases produced by a variety of bacterial pathogens, such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* (9), and metabolized as a source of carbon (53–55). At this time, it has not been clearly demonstrated that *S. aureus* can use HA as a carbon source *in vivo* or whether HysA is involved in this growth mechanism. Both questions are in need of further examination. However, it is possible that the reduction in bacterial burden in a $\Delta hysA$ mutant during pulmonary (Fig. 6B) and soft tissue (28) infections can be explained by an inability of *S. aureus* to utilize an alternative carbon source under nutrition-limited conditions *in vivo*.

This finding is consistent with the findings of previous work showing that CodY regulates other virulence factors to act as a link

between metabolism and virulence (33). For example, the hemolysins (α and β), as well as the secreted proteases SspA and SspB, have been shown to be regulated directly by CodY. HysA was required for the breakdown of HA *in vivo*, and this correlated with a loss of structural integrity and enhanced necrosis of pulmonary tissues, allowing the pathogen access to deeper tissues. In addition to HysA, *S. aureus* possesses other mechanisms for penetrating the host. One example is a secreted cysteine protease, staphopain A, which is able to induce vascular leakage during a guinea pig infection (56), and this effect can be augmented by the addition of another secreted cysteine protease, staphopain B. Additionally, lipases have been observed to enhance *S. aureus* tissue penetration (57). Taken together these results indicate that HysA is part of a class of *S. aureus* secreted proteins that act in a coordinated fashion as spreading factors by breaking down host tissues to facilitate bacterial dissemination (22). Interestingly, the target for each enzyme differs, despite the functional redundancy of these proteins. Considering the dynamic range of infections caused by *S. aureus*, these findings lead us to speculate that these spreading factors may

be a part of a fine-tuned virulence mechanism utilized by this pathogen to nonspecifically break down host barriers and promote dissemination from any site of infection.

Our studies give new insights into the regulation and role of the *S. aureus* virulence factor HysA. This work identified an important link between metabolism and virulence by illustrating a relationship between the metabolic regulator CodY and the virulence factor HysA. By therapeutic targeting of HysA and other spreading factors, it might be possible to prevent localized infections from evolving into more complicated, systemic disease.

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