Hyaluronidase expression and biofilm involvement in *Staphylococcus aureus* UAMS-1 and its *sarA*, *agr* and *sarA agr* regulatory mutants

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In a previous study, two proteins identified as hyaluronidases were detected in spent media by MS and found to be in greater quantity in the *sarA* and *sarA agr* mutant strains when compared with the parent and *agr* mutant strains of *Staphylococcus aureus* UAMS-1. In the present study, spent media and total RNA were isolated from UAMS-1 and its regulatory mutants and analysed for hyaluronidase activity and steady-state hyaluronidase (*hysA*) RNA message levels. Hyaluronidase activity was observed throughout all time points examined regardless of the regulatory effects of *sarA* and *agr* but activity was always substantially higher in the *sarA* and *sarA agr* mutant strains than in the UAMS-1 parent and *agr* mutant strains. Northern analysis did not detect *hysA* message for either the UAMS-1 parent or the *agr* mutant strains at any time point examined, while steady-state *hysA* message levels were detected throughout growth for the *sarA* mutant strain, but only at exponential and early post-exponential growth for the *sarA agr* mutant strain. An *in vitro* biofilm plate assay, pre-coated with human plasma as a source of hyaluronic acid, demonstrated no significant increase in biofilm for a *sarA* mutant strain of *S. aureus* UAMS-1 defective in hyaluronidase activity when compared with the *sarA* mutant strain. These data indicate that, while *hysA* message levels and hyaluronidase activity are elevated in the *sarA* mutant strains of *S. aureus* UAMS-1, the increase in activity did not contribute to the biofilm-negative phenotype observed in the *sarA* mutant strain of *S. aureus* UAMS-1.

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium of both community-associated and hospital-acquired diseases that accounts for 14.9 and 18.8% of all bacterial infections encountered in the clinical setting as either outpatient or inpatient cases, respectively (Styers *et al.*, 2006). Just as important and of great concern is the continual rise in the number of methicillin (oxacillin)-resistant *S. aureus* (MRSA) encountered in the hospital setting and now in the

community (Diekema *et al.*, 2001; Löffler & MacDougall, 2007; Saïd-Salim *et al.*, 2003; Styers *et al.*, 2006). This concern is compounded further by the occurrence of clinical strains resistant to intermediate (Hiramatsu *et al.*, 1997; Srinivasan *et al.*, 2002) and high (Chang *et al.*, 2003; Tenover *et al.*, 2004; Whitener *et al.*, 2004) levels of vancomycin, an antibiotic of last resort for infections involving multiple antibiotic-resistant, hospital-acquired MRSA (Maple *et al.*, 1989).

As an aid to the discovery of new potential targets for the development of alternative approaches for the prevention and treatment of staphylococcal diseases, a number of laboratories have utilized whole-genome sequencing and microarray and proteomic technologies to generate comprehensive databases of *S. aureus* (Hecker *et al.*, 2003;

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Abbreviations: HA, hyaluronic acid; MRSA, methicillin-resistant *S. aureus*.

Lindsay & Holden, 2004). Likewise, we have generated a comprehensive protein database for the clinical osteomyelitis isolate *S. aureus* UAMS-1 and its regulatory mutants, *sarA* and *agr*, using one-dimensional PAGE (1D-PAGE) followed by high-sensitivity, nano-liquid chromatography (nanoLC) coupled to tandem mass spectrometry (MS/MS) (Jones *et al.*, 2008). This approach identified numerous proteins whose abundance in spent media was dependent upon the virulence gene global regulators *sarA* and *agr* (Jones *et al.*, 2008). Two proteins in particular whose abundance was greater in the *sarA* and *sarA agr* mutant strains as compared with the parent strain, UAMS-1, and its *agr* mutant strain, were identified as hyaluronidases (Jones *et al.*, 2008).

Bacterial hyaluronidases (also referred to as hyaluronate lyase) are a group of enzymes that degrade hyaluronic acid (HA) but also have limited ability to degrade chondroitin and chondroitin sulfate (Hynes & Walton, 2000; Stern & Jedrzejewski, 2006). HA is a linear unsulfated glycosaminoglycan polymer made up of β 1–4 alternating units of D-glucuronic acid-[1–3]- β -N-acetylglucosamine (Hynes & Walton, 2000; Stern & Jedrzejewski, 2006). This polymer is 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) and it is a versatile component of many physiological processes, including cell proliferation, differentiation, migration and adhesion, as well as inflammation, wound healing, water homeostasis and viscoelasticity of the extracellular matrix (Girish & Kemparaju, 2007). Hyaluronidases have been found in a variety of Gram-positive and Gram-negative bacteria and a number of these have been implicated in the disease process as a way to invade and penetrate tissues (Hynes & Walton, 2000). For example, the assumption has been that the glycoside hydrolase activity exhibited by hyaluronidase (Mu toxin) and other toxins produced by *Clostridium perfringens* contributes to virulence by destroying connective tissue, which promotes dissemination of the organism and/or facilitates the action of the more potent β -toxin (Rood & Cole, 1991; Smith, 1979). However, the exact role of the clostridial hyaluronidase in virulence has not been determined even though the gene (*nagH*) for HA degradation has been cloned and sequenced (Canard *et al.*, 1994) and the catalytic portion of one (Ficko-Blean & Boraston, 2005) of the four known homologues (Shimizu *et al.*, 2002) has been crystallized. More recently, Starr & Engleberg (2006) demonstrated, using two clinical isolates of *Streptococcus pyogenes* and their corresponding hyaluronidase-defective mutant strains, that hyaluronidase facilitated the dissemination of dextran blue but not *Strep. pyogenes* cells in a mouse model of abscess formation. This allowed Starr & Engleberg (2006) to hypothesize that streptococcal hyaluronidase activity may provide the micro-organism with a potential source of energy, a hypothesis supported by the fact that *Strep. pyogenes* parental strains were able to use HA as a sole source of carbon while hyaluronidase mutants were not. Others have also reported a role for hyaluronidase both in disease and in the acquisition of a suitable carbon

and energy source (Cheng *et al.*, 1995; Homer *et al.*, 1997; Shain *et al.*, 1996).

In a previous study, hyaluronidase expression in *S. aureus* 8325-4 was shown to be activated by *agr* and repressed by *sarA*, as determined by hyaluronidase-specific activity in spent media (Makris *et al.*, 2004). This study also showed that a mutation in the hyaluronidase gene resulted in reduced virulence in a mouse abscess model. However, these studies were conducted with *S. aureus* 8325-4, a laboratory strain known to contain a deletion within the *rsbU* gene (Kullik *et al.*, 1998). The product of the *rsbU* gene is involved in regulating the activity of SigB (Gertz *et al.*, 2000; Giachino *et al.*, 2001; Pané-Farré *et al.*, 2006; Senn *et al.*, 2005), which is the post-exponential sigma factor in *S. aureus* that controls the expression of numerous *S. aureus* virulence factors (Kullik *et al.*, 1998). In addition, DNA genomic databases indicate that at least five *S. aureus* strains contain two open reading frames (ORFs) identified as genes for hyaluronidase (Kersey *et al.*, 2005) whereas *S. aureus* 8325 and the remaining sequenced strains contain only one gene identified as hyaluronidase (Kersey *et al.*, 2005). Given the aforementioned observations and our previous proteomic studies that identified two proteins as hyaluronidases whose abundance was greater in the *sarA* and *sarA agr* mutant strains (Jones *et al.*, 2008), we decided to assess the regulatory role of *sarA* and *agr* for hyaluronidase in *S. aureus* strain UAMS-1. This is a clinical strain isolated from a patient with osteomyelitis (Gillaspy *et al.*, 1995) and does not have an *rsbU* mutation; it is thus safe to assume that this strain is not deficient in SigB activity (Cassat *et al.*, 2006). In addition, because hyaluronidase has been shown to be involved in biofilm dispersal in *Streptococcus intermedius* (Pecharki *et al.*, 2008) and *sarA* mutant strains of *S. aureus* are known to be deficient in biofilm formation (Beenken *et al.*, 2003; Valle *et al.*, 2003) as well as to express elevated amounts of hyaluronidase (Jones *et al.*, 2008; Makris *et al.*, 2004) we examined whether *S. aureus* hyaluronidase is involved in biofilm formation and/or dispersal.

METHODS

Strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Overnight (15–18 h) cultures of *S. aureus*, grown in trypticase soy broth (TSB; Difco Laboratories) containing the appropriate antibiotic [either 50 μ g ml⁻¹ each of kanamycin or neomycin (both Sigma) for the *sarA* mutant strain, 5 μ g tetracycline ml⁻¹ (Sigma) for the *agr* mutant strain, or all three antibiotics for the *sarA agr* mutant strain], were harvested by centrifugation, washed twice in sterile TSB and used to inoculate sterile TSB without antibiotic. Flasks were incubated at 37 °C with rotary aeration (180 r.p.m.) and growth was monitored spectrophotometrically at 550 nm.

Spent media and cell lysate preparation. Spent media were prepared in two ways. For comparison of *S. aureus* UAMS-1 and its regulatory mutant strains at various points in growth, spent media were prepared as described by Jones *et al.* (2008) except precipitation with trichloroacetic acid (TCA) was excluded as staphylococcal hyaluronidase activity is sensitive to TCA (data not shown). Briefly,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source
<i>S. aureus</i>		
Newman	Osteomyelitis isolate (Duthie & Lorenz, 1952)	T. Foster, TC*
Newman (<i>sarA</i>)	<i>sarA::erm</i>	T. Foster, TC
Newman (<i>agr</i>)	Δ <i>agr::tet</i>	T. Foster, TC
RN4220	Nitrosoguanidine-induced restriction mutant	J. J. Iandolo, OUHSC†
Sanger-252	HA-MRSA	NARSA‡
Sanger-252 (<i>hysA1</i>)	Δ <i>hysA1</i>	This study
Sanger-252 (<i>hysA2</i>)	Δ <i>hysA2</i>	This study
UAMS-1	Osteomyelitis isolate, <i>rsbU</i> ⁺	M. S. Smeltzer, UAMS§
UAMS-1 (<i>sarA</i>)	<i>sarA::kan</i>	M. S. Smeltzer, UAMS
UAMS-1 (<i>agr</i>)	Δ <i>agr::tet</i>	M. S. Smeltzer, UAMS
UAMS-1 (<i>sarA/agr</i>)	<i>sarA::kan</i> Δ <i>agr::tet</i>	M. S. Smeltzer, UAMS
UAMS-1 (<i>hysA2</i>)	Δ <i>hysA2</i>	This study
UAMS-1 (<i>hysA1 hysA2</i>)	Δ <i>hysA1</i> Δ <i>hysA2</i>	This study
UAMS-1 (<i>sarA hysA2</i>)	<i>sarA::kan</i> Δ <i>hysA2</i>	This study
UAMS-1 (<i>sarA hysA1 hysA2</i>)	<i>sarA::kan</i> Δ <i>hysA1</i> Δ <i>hysA2</i>	This study
USA300 (LAC)	CA-MRSA	NARSA
USA300 (Miss)	CA-MRSA	NARSA
USA300 (<i>hysA</i>)	Δ <i>hysA</i>	This study
<i>E. coli</i>		
DH5 α	Susceptible to gyrase inhibitor protein coded (<i>ccdB</i>) on pKOR1	Invitrogen
TOP10	Competent strain for plasmid transformation	Invitrogen
Plasmid		
pCR4-TOPO	<i>E. coli</i> cloning vector	Invitrogen
pKOR1	Allelic replacement vector (1)	O. Schneewind, UCII

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40 ml cultures were standardized using optical density determinations with sterile TSB as diluent. Cells were harvested by centrifugation (12 000 g, for 10 min at 4 °C) and spent media were filter-sterilized, quick-frozen with dry ice and ethanol, and stored overnight at –80 °C. Frozen spent media were lyophilized to dryness, reconstituted with water at one-tenth the original volume and combined. Spent media were further concentrated using ultrafiltration [Amicon Ultra-4 filters with 3000 kDa molecular weight cut-off (MWCO); Millipore] and overnight centrifugation (2000 g) at 4 °C. The retentates were recovered and stored at –80 °C until needed.

Spent media prepared for comparative purposes among *S. aureus* strains were prepared as described by Hart *et al.* (2009). Briefly, cell density values of overnight cultures (15–18 h) 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 g, for 10 min at 4 °C) and spent media were filter-sterilized and concentrated by centrifugation (7500 g) for 1 h using ultrafiltration (Centricon Ultracel YM-3 filters with 3000 kDa MWCO; Millipore). Retentates were stored at –80 °C until used.

Cell lysates from *Escherichia coli* strains containing the appropriate *hysA* plasmid clones were prepared as described by Blevins *et al.* (2002). Briefly, cultures were standardized using optical density determinations at 550 nm and sterile TSB as diluent. Portions of 3 ml were removed for isolation of plasmids, and cells from the remaining

culture volume were harvested by centrifugation (12 000 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 of TE. Cell suspensions were pipetted into FastPROTEIN™ BLUE tubes (MP Biochemical) containing silica beads and cells were lysed using a Fast Prep FP120 reciprocator (MP Biochemical) at 6 m s^{–1} for 40 s. Cell lysates were cooled on ice for 15 min followed by centrifugation (10 000 g for 1 min at 4 °C). The supernatants were collected and frozen at –80 °C until used.

PCR and *hysA* cloning. Primers (Table 2) for *hysA* amplification were designed using the completed genome sequence of *S. aureus* Sanger 252 (Holden *et al.*, 2004). Chromosomal DNA isolated from *S. aureus* Sanger 252 and UAMS-1 was used as template and PCR was conducted with the high-fidelity Platinum Taq DNA polymerase as instructed by the manufacturer (Invitrogen). Amplicons (ca. 4.0 kbp in size) consisting of the ORF and approximately 1000 bases up- and downstream were cloned into pCR4-TOPO (Invitrogen) and transformed into *E. coli* TOP10 (Invitrogen). Transformants resistant to carbenicillin (100 µg ml^{–1}) and exhibiting a white to light blue colonial appearance on tripticase soy agar (TSA) containing X-Gal were procured. Plasmids were isolated using a Qiagen Plasmid Mini kit (Qiagen Inc.), amplicon orientation was determined by restriction endonuclease digestion and the identity of the amplicon was confirmed by sequencing the 5' and 3' ends of each cloned insert.

Table 2. PCR primers used in this study

Name	Oligonucleotide sequence (5'→3')*
hysA1F	GAAATACCCACCCATCGGA
hysA1R	CCAAGCAAGTATAGATTCGGGA
hysA2F	AAGCGATTCAATACTGACGCCTGC
hysA2R	AGTGTAGACGATTCAGGTAGCGCA
hysA1FattB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCATCACTCTAATCTCCCCG
hysA1RSacII	GGACCT CCGCGG CGAAAAAGCAAATCAGCGCGAC
hysA1FSacII	GGACCT CCGCGG CTCGGCACTATAACATTTTATAG
hysA1RattB2	GGGACCACTTTGTACAAAGAAAGCTGGGTCTTTTCAGGTCCAATTGAATAAATG
hysA2FattB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCTTCTGCTGTTTGTTCCTG
hysA2RSacII	GGACCT CCGCGG CCAAACATAAAGTAAAGGATAGTC
hysA2FSacII	GGACCT CCGCGG CTTGTAAATCCGCAGACCACATC
hysA2RattB2	GGGGACCACTTTGTACAAAGAAAGCTGGGTGAAGCGAGAGTTTAGTTATTGGC
hysAF	GTGGATTGTTTGACAGTAGACAG
hysAR	CGGTATTTGTAGATTCGGGATTATAG

*The restriction site for *SacII* is indicated by bold type.

hysA mutation. Allelic replacement was accomplished using the method described by Bae & Schneewind (2006) and primers (Table 2) designed from the genomic DNA databases of *S. aureus* Sanger-252 (Holden *et al.*, 2004) and *S. aureus* FPR3757 (USA300) (Diep *et al.*, 2006). Primers hysA1FattB1 and hysA1RSacII and primers hysA1FSacII and hysA1RattB2 were used to generate 628 and 723 bp amplicons, up- and downstream of the ORF of *hysA1*, respectively. Likewise, primers hysA2FattB1 and hysA2RSacII and primers hysA2FSacII and hysA2RattB2 were used to generate 603 and 769 bp amplicons, up- and downstream of the ORF of *hysA2*, respectively. Amplicons were digested with *SacII* and the appropriate amplicon pairs were ligated prior to lambda-mediated integration into plasmid pKOR1 using the Gateway BP Clonase system (Invitrogen). Integration products were transformed into *E. coli* DH5 α , and plasmids isolated from colonies growing in the presence of carbenicillin (100 μ g ml⁻¹) were verified to contain the appropriate insert by PCR. Plasmids were then transformed into *S. aureus* RN4220 by electroporation and subsequently moved into *S. aureus* UAMS-1, Sanger-252 and USA300 (Miss) by ϕ 11-mediated transduction. Plasmid-bearing strains were grown overnight at 43 °C in the presence of chloramphenicol (10 μ g ml⁻¹), followed by a second overnight incubation at 43 °C in the presence of chloramphenicol (7.5 μ g ml⁻¹). Cultures were then diluted and plated on TSA containing chloramphenicol (10 μ g ml⁻¹) and incubated again at 43 °C. Isolated colonies were inoculated into TSB containing chloramphenicol (10 μ g ml⁻¹) and incubated overnight at 30 °C to promote plasmid excision. Cultures were again diluted and plated on TSA containing anhydrotetracycline (1.0 μ g ml⁻¹) to select for *hysA* deletion mutants devoid of plasmids.

RNA isolation and Northern analysis. Isolation of total RNA was done as described by Hart *et al.* (1993). Twofold serial dilutions were made from 5 μ g of isolated RNA and denatured in the presence of glyoxal and DMSO. RNA was resolved by agarose gel electrophoresis and transferred onto neutral nylon membranes (MagnaGraph; Micron Separations) by passive diffusion. Membranes were hybridized overnight at 65 °C with digoxigenin (DIG) 11-UTP (Roche Molecular Biochemicals) -labelled probes of amplicons generated by PCR using either *S. aureus* UAMS-1 or RN6390 (8325 lineage) chromosomal DNA as template and primers hysAF and hysAR (Table 2) designed from the known genomic sequence of *S. aureus* N315. Hybridizing probes were detected by autoradiography using alkaline phosphatase-conjugated antidigoxigenin F(ab')₂ antibody fragments

(Roche Molecular Biochemicals) and the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals). Membranes were then stripped accordingly (Roche Molecular Biochemicals) and hybridized with a DIG-labelled DNA probe specific for 16S rRNA (Snodgrass *et al.*, 1999) to ensure equal RNA loading and transfer to membranes between samples.

HA plate assay for the detection of hyaluronidase activity.

Hyaluronidase activity was determined using the method described by Steiner & Cruce (1992) with modifications described by Hart *et al.* (2009). Briefly, 1% (w/v) BSA (Fraction V; Fisher Scientific), and 0.8 mg HA ml⁻¹ (Sigma, H-1504; potassium salt, from human umbilical cord) in 0.3 M sodium phosphate buffer (pH 5.3) and 1% (w/v) SeaKem LE agarose (Cambrex BioScience) were poured into sterile plastic square plates and allowed to solidify. Wells (4 mm diameter) were made aseptically, and spent medium (20 μ l) was pipetted into each well. Plates were flooded with 2 M acetic acid after overnight (15–18 h) incubation at 37 °C. Zones of clearing were visualized against a background of opaque precipitated HA conjugated to BSA and their diameters were measured in millimetres. Purified bovine testicular hyaluronidase (Sigma, H-3506) was used as a positive control.

Biofilm microtitre plate assay.

Biofilm formation was determined using 96-well flat bottom plates (Costar; Corning Inc.) as described by Beenken *et al.* (2003) except that *S. aureus* strains were grown without antibiotic selection in either filter-sterilized TSB (Difco) or filter-sterilized TSB supplemented with 0.5% (w/v) glucose and 3.0% (w/v) NaCl. TSB containing glucose and NaCl has been shown to enhance biofilm formation in *S. aureus* (Beenken *et al.*, 2003; Götz, 2002). Overnight (15–18 h) cultures were diluted to an OD₅₅₀ of 0.05 with fresh media and dispensed as 0.2 ml portions into each well, which had been coated overnight at 4 °C with 20% human plasma (Sigma, P-9523). Plates were then incubated overnight (ca. 24 h) at 37 °C prior to cell suspension removal and subsequent washing of wells with sterile PBS. Biofilm in each well was stained with 0.2 ml of crystal violet [0.4% (w/v) in water], washed with water and allowed to dry overnight. Stained biofilm was suspended in 0.2 ml of acid alcohol (40% ethanol and 10% acetic acid in water) and diluted 10-fold in water to ensure all optical density values fell within the linear portion of a standard curve of optical density as a function of increasing concentrations of crystal violet. Diluted biofilm suspensions were quantified using a SpectraMax Plus 384 plate reader

(Molecular Devices) and a wavelength of 595 nm. Two independent biological determinations with four replicates per determination ($n=8$) were used to generate geometric least-squares means based on two-way ANOVA of log-transformed data, with effects of strain, date of independent determination and their interaction. Unadjusted pairwise comparisons were performed between all strains within each group (TSB or TSB + glucose + NaCl). All tests were performed with a 0.05 level of significance. Values presented represent the back-transformed least-squares means and their corresponding 95% confidence limits.

RESULTS

Relative hyaluronidase activity of *S. aureus* strains UAMS-1 and Newman and their corresponding regulatory mutant strains

Spent media isolated from overnight (15–18 h) cultures of *S. aureus* strains UAMS-1 and Newman and their corresponding regulatory mutant strains and diluted to OD₅₅₀ ~3 were analysed for hyaluronidase activity using the HA plate assay (Table 3). Hyaluronidase activity of the *S. aureus* UAMS-1 *sarA* and *sarA agr* mutant strains gave zones of clearing approximately twice that of the UAMS-1 wild-type and its *agr* mutant strain (Table 3). Similar results were also observed with *S. aureus* Newman and its isogenic *sarA* and *agr* mutant strains (Table 3).

Table 3. Hyaluronidase activity of *S. aureus* strains and their corresponding *sarA*, *agr* and *hysA* mutant strains

Values represent the mean $\pm$ SD of three independent determinations minus the diameter of the well.

Strain (genotype)	Hyaluronidase activity [diameter (mm) of clearance]
Newman (wild-type)	5.8 $\pm$ 2.5
Newman (<i>sarA::erm</i>)	13.2 $\pm$ 1.0
Newman (Δ <i>agrA::tet</i>)	8.0 $\pm$ 1.0
Sanger-252 (wild-type)	7.2 $\pm$ 2.0
Sanger-252 (Δ <i>hysA1</i>)	7.0 $\pm$ 0.5
Sanger-252 (Δ <i>hysA2</i>)	6.7 $\pm$ 0.6
UAMS-1 (wild-type)	9.0 $\pm$ 1.3
UAMS-1 (<i>sarA::kan</i>)	17.7 $\pm$ 0.3
UAMS-1 (Δ <i>agrA::tet</i>)	9.0 $\pm$ 0
UAMS-1 (<i>sarA::kan</i> Δ <i>agrA::tet</i>)	17.0 $\pm$ 0
UAMS-1 (Δ <i>hysA2</i>)	0 $\pm$ 0
UAMS-1 (Δ <i>hysA1</i> Δ <i>hysA2</i>)	0 $\pm$ 0
UAMS-1 (<i>sarA::kan</i> Δ <i>hysA2</i>)	0 $\pm$ 0
UAMS-1 (<i>sarA::kan</i> Δ <i>hysA1</i> Δ <i>hysA2</i>)	0 $\pm$ 0
USA 300 (LAC)	10.2 $\pm$ 1.6
USA 300 (Miss)	9.8 $\pm$ 0.3
USA 300 (Miss) (<i>hysA</i>)	0 $\pm$ 0

hysA mutant strains of *S. aureus* UAMS-1, Sanger-252 and USA300 (Miss)

In our previous study (Hart *et al.*, 2009 and Table 3) hyaluronidase activity and the number of *hysA* genes present were determined for two pulsed-field type USA300 strains (LAC and Miss). Both strains exhibited similar levels of hyaluronidase activity and were shown by Southern analysis to contain probably one gene for *hysA* (Hart *et al.*, 2009). However, examination of the three available pulsed-field type USA300 genomic databases revealed that two of these strains (FPR3757 and TCH959) contain one gene for *hysA* (Diep *et al.*, 2006; Highlander *et al.*, 2007), whereas the third strain (TCH1516) contains two (Highlander *et al.*, 2007). Given the clonality of the USA300 pulsed-field type (Kennedy *et al.*, 2008) we reasoned that the DNA sequence from FPR3757 could be used to generate the *hysA* mutant in USA300 (Miss). It has been shown previously that *S. aureus* Sanger-252 and UAMS-1 are related (Cassat *et al.*, 2005). Therefore, the DNA sequence of the genomic database for Sanger-252 (Holden *et al.*, 2004) was utilized to generate the *hysA1* and *hysA2* mutations in Sanger-252 and to generate the mutations in UAMS-1. Utilizing these sequences, mutant strains defective in hyaluronidase activity were constructed in *S. aureus* UAMS-1, Sanger-252 and USA300 (Miss) using the method of Bae & Schneewind (2006). Loss of hyaluronidase function was verified using HA plates (Table 3). Deletion of either *hysA1* or *hysA2* in Sanger-252 resulted in hyaluronidase activity similar to that observed for the parent strain while the deletion of the *hysA* in USA300 (Miss) resulted in complete loss of hyaluronidase activity (Table 3). Interestingly, the *S. aureus* UAMS-1 *hysA2* mutant exhibited no detectable hyaluronidase activity, suggesting that the HysA1 protein in UAMS-1 might be defective in activity even though our previous proteomic study detected the protein in spent media (Jones *et al.*, 2008). To determine if the defect in hyaluronidase activity resided within the coding region of *hysA1*, the genes for *hysA1* and *hysA2* from UAMS-1 and Sanger-252 were amplified by PCR and cloned into an *E. coli* plasmid. Cloned amplicons (amplicons included 1000 bp up- and downstream of the ORFs) were verified by restriction endonuclease digestion and sequenced (data not shown). In addition, spent media and cell lysates from several of the *E. coli* clones containing plasmids with either the *hysA1* or the *hysA2* genes from either UAMS-1 or Sanger-252 were generated and assessed for hyaluronidase activity. All *E. coli* clones, except those containing the *hysA1* gene from UAMS-1, exhibited activity (data not shown). Comparative sequence analysis of the coding region of the UAMS-1 *hysA1* gene with other *S. aureus* *hysA1* genes, including the gene from Sanger-252, indicates that the UAMS-1 *hysA1* gene contains a point mutation in a conserved region that results in a glutamic acid to glycine change at amino acid position 480 (data not shown). The Sanger-252 *hysA1* gene cloned on a plasmid was able to restore hyaluronidase activity in the UAMS-1 *hysA1* *hysA2* mutant strain while the UAMS-1 *hysA1* gene was not (data not shown).

Hyaluronidase activity and *hysA* expression of *S. aureus* UAMS-1 and its regulatory mutant strains at various stages of *in vitro* growth

Spent media and total RNA were isolated from *S. aureus* UAMS-1 and its regulatory mutants at 3, 6, 12 and 24 h and assessed for hyaluronidase activity and *hysA* expression (Figs 1 and 2). Spent media were concentrated approximately 200-fold and serially diluted 10-fold prior to assessing activity. The pattern of hyaluronidase activity was similar throughout growth, in that hyaluronidase activity of the UAMS-1 parent and *agr* mutant strains was approximately 100-fold less than the *sarA* and *sarA agr* mutant strains (Fig. 1). A notable change in activity at 24 h was an approximately 10-fold reduction and increase in activity for the *sarA agr* and *agr* mutant strains, respectively, as compared with activity observed at 3, 6 and 12 h (Fig. 1). Whether these observed changes in activity at 24 h are related to the regulatory roles of *sarA* and *agr* is not known, but is currently under investigation. Regardless of the effect of either *sarA* or *agr*, hyaluronidase activity was detected throughout growth.

When total RNA was isolated and hybridized with a probe specific for both *hysA1* and *hysA2*, steady-state *hysA* message was not detected for either UAMS-1 or its *agr* mutant strains at any time point examined (Fig. 2). In contrast, *hysA* message was detected for the *sarA* mutant strain at all time points and for the *sarA agr* mutant strain at the 3 and 6 h time points (Fig. 2). While probes used for Northern analysis did not differentiate between *hysA1* and

hysA2 steady-state message levels, preliminary results obtained by real-time RT-PCR using specific probes for each gene and total RNA isolated from 3 h cultures of UAMS-1 and its mutant strains indicate comparable expression levels for *hysA1* and *hysA2* (data not shown).

Loss of hyaluronidase activity does not increase biofilm formation

Biofilm dispersal in *Strep. intermedius* was shown to involve hyaluronidase (Pecharki *et al.*, 2008). Because *sarA* mutants of *S. aureus* are biofilm deficient (Beenken *et al.*, 2003; Cassat *et al.*, 2005; Valle *et al.*, 2003) and express hyaluronidase at elevated levels (Jones *et al.*, 2008; Makris *et al.*, 2004), we postulated that the elevated levels of hyaluronidase observed in the *sarA* mutant background may contribute to the biofilm-negative phenotype.

Activity of hyaluronidase by *S. aureus* UAMS-1, Sanger-252 and USA300 (Miss) was consistently reduced when grown in TSB supplemented with glucose and NaCl (Fig. 3 and data not shown), a reduction observed more so with the addition of glucose than with NaCl (data not shown). Nevertheless, TSB with and without glucose and NaCl was used to confirm loss of hyaluronidase activity in the UAMS-1 *hysA* mutant strains (Fig. 3) and to determine whether hyaluronidase contributes to the biofilm-negative phenotype of *sarA* mutant strains (Fig. 4). To ensure that hyaluronidase was produced in biofilms, biofilms of *S. aureus* UAMS-1 and its *sarA* mutant strains were harvested and found to contain hyaluronidase activity (data not shown).

Regardless of the growth medium, hyaluronidase activity was not detected in either the UAMS-1 or the *sarA* mutant strains containing a deletion in either the *hysA2* gene or the *hysA1* and *hysA2* genes (Fig. 3). The lack of activity in the *hysA2* mutant strain was addressed above. As with previous reports (Beenken *et al.*, 2003; Cassat *et al.*, 2005; Valle *et al.*, 2003), biofilm formation in TSB containing glucose and NaCl was always more robust than with TSB alone (Fig. 4). Likewise with previous reports (Beenken *et al.*, 2003; Cassat *et al.*, 2005; Valle *et al.*, 2003), the UAMS-1 *sarA* mutant strain was significantly reduced in its ability to form a biofilm as compared with the UAMS-1 parent strain (Fig. 4). However, contrary to our postulate, the loss of hyaluronidase activity in the UAMS-1 *sarA* mutant strains did not significantly increase biofilm formation when compared with the UAMS-1 *sarA* mutant strain alone (Fig. 4). This was true when both TSB and TSB containing glucose and NaCl were used as growth media (Fig. 4). Surprisingly, a significant reduction in biofilm was observed in both the *hysA2* and the *hysA1 hysA2* mutant strains when compared with the UAMS-1 parent strain (Fig. 4). This significant reduction in biofilm formation was only observed in TSB and not in TSB containing glucose and NaCl. Currently, this difference may have significant implications as it relates to biofilm formation

Fig. 1. Hyaluronidase activity of *S. aureus* UAMS-1 and its *sarA*, *agr* and *sarA agr* regulatory mutant strains. Spent media isolated after 3, 6, 12 and 24 h of growth were standardized with respect to optical density at each time point, concentrated and pipetted into wells either undiluted or diluted 10-, 100- or 1000-fold (10^{-1} , 10^{-2} or 10^{-3} , respectively).

Fig. 2. Northern analysis of total RNA isolated from *S. aureus* UAMS-1 and its regulatory mutant strains *sarA*, *agr* and *sarA agr* at 3, 6, 12 and 24 h of growth and hybridized with a digoxigenin-labelled DNA probe generated using the *hysA* amplicon from *S. aureus* UAMS-1. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values represent the reciprocal of the dilution) samples.

and the loss of hyaluronidase activity. This phenomenon is under further investigation.

DISCUSSION

A *S. aureus* hyaluronidase has been purified to homogeneity and crystallized (Rautela & Abramson, 1973), and a gene for hyaluronidase was originally cloned from *S. aureus* 8325-4 with an expected molecular mass of 84 kDa, which agreed with the size of the purified protein (Farrell *et al.*, 1995). Makris *et al.* (2004) has shown in *S. aureus* 8325-4 that hyaluronidase-specific activity occurred and reached a maximum during exponential growth. The activity determined by Makris *et al.* (2004) was in agreement with an earlier report by Taylor & Holland (1991), who demonstrated that hyaluronidase-specific activity in spent media isolated from *S. aureus* FRI1187 increased during the exponential growth phase but gradually decreased during the post-exponential growth phase (Taylor & Holland, 1991). Makris *et al.* (2004) also examined the effects of the global regulators *sarA* and *agr* on hyaluronidase-specific

activity and determined that, while activity was substantially increased in an *S. aureus* 8325-4 *sarA* mutant strain, it was reduced and delayed (i.e. produced later in growth) in the *agr* mutant strain. In addition, staphylococcal hyaluronidase has been implicated as a virulence factor using a *hysA* mutant strain of *S. aureus* 8325-4 (Makris *et al.*, 2004). In this same study, both lesion severity and the number of *hysA* mutant cells recovered from abscesses excised after subcutaneous inoculations in mice were reduced as compared with the 8325-4 parent strain.

In our present study, hyaluronidase activity in spent media isolated from UAMS-1 and its isogenic regulatory mutant strains was detected at 3, 6, 12 and 24 h, which corresponds to the late exponential, early and late post-exponential, and stationary phases of growth (Jones *et al.*, 2008). While Makris *et al.* (2004) reported that in 8325-4 hyaluronidase-specific activity was observed to increase rapidly during exponential growth and gradually decrease with time until approximately 24 h when activity was undetectable, it is difficult to assess whether the differences observed with UAMS-1 and its mutant strains are strain related. This is particularly true given that Makris *et al.* (2004) determined hyaluronidase-specific activity in spent media isolated from cultures grown in a chemically defined medium (Taylor & Holland, 1989) without an apparent step to concentrate the media prior to determining hyaluronidase activity. Therefore, it is reasonable to assume that our detection of hyaluronidase activity throughout growth could be related to our medium selection or the use of concentrated spent media to assess hyaluronidase activity or both.

While hyaluronidase activity was detected for UAMS-1 and its regulatory mutant strains at all time points examined, activity was always substantially higher in the *sarA* and *sarA agr* mutant strains, suggesting that *sarA* has a negative regulatory effect on hyaluronidase expression. This was supported by the fact that *hysA* steady-state message levels were only detected in the *sarA* and *sarA agr* mutant strain

Fig. 3. Hyaluronidase activity of *S. aureus* UAMS-1 and its isogenic *sarA* and *hysA* mutant strains grown in either TSB or TSB supplemented with glucose (0.5%) and NaCl (3.0%).

Fig. 4. *In vitro* biofilm of *S. aureus* UAMS-1 (WT) and its *sarA* and *hysA* mutant strains grown in either TSB or TSB supplemented with glucose (0.5 %) and NaCl (3.0 %). Values represent the back-transformed least-squares means ($n=8$) and their corresponding 95 % confidence limits based on two-way ANOVA of log-transformed data. All pairwise comparisons within a group (TSB or TSB + glucose + NaCl) were statistically significant ($P \leq 0.05$) unless indicated (NS, not significant).

backgrounds as determined by Northern analysis. The exact role of *agr* in the expression of *hysA* in UAMS-1 cannot be ascertained in the current study as *hysA* message was not detected for either UAMS-1 or the *agr* mutant strains. However, *hysA* message was detected for the *sarA* mutant strain at all four time points examined while *hysA* message levels for the *sarA agrA* mutant strain were detectable at 3 and 6 h of growth, barely detectable at 12 h, and undetected at 24 h of growth. This decrease in *hysA* message levels observed with the *sarA agr* mutant strain as compared with the *sarA* mutant strain suggests that *agr* is a positive regulator of *hysA*, as was reported by Makris *et al.* (2004) for 8325-4.

S. aureus sarA mutant strains are defective in biofilm formation (Beenken *et al.*, 2003; Cassat *et al.*, 2005; Valle *et al.*, 2003) and produce elevated levels of hyaluronidase (Jones *et al.*, 2008; Makris *et al.*, 2004), thermostable nuclease and a number of staphylococcal proteases (Beenken *et al.*, 2004; Blevins *et al.*, 2002; Cassat *et al.*, 2006; Karlsson & Arvidson, 2002; Shaw *et al.*, 2004). The involvement of proteases and the thermostable nuclease (*nuc*) in biofilm was assessed in *S. aureus* UAMS-1 using *sarA* and *nuc* mutant

strains in combination with a variety of inhibitors specific for certain classes (serine, cysteine and metalloproteases) of proteases produced by *S. aureus* (Tsang *et al.*, 2008). This study allowed Tsang *et al.* (2008) to conclude that while proteases and nuclease independently contribute to the biofilm-deficient phenotype observed in the UAMS-1 *sarA* mutant strain, their combined effect is significantly greater than the effect of either nuclease or proteases alone. This was clearly evident by the near wild-type levels of biofilm observed when the *sarA nuc* mutant strain was treated with all three protease inhibitors examined (Tsang *et al.*, 2008). While it appears that nuclease and proteases are major contributors to the biofilm-negative phenotype observed in the UAMS-1 *sarA* mutant strain, the inability to completely restore biofilm to wild-type levels when these enzymes are rendered non-functional suggests that other factors are contributory. To ascribe a role for hyaluronidase in biofilm dispersal, we assessed UAMS-1 and its isogenic regulatory mutant strains for the ability to form biofilm. This was particularly relevant given that human plasma contains HA, albeit in low quantities ($10\text{--}100\text{ ng ml}^{-1}$ in healthy individuals) (Laurent & Fraser, 1992), and that the wells of microtitre plates of the *in vitro* biofilm assay used in this

study and others (Beenken *et al.*, 2003) are coated with human plasma. Results from this assessment suggest that the elevated levels of hyaluronidase found in the *sarA* mutant strain of UAMS-1 do not contribute to the biofilm-deficient phenotype observed in this strain. However, the results could also indicate that the effect of the elevated levels of nuclease and proteases is masking the effect of hyaluronidase. Experiments are now under way to assess hyaluronidase activity and biofilm formation in strains deficient in protease and nuclease activity as well as to determine if hyaluronidase is an important component for some aspect of staphylococcal disease.

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