Canadian Journal of Microbiology

A Naturally-Occurring Point Mutation in a Hyaluronidase Gene (*hysA1*) of *Staphylococcus aureus* UAMS-1 Results in Reduced Enzymatic Activity

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2021-0110.R1
Manuscript Type:	Article
Date Submitted by the Author:	30-Aug-2021
Complete List of Authors:	Hu, Haijing; Center for Food Safety and Applied Nutrition, Office of Dietary Supplement Programs Liu, Huanli; Food and Drug Administration Office of Regulatory Affairs, Branch of Microbiology, Arkansas Laboratory Kweon, Ohgew; National Center for Toxicological Research, Division of Microbiology Hart, Mark E.; National Center for Toxicological Research, Microbiology
Keyword:	<i>S. aureus</i> UAMS-1, hyaluronidase, <i>hysA1</i> point mutation, homology modeling
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

SCHOLARONE™
Manuscripts

A Naturally-Occurring Point Mutation in a Hyaluronidase Gene (*hysA1*) of *Staphylococcus aureus* UAMS-1 Results in Reduced Enzymatic Activity

Haijing Hu^{1§}, Huanli Liu^{2§}, Ohgew Kweon^{3§} and Mark E. Hart^{3,4*}

Office of Dietary Supplement Programs, Center for Food Safety and Nutrition, U.S. Food and Drug Administration, College Park, MD 20740¹, Branch of Microbiology, Arkansas Laboratory, Office of Regulatory Affairs² and the Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas, 72079³ and the Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205⁴

Running title: Point mutation in *S. aureus* hyaluronidase

§These authors contributed equally to this study.

***Corresponding author:** Mark E. Hart, Division of Microbiology (HFT-250), National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, Arkansas 72079-9502. mark.hart@fda.hhs.gov

ABSTRACT (347 words)

Hyaluronic acid is a high molecular weight polysaccharide that is widely distributed in animal tissues. Bacterial hyaluronidases degrade hyaluronic acid as secreted enzymes and have been shown to contribute to infection. *Staphylococcus aureus* UAMS-1 is a clinical isolate that codes for two hyaluronidases (*hysA1* and *hysA2*). Previous research has shown the presence of a full-length HysA1 protein from the *S. aureus* UAMS-1 strain with no evidence of enzymatic activity. In this study the coding and upstream promoter regions of *hysA1* from the *S. aureus* UAMS-1 strain were cloned, sequenced, and compared to the *hysA1* gene from the *S. aureus* Sanger 252 strain. A single base change resulting in an E480G amino acid change was identified in the *hysA1* gene from the *S. aureus* UAMS-1 strain when compared to the *hysA1* gene from *S. aureus* Sanger 252. A plasmid copy of *hysA1* from *S. aureus* Sanger 252 transduced into a *S. aureus* UAMS-1 *hysA2* deletion mutant strain restored near wild type levels of enzymatic activity. Homology modeling of the HysA1 hyaluronidase was performed with Swiss-model using hyaluronidase from *Streptococcus pneumoniae* as the template, followed by a series of structural analyses using PyMOL, PLIP, PDBsum, and HOPE servers. This glutamic acid is highly conserved among hyaluronidases from *Staphylococcus* and other Gram-positive bacteria. A series of structural analyses suggested that Glu-480 in the HysA1 is critically responsible for maintaining the structural and functional ensemble of the catalytic and tunnel-forming residues, which are essential for enzyme activity. The missense mutation of Glu-480 to Gly introduces a loss of side chain hydrogen bond interactions with key residues Arg-360 and Arg-364, which are responsible for the tunnel topology, resulting in displacement of the substrate from an ideal position for catalysis through a localized conformational change

of the active site. There is a high degree of relatedness among several Gram-positive bacterial hyaluronidases; the loss of enzymatic activity of HysA1 in the *S. aureus* UAMS-1 strain is most likely caused by the mutation identified in our study. The role of hyaluronidase in staphylococcal infection and the redundancy of this gene have yet to be determined.

Keywords: *S. aureus* UAMS-1, hyaluronidase, *hysA1* point mutation, homology modeling

Draft

1. Introduction

Staphylococcus aureus remains a continuous concern for public health as this bacterial pathogen is endemic to most US hospitals and is now a threat to humans who exhibit no apparent immuno-compromising conditions (Herold et al. 1998; Chambers and DeLeo 2009; DeLeo et al. 2010). This organism, a Gram-positive, facultative anaerobic bacterium, resides primarily as a commensal colonizer on the skin and mucous membranes of both humans and animals (Tong et al. 2012; Yang et al. 2010). Persistent carriage or colonization rates at any given time among healthy human individuals can be as high as 30%, and up to 90% among healthcare workers (Waldvogel 2000). Therefore, it is not surprising that of the estimated two million hospitalizations each year resulting in nosocomial infections, *S. aureus* and the coagulase-negative staphylococci (CoNS) are the most common causative bacterial disease agents (Kallen et al. 2010; Klevens et al. 2007a; Klevens et al. 2007b; Sievert et al. 2013). Also, the number of skin and soft tissue infections (SSTIs) for the years 2001-2003 caused by *S. aureus* was greater than 11 million per year, making this organism the most common cause of SSTIs (McCaig et al. 2006). Often times these relatively superficial infections, along with pneumonia, result in bacteremia, which can lead to more serious metastatic infections such as infective endocarditis, vertebral osteomyelitis, iliopsoas abscess, and septic arthritis (Horino and Hori 2020).

Hyaluronic acid is a high-molecular weight, linear unsulfated glycosaminoglycan polymer made up of β 1-4 alternating units of D-glucuronic acid-[1-3]- β -N-acetylglucosamine (Hynes and Walton 2000; Stern and Jedrzejewski 2006). As a major component of the extracellular matrix of human tissues (e.g., amniotic fluid, articular

cartilage, blood, brain, liver, plasma, skin, synovial fluid and umbilical cord), this polymer contributes significantly to a number of physiological functions, which include wound healing, water homeostasis, and viscoelasticity (Fraser et al. 1997; Hynes and Walton 2000; Stern and Jedrzejewski 2006; Girish and Kemparaju 2007).

The hyaluronidases are a group of naturally-occurring enzymes that degrade hyaluronic acid and can be classified into one of three categories depending upon their source; mammalian, venom, or microbial (Girish and Kemparaju 2007). The bacterial hyaluronidases (also referred to as hyaluronate lyase) have been shown to contribute to infection from a number of different bacterial species; it has been proposed that by degrading the polymer in the extracellular matrix of the host, bacterial pathogens may enhance contact with host cell constituents and facilitate dissemination of bacterial cells and toxins throughout the host (Smith 1979a; Rood and Cole 1991; Cheng et al. 1995; Hynes and Walton 2000; Senn et al. 2005; Starr and Engleberg 2006). In addition, the bacterial pathogen may utilize the degraded polymer product as a suitable energy source (Smith 1979b; Fitzgerald and Repesh 1987; Pettersson et al. 1996; Homer et al. 1997; Shain et al. 1997; Starr and Engleberg 2006).

Type strains of 20 *Staphylococcus* species were screened for the hyaluronidase genes (*hysA*) and their corresponding enzymatic activities and only strains of *S. aureus* coded for hyaluronidases (Hart et al. 2009). These results suggest that hyaluronidase may be yet another potential virulence factor for *S. aureus*; a suggestion supported by results found in two different murine models of virulence (Makris et al. 2004; Ibberson et al. 2014; Ibberson et al. 2016). In addition, hyaluronidase from *S. aureus* USA300 has been shown to be

involved with biofilm dispersal as demonstrated with a catheter implantation model in mice (Ibberson et al. 2016).

Although a hyaluronidase from *S. aureus* was first purified and crystallized in 1958, its molecular structure remains to be determined (Rautela and Abramson 1973). The crystal structures of hyaluronidases from *Streptococcus pneumoniae* and *S. agalactiae*, SpnHL and SagHL, respectively, have been resolved (Li and Jedrzejewski 2001; Jedrzejewski et al. 2002; Mello et al. 2002; Rigden and Jedrzejewski 2003). Both structures consist of two distinct domains, the N-terminal α -helical domain and the C-terminal β -sheet domain, connected by a short linker. This structure supports the function of the enzyme, which is to cleave the β 1,4-glycosidic linkage between the glycan units in the hyaluronic acid polymer to release disaccharide products (Rigden and Jedrzejewski 2003). The amino acid residues in the cleft area that interact with the substrate have been identified (Jedrzejewski et al. 2002; Mello et al. 2002; Rigden and Jedrzejewski 2003; Botzki et al. 2004).

Previous proteomic studies using spent media isolated from the *S. aureus* UAMS-1 strain identified two different proteins as hyaluronidases (Jones et al. 2008), which is consistent with the fact that two hyaluronidase genes (*hysA1* and *hysA2*) exist in the genome of this bacterium (Hart et al. 2009; Sassi et al. 2015). Surprisingly, in a later study, a *S. aureus* UAMS-1 strain containing a *hysA2* deletion mutant exhibited no detectable hyaluronidase activity as determined by a plate assay (Hart et al. 2013), thereby suggesting that the *S. aureus* UAMS-1 *hysA1* gene expresses a nonfunctional enzyme. In addition, it was determined that *S. aureus* UAMS-1 is not the only strain that possesses two determinants for hyaluronidase (Hart et al. 2009). For example, the *S. aureus* Sanger-252 strain also contains two genes for hyaluronidase and the loss of either gene by deletion

results in hyaluronidase activity comparable to wild type levels, thereby suggesting that both hyaluronidases are functional (Hart et al. 2013). In the current study we compared nucleic and amino acid sequences of the *hysA1* gene isolated from *S. aureus* UAMS-1 and Sanger-252 strains and determined that these strains have an average nucleotide identity (ANI) of 99.93 and an average amino identity (AAI) of 99.99. In addition, we discovered a single base change in the open reading frame of the *hysA1* gene from the *S. aureus* UAMS-1 strain. This single base change resulted in an amino acid base change at position 480 from a glutamic acid to a glycine in the *hysA1* gene and most likely accounts for the reduced HysA1 activity in the *S. aureus* UAMS-1 strain, as hyaluronidase activity was restored when the *S. aureus* Sanger-252 *hysA1* gene was expressed from a plasmid in the *S. aureus* UAMS-1 *hysA2* mutant strain.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Staphylococcus aureus* cultures were grown in tryptic soy broth (TSB, BD Bioscience, Franklin Lakes, NJ) with or without chloramphenicol (10 µg/ml; Sigma-Aldrich Chemical Co. St. Louis, MO) as needed. Likewise, cultures of *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) or DH5α (Invitrogen) strains were grown either in Luria broth (LB) or TSB containing carbenicillin (100 µg/ml; Sigma-Aldrich). Both genera were routinely grown at 37°C with rotary aeration (180 rpm) and a flask-to-broth volumetric ratio of 2.5.

2.2. DNA isolation and manipulation

Genomic DNA was isolated from *S. aureus* UAMS-1 and Sanger-252 strains using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) as recommended by the manufacturer for *S. aureus* genomic DNA isolation. Amplicons were generated by PCR using genomic DNA, Platinum® PCR SuperMix HiFi (Invitrogen) and primers, HysA1F (5'-GAAAAGCTTCAACGCCTCTCATGATTCG-3') containing a *Hind*III digestion site and HysA1R (GAAGGTACCCGATGCTTATAAAATCTCGACAC-3') containing a *Kpn*I digestion site. Products of the desired size from triplicate biologically-independent amplifications were gel-purified, ligated into *E. coli* pCR2.1TOPO (Invitrogen) plasmid DNA, and transformed into One Shot® TOP10 chemically-competent *E. coli* cells (Invitrogen) as recommended by the manufacturer. Transformants were selected by growth on LB agar plates containing carbenicillin (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma-Aldrich). Plasmid DNA from *E. coli* transformants was isolated using the Qiagen plasmid mini-prep kit (Qiagen Inc. Valencia,

CA) and sequenced at the University of Arkansas for Medical Sciences core facility using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) to confirm the identity of the inserts.

Plasmid DNA containing the *S. aureus* Sanger-252 *hysA1* gene was digested with *Hind*III and *Kpn*I and the *hysA1*-containing fragment was gel-purified and ligated into the gel-purified, dephosphorylated, *Hind*III/*Kpn*I-digested shuttle plasmid, pCL15 (Luong and Lee, 2006). Ligation mixtures were transformed into electrocompetent *S. aureus* RN4220 as described by Kraemer and Iandolo (1990); transformants growing on TSA plates containing chloramphenicol which also demonstrated hyaluronidase activity on plates containing hyaluronic acid (Sigma-Aldrich, H-1504; potassium salt, from human umbilical cord) were chosen for further study. Plasmid DNA was isolated from transformed *S. aureus* RN4220 using the Qiagen plasmid mini-prep kit with an additional step where cell suspensions were incubated at 37°C for 30 min in the presence of lysostaphin (100 µg/ml; AMBI Productions LLC, Lawrence, NY). Plasmids were isolated from transformants and either digested with *Hind*III/*Kpn*I or used as a template for PCR to verify plasmid inserts. Transducing lysates isolated from the *S. aureus* RN4220 strain containing *hysA1* plasmids were used to transduce the *hysA1* plasmids into the *hysA2* mutant strain of *S. aureus* UAMS-1 using ϕ 11 and the procedure of McNamara and Iandolo (1998) as modified by Hart et al. (2001). Chloramphenicol-resistant transductants containing pCL15*hysA1*⁺ were procured for further study.

2.3. DNA and protein sequence analysis

The entire *hysA1* gene sequence and its approximately 1000 base pair upstream region were constructed using Seqman (Lasergene 9, DNASTAR, Inc., Madison, WI.), compared

for sequence anomalies, and subsequently translated into protein. Protein sequence comparison was performed with Clustal W (MegaAlign, Lasergene 9, DNASTAR, Inc.).

2.4. Cell lysate and spent media preparation

Cell lysates from *E. coli* strains containing their appropriate *hysA* plasmid clones were prepared as described by Blevins et al. (2002). Briefly, cultures were standardized using optical density determinations at 550 nm with sterile TSB as diluent. Three-ml portions were removed for isolation of plasmids, and cells from the remaining culture volume were harvested by centrifugation (12,000 x g, for 10 min at 4°C), washed in an equal volume of TE (10 mM Tris-base, 1 mM EDTA, pH 7.5) buffer, and suspended in 0.4 ml TE. Cell suspensions were pipetted into FastPROTEIN™ BLUE tubes (MP Biochemical, Solon, Ohio) containing silica beads and cells were lysed using a Fast Prep FP120 reciprocator (MP Biochemical) at 6 meters/sec for 40 sec. Cell lysates were cooled on ice for 15 min followed by centrifugation (10,000 x g, for 1 min at 4°C). Supernatants were collected and frozen at -80°C until used.

Spent media from overnight (15 to 18 h, represents a period of time when the cells are in the post-exponential phase of growth) *S. aureus* broth cultures were prepared as described by Hart et al. (2013) except that precipitation with trichloroacetic acid (TCA) was excluded. Cell density values were determined spectrophotometrically at 550 nm and used to dilute each culture to an optical density reading of 3.0 with freshly prepared and sterilized TSB. Cells were pelleted by centrifugation (10,000 x g, for 10 min at 4°C) and spent media were filter-sterilized and concentrated by centrifugation (7,500 x g) for one hour using ultrafiltration (Centricon Ultracel YM-3 filters with 3,000 MWCO, Millipore Corporation, Bedford, ME). Retentates were stored at -80°C until used.

2.5. Hyaluronidase assay (HA)

Hyaluronidase activity was detected using the hyaluronic acid (Sigma) plate assay of Hart et al. (2009). Twenty μ l of sonicated *E. coli* cell lysates or concentrated *S. aureus* spent media were pipetted into each well. Plates were incubated overnight at 37°C prior to precipitating undigested hyaluronic acid conjugated to bovine serum albumin with acetic acid (2 M). Diameters (millimeters) of digested hyaluronic acid were measured and recorded. Purified bovine testicular hyaluronidase (Sigma, H-3506) was used as a positive control.

2.6. Homology modeling and bioinformatics analysis

The crystal structure (Protein Data Bank [PDB] 1LOH) of hyaluronidase (SpnHL) from *Streptococcus pneumoniae* serotype 4 strain (ATCC BAA-334/TIGR4) was used as a template for computational modeling and analysis (Jedrzejewski et al. 2002; Nukui et al. 2003). Homology three-dimensional models for the *S. aureus* HysA1 wild type and mutant proteins were generated using SWISS-MODEL (<http://swissmodel.expasy.org/workspace/index.php>) with default parameters. SAS (<https://www.ebi.ac.uk/thornton-srv/databases/sas/>) was used for sequence alignment analysis of the HysA1 from *S. aureus* UAMS-1 against all protein sequences in the PDB. The PDB structure files and their at-a-glance structural overview information were obtained from the databases RCSB Protein Data Bank (RCSB PDB) (<http://www.rcsb.org/pdb/home/home.do>) and PDBsum (<http://www.ebi.ac.uk/pdbsum/>) (Rose et al. 2011). The receptor-ligand docking program, DockThor (<https://www.dockthor.lncc.br/v2/>) was used to identify the binding site and the best binding mode of the substrate hyaluronan using the blind docking approach with

default parameters (Santos et al. 2020). To identify the best binding pose, the hyaluronan bound to the *S. pneumoniae* SpnHL was used to compare docking poses as a reference conformation. SuperPose (version 1.0) was used for structural superposition and Root-Mean-Square-Deviation (RMSD) calculations of the wild-type and mutant proteins (Maiti et al. 2004). For automatic mutant analysis and for prediction of changes in protein stability after point mutation, HOPE server (Venselaar et al. 2010) and CUPSAT (<http://cupsat.tu-bs.de/>) were adopted (Parthiban et al. 2006), respectively. CSU server was used to identify the interaction of the residue at position 480 in wild type and mutant proteins (Sobolev et al. 1999). To identify non-covalent interactions between hyaluronidase and the substrate hyaluronan, PLIP (<https://projects.biotec.tu-dresden.de/plipweb/plip/index>) was used (Salentin et al. 2015). PyMOL (0.99RC6) (<http://www.pymol.org>) was used for structural analysis, visualization, and RMSD calculations between two ligands.

The ANI between *S. aureus* UAMS-1 and Sanger 252 strains was calculated using the OrthoANiU algorithm, an improved version of the original OrthoANI algorithm, which uses USEARCH instead of BLAST (Yoon 2017). In addition, we also calculated the AAI from the core proteins of the two strains using the functional module for pan-genome analysis in the CYPminer (v1) (Kweon et al. 2020). To check the occurrence of the mutation (E480G) of HysA1 that occurs in *S. aureus* UAMS-1 strain, we conducted a protein-protein BLAST search using PATRIC [<https://www.patricbrc.org/>] and NCBI [<https://www.ncbi.nlm.nih.gov/>] databases with a query sequence of the conserved sequence (ENLKGWHTGAGM) or full protein sequence of HysA1 from the *S. aureus* UAMS-1 strain. In the PATRIC database analysis, we searched greater than 2,400 (completed and draft genomes) sequences of *S. aureus* strains. We also used the NCBI

database containing 322,214,535 sequences and all non-redundant GenBank CDS translations + PDB + SwissProt + PIR+PRF excluding environmental samples from WGS projects, to cover all possible hyaluronidase sequences. We used default algorithm parameters for the blast searches.

Draft

3. Results

3.1. A point mutation was identified in the *hysA1* gene of the *S. aureus* UAMS-1 strain

Sequences of *hysA1* and *hysA2* genes from the *S. aureus* UAMS-1 strain showed 80% nucleotide identity (75% protein sequence identity) and the sequenced regions were identical to published sequences of *hysA1* and *hysA2* from the *S. aureus* Sanger 252 and UAMS-1 strains whose genomes have been completely sequenced and annotated (Holden et al. 2004; Sassi et al. 2015). A deletion mutation of the *hysA2* gene in the *S. aureus* UAMS-1 strain resulted in no detectable hyaluronidase activity on HA plates, suggesting loss of HysA1 function (Hart et al. 2013). To determine if the defect in hyaluronidase activity resided within the coding region of *hysA1*, the published sequence of the *S. aureus* Sanger-252 was utilized to design primers to amplify the entire coding region and the upstream region (approximately 1000 base pairs) of the *hysA1* and *hysA2* genes from the *S. aureus* UAMS-1 strain. Amplicons were cloned into *E. coli* plasmids and sequenced to verify their identity and correctness of sequence (data not shown). In addition, spent media and cell lysates from several of the *E. coli* clones containing plasmids with either the *hysA1* or *hysA2* genes from either the *S. aureus* UAMS-1 or Sanger-252 strains were generated and assessed for hyaluronidase activity. All *E. coli* clones exhibited activity, except those containing the *hysA1* gene from the *S. aureus* UAMS-1 strain. Sequenced regions were then compared to those of the *S. aureus* Sanger-252 region, and a single base pair difference (G to A) at position 1439 was discovered. This point mutation resulted in an E480G change in the amino acid sequence, and further comparisons with additional HysA sequences from *S. aureus* strains suggested that the glutamic acid at position 480 is a unique point mutation

only observed in a highly conserved region of the *hysA1* gene of the *S. aureus* UAMS-1 strain (Figure 1).

3.2. A copy of *hysA1* gene from *S. aureus* Sanger 252 restored the hyaluronidase activity in the *S. aureus* UAMS-1 Δ *hysA2* mutant strain

As shown in the sequence alignment and percent identity matrix of the four protein sequences of hyaluronidases, the HysA1 and HysA2 from *S. aureus* UAMS-1 and the HysA1 and HysA2 from *S. aureus* Sanger-252 (Figure 1 supplementary and Table 1 supplementary), they have two hyaluronidases (i.e., HysA1 and HysA2). The protein sequences of HysA2 from both strains exhibit 100% identity. The protein sequences of HysA1 from the two strains show 99.9% sequence identity. In other words, the two HysA1 protein sequences are essentially identical, except for the residue at position 480; a glycine at 480 for the UAMS-1 HysA1 protein and a glutamic acid at 480 for the Sanger-252 HysA1 protein (Figure 1 supplementary and Table 1 supplementary). The *S. aureus* Sanger-252 *hysA1* gene was cloned into pCL15 and transduced into the *hysA2* deletion mutant strain of *S. aureus* UAMS-1. While the gene was cloned downstream of the pSPAC inducible promoter of pCL15, induction was not performed thereby indicating expression occurred by the *hysA1* natural promoter presumably located within the cloned upstream region. Spent media from the transductants were isolated and assessed for hyaluronidase activity on HA plates (Figure 2). Only the *S. aureus* UAMS-1 wild type and the Δ *hysA2* mutant strain containing the pCL15*hysA1*⁺ exhibited hyaluronidase activity.

3.3. Homology modeling of the *S. aureus* UAMS-1 HysA1 protein suggests that the glutamic acid at 480 is a highly conserved residue with structural responsibility for catalytic activity

For structural understanding of the reduced enzyme activity in the *S. aureus* UAMS-1 HysA1 mutant (E480G) protein, homology models of the wild type and mutant protein structures were generated using the SpnHL (PDB 1LOH; 36.36% amino acid sequence identity) from *S. pneumoniae* as a template (Jedrzejewski et al. 2002). Blind docking simulations suggested that the structural arrangements of the hexasaccharide hyaluronan were bound in the catalytic cleft of the *S. aureus* UAMS-1 HysA1 protein. Figure 3 shows the overall domain organization of *S. aureus* UAMS-1 HysA1 and the structural arrangements of the hexasaccharide hyaluronan bound in the catalytic cleft.

The wild-type *S. aureus* UAMS-1 HysA1 protein consisted of two distinct structural domains; the N-terminal α -helical domain (α -domain) and the C-terminal β -sheet domain (β -domain) connected by a short linker (Asp-430 to Gln-440) (Figure 3A). The catalytic cleft of the HysA1, the place where hyaluronan binds and is degraded, existed between the α - and β -domains (Figure 3B). In the local structural environment of the catalytic cleft with a site volume of $\sim 3,792 \text{ \AA}^3$, the catalytic residues (Asn-242, His-298, and Tyr-307) and the residues of the hydrophobic (Trp-184 and Trp-185) and the negative (Asp-297 and Gln-299) patches, which are crucial for the enzyme activity, were highly conserved (Figure 3C). In the catalytic cleft, the substrate hexasaccharide hyaluronan was stabilized by approximately 16 residues, which include functional residues that formed an interaction network of hydrogen bonds and hydrophobic interactions (data not shown). As shown in Figure 3C, among the six sugar rings of the hexasaccharide, three sugar rings (the second,

third, and fourth sugar rings from the reducing end) of the substrates bound to the wild-type and mutant HysA1 proteins were superimposed on those of the SpnHL, indicating its active binding mode. As shown in the *S. pneumoniae* SpnHL, the second and third sugars were stacked between two hydrophobic residues (Trp-184 and Trp-185) and two, negatively-charged residues (Arg-360 and Arg-364) from the α -domain of the HysA1 protein. The Asn-478 residue participated in the formation of one edge of the cleft and made the shortest cross-distance of the cleft with Asn-183 over the second sugar ring, forming a tunnel (Figures 3B and 3C). Asn-478 was the only residue from the β -domain in direct contact with the substrate (the second sugar ring) in our substrate modeling studies.

The amino acid Glu-480 was located in the isolated beta-bridge with solvent accessibility of 10.5% in the cleft, and was highly conserved in the related proteins of known three-dimensional structure in the PDB (Figure 2 supplemental). The residue Glu-480 formed hydrogen bonds with the six residues of Arg-360 (2.7 Å), Arg-364 (2.9 Å), Asn-476 (2.6 Å), Ile-477 (3.0 Å), Asn-481 (1.3 Å), and Asn-658 (2.8 Å). However, the mutation from the negative, long-side chain of Glu to a neutral short side chain Gly, at position 480 caused loss of hydrogen bonding with the three residues Arg-360, Arg-364, and Asn-658, resulting in an overall destabilization ($\Delta\Delta G = -0.98$). The RMSD (α carbons) between the structures of the wild type and the mutant HysA1 was 0.138 Å. The substrate hyaluronan in the mutant HysA1 showed a similar binding pose to that of the wild-type HysA1 (RMSD of 0.818) (Figure 3C).

4. Discussion

Staphylococcus aureus is a major concern for public health due to its ability to cause numerous disease syndromes and its propensity to acquire multiple antibiotic resistance determinants (Herold et al. 1998; Chambers and DeLeo 2009; DeLeo et al. 2010). Various virulence factors, which are under strict regulation by global regulators SarA and Agr have been identified in this species (Dunman et al. 2001; Cheung et al. 2008). Although the exact role of hyaluronidase in staphylococcal infections has not been determined, there is evidence showing that this secreted enzyme is a potential virulence factor (Makris et al. 2004; Ibberson et al. 2014; Ibberson et al. 2016).

Staphylococcus aureus UAMS-1 contains two gene copies of hyaluronidase, *hysA1* and *hysA2*, and both genes are transcribed and translated into proteins (Jones et al. 2008). Previous studies utilizing a *hysA2* deletion mutant of the *S. aureus* UAMS-1 strain demonstrated no detectable levels of hyaluronidase activity (Hart et al. 2013). Sequencing analysis indicated a naturally occurring point mutation within the coding region of the *hysA1* gene; it is a mutation complemented by introducing, *in trans*, the *hysA1*⁺ gene isolated from the *S. aureus* Sanger-252 strain.

Hyaluronidase, like coagulase and DNase, appears to be a predominant enzyme found among *S. aureus* strains (Choudhuri and Chakrabarty 1969; Essers and Radebold 1980; Hart et al. 2009). Earlier studies, primarily examining *S. aureus* vaginal toxic shock syndrome isolates and clinical strains isolated from abscesses, wounds, and blood, determined that while not all strains exhibited hyaluronidase activity, they all contained at least one *hysA* gene (Hart et al. 2009). Interestingly, over half of these isolates contained

two independent determinants for *hysA*. However, within this group of *S. aureus* strains, hyaluronidase redundancy does not appear to afford an increased level of activity over that of strains, which contain only one determinant (Hart et al. 2009; Hart et al. 2013). Whether this redundancy is the result of the presence of a mobile element is currently unknown and under investigation.

As revealed by the structural comparison of *S. pneumoniae* SpnHL and *S. aureus* UAMS-1 HysA1, the highly conserved catalytic residues and the residues of the hydrophobic negative patches in the catalytic cleft indicate that they share structure and function in terms of hyaluronan degradation (Nukui et al. 2003; Jedrzejewski et al. 2002; Rigden and Jedrzejewski 2003). A hexasaccharide hyaluronan seems to be the longest hyaluronan unit which fits into the catalytic cleft of the *S. aureus* UAMS-1 HyA1 protein. In addition to the three catalytic residues (Asn-242, His-298, and Tyr-307) in HysA1, the six residues (Asn-183, Trp-184, Trp-185, Arg-360, Arg-364, and Asn-478) in HysA1 responsible for binding the second and third sugar rings were perfectly conserved in the primary and tertiary structures of HysA1 and SpnHL (Figure 3C). Considering that the SpnHL mechanism in which His-399 extracts the acidic C5 proton on the glucuronate residue, results in the formation of a double bond between C4 and C5 and Tyr-408 provides a proton to the glycosidic oxygen which breaks the glycosidic linkage (Jedrzejewski et al. 2002), the six conserved residues should play important roles in binding the hexasaccharide substrate within reactive distance from the catalytic residues, allowing successful proton extraction and donation. In the cleft the second and third sugar rings of the hexasaccharide were sandwiched between two hydrophobic residues (Trp-184 and Trp-185) and two negatively charged residues (Arg-360 and Arg-364) from the α -domain.

Additionally, the two residues Asn-183 (α -domain) and Asn-478 (β -domain) bridged the cleft over the second sugar ring (Figures 3B and 3C). Therefore, such a topological tunnel feature of the active site in the cleft could be the most ideal structural environment for accurately holding the cleavage sites on the substrate chain, anchoring them into their correct positions for optimal enzyme activity. As shown in the superposition of hexasaccharides bound to the SpnHL and HysA1 (Figure 3C), and despite that the first, fifth, and sixth sugar rings showed different binding poses, the three sugar rings (from the sugar 2 to the sugar 4) were almost superimposed. Conclusively, the structural and functional ensemble of the catalytic residues and tunnel-forming residues seems essential for enzyme activity, i.e., productive hyaluronan degradation.

Despite accumulated biochemical and three-dimensional structural information and advanced bioinformatics tools, predicting mutational effect(s) of a residue on the enzyme (i.e., mutational phenotypic feedback) remains challenging, in part, due to the pleiotropic and epistatic behavior of a residue in terms of structure and function. Pleiotropic mutational effects of a residue depend on various factors, including the functional and/or structural responsibility of residues in the protein (Sun et al. 2017). Here, the physicochemical properties of the residue in context (i.e., nature of the mutation) are known to largely govern the degree of phenotypic feedback (Sun et al. 2017). In this perspective, an apparent loss of activity of the mutant HysA1 (E480G) is intriguing. In the proposed enzyme mechanism for the SpnHL, the β -domain is more important in modulating SpnHL activity rather than directly degrading HA (Li et al. 2000). Considering its indirect contribution to substrate binding and enzyme catalytic reaction, the functional responsibility of Glu-480 appears insignificant. Therefore, despite such a dramatic

physicochemical change in the side chain, its functional responsibility-centric explanation seems to be an incomplete explanation for the apparent loss of activity of the mutant HysA1. On the other hand, in the HysA1, the residue Glu-480 forms an interaction network via hydrogen bonds with approximately six residues (Arg-360, Arg-364, Asn-476, Ile-477, Asn-481, and Asn-658). A loss of the negatively charged, long side chain at position 480 causes a loss of hydrogen bond interactions with the three residues Arg-360, Arg-364, and Asn-658. Such a decreased pleiotropic contribution at that position results in an overall destabilization of HysA1. As explained above, the two residues Arg-360 and Arg-364 are primarily responsible for the tunnel topology and also interact directly through hydrogen bonds with the second and third sugar rings of the hexasaccharide.

Overall, the following hypothesis for the intolerant mutation seems plausible: the missense mutation of Glu-480 to Gly introduces a loss of side chain hydrogen bond interactions with several residues (i.e., decreased pleiotropic contribution), including Arg-360 and Arg-364 which are tunnel-forming residues responsible for productive substrate binding, causing a localized conformational change to the active site in the catalytic cleft (i.e., detrimental structural change), resulting in displacement of the substrate from an ideal position for catalysis (i.e., dissonance in its structural and functional relationship). Several mutational cases of hyaluronidase support this hypothesis (Hynes et al. 2009; Li et al. 2009). As expected, the site-directed mutants (N349A, H399A, and Y408F) of catalytic residues with high SpnHL functional responsibility show significant or complete loss of enzyme activity (Li et al. 2000). On the other hand, enzyme activities of two mutants, R243V and N580G, with relatively high structural responsibility (i.e., involved in the substrate binding but outside of the active center) were 67% and 115%, respectively,

relative to SpnHL enzyme activity. In the case of mutant N580G, the Asn-580 residue plays a key role in controlling substrate entrance structure into the cleft of the SpnHL; swapping Asn-580 to Gly creates a wider entrance into the cleft, allowing substrate to reach the active residues more easily. This observation encourages us to include in our hypothesis that the detrimentally changed tunnel topology would prevent passage of the substrate, resulting in reduced enzyme activity. However, comparative analysis of the wild type and mutant HysA1 homology models showed no meaningful difference in the structure and substrate binding pose of the hexasaccharide. An apparent limitation to the presented approach is a lack of a suitable template protein (with high sequence identity) for the homology modeling process. Experimentally determined structural data for HysA1 should be generated to prove this hypothesis. Hynes et al. (2009) reported another intriguing mutational case; an amino acid change at position 199 (Asp to Val) of the hyaluronidase was present in a highly-conserved region of the protein in *Streptococcus pyogenes* isolates that do not produce active enzyme. Interestingly, the residue is involved in substrate binding (i.e., the fourth sugar ring) and not catalysis.

The point mutation in the *hysA1* gene of *S. aureus* UAMS-1 appears to be a rare event among multiple strains of *S. aureus*; a mutation that renders enzyme activity undetectable as determined with a hyaluronic acid plate assay. However, the loss of HysA1 in *S. aureus* UAMS-1 doesn't appear to impede its ability to cause musculoskeletal disease in humans, rabbits, and mice, at least to the level examined (Gillaspy et al. 1995; Blevins et al. 2003). Nevertheless, the hyaluronidase coded for by *hysA1* in *S. aureus* is important in some aspect of disease as demonstrated by studies conducted in mouse models of pneumonia and subcutaneous infection (Makris et al. 2004; Ibberson et al. 2014; Ibberson et al. 2016).

These virulence studies were conducted with a *S. aureus* USA300 strain (Diep et al. 2006), a prominent community-associated methicillin resistant *S. aureus* (CA-MRSA) strain isolated primarily from skin and soft tissue infections in the US (Tenover et al. 2006), and *S. aureus* 8325-4 (Peng et al. 1988); strains of *S. aureus* that code for only one hyaluronidase namely, HysA1. Whether or not HysA1 is important for pathogenesis in the *S. aureus* UAMS-1 strain remains to be seen, as it is possible, that the presence of a second functional hyaluronidase (HysA2) may provide the necessary activity needed for disease in the absence of a functional HysA1.

Draft

Data availability statement

All datasets generated for this study are included in the article/Supplementary Material.

Author contributions

MEH and HH were involved in the design of the study. HH, HL, OK, and MEH were involved in the execution of the study the analysis, and the interpretation of the results. HH and MEH wrote the primary manuscript. All authors reviewed and edited the manuscript.

Declaration of competing interest

The authors have no conflict of interest to declare.

Acknowledgements, funding, and disclaimer

This research was supported by protocol E-07175.01 awarded to M. E. Hart by the National Center for Toxicological Research (NCTR) of the United States Food and Drug Administration. Special thanks to Drs. Carl Cerniglia, Steve Foley, and Robin Stingley for their editorial comments.

The study was supported in part by the Center for Microbial Pathogenesis and Host Inflammatory Responses grant P20GM103625 through the NIH National Institute of General Medical Sciences Centers of Biomedical Research Excellence. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The views presented in this manuscript do not necessarily reflect those of the U. S. Food and Drug Administration.

Appendix A. Supplementary data

The Supplementary Material for this article can be found online at:

References

- Berry, A.M., R.A. Lock, S.M. Thomas, D.P. Rajan, D. Hansman, and J.C. Paton.** 1994. Cloning and nucleotide sequence of the *Streptococcus pneumoniae* hyaluronidase gene and purification of the enzyme from recombinant *Escherichia coli*. *Infection and Immunity*, **62**:1101-1108.
- Blevins, J.S, K.E. Beenken, M.O. Elasri, B.K. Hurlburt, and M.S. Smeltzer.** 2002. Strain-dependent differences in the regulatory roles of *sarA* and *agr* in *Staphylococcus aureus*. *Infection and Immunity*, **70**:470-480.
- Blevins, J.S., M.O. Elasri, S.D. Allmendinger, K.E. Beenken, R.A. Skinner, J.R. Thomas, and M.S. Smeltzer.** 2003. Role of *sarA* in the pathogenesis of *Staphylococcus aureus* musculoskeletal infection. *Infection and Immunity*, **71**:516-523.
- Botzki, A., D.J. Rigden, S. Braun, M. Nukui, S. Salmen, J. Hoechstetter, G. Bernhardt, S. Jedrezejas, and A. Buschauer.** 2004. L-Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes. *Journal of Biological Chemistry*, **279**:45990-45997.
- Chambers, H.F. and, F.R. DeLeo.** 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, **7**: 629-641.
- Cheng, Q., M.C. Yu, A.R. Reeves, and A.A. Salyers.** 1995. Identification and characterization of a *Bacteroides* gene, *csuF*, which encodes an outer membrane protein that is essential for growth on chondroitin sulfate. *Journal of Bacteriology*, **177**:3721-3727.
- Cheung, A.L., K. Nishina, A.C. Manna.** 2008. SarA of *Staphylococcus aureus* binds to the *sarA* promoter to regulate gene expression. *Journal of Bacteriology*, **190**:2239-2243.

- Choudhuri, K.K., and A.N. Chakrabarty.** 1969. Hyaluronate lyase activity of *Staphylococci*. *Indian Journal of Experimental Biology*, **7**:183-185.
- DeLeo, F.R., M. Otto, B.N. Kreiswirth, and H.F. Chambers.** 2010. Community-associated methicillin-resistant *Staphylococcus aureus*. *The Lancet*, **375**:1557-1568.
- Diep, B.A., S.R. Gill, R.F. Chang, T.H. Phan, J.H. Chen, M.G. Davidson, F. Lin, J. Lin, H.A. Carleton, E.F. Mogodin, G.F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet*, **367**:731-739.
- Dunman, P.M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E.L. Brown, R.J. Zagursky, D. Shlaes, and S.J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *Journal of Bacteriology*, **183**: 7341-7353.
- Essers, L., and K. Radebold.** 1980. Rapid and reliable identification of *Staphylococcus aureus* by a latex agglutination test. *Journal of Clinical Microbiology*, **12**:641-643.
- Fitzgerald, T.J., and L.A. Repesh.** 1987. The hyaluronidase associated with *Treponema pallidum* facilitates treponemal dissemination. *Infection and Immunity*, **55**:1023-1028.
- Fraser, J.R., T.C. Laurent, and U.B. Laurent.** 1997. Hyaluronan: its nature, distribution, functions and turnover. *Journal of Internal Medicine*, **242**: 27-33.
- Gillaspy, A.F., S.G. Hickmon, R.A. Skinner, J.R. Thomas, C.L. Nelson, and M.S. Smeltzer.** 1995. Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. *Infection and Immunity*, **63**:3373-3380.

- Girish, K.S., and K. Kemparaju.** 2007. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. *Life and Science*, **80**:1921-1943.
- Hart, M.E., R.M. Crum, M.A. St. John-Tidwell, and C.A. Malakowsky.** 2001. Isolation of stable hemolysin and catalase variants of *Staphylococcus aureus* S6C includes one with an exoprotein-deficient phenotype. *Current Microbiology*, **43**:134-139.
- Hart, M.E., M.J. Hart, and A.J. Roop.** 2009. Genotypic and phenotypic assessment of hyaluronidase among type strains of a select group of staphylococcal species. *International Journal of Microbiology*, Volume 2009, Article ID 614371.
- Hart, M.E., L.H. Tsang, J. Deck, S.T. Daily, R.C. Jones, H. Liu, H. Hu, M.J. Hart, and M.S. Smeltzer.** 2013. Hyaluronidase expression and biofilm involvement in *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr* and *sarA agr* regulatory mutants. *Microbiology*, **159**: 782-91.
- Herold, B.C., L.C. Immergluck, M.C. Maranan, D.S. Lauderdale, R.E. Gaskin, S. Boyle-Vavra, C.D. Leitch, and R.S. Daum.** 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *Journal of the American Medical Association*, **279**:593-598.
- Holden, M.T.G., E.J. Feil, J.A. Lindsay, S.J. Peacock, N.P.J. Day, M.C. Enright, T.J. Foster, C.E. Moore, L. Hurst, R. Atkin, A. Barron, N. Bason, S.D. Bentley, C. Chillingworth, T. Chillingworth, C. Churcher, L. Clark, C. Corton, A. Cronin, J. Doggett, L. Dowd, T. Feltwell, Z. Hance, B. Harris, H. Hauser, S. Holroyd, K. Jagels, K.D. James, N. Lennard, A. Line, R. Mayes, S. Moule, K. Mungall, D. Ormond, M.A. Quail, E. Rabinowitsch, K.M. Rutherford, M. Sanders, S. Sharp, M. Simmonds, K. Stevens, S. Whitehead, B.G. Barrell, B.G. Spratt, and J. Parkhill.**

2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proceedings of the National Academy of Sciences USA*, **101**:9786-9791.

Homer, K., H. Shain, and D. Beighton. 1997. The role of hyaluronidase in growth of *Streptococcus intermedius* on hyaluronate. *Advances in Experimental Medicine and Biology*, **418**:681-683.

Horino, T., and S. Hori. 2020. Metastatic infection during *Staphylococcus aureus* bacteremia. *Journal of Infection and Chemotherapy*, **26**:162-169.

Hynes, W.L., and S.L. Walton. 2000. Hyaluronidases of gram-positive bacteria. *Federation of European Microbiological Societies Microbiology Letters*,

Hynes, W., C. Johnson, and M. Stokes. 2009. A single nucleotide mutation results in loss of enzymatic activity in the hyaluronate lyase gene of *Streptococcus pyogenes*. *Microbial Pathogenesis*, **47**:308-313.

Ibberson, C.B., C.L. Jones, S. Singh, M.C. Wise, M.E. Hart, D.V. Zurawski, and A.R. Horswill. 2014. *Staphylococcus aureus* hyaluronidase is a CodY-regulated virulence factor. *Infection and Immunity*, **82**:4253-4264.

Ibberson, C.B., C.P. Parlet, J. Kwiecinski, H.A. Crosby, D.K. Meyerholz, and A.R. Horswill. 2016. Hyaluronan modulation impacts *Staphylococcus aureus* biofilm infection. *Infection and Immunity*, **84**:1917-1929.

Jedrzejewski, M.J., L.V. Mello, B.L. de Groot, and S. Li. 2002. Mechanism of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. *Journal of Biological Chemistry*, **277**:28287-28297.

- Jones, R.C., J. Deck, R.D. Edmondson, and M.E. Hart.** 2008. Relative quantitative comparisons of the extracellular protein profiles of *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr*, and *sarA agr* regulatory mutants using one-dimensional polyacrylamide gel electrophoresis and nanocapillary liquid chromatography coupled with tandem mass spectrometry. *Journal of Bacteriology*, **190**:5265-5278.
- Kallen, A.J., Y. Mu, S. Bulens, A. Reingold, S. Petit, K. Gershman, S.M. Ray, L.H. Harrison, R. Lynfield, G. Dumyati, J.M. Townes, W. Schaffner, P.R. Patel, S.K. Fridkin, and Active bacterial core surveillance, M.I.o.t.E.I.P.** 2010. Health care-associated invasive MRSA infections, 2005-2008. *Journal of the American Medical Association*, **304**:641-648.
- Klebens, R.M., J.R. Edwards, C.L. Richards Jr., T.C. Horan, R.P. Gaynes, D.A. Pollock, and D.M. Cardo.** 2007. Estimating health care-associated infections and deaths in U.S. hospitals, 2002. *Public Health Reports*, **122**:160-166.
- Klebens, R.M., M.A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L.H. Harrison, R. Lynfield, G. Dumyati, J.M. Townes, A.S. Craig, E.R. Zell, G.E. Fosheim, L.K. McDougal, R.B. Carey, S.K. Fridkin, and Active Bacterial Core Surveillance, M.I.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Journal of the American Medical Association*, **298**:1763-1771.
- Kraemer, G.R., and J.J. Iandolo.** 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. *Current Microbiology*, **21**:373-376.
- Kweon, O., S.-J. Kim, J.H. Kim, S.W. Nho, D. Bae, J. Chon, M. Hart, D.-H. Baek, Y.-C. Kim, W. Wang, S.-K. Kim, J.B. Sutherland, and C.E. Cerniglia.** 2020.

CYPminer: An automated cytochrome P450 identification, classification, and data analysis tool for genome data sets across kingdoms. *BioMed Central Bioinformatics*, **21**:160.

Li, S., and M.J. Jedrzejewski. 2001. Hyaluronan binding and degradation by *Streptococcus agalactiae* hyaluronate lyase. *Journal of Biological Chemistry*, **276**: 41407-41416.

Li, S., S.J. Kelly, E. Lamani, M. Ferraroni, and M.J. Jedrzejewski. 2000. Structural basis of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. *European Molecular Biology Organization Journal*, **19**:1228-1240.

Luong, T.T., and C.Y. Lee. 2006. The *arl* locus positively regulates *Staphylococcus aureus* type 5 capsule via an *mgrA*-dependent pathway. *Microbiology*, **152**:3123-3131.

Madeira, F., Y. mi Park, J. Lee, N. Buso, T. Gur, N. Madhusoodanan, P. Basutkar, A.R.N. Tivey, S.C. Potter, R.D. Finn, and R. Lopez. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, **47**:W636-W641.

Maiti, R., G.H. Van Domselaar, H. Zhang, and D.S. Wishart. 2004. SuperPose: a simple server for sophisticated structural superposition. *Nucleic Acids Research*, **32**:W590-594.

Makris, G., J.D. Wright, E. Ingham, and K.T. Holland. 2004. The hyaluronate lyase of *Staphylococcus aureus* - a virulence factor? *Microbiology*, **150**:2005-2013.

McCaig, L.F., L.C. McDonald, S. Mandal, and D.B. Jernigan. 2006. *Staphylococcus aureus*-associated skin and soft tissue infections in ambulatory. *Emerging Infectious Diseases*, **12**:1715-1723

- McNamara, P.J., and J.J. Iandolo.** 1998. Genetic instability of the global regulator *agr* explains the phenotype of the *xpr* mutation in *Staphylococcus aureus* KSI9051. *Journal of Bacteriology*, **180**:2609-2615.
- Mello, L.V., B.L. De Groot, S. Li, and M.J. Jedrzejewski.** 2002. Structure and flexibility of *Streptococcus agalactiae* hyaluronate lyase complex with its substrate. Insights into the mechanism of processive degradation of hyaluronan. *Journal of Biological Chemistry*, **277**:36678-36688.
- Nukui, M., K.B. Taylor, D.T. McPherson, M.K. Shigenaga, and M.J. Jedrzejewski.** 2003. The function of hydrophobic residues in the catalytic cleft of *Streptococcus pneumoniae* hyaluronate lyase. Kinetic characterization of mutant enzyme forms. *Journal of Biological Chemistry*, **278**:3079-3088.
- Parthiban, V., M.M. Gromiha, and D. Schomburg.** 2006. CUPSAT: prediction of protein stability upon point mutations. *Nucleic Acids Research*, **34**:W239-242.
- Peng, H.-L., R.P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *Journal of Bacteriology*, **170**:4365-4372.
- Pettersson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K.E. Magnusson, and H. Wolf-Watz.** 1996. Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**:1231-1233.
- Rautela, G.S., and C. Abramson.** 1973. Crystallization and partial characterization of *Staphylococcus aureus* hyaluronate lyase. *Archives of Biochemistry and Biophysics*, **158**:687-694.

- Rigden, D.J., and M.J. Jedrzejewski.** 2003. Structures of *Streptococcus pneumoniae* hyaluronate lyase in complex with chondroitin and chondroitin sulfate disaccharides. *Journal of Biological Chemistry*, **278**:50596-50606.
- Rood, J.I., and S.T. Cole.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiological Reviews*, **55**:621-648.
- Rose, P.W., B. Beran, C. Bi, W.F. Bluhm, D. Dimitropoulos, D.S. Goodsell, A. Prlic, M. Quesada, G.B. Quinn, J.D. Westbrook, J. Young, B. Yukich, C. Zardecki, H.M. Berman, and P.E. Bourne.** 2011. The RCSB Protein Data Bank: redesigned web site and web services. *Nucleic Acids Research*, **39**:D392-401.
- Salentin, S., S. Schreiber, V.J. Haupt, M.F. Adasme, and M. Schroeder.** 2015. PLIP: fully automated protein-ligand interaction profiler. *Nucleic Acid Research*, **43**(W1):W443-447.
- Santos, K.B., I.A. Guedes, A.L.M. Karl, and L.E. Dardenne.** 2020. Highly flexible ligand docking: benchmarking of the DockThor program on the LEADS-PEP protein-peptide data set. *Journal of Chemical Information and Modeling*, **60**:667-683.
- Sassi, M., D. Sharma, S.R. Brinsmade, B. Felden, and Y. Augagneur.** 2015. Genome sequence of the clinical isolate *Staphylococcus aureus* subsp. *aureus* Strain UAMS-1. *Genome Announcements* 3.
- Senn, M.M., P. Giachino, D. Homerova, A. Steinhuber, J. Strassner, J. Kormanec, U. Fluckiger, B. Berger-Bachi, and M. Bischoff.** 2005. Molecular analysis and organization of the σ B operon in *Staphylococcus aureus*. *Journal of Bacteriology*, **187**: 8006-8019.

Shain, H., K.A. Homer, J. Aduse-Opoku, and D. Beighton. 1997. A conserved region of a hyaluronidase gene from *Streptococcus intermedius*. *Advances in Experimental Medicine and Biology*, **418**:769-772.

Sievert, D.M., P. Ricks, J.R. Edwards, A Schneider, J. Patel, A. Srinivasan, A. Kallen, B. Limbago, S. Fridkin, and the National Healthcare Safety Network (NHSN) Team and Participating NHSN Facilities. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection Control and Hospital Epidemiology*, **34**:1-14.

Smith, L.D. 1979a. *Clostridium botulinum*: characteristics and occurrence. *Reviews of Infectious Diseases*, **1**:637-641.

Smith, L.D. 1979b. Virulence factors of *Clostridium perfringens*. *Reviews of Infectious Disease*, **1**: 254-262.

Sobolev, V., A. Sorokine, J. Prilusky, E.E. Abola, and M. Edelman. 1999. Automated analysis of interatomic contacts in proteins. *Bioinformatics*, **15**:327-332.

Starr, C.R., and N.C. Engleberg. 2006. Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus. *Infection and Immunity*, **74**:40-48.

Stern, R., and M.J. Jedrzejewski. 2006. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chemical Reviews*, **106**:818-839.

Sun, J., O. Kweon, J. Jin, G.-X. He, X. Li, C.E. Cerniglia, and H. Chen. 2017. Mutation network-based understanding of pleiotropic and epistatic mutation behavior of *Enterococcus faecalis* FMN-dependent azoreductase. *Biochemistry and Biophysics Reports*, **12**:240-244.

- Tenover, F.C., L.K. McDougal, R.V. Goering, G. Killgore, S.J. Projan, J.B. Patel, and P.M. Dunman.** 2006. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. *Journal of Clinical Microbiology*, **44**:108-118.
- Tong, S.Y., L.F. Chen, and V.G. Fowler, Jr.** 2012. Colonization, pathogenicity, host susceptibility, and therapeutics for *Staphylococcus aureus*: what is the clinical relevance? *Seminars in Immunopathology*, **34**:185-200.
- Venselaar, H., T.A. Te Beck, R.K. Knipers, M.L. Hekkelman, and G. Vriend.** 2010. Protein structure analysis of mutations causing inheritable diseases. an e-science approach with life scientist friendly interfaces. *BioMed Central Bioinformatics*, **11**:548.
- Waldvogel, F.A.** 2000. *Staphylococcus aureus* (including staphylococcal toxic shock), p 2069-2092. *In Principles and Practice of Infectious Diseases. Edited by G.L. Mandell, J.E. Bennett, and R. Dolin.* Churchill Livingstone, Philadelphia.
- Yang, E.S., J. Tan, S. Eells, G. Rieg, G. Tagudar, and L.G. Miller.** 2010. Body site colonization in patients with community-associated methicillin-resistant *Staphylococcus aureus* and other types of *S. aureus* infections. *European Society of Clinical Microbiology and Infectious Diseases*, **16**:425-431.
- Yoon, S.-H., S.-M. Ha, J. Lim, S. Kwon, and J. Chun.** 2017. A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie van Leeuwenhoek*, **110**:1281–1286.

FIGURE LEGENDS

Fig. 1. A phylogenetic tree with multiple sequence alignment of 110 hyaluronidase sequences from *Staphylococcus aureus* strains known to have publicly available complete genome sequences. The hyaluronidase sequences show >82% identity to the reference sequence of HysA1 from strain UAMS-1. The tree was re-rooted using the reference sequence of HysA1 from strain UAMS-1 (top in the tree), and the G-480 residue of the HysA1 corresponds to position 519 in the aligned sequences. The labels consist of locus tag and strain name (i.e., fig|locus_tag [gene/protein name]|strain name). The completed genome sequences were retrieved from PATRIC (<https://www.patricbrc.org/>), and Clustal Omega of EMBL-EBI (Madeira et al. 2019) was used for sequence alignment and phylogenetic tree construction (neighbor-joining method). ITOL (<https://itol.embl.de/>) was used to visualize a phylogenetic tree with sequence alignment.

Fig. 2. Hyaluronidase activity of *S. aureus* UAMS-1 wild type (**A**) and mutant strains; UAMS-1 ($\Delta hysA2$) (**B**), UAMS-1 ($\Delta hysA1 \Delta hysA2$) (**C**), UAMS-1 ($\Delta hysA2$, pCL15) (**D**), UAMS-1 ($\Delta hysA2$, pCL15 $hysA1^+$) (**E**), and bovine testicular hyaluronidase (**F**).

Fig. 3. Three-dimensional structure of the *S. aureus* UAMS-1 HysA1 protein.

Schematic view of *S. aureus* UAMS-1 wild type HysA1 with a hexasaccharide substrate (shown as a stick model) (**A**). Surface view of the wild type HysA1 with close-up views of the tunnel structure at the active site in the catalytic cleft (**B**). Close-up view of the SpnHL protein and the *S. aureus* UAMS-1 wild type HysA1 containing the hexasaccharide substrates (green for SpnHL, red for HysA1 wild type protein, and blue

for HysA1 mutant protein, respectively). Catalytic residues (Asn-242, His-298, and Tyr-307) and the tunnel-forming residues (Asn-183, Trp-184, Trp-185, Arg-360, Arg-364, and Asn-478) of the HysA1 wild type are shown in stick form (C).

Draft

Table 1
Strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source
<i>S. aureus</i>		
RN4220	Nitrosoguanidine-induced restriction mutant	J.J. Iandolo, OUHSC ^a
Sanger-252	<i>hysA1</i> ⁺ , <i>hysA2</i> ⁺ , hyaluronidase activity detected	NARSA ^b
UAMS-1	<i>hysA1</i> , <i>hysA2</i> ⁺ , hyaluronidase activity detected	M.S. Smeltzer, UAMS ^c
UAMS-1 (Δ <i>hysA2</i>)	<i>hysA1</i> , Δ <i>hysA2</i> , no detectable hyaluronidase activity	Hart et al. 2013
UAMS-1 (Δ <i>hysA1</i> Δ <i>hysA2</i>)	Δ <i>hysA1</i> , Δ <i>hysA2</i> , no detectable hyaluronidase activity	This study
UAMS-1 (Δ <i>hysA2</i> , pCL15)	<i>hysA1</i> , Δ <i>hysA2</i> , containing pCL15, no hyaluronidase activity detected	This study
UAMS-1 (Δ <i>hysA2</i> , pCL15 <i>hysA1</i> ⁺)	<i>hysA1</i> , Δ <i>hysA2</i> , containing pCL15 <i>hysA1</i> ⁺ , hyaluronidase activity detected	This study
<i>E. coli</i>		
DH5 α	Competent strain for plasmid transformation	Invitrogen
TOP10	Competent strain for plasmid transformation	Invitrogen
Plasmids		
pCR2.1TOPO	<i>E. coli</i> cloning vector	Invitrogen
pCL15	Shuttle vector	C.L. Lee, UAMS ^c
pCL15 <i>hysA1</i> ⁺	Shuttle vector with Sanger 252 <i>hyaA1</i> ⁺	This study

^aOUHSC, Oklahoma University Health Science Center.
^bNARSA, Network for Antimicrobial Resistant *Staphylococcus aureus*.
^cUAMS, University of Arkansas for Medical Sciences.

Supplemental Table 1

	HysA2 (Sanger-252)	HysA2 (UAMS-1)	HysA1 (UAMS-1)	HysA1 (Sanger-252)
HysA2 (Sanger-252)	100.00	100.00	75.09	75.22
HysA2 (UAMS-1)	100.00	100.00	75.09	75.22
HysA1 (UAMS-1)	75.09	75.09	100.00	99.88
HysA1 (Sanger-252)	75.22	75.22	99.88	100.00

Draft

SUPPLEMENTAL FIGURE 1. A sequence alignment of the four protein sequences of hyaluronidases from *S. aureus* strains UAMS-1 and Sanger-252. The two strains have two hyaluronidases (i.e., HysA1 and HysA2). The residues Glu (E) and Gly (G) at the mutation position corresponding to the amino acid 480 at HysA1 is highlighted in yellow and red, respectively.

SUPPLEMENTAL FIGURE 2. A sequence alignment produced by SAS analysis (<https://www.ebi.ac.uk/thornton-srv/databases/sas/>) of the HysA1 protein sequence from *S. aureus* UAMS-1 against all protein sequences in the PDB. Sequence IDs are PDB codes except for HysA1. Sequences are amino acids corresponding to the residues 469-491 of the HysA1. Residues that are conserved across all sequences and residues in contact to ligand are denoted by asterisk (*) and black circle, respectively. The residue Glu at the mutation position (Glu-480 in HysA1) is highlighted in red. For more detailed structural information of the retrieved sequences, refer to RCSB Protein Data Bank (RCSB PDB) (<http://www.rcsb.org/pdb/home/home.do>).

Figure 1.

Figure 2.

Figure 3.

Supplemental Figure 1

HysA2_Sanger-252_UPI0001B70206_Sanger-252	MTYRMKKWKQLSTITLLMAGVITLNGGEFRSIDKHQIADVTNVQTTDYEKLRNIWLDVN	60
HysA2_fig 46170.369.peg.2120_UAMS-1	MTYRMKKWKQLSTITLLMAGVITLNGGEFRSIDKHQIADVTNVQTTDYEKLRNIWLDVN	60
HysA1_fig 46170.369.peg.1707_UAMS-1	MTNRMKRWKQLSTITLLMTGVIALNNGEFRNVDRHQIADVTNVQTPDYEKLRKIWLDVN	60
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	MTNRMKRWKQLSTITLLMTGVIALNNGEFRNVDRHQIADVTNVQTPDYEKLRKIWLDVN	60
	*****:*****.*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	YGYDKYDENNPIMKKKFEATENEAEKLLKEMKTESDRKYLWESSKDLTKSADMTKTYRN	120
HysA2_fig 46170.369.peg.2120_UAMS-1	YGYDKYDENNPIMKKKFEATENEAEKLLKEMKTESDRKYLWESSKDLTKSADMTKTYRN	120
HysA1_fig 46170.369.peg.1707_UAMS-1	YGYDQYDENNQIMKKKFDAKEKEAKLLDDMKTDNRTYLSWGAENLETNSHMTKTYRN	120
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	YGYDQYDENNQIMKKKFDAKEKEAKLLDDMKTDNRTYLSWGAENLETNSHMTKTYRN	120
	*****:*****:*****.*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	IEKI SEAMKHKNTKLKT DENKTKVKDALEWLHKNAYGKE PDKKVADLT SNFNK- TSRT	179
HysA2_fig 46170.369.peg.2120_UAMS-1	IEKI SEAMKHKNTKLKT DENKTKVKDALEWLHKNAYGKE PDKKVADLT SNFNK- TSRT	179
HysA1_fig 46170.369.peg.1707_UAMS-1	IEKIAESMQHKNTVLKTVENKLIK EALDWMHKNVYGNPSQKVEDLTKNRKGQTPKNN	180
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	IEKIAESMQHKNTVLKTVENKLIK EALDWMHKNVYGNPSQKVEDLTKNRKGQTPKNN	180
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	NLNWWDYEIGT PRALNTLILLQE DFTDEEKKYITAP IKT FAPDSDKI LSSVGKSEPAKG	239
HysA2_fig 46170.369.peg.2120_UAMS-1	NLNWWDYEIGT PRALNTLILLQE DFTDEEKKYITAP IKT FAPDSDKI LSSVGKSEPAKG	239
HysA1_fig 46170.369.peg.1707_UAMS-1	SLNWWDYEIGT PRALNTLLMDMLTKDEMKNYSKP ISTYAPSSDKI LSSVGSEDAKG	240
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	SLNWWDYEIGT PRALNTLLMDMLTKDEMKNYSKP ISTYAPSSDKI LSSVGSEDAKG	240
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	GNLVDISKVKLLESIEEDKIMMKKS IDS FNTVFTYAQNSATGKERNGFYKDSYIDHQD	299
HysA2_fig 46170.369.peg.2120_UAMS-1	GNLVDISKVKLLESIEEDKIMMKKS IDS FNTVFTYAQNSATGKERNGFYKDSYIDHQD	299
HysA1_fig 46170.369.peg.1707_UAMS-1	GNLVDISKVKLLESVIEEDVIMLKKS IDS FNVKFTYVQDSATGGRNGFYKDSYIDHQD	300
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	GNLVDISKVKLLESVIEEDVIMLKKS IDS FNVKFTYVQDSATGGRNGFYKDSYIDHQD	300
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	VPYTGAYGVVLEIGISOMPMIKETPFNDSNQNDITLKS WIDDGFMPLIYKGEMMDLSRG	359
HysA2_fig 46170.369.peg.2120_UAMS-1	VPYTGAYGVVLEIGISOMPMIKETPFNDSNQNDITLKS WIDDGFMPLIYKGEMMDLSRG	359
HysA1_fig 46170.369.peg.1707_UAMS-1	VPYTGAYGVVLEIGISOMPMIKESPFKTT-QDNATLSNWIDEGFMPLIYKGEMMDLSRG	359
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	VPYTGAYGVVLEIGISOMPMIKESPFKTT-QDNATLSNWIDEGFMPLIYKGEMMDLSRG	359
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	RAISRENETSHSASATVMKSLRLSDTMDKSTKAKYKKIVKTSVESDSSYKQTDYLSYS	419
HysA2_fig 46170.369.peg.2120_UAMS-1	RAISRENETSHSASATVMKSLRLSDTMDKSTKAKYKKIVKTSVESDSSYKQTDYLSYS	419
HysA1_fig 46170.369.peg.1707_UAMS-1	RAISRENETSHSASATVMKSLRLNDTMDGSTKTRKQIVKTSVNSDSSYNQNNYINSYS	419
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	RAISRENETSHSASATVMKSLRLNDTMDGSTKTRKQIVKTSVNSDSSYNQNNYINSYS	419
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	DISKMKSLMEDSTISINGLTQQLKIYNDMDRVTYHNKGLDFAFGLSMTSKNARYESING	479
HysA2_fig 46170.369.peg.2120_UAMS-1	DISKMKSLMEDSTISINGLTQQLKIYNDMDRVTYHNKGLDFAFGLSMTSKNARYESING	479
HysA1_fig 46170.369.peg.1707_UAMS-1	DIAMKKKLMNDSTISRNDLTQQLKIYNDMDRVTYHNKGLDFAFGLSMTSKNARYENING	479
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	DIAMKKKLMNDSTISRNDLTQQLKIYNDMDRVTYHNKGLDFAFGLSMTSKNARYENING	479
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	NLKGWHTGAGMSYLYNSDVGHYRDNFWATADMRLAGTTTLNDEEPKSTDVKKSKITFV	539
HysA2_fig 46170.369.peg.2120_UAMS-1	NLKGWHTGAGMSYLYNSDVGHYRDNFWATADMRLAGTTTLNDEEPKSTDVKKSKITFV	539
HysA1_fig 46170.369.peg.1707_UAMS-1	NLKGWHTGAGMSYLYNSDVGHYRDNFWATADMRLAGTTTLNDEEPKSTDVKKSKITFV	536
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	NLKGWHTGAGMSYLYNSDVGHYRDNFWATADMRLAGTTTLNDEEPKSTDVKKSKITFV	536
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	GGTKFDQHASIGMDFENQDKTLTAKKSYFILNKKIVFLGTGIKSTDSSKNPVTIENRK	599
HysA2_fig 46170.369.peg.2120_UAMS-1	GGTKFDQHASIGMDFENQDKTLTAKKSYFILNKKIVFLGTGIKSTDSSKNPVTIENRK	599
HysA1_fig 46170.369.peg.1707_UAMS-1	GGTKLNNKYASIGMDFENQDKTLTAKKSYFILNKKIVFLGTGIKSTDSSKNPVTIENRK	596
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	GGTKLNNKYASIGMDFENQDKTLTAKKSYFILNKKIVFLGTGIKSTDSSKNPVTIENRK	596
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	ANDYKLYKDDTQTINSN--NQEINSLFLESTNSTQNNIGYHFLNESKITVKKESHTGKWS	657
HysA2_fig 46170.369.peg.2120_UAMS-1	ANDYKLYKDDTQTINSN--NQEINSLFLESTNSTQNNIGYHFLNESKITVKKESHTGKWS	657
HysA1_fig 46170.369.peg.1707_UAMS-1	ANGYKLFKDDIEITSDVNAQETHSVFLES-NDTKRNIGYHFLDKPKITVKKESHTGKWS	655
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	ANGYKLFKDDIEITSDVNAQETHSVFLES-NDTKRNIGYHFLDKPKITVKKESHTGKWS	655
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	DINKSQDKIQKTDEYEVITQKHSNTDSKYAYVLYPGLSKDVFKSKASKVTIVKQEDDFHV	717
HysA2_fig 46170.369.peg.2120_UAMS-1	DINKSQDKIQKTDEYEVITQKHSNTDSKYAYVLYPGLSKDVFKSKASKVTIVKQEDDFHV	717
HysA1_fig 46170.369.peg.1707_UAMS-1	EINKSQKDDKDEYEVITQTHNTSDSKYAYVLYPGLSKSDFKSKNNVSVIVKQEDDFHV	715
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	EINKSQKDDKDEYEVITQTHNTSDSKYAYVLYPGLSKSDFKSKNNVSVIVKQEDDFHV	715
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	VKDNESVWAGINYSDSAKT FEINNITKVEVKAGMFILTKKDDNTYEC SFYNPESTNSVSD	777
HysA2_fig 46170.369.peg.2120_UAMS-1	VKDNESVWAGINYSDSAKT FEINNITKVEVKAGMFILTKKDDNTYEC SFYNPESTNSVSD	777
HysA1_fig 46170.369.peg.1707_UAMS-1	IKNDGVFAGVNYSDNTKSF DINGITVELKEKGMFVILKKDDKAYKCS FYNPETTNASN	775
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	IKNDGVFAGVNYSDNTKSF DINGITVELKEKGMFVILKKDDKAYKCS FYNPETTNASN	775
	*****:*****:*****:*****:*****:*****:*****:*****:*****	
HysA2_Sanger-252_UPI0001B70206_Sanger-252	IESKISMIGYSIINKNTSTSNESGVR FELTK	808
HysA2_fig 46170.369.peg.2120_UAMS-1	IESKISMIGYSIINKNTSTSNESGVR FELTK	808
HysA1_fig 46170.369.peg.1707_UAMS-1	IESKIFIKGYTITNKSVINSDAGVNFELTK	806
HysA1_Sanger-252_UPI0001C0B4F1_Sanger-252	IESKIFIKGYTITNKSVINSDAGVNFELTK	806
	*****:*****:*****:*****:*****:*****:*****:*****:*****	

Supplemental Figure 2

Table

Strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source
<i>S. aureus</i>		
RN4220	Nitrosoguanidine-induced restriction mutant	J.J. Iandolo, OUHSC ^a
Sanger-252	<i>hysA1</i> ⁺ , <i>hysA2</i> ⁺ , hyaluronidase activity detected	NARSA ^b
UAMS-1	<i>hysA1</i> , <i>hysA2</i> ⁺ , hyaluronidase activity detected	M.S. Smeltzer, UAMS ^c
UAMS-1 ($\Delta hysA2$)	<i>hysA1</i> , $\Delta hysA2$, no detectable hyaluronidase activity	Hart et al., 2013
UAMS-1 ($\Delta hysA1 \Delta hysA2$)	$\Delta hysA1$, $\Delta hysA2$, no detectable hyaluronidase activity	This study
UAMS-1 ($\Delta hysA2$, pCL15)	<i>hysA1</i> , $\Delta hysA2$, containing pCL15, no hyaluronidase activity detected	This study
UAMS-1 ($\Delta hysA2$, pCL15 <i>hysA1</i> ⁺)	<i>hysA1</i> , $\Delta hysA2$, containing pCL15 <i>hysA1</i> ⁺ , hyaluronidase activity detected	This study
<i>E. coli</i>		
DH5 α	Competent strain for plasmid transformation	Invitrogen
TOP10	Competent strain for plasmid transformation	Invitrogen
Plasmids		
pCR2.1TOPO	<i>E. coli</i> cloning vector	Invitrogen
pCL15	Shuttle vector	C.L. Lee, UAMS ^c
pCL15 <i>hysA1</i> ⁺	Shuttle vector with Sanger 252 <i>hyaA1</i> ⁺	This study

^aOUHSC, Oklahoma University Health Science Center.^bNARSA, Network for Antimicrobial Resistant *Staphylococcus aureus*.^cUAMS, University of Arkansas for Medical Sciences.

Figure 1

Fig. 1. A phylogenetic tree with multiple sequence alignment of 110 hyaluronidase sequences from *Staphylococcus aureus* strains known to have publicly available complete genome sequences. The hyaluronidase sequences show >82% identity to the reference sequence of HysA1 from strain UAMS-1. The tree was re-rooted using the reference sequence of HysA1 from strain UAMS-1 (top in the tree), and the G-480 residue of the HysA1 corresponds to position 519 in the aligned sequences. The labels consist of locus tag and strain name (i.e., fig|locus_tag [gene/protein name]|strain name). The completed genome sequences were retrieved from PATRIC (<https://www.patricbrc.org/>), and Clustal Omega of EMBL-EBI (Madeira et al., 2019) was used for sequence alignment and phylogenetic tree construction (neighbor-joining method). ITOL (<https://itol.embl.de/>) was used to visualize a phylogenetic tree with sequence alignment.

Fig. 2. Hyaluronidase activity of *S. aureus* UAMS-1 wild type (A) and mutant strains; UAMS-1 ($\Delta hysA2$) (B), UAMS-1 ($\Delta hysA1 \Delta hysA2$) (C), UAMS-1 ($\Delta hysA2$, pCL15) (D), UAMS-1 ($\Delta hysA2$, pCL15hysA1+) (E), and bovine testicular hyaluronidase (F).

92x109mm (600 x 600 DPI)

Figure 3

Fig. 3. Three-dimensional structure of the *S. aureus* UAMS-1 HysA1 protein. Schematic view of *S. aureus* UAMS-1 wild type HysA1 with a hexasaccharide substrate (shown as a stick model) (**A**). Surface view of the wild type HysA1 with close-up views of the tunnel structure at the active site in the catalytic cleft (**B**). Close-up view of the SpnHL protein and the *S. aureus* UAMS-1 wild type HysA1 containing the hexasaccharide substrates (green for SpnHL, red for HysA1 wild type protein, and blue for HysA1 mutant protein, respectively). Catalytic residues (Asn-242, His-298, and Tyr-307) and the tunnel-forming residues (Asn-183, Trp-184, Trp-185, Arg-360, Arg-364, and Asn-478) of the HysA1 wild type are shown in stick form (**C**).