

Secondary Bacterial Pneumonia by *Staphylococcus aureus* Following Influenza A Infection Is SaeR/S Dependent

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Staphylococcus aureus is a predominant cause of fatal pneumonia following influenza A virus (IAV) infection. Herein we investigate the influence of antecedent IAV infection on *S. aureus* virulence gene expression. Using a murine model, comparing the USA300 and USA300 Δ saeR/S strains, we demonstrate that *S. aureus* pathogenesis following IAV infection is SaeR/S dependent. Furthermore, we show that IAV modulates the lung environment to rapidly up-regulate *S. aureus* virulence factors containing the SaeR-binding domain. Data demonstrate that the pathogen response to IAV infection impacts host outcome and provides evidence that the ability of *S. aureus* to sense and respond to the lung environment determines severity of pneumonia.

Keywords. *Staphylococcus aureus*; SaeR/S; influenza A; coinfection; superinfection; pneumonia.

Influenza A virus (IAV) infections are associated with increased susceptibility to secondary bacterial infections, wherein morbidity and mortality increase significantly. Retroactive studies of samples from the 4 pandemic influenza outbreaks of the last century identified that secondary bacterial infections were associated with 40%–95% of fatal cases [1]. In the previous decade, influenza coinfections have displayed mortality rates ranging from 11- to 15-fold higher than those of influenza alone, and have consistently ranked within the top 10 leading causes of death in the United States [2, 3].

In the early 20th century, *Streptococcus pneumoniae* was the major cause of bacterial superinfection following IAV. However, since the 1950s, a shift toward *Staphylococcus aureus*

as a causative agent in secondary infections was observed [1, 4]. *Staphylococcus aureus* is now recognized as one of the most frequent causes of severe bacterial coinfections [1]. Interestingly, *S. aureus* colonizes the nares of approximately 50% of the general population [1, 5]. Despite this close proximity to the lower respiratory tract, *S. aureus* acting as a primary infectious agent of community-associated pneumonia in healthy individuals occurs infrequently. Recent work describing the interactions of upper respiratory tract commensals has demonstrated that members of the upper respiratory tract transiently encounter the lower respiratory tract [6]. Taken together, these observations imply that *S. aureus* frequently encounters the lung environment in a significant portion of the population without leading to disease.

The innate ability of *S. aureus* to sense and respond to environments within a human host is dependent on tightly controlled gene regulatory systems [5]. SaeR/S is a 2-component gene regulatory system that controls transcription of virulence genes essential in neutrophil evasion and *S. aureus* pathogenesis [5]. In the current study, we use a murine model of secondary pneumonia to demonstrate that *S. aureus* pathogenesis following antecedent IAV infection is SaeR/S dependent. Furthermore, we provide evidence that IAV modulates the host lung environment to rapidly up-regulate *S. aureus* virulence factors containing the SaeR-binding domain.

MATERIALS AND METHODS

Bacterial Cultures

Staphylococcus aureus strains USA300, USA400, and isogenic saeR/S deletion mutant strains (Δ saeR/S) were grown and harvested as described previously [7]. Bacterial inocula were resuspended in sterile Dulbecco's phosphate-buffered saline.

IAV Strains

IAV/WSN/1933(H1N1) (hereafter “WSN”) was propagated in the Obar laboratory (Geisel School of Medicine at Dartmouth), and IAV/PR/8/1934 (PR8) was purchased from Charles River Laboratories. IAV infections were performed using a total volume of 50 μ L sterile phosphate-buffered saline (PBS) containing 750 plaque-forming units (PFU) for WSN or 100 PFU for PR8.

Mouse Models of Pneumonia and In Vivo *S. aureus* Gene Expression Analysis

All studies conformed to National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University, Bozeman (MSU). Male and female BALB/c mice (8–10 weeks old) were either bred at the MSU Animal Resources Center or were commercially acquired.

Received 21 December 2017; editorial decision 5 April 2018; accepted 11 April 2018; published online April 13, 2018.

Presented in part: 49th Annual Meeting of the Society of Leukocyte Biology, Raleigh, North Carolina, 2015; Northwest Branch Meeting of the American Society for Microbiology, Seattle, Washington, 2015; International Society for Microbial Ecology General Meeting, Montreal, Canada, 2016; and 50th Annual Meeting of the Society for Leukocyte Biology, Vancouver, Canada, 2017.

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The Journal of Infectious Diseases® 2018;218:809–13

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All challenges with *S. aureus* were performed on day 6 post-IAV infection. WSN-infected mice were challenged via intranasal administration of *S. aureus* with 5×10^8 for gene expression assays and for morbidity and mortality studies. PR8-infected mice were challenged via intratracheal administration of *S. aureus* at 5×10^8 colony-forming units (CFU)/50 μ L for gene expression, or 2.5×10^8 for morbidity and mortality assays. Doses were determined by pilot studies and are in accordance with trends observed in Miller et al [8].

Mouse models of intranasal infections were adapted from Lee et al [3]. Mice were slightly anesthetized with isoflurane and scruffed in an upright position while a 50- μ L volume was administered directly to mouse nares. After aspiration, mice were held upright for an additional 60 seconds to ensure distribution into the lower respiratory tract. For intratracheal infection, mice were deeply anesthetized with isoflurane and suspended from front incisors at a 60°–70° angle. A blunt-tipped, bent 18-g needle was inserted through the trachea and suspended above the carina to administer 50 μ L directly into the lungs. Mouse nostrils were then blocked until a large gasp was observed. Mice were euthanized using a morbidity scale based on activity, feeding, and respiratory distress in combination with weight loss exceeding 20% of initial body mass.

Mice were euthanized at 4, 6, and 8 hours post-*S. aureus* challenge and lungs were homogenized in 2 mL of PBS. Aliquots (100 μ L) were used for CFU determination and the remaining sample was centrifuged at 4000 rpm (3724 \times g) for 10 minutes at 4°C. Supernatant was removed and pelleted lung tissue was resuspended in buffer RLT (Qiagen). *Staphylococcus aureus* RNA was purified as described previously [9]. Purified RNA from individual mice within each treatment group (3–4 per experiment) was pooled and adjusted to a final concentration of 5 ng/ μ L and subjected to Quantigene 2.0 assays (Affymetrix) or TaqMan reverse-transcription polymerase chain reaction (RT-PCR) (Supplementary Table 1) [7].

RESULTS AND DISCUSSION

Previous studies have suggested that dysregulated lung physiology, including impairment of mucociliary clearance as well as modulation of the host immune system in response to IAV, is a major contributor to increased host susceptibility to secondary *S. aureus* infections [1]. Specifically, IAV has been demonstrated to influence type 1 interferon responses, nicotinamide adenine dinucleotide phosphate oxidase activity, and antimicrobial peptide production [1].

These efforts have provided insight into disease progression by identifying a role for IAV infection in the dissemination of *S. aureus* from the upper respiratory tract to the lower respiratory tract [10]. Additionally, the expression of specific *S. aureus* toxins has been linked to the severity of pneumonia [3]. However, the contribution of IAV infection on *S. aureus*

virulence regulation remains incompletely defined. To that end, we investigated the impact of an antecedent IAV infection on *S. aureus* pathogenesis during secondary bacterial pneumonia.

Mice were infected intranasally with WSN followed by subsequent challenge with USA300 or USA300 Δ *saeR/S* (Δ *saeR/S*) at 6 days post-primary IAV infection [3]. Consistent with published observations, infection with IAV or *S. aureus* only had minimal impact on survival. However, coinfection with USA300 led to a significant increase in murine mortality (Figure 1A) [1, 4]. In contrast, mice infected with WSN but challenged with Δ *saeR/S* retained a survival rate of 100%, suggesting that *SaeR/S* is a key contributor to *S. aureus* secondary pneumonia (Figure 1A).

To confirm that the increased susceptibility to USA300 following IAV infection was not due to responses specific to WSN or route of infection, we infected mice with IAV PR8 via an intratracheal route (Supplementary Figure 1) [8]. Similar to the results of mice coinfecting with WSN and *S. aureus*, PR8-infected mice challenged with USA300 had significant increases in mortality compared with mice infected with USA300 alone (Supplementary Figure 1).

Given the relative infrequency of *S. aureus* acting as a primary infectious agent in the lung environment, in combination with the observed absence of mortality in mice challenged with IAV and Δ *saeR/S*, we hypothesized that *SaeR/S*-regulated virulence factors were induced during coinfection with IAV. Thus, we investigated the influence of antecedent IAV infection on the expression of several *SaeR*-regulated virulence genes with described roles in lung infections (*hlgA*, *hla*, and *lukA*) [11]. TaqMan quantitative RT-PCR was used to measure the transcript abundance in mice infected with *S. aureus* alone or coinfecting with WSN or PR8 (Figure 1B and 1C) at 4 hours following bacterial challenge. Indeed, antecedent infection with IAV led to increased expression of all virulence genes assayed compared to infection with *S. aureus* alone. To ensure the observed upregulation of *SaeR*-target genes was not specific to coinfection with USA300, we repeated the experiment using USA400. Challenge with USA400 following IAV demonstrated similar increased expression of *saeR* target genes compared to challenge with USA400 alone (Figure 1D).

To substantiate the impact of antecedent IAV infection on the regulation of *saeS* and *saeR*, we evaluated *S. aureus* gene expression in vivo at 4, 6, and 8 hours post-*S. aureus* challenge (Figure 2). Expression of select *S. aureus* virulence genes and regulatory systems were determined (Figure 2A–C; Supplementary Table 2) [9]. Quantigene 2.0 analyses of *S. aureus* gene expression as a primary or secondary infectious agent during lung infection demonstrated that antecedent IAV infection rapidly increased expression of *saeR*, *saeS*, and *SaeR*-regulated virulence genes in mice coinfecting with USA300 compared to expression of these genes during pneumonia caused by USA300 alone. Specifically, at 4 hours post-*S. aureus* challenge, virulence genes containing

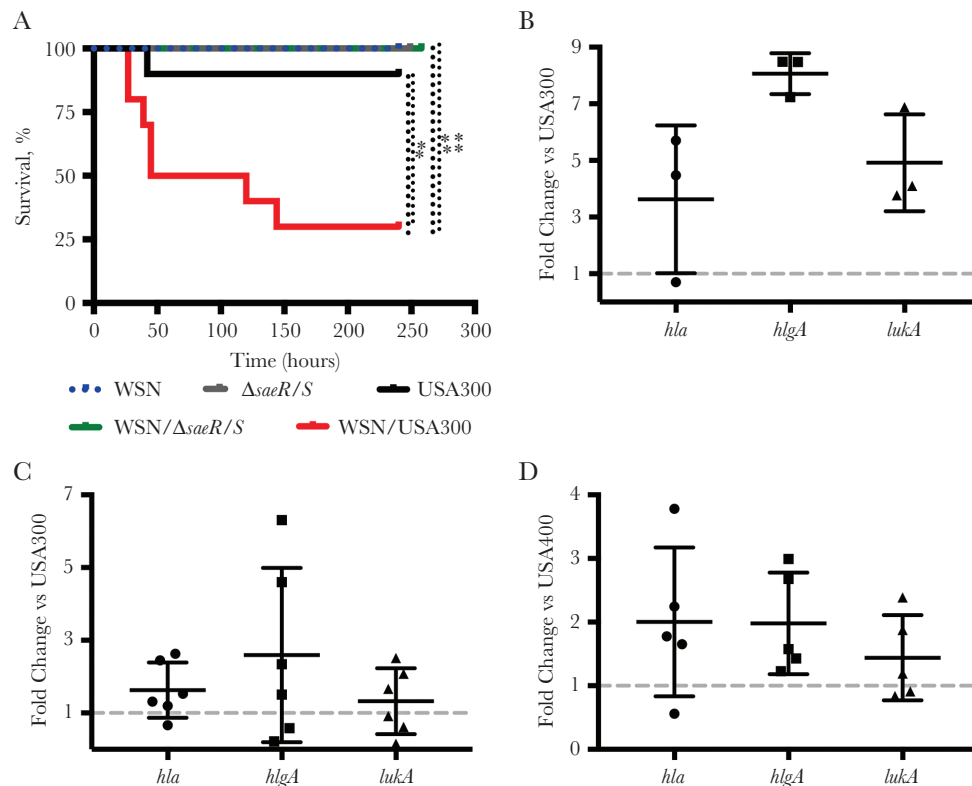


Figure 1. *Staphylococcus aureus* bacterial pneumonia following antecedent influenza A virus (IAV) infection is *saeR/S* dependent. Mice were intranasally mock-infected with phosphate-buffered saline or infected with IAV. Six days postinfection, mice were challenged with *S. aureus*. A, Survival assay of mice infected with IAV/WSN/1933(H1N1) (WSN) (750 plaque-forming units/50 μ L) and challenged with USA300 (5×10^8 colony-forming units [CFU]/50 μ L), $n = 10$ mice per group. * $P < .05$; ** $P < .01$, log-rank (Mantel-Cox) test. B–D, TaqMan reverse-transcription polymerase chain reaction quantification of select *SaeR*-target genes. Four hours after *S. aureus* challenge (5×10^8 CFU/50 μ L), RNA was isolated from 3–6 mice per treatment group. Data displayed are the mean fold change of *S. aureus* expression levels from coinfecting lungs compared to lungs infected with *S. aureus* alone. Gene transcripts were normalized to *gyrB* expression and calibrated to expression in *S. aureus*-only infection. B, WSN and USA300. C, PR8 and USA300. D, PR8 and USA400.

the *SaeR*-binding domain including *lukA*, *ssL7*, *sbi*, *hlgA*, *hlgB*, *hlgC*, and *hla* were up-regulated in coinfecting mice (Figure 2A). The bicomponent γ -hemolysins *hlgA*, *hlgB*, and *hlgC* displayed the greatest fold-increase in expression compared to USA300 infection alone and expression decreased by 6 hours, suggesting that early activation of these toxins may be critical for *S. aureus* to establish a productive infection within the lung environment. While no comprehensive studies of *S. aureus* gene expression from patients following IAV have been conducted, efforts using rabbit models of pneumonia—where *S. aureus* toxin production is believed to be more indicative of a human infection—have demonstrated that active immunization against secreted γ -toxins resulted in protection of >97% of all subjects tested compared to survival of <1% in those unvaccinated [12, 13], suggesting that our observation of early production of γ -toxins may correlate with secondary *S. aureus* pneumonia in humans.

Mice infected with Δ *saeR/S* showed no differences in expression of these virulence genes due to preceding IAV infection and were below the level of detection at all time points (Figure 2A–C), demonstrating a predominant role for *SaeR/S* in regulating *lukA*, *sbi*, *hlgA*, *hlgB*, *hlgC*, and *hla* in vivo and

implicating these factors in disease outcome. Expression of *agrA* and *sarA* (genes that do not contain the *SaeR*-recognition sequence) were increased in USA300 following IAV infection. In our studies, bacterial CFUs were equal between USA300- and Δ *saeR/S*-infected mice at 4 hours postchallenge and, therefore, differences in pathogen burden did not impact gene expression results (Supplementary Figure 2A and 2B). Bacterial burdens recovered at 8 hours postchallenge were lower in Δ *saeR/S*- compared with USA300-infected mice; however, CFUs were equal between both coinfecting treatment groups. Interestingly, *agrA* and *sarA* were increased during infection with Δ *saeR/S* even without antecedent IAV infection. Bloes et al demonstrated the importance of Arg-regulated phenol-soluble modulins in secondary *S. aureus* pneumonia by challenging mice with USA300- and PSM-deficient mutant 2 days post-IAV infection [14]. However, our data suggest that *SaeR/S* is the predominant regulator initiating virulence factor expression following IAV.

Over time, the impact of IAV on *S. aureus* virulence gene expression in USA300-infected mice was reduced (Figure 2B and 2C; Supplementary Figure 3; Supplementary Table 3). As previous studies have demonstrated that *SaeR/S* influences

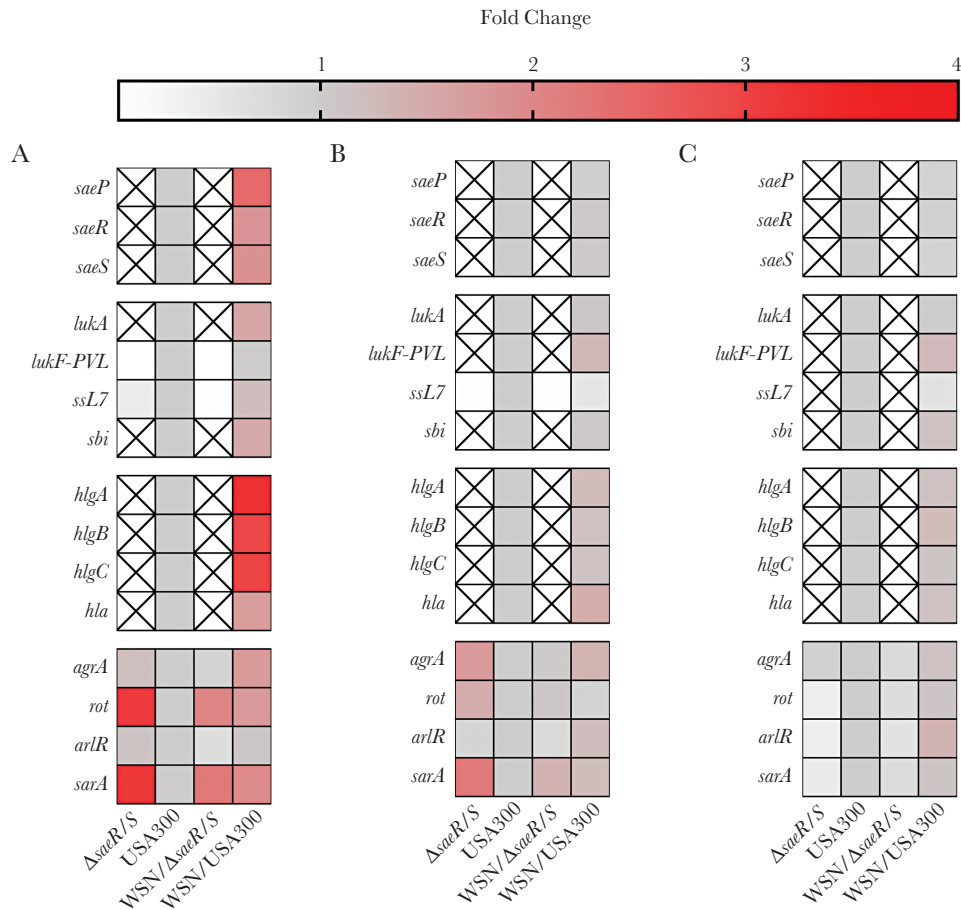


Figure 2. Influenza A virus (IAV) infection promotes rapid up-regulation of *Staphylococcus aureus* SaeR-regulated genes. Mice were infected intranasally with IAV/WSN/1933(H1N1). Six days post-IAV infection, mice were challenged with USA300 or Δ saeR/S. Four (A), 6 (B), or 8 (C) hours post-*S. aureus* challenge, mouse lungs were harvested. RNA was purified from harvested lungs and treatment groups were pooled (n = 4–5 mice/treatment group/experiment). Gene transcripts were normalized to *gyrB* and calibrated to the expression levels of USA300-only infection at corresponding time points. Differential regulation of SaeR target genes was assessed by Quantigene 2.0 assays. “X” indicates transcript levels below the limit of detection. Data are reported as mean fold change of 2 separate experiments.

primary *S. aureus* pneumonia, we analyzed *S. aureus* gene expression in USA300 over time to determine if SaeR-regulated genes were differentially regulated (Supplementary Figure 3; Supplementary Table 3) [15]. Indeed, SaeR/S-regulated genes increased in expression over time in USA300-infected mice even without antecedent IAV infection. In addition, unlike the increased expression of the bicomponent γ -hemolysins at 4 hours, increased expression of *lukF-PVL*, a toxin linked to the severity of *S. aureus* lung infections, was not observed until 6 hours after *S. aureus* challenge in coinfecting mice (Figure 2B and 2C; Supplementary Table 2). These observations, combined with the mortality data (Figure 1A; Supplementary Figure 1) and data from other studies, suggest that the timing of induction of virulence genes regulated by SaeR is critical to initiation of infection [1, 10, 14]. Based on these data, we hypothesize that components of the IAV-infected lung environment are recognized by SaeS, leading to early up-regulation of SaeR-regulated toxins. Conversely, when *S. aureus* encounters a healthy lung environment, no immediate activation of SaeR/S occurs.

The delay in toxin production in absence of IAV may provide sufficient time for detection by innate responses, leading to clearance of *S. aureus* from the lung environment.

In conclusion, these data provide new understanding of *S. aureus* pathogenesis following antecedent IAV infection and suggest that the initiation and severity of secondary infection by *S. aureus* in the lung environment are *saeR/S* dependent. We acknowledge that our choice of IAVs represents seasonal influenza strains, and additional studies are necessary to determine if our results are applicable to pandemic IAVs. Further efforts focused on *S. aureus* sensing and responding to the surrounding lung environment during IAV infection will provide insight into environmental changes due to IAV that up-regulate *S. aureus* virulence during secondary pneumonia.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to

benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. This work was supported by the US National Institutes of Health (grant numbers R01A1090046, R01AI103353, GM110732, and U54GM115371); funds from the Montana University System Research Initiative (51040-MUSRI2015-03) and Montana State University Agriculture Experiment Station; and an equipment grant from the Murdock Charitable Trust.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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