Using GFP Fluorescence as a Proxy for γ-hemolysin Expression Following Neutrophil Phagocytosis of *S. aureus*

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ABSTRACT

Background and Objective: The *Staphylococcus aureus* (*S. aureus)* exoprotein secretion system (SaeR/S) is a two-component protein system within *Staphylococcus aureus* that has been linked to this pathogen's ability to survive within human neutrophils (polymorphonuclear leukocytes or PMNs). Prior studies have shown that an extracellular (EC) loop, consisting of nine amino acid residues on SaeS, is vital for *S. aureus* to sense and respond to extracellular stimuli—specifically components of human PMNs. Additionally, γ-hemolysin (*hlgA*) is a predominant virulence factor that targets immune and red blood cells. This toxin has been shown to be regulated by SaeR/S. New *hlgA*-GFP *S. aureus* cell strains—including point mutations of the residues on the EC loop—have been developed in order to study the role of each residue in *S. aureus* survival. All strains contained a plasmid on which the *hlgA* gene was linked with the GFP reporter. The current study sought to both characterize the activity of these strains in the presence of human PMNs as well as determine if *hlgA*-GFP fluorescence was a legitimate proxy for measuring *hlgA* expression.

Methods: We investigated the expression of *hlgA* following neutrophil phagocytosis of *S. aureus* using *hlgA*-GFP reporter strains. Flow cytometry was used to measure GFP fluorescence within samples after being incubated for varying lengths of time.

Results: Our findings suggest that the *hlgA*-GFP reporter can be used to show *hlgA* expression at later time points (4-6 hr). However, at earlier time points (0.5-2 hr) the *hlgA*-GFP reporter was not sensitive enough to assess *hlgA* transcription. This is likely because GFP was not present in high enough quantities to be detected.

Discussion and Conclusions: The data collected in this study demonstrate that the *hlgA*-GFP reporter can be used as a proxy for *hlgA* transcription during neutrophil interaction. However, it is not sensitive enough to be used at time points earlier than four hours. Additionally, our data imply that at later time points (4-6 hr) *hlgA* may be controlled by a regulatory system within *S. aureus* other than SaeR/S. To date, there is no research outlining the regulation of *hlgA* at later time points.

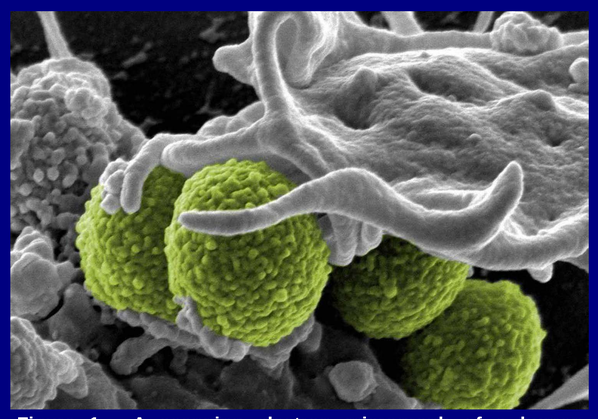

Figure 1. A scanning electron micrograph of a human neutrophil interacting with *Staphylococcus aureus* (green). Previous studies have shown that the ability of *S. aureus* to sense the neutrophil dictates outcome of infection.

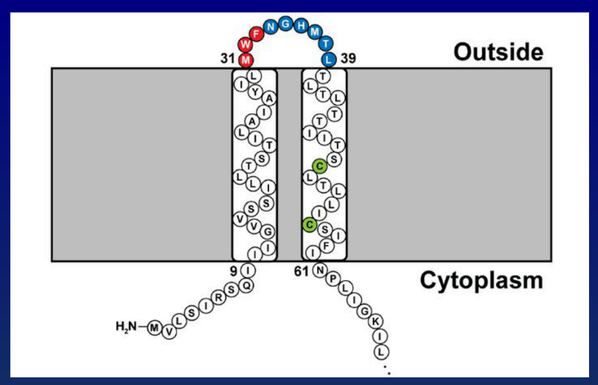

Figure 2. The nine residue EC loop of SaeS. Previous studies have identified this loop as the sensing domain of SaeS.

METHODS

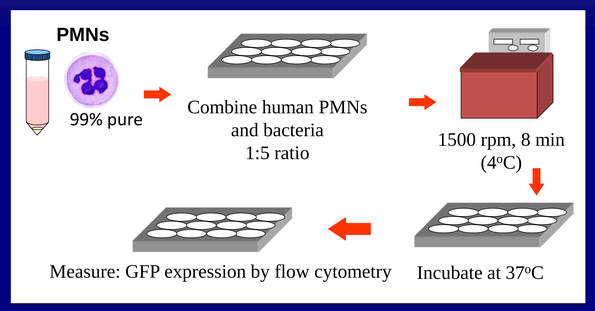

Figure 3. A schematic of the experimental setup used to measure *hlgA expression*

***S. aureus* strains**

- Wild Type *saeS* (MRSA, WT)
- Knockout—*ΔsaeS*
- Complement—*ΔsaeS* + WT *saeS*
- Point Mutants
  - M31A
  - W32A
  - F33A
  - N34A

Each point mutant has *ΔsaeS* + WT *saeS* where the designated amino acid residue on the EC loop of SaeS is mutated to an alanine.

***hlgA*-GFP assay**: Cultures were grown to OD 1.5 (at 600 nm). The resuspended cultures were then synchronized with neutrophils in a 96 well plate. The plates were incubated at 37˚C with CO_2 . GFP fluorescence was measured on the flow cytometer

Growth Curves: To determine proper PMN to bacteria ratio growth curves were conducted. Cultures were grown for 6 hours. Samples were taken every hour—the optical density (OD) was measured and samples were plated on TSA plates. Colonies were counted the following day.

RESULTS

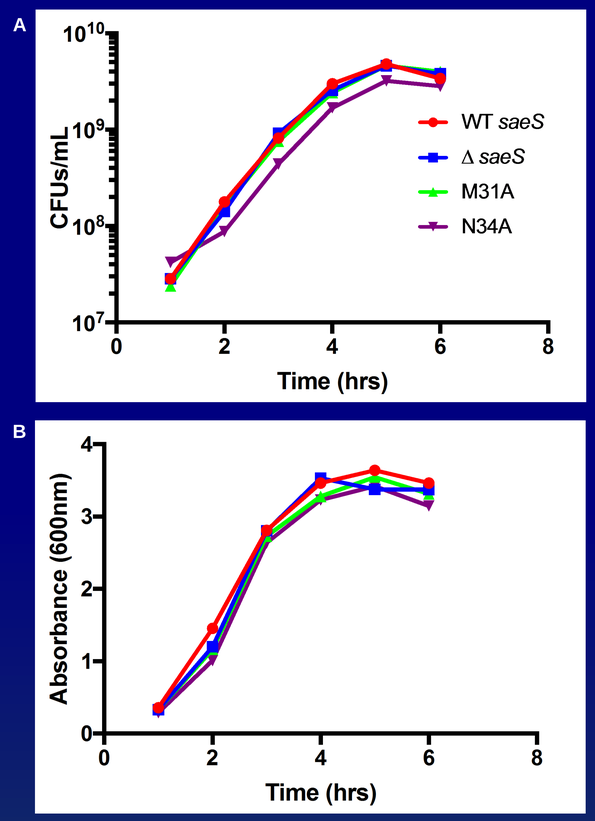

Figure 4. Growth curves of four *hlgA*-GFP *S. aureus* strains. A . Colony forming units (CFUs) per mL culture. These results verified that no strains have a growth defect. B. Optical Density (OD) as measured at 600 nm. A & B provide a means for determining bacterial concentration of specific cultures.

RESULTS CONTINUED

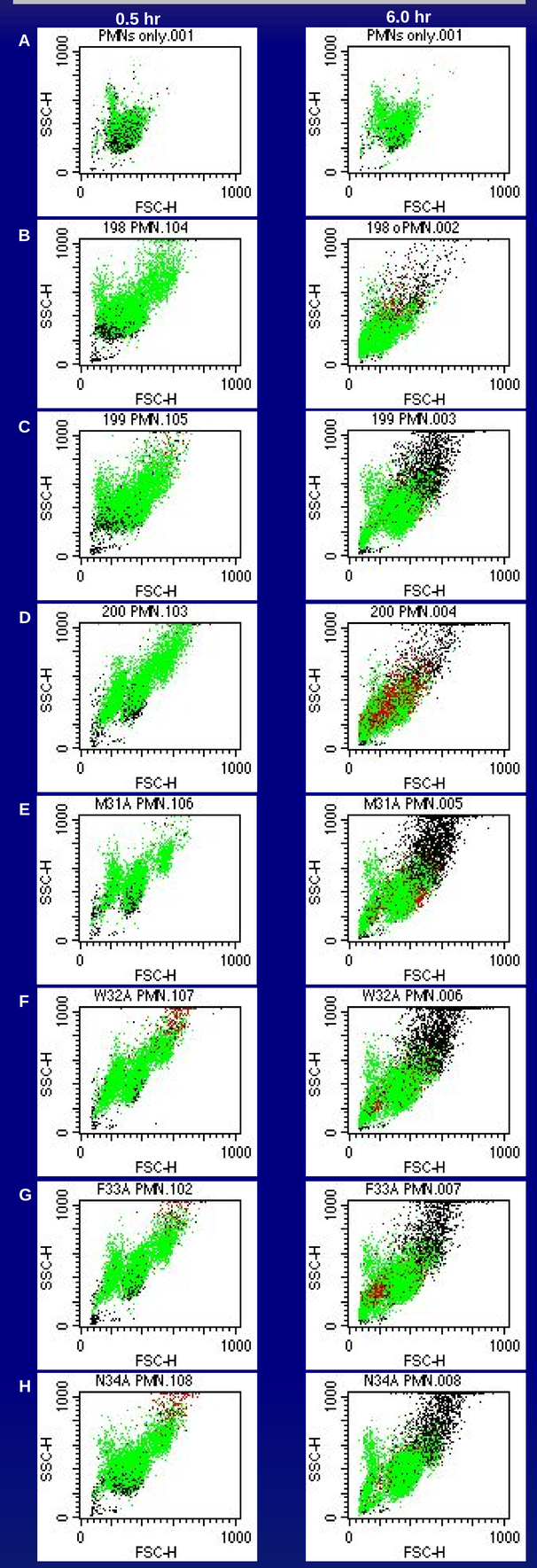

Figure 5. Flow cytometry analysis. Forward scatter-Side scatter dot plots at 30 minutes and 6 hours. A. PMNs only. B. WT *saeS*. C. *ΔsaeS*. D. *ΔsaeS* + WT (complement). E-H. Strains with amino acid point mutations in the SaeS extracellular loop. E.M31A. F. W32A. G. F33A. H. N34A.

RESULTS CONTINUED

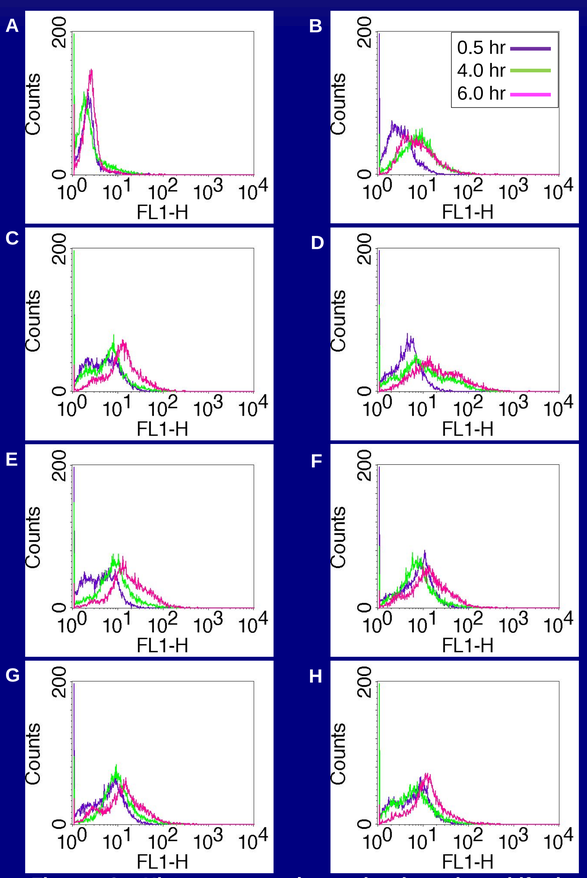

Figure 6. Histogram overlays showing the shift in fluorescence over time of the *hlgA*-GFP reporter. A. PMNs only. B. WT *saeS*. C. *ΔsaeS*. D. Complement *ΔsaeS* + WT. E. M31A. F. W32A. G. F33A. H. N34A.

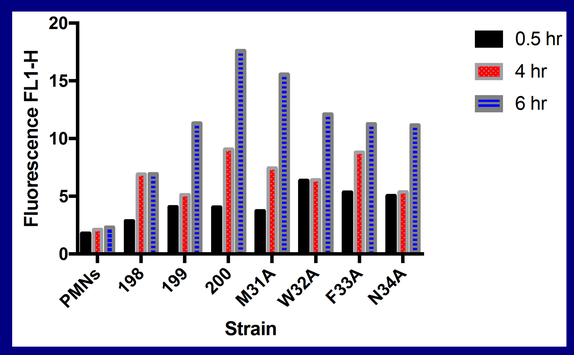

Figure 7. Change in GFP fluorescence over time. Results are shown as the geometric mean of fluorescence. By 6 hours, all strains exhibit increases in fluorescence.

CONCLUSIONS/DISCUSSION

- The *hlgA*-GFP reporter is a viable proxy for *hlgA* expression.
- The reporter is not sensitive enough at early time points (0.5-2 hr) to be accurately measured on the flow cytometer.
- The knockout strain *ΔsaeS and point mutant strains* also demonstrate a shift in fluorescence at six hours.
- It is probable that an alternate system within *S. aureus* regulates *hlgA* at time points greater than four hours.

ACKNOWLDGEMENTS

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