INITIATION AND PATHOGENESIS OF STAPHYLOCOCCUS AUREUS PNEUMONIA FOLLOWING INFLUENZA A INFECTION

by

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DEDICATION

The completion of this dissertation and degree is dedicated to my family.

First and foremost, to my wife Joanna Borgogna, who has been alongside me to experience every failure and triumph of this journey. You are the one I love to celebrate with. My heart is overwhelmed with a good theme.

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ABSTRACT

Infection influenza A virus (IAV) leads to increased host susceptibility to secondary bacterial pneumonia. In cases such as these, *Staphylococcus aureus* (*S. aureus*) has emerged as the dominant bacterial pathogen associated with severe infection outcomes. *S. aureus* is a common commensal of the anterior nares and is frequently trafficked into the lower respiratory tract through inhalations, micro-aspirations, and direct mucosal dispersion. Despite recurrent exposure to the lungs and the capacity to cause severe disease, cases of *S. aureus* pneumonia are rare in immunocompetent hosts. Previous efforts interrogating *S. aureus* secondary bacterial pneumonia have largely focused on the immunomodulation that occurs during the antecedent influenza infection and have ignored the virulence contributions of the bacterial pathogen. To that end, we developed a murine model of secondary pneumonia to investigate *S. aureus* pathogenesis following influenza A infection. We identify that secondary bacterial pneumonia by *S. aureus* is dependent on the activation of the two-component regulatory system (TCS) SaeR/S. Further, studies demonstrated that in the absence of IAV infection the healthy lung environment suppresses virulence gene expression. Characterization of the lung environment revealed that the lipid constituents of pulmonary surfactant suppress *S. aureus* virulence production. Our data provide a model of secondary bacterial pneumonia wherein infection with IAV significantly reduces surfactant lipid concentrations within the lungs. The reduction of pulmonary surfactant lipids leads to a loss of *S. aureus* virulence suppression and rapid activation of the major virulence regulator *saeR/S*. Taken together, these data provide a strong rational for the low incidence of primary *S. aureus* pneumonia and the increased severity of *S. aureus* pneumonia following antecedent influenza A infection. Furthermore, these data highlight possible pulmonary surfactant replacement therapies that may significantly alleviate secondary bacterial pneumonia morbidity and mortality.

CHAPTER ONE

INTRODUCTION TO *STAPHYLOCOCCUS AUREUS*, INFLUENZA, AND CO-INFECTION

Staphylococcus aureus

In a foreshadowing of the collaborative efforts necessary to combat the diseases that would become its hallmarks, the discovery of *Staphylococcus aureus* was a culmination of inspiration, debate, and experimentation. Scottish surgeon Sir Alexander Ogston was increasingly dissatisfied with the mortality rates accompanying post-operative patients[1]. He soon became intrigued with post-surgical success rates accompanying Joseph Lister's implementation of antiseptic technique but was not satisfied with Lister's explanation that suppuration was a result of putrefaction from the air[1,2]. Ogston rejected Lister's notion that tissues were normally sterile and hypothesized that suppuration was caused by "some special germ"[1]. In attempt to substantiate his hypothesis Ogston examined the pus from a knee abscess under his microscope. In a description of his observations Ogston writes "…there were revealed to me beautiful tangles, and tufts and chains of round organisms in great numbers..."[1,3]At the time of his discovery Ogston described these organisms simply has micrococci but soon after he accurately distinguished two distinct growth characteristics – 1) growth by these organisms in chains which had previously been described as *Streptococci* (Billroth, 1874), and 2) the novel growth characteristic of bunches, to which he termed "*Staphylococci*" [4,5].

The debate between Lister and Ogston regarding the explanation of suppuration typified conversations within bacteriology during this era. The causal relationships between bacteria and disease were currently being identified and popularized. Inspired by a recent publication by Robert Koch, Ogston purposed to demonstrate that the organisms collected from the purulent discharge of the knee abscess could recapitulate the same phenotype when infected into healthy animals[6,7]. Upon infection, pus derived from abscesses deficient in micrococci lead to no new abscess formation, however infection of pus "swarming with micrococci" lead to severe animal morbidity[8]. Satisfied that his efforts had confronted the dissolutions of his contemporary Lister, and validated the work of Koch, Ogston presented his findings at the Ninth Surgical Congress in Berlin Germany[7]. Despite his progress and identification of the genus *Staphylococcus*, Ogston viewed his work as incomplete. In an article entitled "Report upon Microorganisms in Surgical Diseases" Ogston notes:

> It will not have escaped the reader that my report is contradictory. Why are micrococci so deleterious when injected, and so harmless on the surface of wounds and ulcers. It is no doubt possible that there exist micrococci precisely similar in appearance and growth and yet widely different in the effects they are capable of producing[9].

While Ogston was not able to reconcile the differing pathologies he observed, his suggestion that two phenotypically similar organisms may convey vastly different diseases would soon be confirmed. Several years later work by the German physician Anton J. Rosenbach would distinguish staphylococcal species on the basis of colony pigment production. Organisms that produced white colonies he termed "*Staphylococcus*

albus" to which we now refer as "*Staphylococcus epidermidis";* the other organism produced a gold pigment, to which he termed St*aphylococcus aureus*[5].

Since the inaugural characterizations of *Staphylococcus aureus* (*S. aureus*) there have been many successes and failures accompanying scientific progress towards understanding this common pathogen. The most exemplary of these is the discovery and implementation of penicillin as it was heralded to end the war on infectious disease only to be thwarted by the emergence of penicillin resistant *S. aureus* strains within one-year of use[10]. Antibiotic resistance has become a defining characteristic of *S. aureus* as a species. In a clinical version of the red queen hypothesis, each new generation of antimicrobial therapeutics has been quickly countered by the emergence of resistance. In 1959 methicillin was introduced to treat penicillin-resistant *S. aureus*[11,12]. As soon as 1961, resistance to methicillin was detected giving rise to the lineage of methicillin resistant *Staphylococcus aureus* (MRSA) strains[11,12]. Unlike previously acquired resistance mechanisms wherein resistance was conferred towards a select antibiotic therapy, resistance to methicillin conferred resistance to an entire class of antibiotics, the beta-lactams. Methicillin resistance was acquired though the acquisition of the Staphylococcal cassette chromosome *mec* (SCC*mec*). SSC*mec* encodes for the *mecA* gene whose product is the peptidoglycan synthesis enzyme – penicillin binding protein (PBP) 2a[13]. PBP2a has a lower binding affinity for beta-lactam antibiotics than native PBP proteins, thus organisms carrying this cassette are resistant to the entire class of beta lactam antibiotics.

Since its detection in the early 1960s and into the 1970s prevalence of MRSA was relatively rare and almost entirely relegated to hospital settings[14]. As time progressed into the 1980s, MRSA remained largely confined within hospital settings but prevalence rates increased from as low as 5% in the previous decade to as high as 40% within European and American hospitals[14]. A major change in MRSA epidemiology occurred in the 1990s with the emergence of community-acquired MRSA (CA-MRSA) strains[11]. Whereas previous MRSA infections had mainly been associated with nosocomial infections in immunocompromised hosts, cases of CA-MRSA infections were being reported from otherwise healthy individuals unaffiliated with hospital settings[12]. By the 2000s CA-MRSA had become recognized as a major cause of diseases ranging in severity from mild soft-tissue infections to life threatening necrotizing pneumonia[12,15].

The diverse range of infections cause by *S. aureus* is due to the innate ability of the bacterium to tightly regulate its gene expression in response to environmental signals[16–18]. Understanding how *S. aureus* senses and responds to environmental stimuli that in turn regulate the expression of its virulence factors is imperative towards developing a more complete understanding of the organism's pathogenesis. The *S. aureus* genome contains 16 two-component regulatory systems (TCS) that sense the surrounding environment and regulate gene expression accordingly[19,20]. The SaeR/S TCS has been identified to directly influence *S. aureus* pathogenesis by regulating the expression of virulence factors involved in toxin expression and immune evasion[16–18,21–25]. For example, induction of the SaeR/S targets alpha-toxin (Hla) and toxic-shock syndrome toxin -1 (TSST-1) directly influence abscess formation and toxic shock syndrome while

other targets such as leukotoxin G/H (LukGH) and staphylococcal peroxidase inhibitor (SPIN) confer immune evasion strategies though pore formation or neutralization of antimicrobial strategies[11,26–30].

SaeR/S is typical of two component systems in that it contains a transmembrane histidine sensor kinase (SaeS) and a cognate intracellular response regulator (SaeR). Upon stimulation, the histidine sensor kinase is auto-phosphorylated. The phosphate group is then transferred to the cognate response regulator and genes containing a SaeR consensus sequence within the promoter region are transcribed[31]. Previous studies have primarily focused on host factors (whole blood, HNP-1, and H_2O_2) that activate the TCS leading to differential stimulation of virulence factors[17,18,22,32]. However, several recent transcriptomics analyses of *S. aureus* grown in the presence of fatty acids have revealed an inverse relationship between SaeR/S and several gene classes. This observation suggests that in addition to acting as transcription inducer, SaeR/S may also suppress transcription[33–36] Further studies are needed to understand how host tissues and products may govern *saeR/S* expression.

Influenza

The ability of influenza to rapidly evolve has enabled it to remain within human and animal populations for centuries. Though the technologies necessary for its isolation and characterization would not arrive until the mid-1900s, influenza has been symptomatically described reaching as far back as Hippocrates[37]. Similar reports are found through nearly each century of the middle ages. As early as 1357 citizens of Florence, Italy recognized a “flu season” by associating an increase in disease burden

within their community during colder months of the year. Community members referred to the disease as *"influenza di freddo*" or "cold influence" from which now refer to the disease as "influenza."

Symptoms of influenza-like disease continue to appear throughout history[38,39]. It is generally accepted that during his second journey to the new world in 1493, Christopher Columbus and his crew were suffering from influenza[40,41]. Upon reaching the Antilles influenza spread from the crew to the native population wiping out an estimated 90% of the indigenous residents[40,41]. Reports of influenza continue to be found into the 20th century, however it was the devastating impact of the influenza pandemic of 1918 that would forever shape modern influenza virology [38].

The 1918 influenza pandemic has been referred to as "the greatest medical holocaust in history".[38] Conservative estimates report the 1918 influenza strain led to 50 million deaths globally while others suggest the death toll could have reached as many as 100 million. At the time of the 1918 outbreak, the etiological agent of influenza had yet to be correctly identified. In the previous decades, Richard Pfeiffer had isolated a rod-shaped bacterium from the nose of flu-infected patients that he believed to be the causative agent of influenza[42]. Pfieffer named the bacterium *Bacillus influenzae* which would later come to be known as *Haemophilus influenzae*[42]. Though Pfieffer's work was widely accepted, the devastation accompanying the 1918 pandemic caused renewed vigor in influenza research that ultimately called into question the validity of Pfieffer's claims. In 1921 Peter Olitsky and Fredericck Gates took nasal secretions from patients infected the 1918 strain and passed them through a Berkefeld filter. The filtrate was then exposed

to rabbits wherein the rabbits demonstrated symptoms indicative of influenza infection[42,43]. Olitsky and Gates' studies were the first to suggest the causative agent of influenza was not of bacterial origin, but their work became heavily criticized as others struggled to repeat it. It wasn't until 1929 that Richard Shope who followed Olitksy and Gates' filtration method using lung samples from an influenza infected pig who would confirm that the filterable agent was the cause of the influenza-like disease within pigs[42,44]. Inspired by Shope's finding, a group from the National Institute for Medical Research in the United Kingdom soon applied Shope's protocol to humans and were able to successfully isolate human influenza virus. Pandemic influenza strains would continue to persist through the 20th and into the 21st century, (notably during 1957, 1968, and 2009) however the isolation of the influenza virus now enabled the characterization of viral properties and pathogenesis.

Influenza viruses are members of the Orthomyxoviridae family and consist of influenza A, B, C, and D. Of the four influenza types, only influenza A and B are of notable to concern to human health. The genome of influenza consists of eight negative-sense, single-stranded RNA segments[45]. The nature of the influenza genome enables the virus to undergo antigenic drift and antigenic shift. RNA viruses display high mutation frequencies due to the reduced fidelity of RNA polymerase and the lack of 3' exonuclease proofreading mechanisms otherwise found within DNA genomes[46]. Projecting from the viral envelope are two glycoproteins, haemagglutinin (HA) and neuraminidase (NA). HA and NA have essential roles in the viral replication cycle as they facilitate viral attachment and escape from host cells. HA binds to the host cell

receptor alpha-2,6-linked sialic acid or alpha-2,3-linked sialic acid. As mutations accumulate within the HA gene segment antigenic shift can occur leading to changes in the HA subtype and affinity for sialic acid moieties[45,47].

Influenza A virus (IAV) is known to infect mammalian and avian species, the most notable being human, swine, and domestic poultry populations[45,48,49]. While influenza strains tend to circulate within one host species, observations of strains crossing species barriers is well documented[50,51]. Host receptor availability in combination with HA sub-type affinity contribute to virus infectivity. The distribution of alpha-2,6-linked sialic acid and alpha-2,3-linked sialic acid vary in anatomical locations among hosts. The bronchial epithelial cells of the human upper respiratory tract primarily display alpha-2,6-linkied sialic acid moieties while cells within the lower respiratory tract display the alpha-2,3-linked moiety. Within avian populations influenza typically infects the intestinal tract where the dominant receptor present is alpha-2,3-linked sialic acid. In contrast, Swine display and nearly even distribution of alpha-2,6-linked sialic acid and alpha-2,3-linked sialic acid within the trachea[45,49]. The receptor availability of both 2,3 and 2,6 linked sialic acid within swine can facilitate antigenic shift within influenza strains. Antigenic shift can occur when multiple viruses with differing receptor affinities simultaneously infect a host leading to a reassortment of viral gene segments and the production of a genetically distinct strain. This phenomenon, in combination with antigenic drift, contribute to looming threat of a novel influenza strain that would mirror the effects of the 1918 influenza pandemic.

Co-infection

Influenza A virus, (herein referred to as IAV) infection typically presents mild morbidity in immunocompetent hosts and is often resolved within two weeks of infection onset[52]. Within the last decade in the United State, influenza burdens have ranged from ~9-50 million confirmed cases annually. In contrast to the overwhelming number of illnesses, death caused by influenza alone is relatively uncommon[53]. Regardless of national annual influenza burdens, mortality totals rarely exceed 7 thousand and often occur in immunocompromised populations such as the young (< 5 years of age) and elderly (> 65 years of age)[52,54]. Despite low mortality rates from influenza infection alone, infection with influenza leads to an increased susceptibility to secondary bacterial pneumonia[55]. Bacterial pneumonia following antecedent influenza infections typically displays mortality rates between 10 to 15-fold greater than those of influenza alone[56–58]. This pathology has consistently been recognized as the 8th leading cause of death within the United States and was recently acknowledged as the primary infectious disease responsible for the recent decline in life expectancy at birth for US residents[58].

The first associations between primary influenza infection and the increased severity of secondary infections occurred early in the 20th century. In an observation of patients during the 1918 outbreak, Louis Cruveilhier made the confession "If grippe condemns, the secondary infections execute.[55]" Following his description of influenza etiology in swine, Robert Shope published an additional paper wherein he described an increased susceptibility to bacterial infection following influenza[42,44]. During the influenza pandemic of 1957 Donald Louria published his observations of three distinct

patient phenotypes 1) viral infection alone, 2) viral infection followed by late bacterial infection, and 3) simultaneous viral and bacterial infection; he noted the latter group displayed the highest mortality rates[59]. Recent retroactive studies performed by David Morens analyzed samples from each influenza pandemic of the previous century (1918, 1957, and 1968)[55]. Deaths from each pandemic strain were highly associated with secondary bacterial infections, particularly by *Streptococcus pneumoniae* and *S. aureus*. During the most recent 2009 pandemic, influenza co-infections were identified in >50% of all fatal cases[60,61]. The most prominent cause of fatal secondary bacterial pneumonia was identified as *S. aureus*[60].

S. aureus persistently colonizes the nares of 30% of the population[62]. Studies interrogating the contributions of respiratory commensals on disease have identified residents of the upper respiratory tract are frequently trafficked into the lungs via inhalation, microaspirations, and direct mucosal dispersion[63,64]. Despite recurrent exposure into the lower respiratory tract, *S. aureus* is rarely an etiological agent of lung disease in immunocompetent hosts; yet, it is the leading cause of fatal secondary pneumonia following infection with antecedent influenza[60,61]. Efforts to define the increased pathogenesis of *S. aureus* during secondary pneumonia have largely focused on immunomodulation occurring during the incumbent infection[65–68]. Despite progress demonstrating disruptions in the T_H17 pathway, Type I IFN, and innate immune cell function, studies examining secondary bacterial pneumonia by *S. aureus* remain incomplete[69–72]. Little attention has been directed towards defining how these host changes directly impact *S. aureus* virulence regulation and disease outcome[73].

In a unique model of *S. aureus* superinfection, Reddinger et al. demonstrated that infection with IAV facilitated dispersion of *S. aureus* nasal biofilms into the lower respiratory tract. In this study the authors suggest that changes in expression patterns, as *S. aureus* transitions from biofilm to planktonic growth, likely contribute to the increased incidences of *S. aureus* pneumonia following IAV infection[73]. Of note, *S. aureus* biofilm growth is characterized by decreased expression of toxins directly regulated by the *SaeR/S* TCS[74]. Additional studies examining the effect of IAV on *S. aureus* pathogenesis have suggested that IAV can directly bind to *S. aureus* peptidoglycan causing enhanced binding of *S. aureus* to epithelial cells[75].

Once within the lung, *S. aureus* persistence and disease has been linked to Hla dependent microaggregate formation; however, induction of *hla* expression within the lungs is debated[76]. RNA-seq analysis of *S. aureus* strain Newman grown in the presence of semi-synthetic bovine pulmonary surfactant (Surfacten®) demonstrated expression of *hla* and other *saeR/S* regulated virulence genes were slightly upregulated compared to growth in tryptic soy broth (TSB)[35]. In contrast, others have shown growth of *S. aureus* in host-fatty acids commonly associated with epithelial cells inhibit the expression of *saeR/S*[34,36]. Taken together, these studies demonstrate the ability of *S. aureus* to regulate transcription in response to the lung environment and highlight the importance of specific virulence factors in disease; nonetheless, they remain incomplete in describing how IAV infection directly impacts *S. aureus* pathogenesis during co-infection. Until recently, models to interrogate the bacterial contribution to secondary bacterial pneumonia were inefficient and lacked reproducibility. We recently refined a murine model of co-infection

to directly address the mechanisms defining the initiation and increased pathogenesis of secondary *S. aureus* pneumonia (chapter 2).

Hypothesis

S. aureus frequently encounters the lower respiratory environment yet cases of primary *S. aureus* pneumonia in immunocompetent hosts are rare. In contrast, *S. aureus* is a prominent cause of fatal secondary pneumonia when following an antecedent IAV infection. Efforts to resolve this pathology have attributed the increased susceptibility to *S. aureus* pneumonia to host immune changes resulting from the incumbent IAV infection. Despite the ability of *S. aureus* to sense and respond to its surrounding environment as it transitions from an asymptomatic commensal to an invasive pathogen, little attention has been aimed at understanding the impact of IAV infection on *S. aureus* virulence regulation. Thus, the overarching hypothesis governing the work herein is that **changes within the lower respiratory environment during IAV infection directly impact *S. aureus* virulence production and subsequent lung pathogenesis**.

In order to accurately assess the contributions of both IAV and *S. aureus* towards disease outcome it was necessary to develop a murine model of infection that enabled the delivery of precise pathogen doses into the lower respiratory tract. Chapter 2 discusses the benefits of our murine model of secondary pneumonia and demonstrates the advantages of this tool for studying transcriptional changes in the pathogen. Chapter 3 directly explored the hypothesis that *infection with antecedent IAV leads to increased expression of saeR/S-regulated toxins*. Data from this study identified that secondary bacterial pneumonia by *S. aureus* following IAV infection is *saeR/S* dependent. In

addition, these studies revealed toxin expression was delayed upon exposure to the healthy lung environment. Analysis of virulence expression patterns determined in chapter 3 led to the hypothesis that *components of the healthy lung environment suppress S. aureus virulence production* and this is explored in chapter 4. Results from this study demonstrate that, in healthy hosts, the lipid constituents of pulmonary surfactant suppress *S. aureus* cytotoxicity. Collectively, my dissertation research presents novel findings of the pathogenesis of secondary bacterial pneumonia by *S. aureus* wherein infection with IAV reduces pulmonary surfactant lipid concentrations leading to a loss of *S. aureus* virulence suppression and activation of *saeR/S*.

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CHAPTER TWO

A PRECISE PATHOGEN DELIVERY AND RECOVERY SYSTEM FOR MURINE MODELS OF SECONDARY BACTERIAL PNEUMONIA

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Video Article

A Precise Pathogen Delivery and Recovery System for Murine Models of Secondary Bacterial Pneumonia

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Abstract

Secondary bacterial pneumonias following influenza infections consistently rank within the top ten leading causes of death in the United States. To date, murine models of co-infection have been the primary tool developed to explore the pathologies of both the primary and secondary infections. Despite the prevalence of this model, considerable discrepancies regarding instillation procedures, dose volumes, and efficacies are prevalent among studies. Furthermore, these efforts have been largely incomplete in addressing how the pathogen may be directly influencing disease progression post-infection. Herein we provide a precise method of pathogen delivery, recovery, and analysis to be used in murine models of secondary bacterial pneumonia. We demonstrate that intratracheal instillation enables an efficient and accurate delivery of controlled volumes directly and evenly into the lower respiratory tract. Lungs can be excised to recover and quantify the pathogen burden. Following excision of the infected lungs, we describe a method to extract high quality pathogen RNA for subsequent transcriptional analysis. This procedure benefits from being a non-surgical method of delivery without the use of specialized laboratory equipment and provides a reproducible strategy to investigate pathogen contributions to secondary bacterial pneumonia.

Video Link

The video component of this article can be found at https://www.jove.com/video/59566/

Introduction

Secondary bacterial pneumonia following influenza infection is a leading cause of death in the United States and an active area of research[1,2]. Despite numerous studies using murine models of secondary bacterial pneumonia, inconsistencies regarding pathogen instillation and interrogation remain[3,4,5]. In addition, while many previous efforts have focused on the immunomodulatory effects of influenza that lead to an increased susceptibly to secondary bacterial infection, more recent data suggests virulence regulation of the bacterial pathogen is an equal contributor towards the establishment of disease[6,7,8,9]. These new data necessitate a more precise method to explore secondary bacterial pneumonia in murine models of co-infection that facilitate investigation into the pathogen response.

Unique to influenza co-infection models, host organisms are intentionally immunocompromised by a primary influenza infection prior to the administration of the secondary bacterial agent. In order to best replicate the disease pathogenesis observed in human hosts, it is imperative that the pathogen load of both primary and secondary agents be controlled so as to observe the individual and combinatorial effects of each infectious agent. Most commonly, respiratory infections in mice have been established through an intranasal administration[3,4,5,6,10]. Inasmuch as this route is noted for being technically simple and can be appropriate in some single-agent infection applications, it is unsuitable for co-infection models, as instillation procedures, dose volumes, and efficacy are highly variable within published literature[3,4,5,6].

To gain a more complete understanding of secondary bacterial pneumonia pathogenesis, contributions of both the host and the pathogen must be considered. To that end, we have developed a straightforward and reproducible approach for recovery of viable bacteria and pathogen RNA from infected lungs. This method uses a simplified, non-invasive intratracheal instillation procedure followed by subsequent isolation of bacterial RNA. The intratracheal instillation procedure described herein is similar to previously described methods and is not limited to pathogen delivery[11,12,13]. Use of this particular procedure benefits from being low-cost and does not require the use of specialized equipment such as cannulas, guide wires, or fiber optic cable; furthermore, because this procedure is non-invasive it insures minimal stress on murine subjects, minimizes an inflammatory response from the inoculation mechanics, and provides an efficient delivery route for the infection of multiple subjects. Briefly, isoflurane anesthetized mice are suspended from the incisors. Forceps are used to gently grasp the tongue followed by insertion of a pre-loaded bent, blunt-tipped, 21-gauge needle into the trachea and delivery of pathogen load. Validation of this procedure is demonstrated by visual confirmation of dye equally distributed into the pulmonary compartment and recovery of bacterial load. We then demonstrate how to recover viable *Staphylococcus aureus* (*S. aureus*) from infected lungs and describe a reproducible method to isolate high quality pathogen RNA.

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Protocol

All methods conform to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Montana State University.

1. Intratracheal Instillation

1. Prepare a workspace containing the following supplies: an intubation platform, a sterile 1 mL syringe, sterile blunt-tipped forceps, sterile 21-gauge blunt-tipped needle, and two conical tubes to store the syringe and forceps.
   NOTE: Intubation platforms can be purchased commercially or constructed in-house. The intubation platform used here was constructed using 0.25 inch plexiglass. Briefly, heat was applied to the plexiglass and the board is bent to an approximate 65° interior angle. On the exterior side of the intubation platform, two machine screws were seeded 3 inches apart and a rubber-band was suspended between the two screws.
2. Prepare the pathogen inoculum by serially diluting the sample so that the desired final concentration is obtained in a total volume of 50 µL. Store the inoculum on ice.
3. Wearing sterile gloves, bend a 21 G blunt-tipped needle to approximately 35°.
   NOTE: A separate needle is needed for each mouse.
4. Fix the bent 21 G blunt-tipped needle to a 1 mL syringe. Draw a 100 µL air cushion into the syringe followed by 50 µL of the infectious agent. Place the loaded needle and syringe in a location easily accessible by the dominant hand.
5. Deeply anesthetize a mouse using a 4% isoflurane/oxygen mixture or similar IACUC approved method of anesthesia. Proper anesthesia depth is typically obtained when respiration rates slow to approximately 1 inhalation per 5–8 s and can be confirmed by pinching an appendage and observing no reaction from the mouse.
   NOTE: This method of anesthesia provided a sufficient anesthetization duration of approximately 1.5 min. This was sufficient for members in our lab to learn and perform this instillation procedure; however, other institutionally approved anesthesia methods may be employed[11,12,13].
6. Remove the anesthetized mouse from the anesthesia chamber and suspend the mouse on the intubation platform from the maxillary incisors.
7. To open a clear passage into the trachea, use the blunt-tipped forceps to gently grasp and extend the tongue outside of the mouth. Transfer the tongue from the forceps into the thumb and index finger of the non-dominant hand.
8. Remain holding the tongue in the non-dominant hand and pick up the pre-loaded syringe. Point the bent end of the needle away from the body and insert the needle into the oral cavity to the base of the tongue.
9. With the needle at the base of the tongue, gently angle the wrist to cause a slight push of the needle away from the body. This step insures the needle will be inserted into the trachea and not the esophagus.
10. Slowly guide the needle down into the trachea; often a slight tick is felt as the needle passes through the vocal fold. Pass the needle through the trachea until slight resistance is felt as the needle encounters the carina.
11. Slightly lift the needle in the proximal direction to suspend the needle above the carina. This enables delivery into the two primary bronchi. Fully depress the syringe's plunger to deliver the infectious load. Remove the needle from the trachea by lifting upward and discard.
12. Remove the mouse from the intubation platform by depressing the rubber band but maintain holding the mouse in an upright position. Obstruct the nasal airways by placing a finger directly over the nares. Hold this position for approximately 1 min or until several deep breathes are observed.
    NOTE: This last step ensures that the total inoculum volume is delivered into the lower respiratory tract.
13. Return the mouse to its cage and observe a complete recovery from the anesthesia.

2. Excision of Infected Lungs

1. Working within a laminar flow hood to maintain sterility, prepare a workspace with the following items: a scale and sterile weigh boat, a dissection platform, a dissection kit containing scissors and forceps, sterile RNAse-free PBS, and sterile gauze.
2. Euthanize an infected mouse with CO_2 or similar IACUC approved method of euthanasia.
3. Place an infected mouse on a dissection platform and secure the mouse using pins at the end of each appendage. Spray the mouse with ethanol to help maintain sterility of the working surfaces.
4. Beginning at the umbilicus, use a pair of forceps to lift the skin and make an incision up to the base of the trachea. Grasping the skin on either side of the initial incision, pull the skin away from the body and cut through the tissue connections.
   NOTE: It can be helpful to make several lateral cuts across the skin to reveal more of the thoracic region.
5. Starting at the base of the xiphoid process, make a small incision with the intention of puncturing the diaphragm. This results in an increase in pressure in the thoracic cavity that causes the lungs to retract. With the lungs retracted continue the incision to free the diaphragm.
6. Remove the ribs by making an incision along both sides of the rib cage. This will fully expose the thoracic cavity and lungs.
7. To excise the lungs, grasp the base of the heart with the forceps and lift upward. Place the scissors behind the lungs and begin to make small incisions through the tissue connections while continuing to the lift the heart upward.
8. Once the lungs have been excised, place them onto the sterile gauze and remove the heart. The heart can be easily removed by lifting it away from the lungs with the forceps and cutting the remaining tissue connections.
9. Transfer the lungs to a pre-tared weigh boat and record the weight.
10. After the lungs have been weighed, transfer them into sterile phosphate buffered saline (PBS) and temporarily store them on ice until moving forward to the pathogen recovery and analysis steps.

3. Pathogen Recovery and Analysis

1. Continuing to work within a laminar flow hood, prepare a workspace with the following supplies: a tissue grinder, sterile RNAse-free PBS, Buffer RLT supplemented with ß-mercaptoethanol (1:100), and 1.5 mL RNAse-free microcentrifuge tubes.

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CAUTION: ß-mercaptoethanol can be hazardous in the case of skin contact, ingestion, eye contact, or inhalation. Dilution of ß-mercaptoethanol into buffer RLT should be done in a fume hood.

2. Begin by first adding 1 mL of sterile RNAse-free PBS to the tissue grinder. Once filled, store the tissue grinder on ice.
3. Prepare a series of sterile RNAse-free serial dilution tubes that will be used to quantify the bacterial load recovered from the infected lungs. This is most easily accomplished by aliquoting 900 µL of sterile water into each microcentrifuge tube followed by serial dilution of the pathogen inoculum.
   NOTE: The number of dilution tubes necessary for accurate pathogen enumeration will vary on initial pathogen input and duration of infection. This must be empirically determined.
4. Using sterile forceps, transfer the lungs into the prepared tissue grinder and thoroughly homogenize the tissue by pressing down on the pedestal while rotating.
5. Open the tissue grinder and aliquot 100 µL of the homogenized sample into the first of the prepared serial dilution tubes. Serially dilute the samples and enumerate CFUs by plating onto nutrient agar. CFUs can be recorded as CFUs/mL or CFUs/mL/mg of lung tissue.
   NOTE: At this step an additional 200 µL of the homogenized sample can be removed for purification of viral RNA (see **Table of Materials**) or stored at -80°C for future analysis.
6. After the aliquots have been removed for CFU determination and/or viral RNA isolation, replace the grinding pedestal and pellet the homogenized tissue by centrifugation at 4,000 rpm (3724 x *g*) for 10 min at 4 °C.
7. Using a pipette, remove the supernatant from the pelleted sample and place into a 1.5 mL microcentrifuge tube.
   NOTE: The supernatant is free of bacteria and can be stored at -80 °C for analysis of soluble host and secreted pathogen factors.
8. Resuspend the homogenized tissue pellet by pipetting or vortexing in 700 µL of buffer RLT-ß-mercaptoethanol and transfer the sample to a sterile RNAse-free 1.5 mL microcentrifuge tube.
   NOTE: At this point the samples can be stored at -80 °C for several months.
9. Purify RNA using slight modifications to the manufacturer's protocol of a purification kit (see **Table of Materials**); see Voyich et al. 2008[14].
   1. Transfer the homogenized lung slurry into a 2 mL microcentrifuge tube containing 0.1 mm silica beads and processed via a bead beater for 20 s at 6 m/s.
   2. Centrifuge the sample at 1,500 rpm for 3 minutes. After centrifugation, pipette the supernatant into new 1.5 mL microcentrifuge tube.
   3. Add 350 µL of 96%–100% ethanol to the sample and thoroughly mix by pipetting or inversion.
   4. Transfer 700 µL of the sample to a purification column placed in a 2 mL collection tube. Centrifuge the sample at ≥ 8,000 x *g* for 15 s and discard the flow-through. Run any excess sample through the same column as described above.
   5. Wash the column with 700 µL of buffer RW1 and centrifuge sample at ≥ 8,000 x *g* for 15 s. Discard the flow-through and transfer the silica membrane column into a new 2 mL collection tube.
   6. Apply 500 µL of buffer RPE to the column. Wash the column by centrifugation at ≥ 8,000 x *g* for 15 s. Discard the flow-through.
   7. Apply an additional 500 µL of buffer RPE to the RNeasy column and centrifuge at 8,000 x *g* for 2 min. Discard flow-through and centrifuge the column at ≥10,000 x *g* for 1 min.
   8. To elute purified RNA, begin by transferring the column to a new 1.5 mL RNAse-free microcentrifuge tube. Pipette 50 µL of RNase-free water directly onto the silica-gel membrane of the column and centrifuge at ≥8000 x *g* for 1 min.
      NOTE: The eluted RNA can be run over the same column again to increase RNA yield.
   9. Add 50 µL of RNase-free water to the purified RNA to bring the total volume to 100 µL. Aliquot 350 µL RLT-ß-mercaptoethanol followed by 250 µL of 96%–100% ethanol to the sample and mix thoroughly by pipetting or inversion.
   10. Apply the sample to a purification column placed in a collection tube and centrifuge at ≥8000 x *g* for 15 s. Discard the flow-through and replace the collection tube.
   11. Wash the column by adding 350 µL of buffer RW1 followed by centrifugation at ≥8000 x *g* for 15 s.
   12. Prepare a DNase solution containing approximately 27 Kunitz units by adding 10 µL of DNase stock (750 Kunitz units/mL) to 70 µL of buffer RDD. Add 80 µL of DNase solution directly onto the silica-gel membrane and incubate the sample for 15 min at room temperature.
   13. Wash the column with 350 µL of buffer RW1 and centrifuge at ≥8000 x *g* for 15 s and discard the flow-through.
   14. Repeat steps 3.9.6–3.9.8 to purify RNA.
       NOTE: It is recommended to repeat the RNA clean-up procedure by following steps 3.9.9–3.9.10 and 3.9.6–3.9.8 (skip DNase steps 3.9.11–3.9.13). This additional clean-up it results in very high-quality RNA.
10. RNA yield can be quantified using a spectrophotometer with readings at 260 nM for determining concentration and 260:280 nM for purity. After RNA yield is obtained, standardize the concentration of each RNA sample to 50 ng/µL.
    NOTE: It is recommended to make multiple aliquots at a concentration of 50 ng/µL to avoid freeze/thaw cycles that can lead to RNA degradation.
11. Store the purified RNA at -80 °C or use immediately for transcript analysis.
    NOTE: In previous publications RNA from 3–5 mice was pooled for transcript analysis. Through optimization of the above technique, RNA from 1 mouse has been empirically determined as sufficient for transcript analysis (80–120 ng/µL).

Representative Results

Figure 1 utilizes a 0.1% weight/volume Coomassie brilliant blue solution to demonstrate that an intratracheal instillation delivers the inoculum directly and evenly within the lower respiratory tract. **Figure 2** shows that the bacteria (*S. aureus*) CFUs recovered directly from homogenized lung tissue. **Figure 3** demonstrates the use of this system for precise delivery and recovery of inoculum in the lower respiratory tract by plotting the input and recovery CFUs from individual mice. **Figure 4** shows the qRT-PCR amplification curve of the bacterial housekeeping gene *gyrB* to demonstrate that bacterial RNA can be extracted directly from infected lung tissue with minimal DNA contamination. **Figure 5** shows the construction of a standard curve using qRT-PCR amplification of the influenza A virus M-segment to demonstrate viral RNA can be extracted directly from infected lung tissue.

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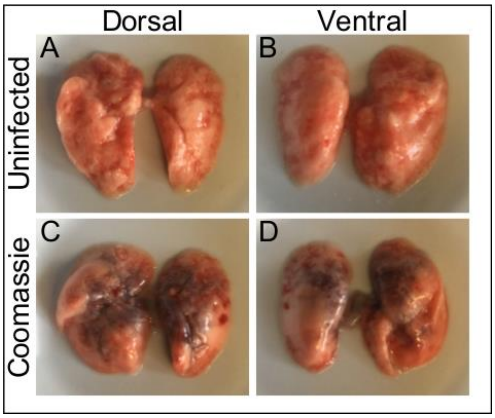


Figure 1: Intratracheal instillation enables even distribution into the lower respiratory tract. (**A**, **B**) Uninfected lungs were excised from a healthy mouse following intratracheal instillation of sterile PBS and photographed from (**A**) dorsal and (**B**) ventral perspectives. (**C**, **D**) 50 µL of a 0.1% Coomassie brilliant blue solution were administered to an anesthetized mouse via intratracheal administration. (**C**) Dorsal. (**D**) Ventral. Please click here to view a larger version of this figure.

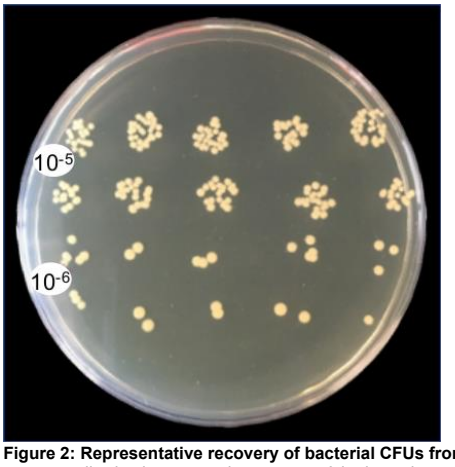


Figure 2: Representative recovery of bacterial CFUs from infected lung homogenate. Lungs were excised one day post-challenge with *S. aureus*. Following homogenation, 100 µL of the lung slurry was serially diluted through 10^{-6}. To enumerate CFUs recovered, 10 µL drops were plated from the 10^{-5} and 10^{-6} dilutions onto tryptic soy agar (TSA) and incubated at 37 °C with 5% $C0_2$ overnight. Please click here to view a larger version of this figure.

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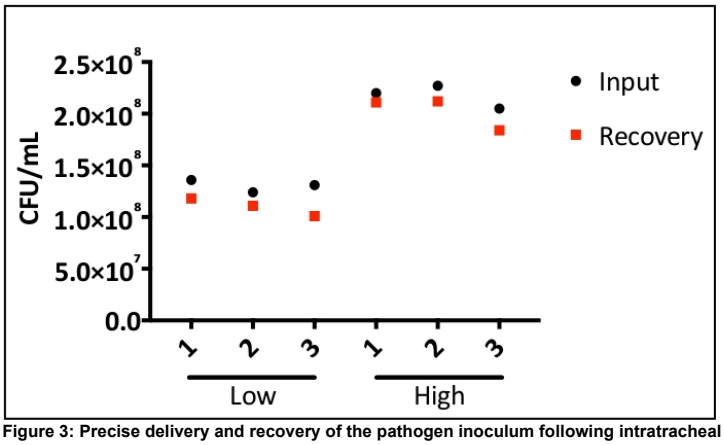


Figure 3: Precise delivery and recovery of the pathogen inoculum following intratracheal administration. Mice were divided into two groups containing three mice per group. Mice were subjected to intratracheal instillation of *S. aureus* at 1 x 10^8 (low) and 2 x 10^8 (high) CFU/mL. One hour post-infection, mice were euthanized and the lungs were excised to demonstrate the precision of the instillation and recovery. Bacterial inoculum and bacteria recovered from the lung homogenate were plated on TSA (tryptic soy agar). No significant differences were reported between bacterial input and recovery. Please click here to view a larger version of this figure.

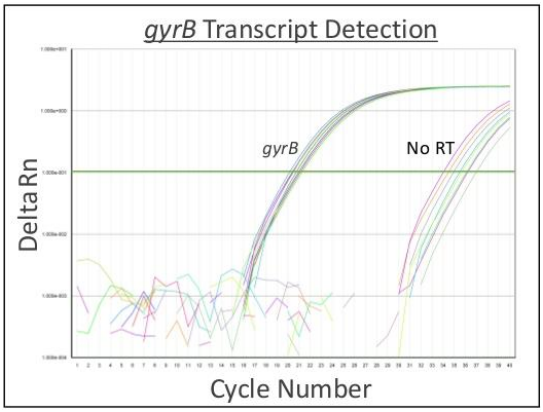


Figure 4: Representative bacterial RNA recovery and purity. Six-hours following intratracheal instillation with 1 x 10^8 CFU/50 µL of *S. aureus*, mice were euthanized. Lungs were excised and homogenized followed by resuspension of the lung slurry in buffer RLT-ß-mercaptoethanol. RNA was purified as described in step 3.9[14]. qRT-PCR was used to detect transcripts of the bacterial housekeeping gene *gyrB*. A control containing no reverse transcriptase (nRT) was included to demonstrate the purity of the RNA recovered. At a threshold of 0.1, *gyrB* transcripts were detected at an average cycle of 21.1, 20.3, and 20.5. nRT controls were not detected until cycle averages 35.9, 35.5, and 35.0. n = 3 biological replicates, containing 3 technical replicates/biological replicate. Please click here to view a larger version of this figure.

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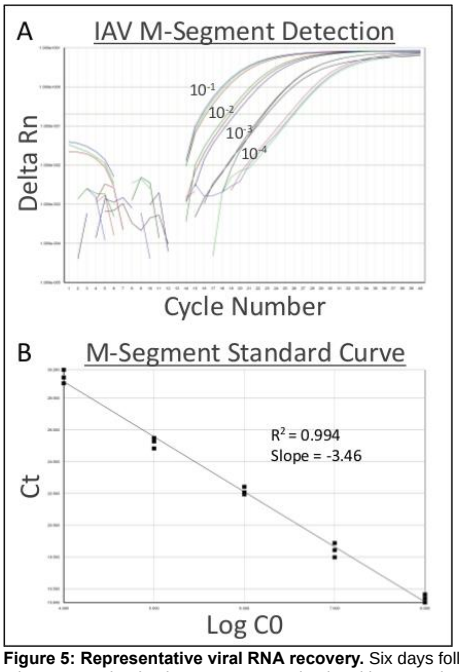


Figure 5: Representative viral RNA recovery. Six days following intratracheal instillation with 100 PFU/50 µL of influenza A/PR/8/1934(H1N1), mice were euthanized. Lungs were excised and homogenized, and 200 µL of the homogenized slurry was collected and passed through a 70 µm cell strainer prior to viral RNA purification. Purified RNA was serially diluted (10^{-1}-10^{-4}) followed by amplification of influenza A M-segment. (**A**) Amplification plot of influenza A RNA recovered from an infected lung and diluted 10^{-1}–10^{-4}. (**B**) Standard curve of influenza A M-segment. Threshold = 0.2, R^2 = 0.994, slope = -3.46. Please click here to view a larger version of this figure.

Discussion

Use of this model provides a highly efficient and reproducible method to study secondary bacterial infections. The ability to tightly control the delivery of the pathogen inoculum enables more precise observations of the individual and combinatorial effects of each pathogen. Inefficiencies in the more common intranasal instillation route have likely contributed to the discrepancies in dose volumes and concentrations present in the literature. It is reasonable that the lack of a precise murine system to study secondary bacterial pneumonia has delayed findings identifying bacterial specific responses that contribute to the severity of pulmonary co-infections. Developing a reproducible model to study virulence expression during secondary bacterial infections could lead to the identification of vaccine or drug targets to ameliorate these infections.

The intratracheal instillation step is critical to successfully establish a lower respiratory tract infection and any down-stream analysis of the pathogens. When learning this technique, it may be helpful to practice using a dye (as described in the methods) prior to administering infectious material. Using a dye allows for the direct visualization of the inoculum into the respiratory tract. A common mistake that can occur is insertion of the blunt-needle into the esophagus rather than the trachea. This will result in delivery of the inoculum into the stomach rather than the lungs. To correct this mistake, angle the needle further away from the body and pass it down into the trachea. Once mastered, this procedure is very efficient and can be used to conduct experiments with large numbers of mice. Working in batches to anesthetize mice, the intratracheal instillation can be completed in approximately 30 seconds per mouse. In addition, the excision of the lungs can be completed in 2 to 3 minutes per mouse.

Recovery of viable and pure bacterial RNA from infected tissues is critical for transcript analysis. RNases are ubiquitous and can quickly ruin an experiment[15]. Some methods include using RNase inhibitors; however, we have found that freezing the sample at -80 °C in RLT-ß-mercaptoethanol or immediately processing the sample for RNA isolation using all RNase free tubes and reagents are effective at reducing RNase contamination. Additionally, we recommend that a maximum of six samples be purified at one time. Including more than six samples can result in prolonged latencies between protocol steps that can culminate in RNA degradation. Once purified, care should also be taken to avoid any unnecessary freeze-thaw cycles. Thus, if multiple analyses will be done on one sample, aliquoting purified RNA for storage at -80 °C is recommended.

In addition to the techniques reviewed herein, this method can be supplemented by performing bronchial alveolar lavage prior to the excision and homogenation of the lungs[16]. This can be accomplished by lavage of the entire lower respiratory tract or by using suture thread to restrict one branching arm of the bronchial tree followed by lavage through the remaining branch. Often this leads to a reduction in the recovery of the pathogen load but provides a sample whereupon information such as lactate dehydrogenase activity, cellular population identity, and cytokine

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profiles can be obtained[16]. Together these data can form a more complete understanding of the host-pathogen interactions occurring during secondary bacterial pneumonia.

While the methods discussed have been within the context of secondary bacterial pneumonia, they are suitable to be extended to any murine model of lower respiratory infection; specifically, those that would benefit from tightly controlled delivery and recovery of the installed inoculum. Furthermore, like many other infection routes, the intratracheal instillation can be utilized in non-infectious applications, such as the administration of therapeutics and environmental compounds[12].

Disclosures

The authors have nothing to disclose.

Acknowledgments

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CHAPTER THREE

SECONDARY BACTERIAL PNEUMONIA BY *STAPHYLOCOCCUS AUREUS* FOLLOWING INFLUENZA A INFECTION IS SAER/S DEPENDENT

Contribution of Authors and Co-Authors

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Contributions: project design and experimental procedures

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Contributions: oversight, project design, data analysis, figure presentation, and manuscript writing

Manuscript Information

Timothy R. Borgogna, Bennett Hisey, Emily Heitman, Joshua J. Obar, Nicole Meissner, and Jovanka M. Voyich

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BRIEF REPORT

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 USA300Δ*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 preformed using a total volume of 50 μL 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.

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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 µL 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-µL 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 µL 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 µL) were used for CFU determination and the remaining sample was centrifuged at 4000 rpm (3724 × 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/µL 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 USA300Δ*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 *ΔsaeR/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 coinfected 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 *ΔsaeR/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 coinfected 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 coinfected 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

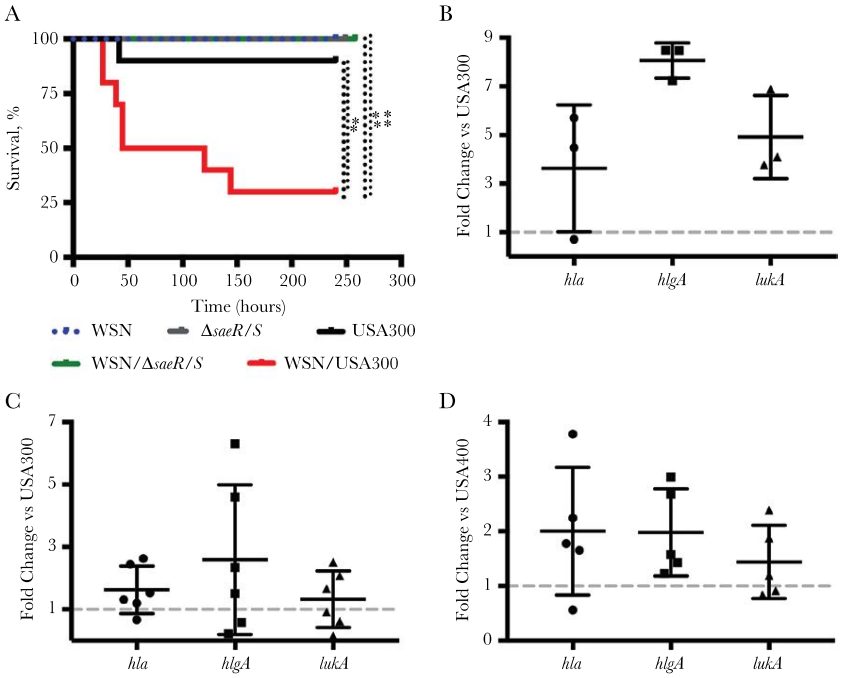


Figure 1. *Staphylococcus aureus* bacterial pneumonia following antecedent influenza A virus (IAV) infection is *saeR/S* dependent. Mice were intranasally mock-infected with phosphate-buffered saline or infected with IAV. Six days postinfection, mice were challenged with *S. aureus*. *A*, Survival assay of mice infected with IAV/WSN/1933(H1N1) (WSN) (750 plaque-forming units/50 µL) and challenged with USA300 (5×10^8 colony-forming units [CFU]/50 µL), 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 µL), RNA was isolated from 3–6 mice per treatment group. Data displayed are the mean fold change of *S. aureus* expression levels from coinfected 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 coinfected 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 2*A* and 2*B*). Bacterial burdens recovered at 8 hours postchallenge were lower in Δ*saeR/S*- compared with USA300-infected mice; however, CFUs were equal between both coinfected 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

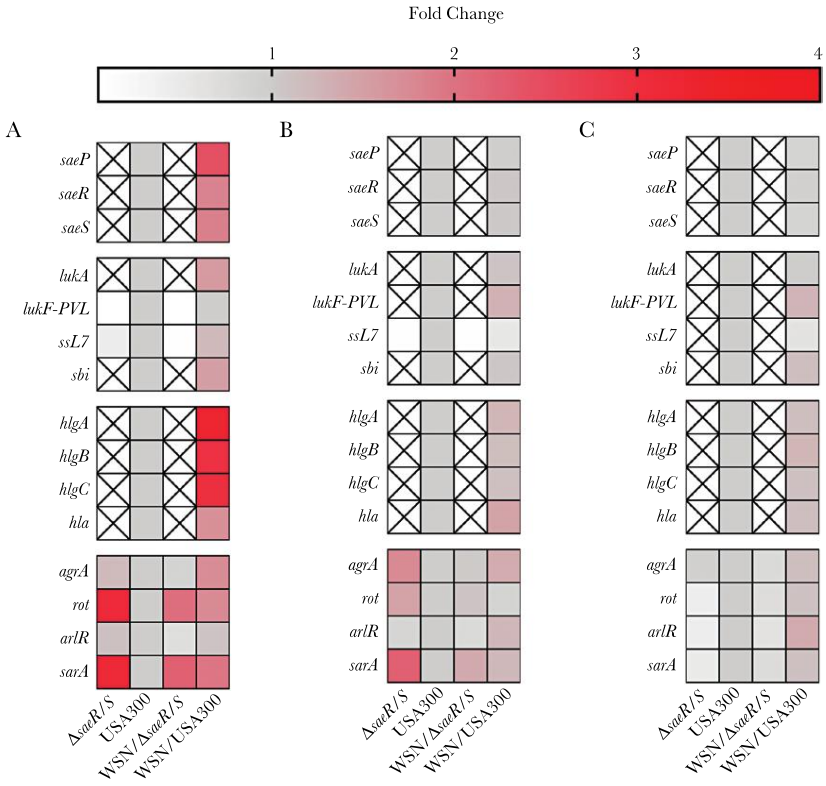


Figure 2. Influenza A virus (IAV) infection promotes rapid up-regulation of *Staphylococcus aureus* SaeR-regulated genes. Mice were infected intranasally with IAV/WSN/1933(H1N1). Six days post-IAV infection, mice were challenged with USA300 or Δ*saeR/S*. Four (*A*), 6 (*B*), or 8 (*C*) hours post–*S. 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 coinfected 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

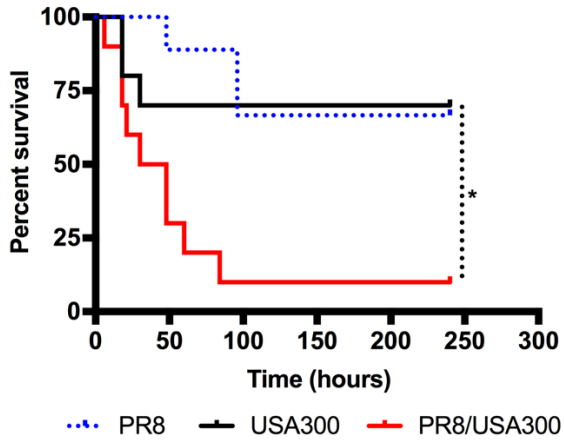
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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript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

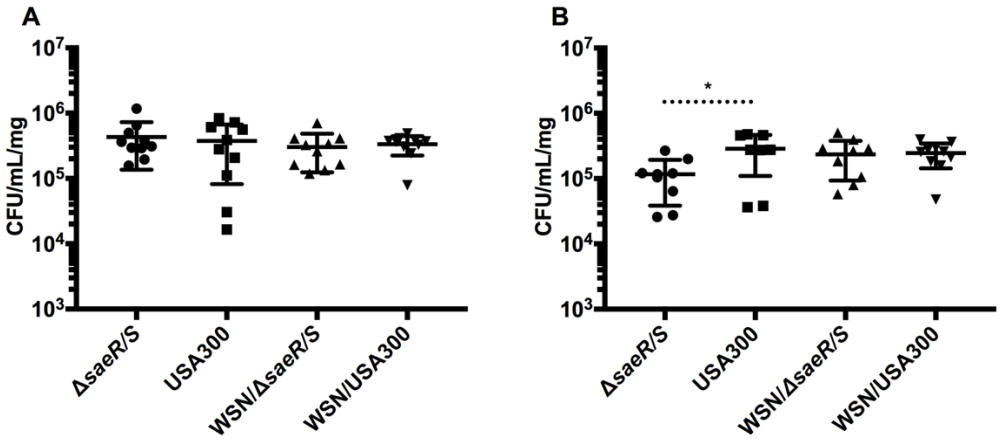
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SUPPLEMENTAL FIGURES

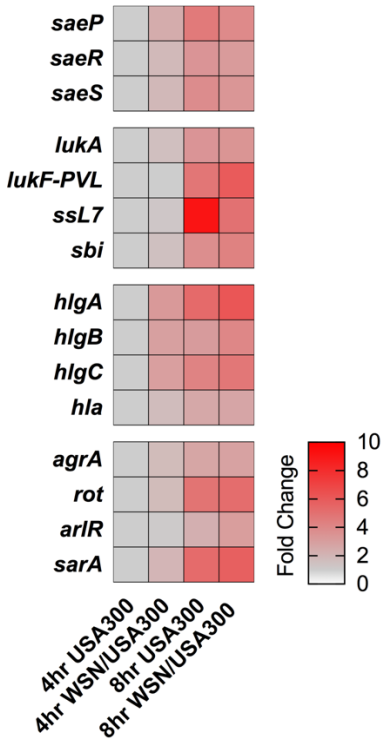


Supplemental Figure 1. Antecedent infection with IAV strain PR8 leads to increased *S. aureus* mortality compared to infection with IAV or *S. aureus* alone. Mice were given PBS (50 µL) or infected with PR8 intratracheally (100 PFU/50 µL). 6-days post infection mice were challenged with *S. aureus* ($2.5x10^8$ CFU/50uL) via the same route. n = 9-10 mice per group. *$p < 0.05$, log-rank (Mantel-Cox) test.



Supplemental Figure 2. SaeR/S does not impact bacterial burden during pneumonia. Bacterial burdens recovered from treatment groups at (A) 4 and (B) 8-hours post *S. aureus* infection. Infected lungs were homogenized in 2 ml of PBS, serially diluted, and plated on tryptic soy agar. Bacterial colonies were enumerated the following day to

calculate CFUs/mL. Total CFU/mL data reported as CFU/mL/mg of harvested lung tissue. Data displayed are from two separate experiments, n = 4-5 mice per treatment group. *p = 0.05 using ANOVA followed by Tukey's multiple comparison test.



Supplemental Figure 3. SaeR/S and SaeR-target genes are upregulated late in primary *S. aureus* pneumonia. Mice were infected intranasally with WSN (750 PFU/50 µL). Six days post influenza A infection mice were challenged with USA300 or Δ*saeR/S.* 4 and 8-hours-post *S. aureus* challenge mouse lungs were harvested. RNA was purified from harvested lungs and treatment groups were pooled (n=4-5 mice per treatment group). Gene transcripts were normalized to *gyrB* and calibrated to the expression levels in USA300 only infection at 4 hours. Differential regulation of SaeR-target genes was assessed by Quantigene 2.0 assays. Data reported as mean fold-change of two experiments.

SUPPLEMENTAL TABLES

Supplemental Table 1. qRT-PCR primers and probe sequences.

RT-PCR Primers and Probes	**Sequence**
gyrB fwd	5’ - CAAATGATCACAGCTTTGGTACAG - 3’
gyrB rvs	5’ - CGGCATCAGTCATAATGACGAT - 3’
gyrB probe	5’ - AATCGGTGGCGACTTTGATCTAGCGAAAG - 3’
hla fwd	5’ - CAACAACACTATTGCTAGGTTCCATATT - 3’
hla rvs	5’ - CCTGTTTTTACTGTAGTATTGCTTCCA - 3’
hla probe	5’ - ATGAATCCTGTCGCTAATGCCGCAGA - 3’
hlgA fwd	5’ - ACTTATTTGACAAGACCCAACTG - 3’
hlgA rvs	5’ - CCACTTTGAATTAAAGGAGGTAATTGAT - 3’
hlgA probe	5’ - CAGCAGCAAGAGACTATTTCGTCCCAG - 3’
lukA fwd	5’ - GCGTCATCATTATCATGTGCAA - 3’
lukA rvs	5’ - TCTTTCTTATTTTGGTVTTGAGAGTCTT - 3’
lukA probe	5’ - CAGCAACGACTCAAGCAAATTCAGCTCA - 3’
lukF-PVL fwd	5’ - TTGCTTTTGCTATCCAATACAGTTG - 3’
lukF-PVL rvs	5’ - TCGGAATCTGATGTTGCAGTTG - 3’
lukF-PVL probe	5’ - TGCAGCTCAACATATCACACCTGTAAGT - 3’

Supplemental Table 2. Immediate increase in select *S. aureus* gene transcripts in secondary *S. aureus* pneumonia. Fold-change expression of USA300 targets in co-infected mice compared to mice infected with USA300. Quantigene 2.0 assays of select *S. aureus* gene transcripts. Transcripts were normalized to *gyrB* expression and calibrated to the expression levels in USA300 only infection at 4, 6, and 8 hours. Data displayed is the time-matched, fold-change expression of target genes at 4, 6, and 8 hours post *S. aureus* challenge compared to expression levels in USA300 only infection at 4, 6, and 8 hours post *S. aureus* challenge. *Indicates target gene not included in heatmap analysis. SEM = Standard error of the mean of 2 experiments.

Locus	**4 Hour**	**SEM**	**6 Hour**	**SEM**	**8 Hour**	**SEM**
saeP	2.40	+/- 0.32	0.96	+/- 0.11	0.86	+/- 0.08
saeR	1.80	+/- 0.30	1.03	+/- 0.24	0.92	+/- 0.02
saeS	1.85	+/- 0.34	1.03	+/- 0.20	0.89	+/- 0.01
lukA	1.56	+/- 0.56	1.09	+/- 0.53	0.99	+/- 0.14
lukF-PVL	0.98	+/- 0.22	1.29	+/- 0.64	1.25	+/- 0.16
ssL7	1.20	+/- 0.49	0.53	+/- 0.29	0.60	+/- 0.14

sbi	1.50	+/-	0.46	1.04	+/-	0.43	1.14	+/-	0.14
hlgA	3.25	+/-	1.31	1.25	+/-	0.25	1.13	+/-	0.15
hlgB	2.92	+/-	1.22	1.15	+/-	0.17	1.24	+/-	0.20
hlgC	2.95	+/-	1.12	1.12	+/-	0.20	1.11	+/-	0.05
hla	1.66	+/-	0.25	1.44	+/-	0.33	1.14	+/-	0.46
agrA	1.72	+/-	0.16	1.35	+/-	0.50	1.14	+/-	0.28
rot	1.75	+/-	0.17	0.88	+/-	0.08	1.10	+/-	0.22
arlR	1.07	+/-	0.05	1.23	+/-	0.55	1.36	+/-	0.16
sarA	1.94	+/-	0.10	1.21	+/-	0.32	1.11	+/-	0.25
mecA*	1.31	+/-	0.14	1.23	+/-	0.03	1.08	+/-	0.04
splA*	0.88	+/-	0.01	0.97	+/-	0.78	1.20	+/-	0.40
dltA*	1.12	+/-	0.00	0.99	+/-	0.07	0.93	+/-	0.01

Supplemental Table 3. Late induction of SaeR/S and SaeR-target genes in primary *S. aureus* pneumonia. Fold-change expression of USA300 targets in co-infected mice compared to mice infected with USA300. Quantigene 2.0 assays of select *S. aureus* gene transcripts. Transcripts were normalized to *gyrB* expression and calibrated to the expression levels in USA300 only infection at 4 hours. Data displayed is the fold-change expression of target genes at 4, 6, and 8 hours post *S. aureus* challenge compared to expression levels in USA300 only infection at 4 hours post *S. aureus* challenge. *Indicates target gene not included in heatmap analysis. SEM = Standard error of the mean of 2 experiments.

Locus	**4 Hr USA300**	**SEM**	**4 Hr WSN/USA300**	**SEM**	**8 Hr USA300**	**SEM**	**8 Hr WSN/USA300**	**SEM**
saeP	1.00	0.00	2.40	+/- 0.32	4.62	+/- 0.83	3.90	+/- 0.18
saeR	1.00	0.00	1.80	+/- 0.30	3.39	+/- 0.29	3.13	+/- 0.16
saeS	1.00	0.00	1.85	+/- 0.34	3.81	+/- 0.30	3.38	+/- 0.17
lukA	1.00	0.00	1.56	+/- 0.56	3.41	+/- 0.55	3.46	+/- 0.52
lukF-PVL	1.00	0.00	0.98	+/- 0.22	4.76	+/- 0.13	5.94	+/- 0.30
ssl7	1.00	0.00	1.20	+/- 0.49	9.12	+/- 3.76	4.97	+/- 0.49
sbi	1.00	0.00	1.50	+/- 0.46	3.70	+/- 0.17	4.25	+/- 0.36

hlgA	1.00	0.00	3.25	+/- 1.31	5.28	+/- 1.33	6.18	+/- 1.14
hlgB	1.00	0.00	2.92	+/- 1.22	3.10	+/- 0.96	4.04	+/- 0.90
hlgC	1.00	0.00	2.95	+/- 1.12	4.20	+/- 1.34	4.72	+/- 0.84
hla	1.00	0.00	1.66	+/- 0.26	2.56	+/- 0.52	2.69	+/- 0.29
agrA	1.00	0.00	1.72	+/- 0.16	2.64	+/- 0.57	2.84	+/- 0.05
rot	1.00	0.00	1.75	+/- 0.17	4.85	+/- 0.72	5.16	+/- 0.14
arlR	1.00	0.00	1.07	+/- 0.05	2.21	+/- 0.07	3.00	+/- 0.22
sarA	1.00	0.00	1.94	+/- 0.10	5.27	+/- 0.38	5.76	+/- 0.45
mecA*	1.00	0.00	1.31	+/- 0.07	2.06	+/- 0.71	2.20	+/- 0.34
splA*	1.00	0.00	0.88	+/- 0.01	2.88	+/- 0.67	3.73	+/- 0.98
dltA*	1.00	0.00	1.16	+/- 0.02	1.52	+/- 0.05	1.41	+/- 0.03

CHAPTER FOUR

UNCOVERING THE EXECUTIONER: DISRUPTION OF PULMONARY SURFACTANT BY INFLUENZA A TRIGGERS *STAPHYLOCOCCUS AUREUS* PNEUMONIA

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Contributions: project oversight, project design, data analysis, figure presentation, and manuscript writing

Manuscript Information

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____ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal

ABSTRACT

Staphylococcus aureus (*S. aureus*) commonly colonizes the anterior nares and is frequently trafficked from the upper respiratory tract into the lungs. Despite recurrent exposure to the lower respiratory tract, *S. aureus* is rarely an etiological agent of lung disease in immunocompetent hosts. Infection with influenza A virus (IAV) drastically increases host susceptibility to lethal *S. aureus* pneumonia, yet the mechanisms describing the pathogenesis of *S. aureus* pneumonia following IAV infection have remained incompletely defined. Herein we test the hypothesis that apart of IAV induced injury, components of pulmonary surfactant suppress *S. aureus* virulence. USA300 exposed to murine pulmonary surfactant showed reduced expression of the bi-component γ-toxin *hlgA* and an inability to lyse human polymorphonuclear cells (PMNs). Use of a plasmid based USA300-$_{hlgA}$-sGFP reporter assay identified that growth of USA300 in the presence of palmitate mirrored the suppressive effects of pulmonary surfactant. We demonstrate that infection with IAV leads to a reduction in total lung fatty acid concentrations. Lipidomic analysis of IAV infected lungs revealed 25.88% of phosphatidylcholine and 69.35% of phosphatidylglycerol lipid analytes were significantly reduced compared to mock infected lungs. Together, these data provide a novel model of secondary bacterial pneumonia by *S. aureus* wherein infection with IAV reduces pulmonary surfactant lipid concentrations leading to a loss of *S. aureus* virulence suppression and onset of disease.

INTRODUCTION

Bacterial co-infection following antecedent IAV infection is the leading cause of IAV associated deaths within the United States[1]. Modern studies examining samples from the four most recent pandemic influenza outbreaks (1918, 1957, 1968, and 2009) demonstrate up to 95% of fatal cases were associated with secondary bacterial infections[2–4]. In non-pandemic years, mortality rates of IAV infection followed by bacterial infection remain greater than 10-fold higher than IAV infection alone[1,5,6]. Furthermore, IAV leading to pneumonia has been identified as the primary infectious disease responsible for the recent decline in life expectancy at birth for US residents, a trend not previously observed since the World War I era[1].

Studies defining the mechanisms associated with the increased susceptibility to secondary bacterial pneumonia have heavily focused on the dysregulation of host-defenses during the incumbent infection[7,8]. Despite progress identifying IAV specific immunomodulations, such as disruptions in the T_H17 pathway, type I IFN production, and phagocyte cell function, investigations examining the reciprocal interplay between host and pathogens remain incomplete[9–13]. Only recently have efforts been made to describe the direct contributions of the bacterial pathogen to the increased morbidities and mortalities associated with bacterial co-infections[14,15].

Three pathogens have been identified as the predominant etiologic agents of secondary bacterial pneumonia, *Haemophilus influenzae* (*H. influenzae*), *Streptococcus pneumoniae (S. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*)[4,16]. While *S. pneumoniae* is often recognized as the most prevalent, *S. aureus* has emerged as the most

common pathogen associated with severe and life-threatening secondary pneumonia[3,15,16]. *S. aureus* is common colonizer of the upper respiratory tract and is estimated to colonize 50-60% of the population[17,18]. Its residence in the anterior nares allows for it to be frequently trafficked into the lungs through inhalations, microaspirations, and direct mucosal dispersions[19]. Despite recurrent exposure to the lower respiratory tract, *S. aureus* is rarely an etiological agent of lung disease in immunocompetent hosts; however, infection with IAV greatly increases host susceptibility to severe secondary bacterial pneumonia by *S. aureus*. Previous studies examining IAV and *S. aureus* co-infections have overwhelmingly focused on host immune responses during the antecedent infection, yet the factors governing *S. aureus* pathogenesis in the lungs have been largely ignored[20–22].

Pulmonary surfactant is a lipid rich complex (~90% lipid by dry weight) that can acts as the first line of defense against invading organisms as they progress through the bronchial tree and into the lungs. The vast majority of lipids composing surfactant are phosphatidylcholines, with dipalmitoylphosphatidylcholine (DPPC) being the most abundant, and phosphatidylglycerols[23,24]. Palmitic acid has been reported as the most prevalent free fatty acid and is required for the synthesis of DPPC[23,25]. The remaining 10% of pulmonary surfactant is composed of four surfactant proteins: SP-A, SP-B, SP-C, and SP-D. SP-A and SP-D are hydrophilic proteins known to function as collectins while SP-B and SP-C are two hydrophobic proteins that facilitate surfactant dispersal across the alveolar surface[24,26]. The antimicrobial effects of the fatty acids and collectins have been

well documented; however, only recently have studies suggested that host lipids may influence *S. aureus* gene expression[23,26–29].

Recently, we reported *S. aureus* virulence production within the lungs of healthy mice is delayed compared to mice infected with IAV. This observation suggests that infection by IAV may disrupt components of the lung environment that contribute to toxin suppression[15]. During lower respiratory tract infections IAV preferentially binds to type II pneumocytes[30]. Type II pneumocytes are the primary site of pulmonary surfactant synthesis and secretion. Studies investigating the impact of IAV infection on type II pneumocyte function have revealed that IAV may disrupt pulmonary surfactant lipid produciton[31,32]. Of note, separate studies examining surfactant composition during bacterial pneumonia have noted reductions in surfactant lipids during infection[33].

Given the increased severity of *S. aureus* following IAV infection and the innate ability of *S. aureus* to sense and respond to environmental stimuli, our recent efforts explore the hypothesis that components of pulmonary surfactant suppress *S. aureus* virulence[34–37]. In the current study we provide evidence that pulmonary surfactant from healthy mice inhibits virulence expression and production. Experiments identify that growth of *S. aureus* in palmitate mirrors the suppressive effect of treatment with whole surfactant. Antecedent infection with IAV reduces the availability of free-fatty acids within the lungs and leads to a significant reduction in the major pulmonary surfactant lipids constituents: phosphatidylcholine and phosphatidylglycerol. Taken together, our data provide a rationale for the increased susceptibility to secondary bacterial pneumonia following IAV infection wherein infection with IAV removes the suppressive effect of

pulmonary surfactant enabling the increased production of *S. aureus* virulence factors and initiation of disease. In addition, these data may highlight a novel treatment strategy for secondary bacterial pneumonia.

MATERIALS AND METHODS

Bacterial Cultures

S. aureus pulse-field gel electrophoresis-type USA300 (strain LAC) was grown in tryptic soy both (TSB) supplemented in 0.5% glucose and harvested as previously described[38].

Influenza A

IAV/PR/8/1934 (PR8) was purchased from Charles River Laboratories. IAV infections were preformed using a total volume of 50 μL of sterile Dulbecco's phosphate buffered saline (DPBS) containing 100 plaque-forming units (PFU).

Sodium Palmitate

Sodium Palmitate (palmitate) was purchased from TCI (CAS: 408-35-5). For each experimental day, palmitate was dissolved at 60°C in 50% ethanol (ETOH) and DPBS at a final concentration of 0.18 M. Working concentrations were dissolved in DPBS and maintained at 37°C until use.

Mouse Model of Lung Infection

All studies conformed to National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman, MT. Male and female BALB/c mice (8 to 10-week) were bred at the Animal Resources Center.

Intratracheal infections and lung harvest were performed as previously described[39]. Mice were euthanized at 6-days post IAV infection. For viral infections, intratracheal administrations were performed with 50 µL containing 100 PFU/50 µL. For bacterial infections, intratracheal administrations were performed with 50 µL containing 5 x 10^8 colony-forming units (CFU)/50µL.

Transcriptional Analysis

Transcriptional analysis by TaqMan™ qRT-PCR was performed as previously described[15,38]. Primers and probes are listed in Supplemental Table 1.

Human PMN Isolation and Plasma Membrane Damage Assay

Human polymorphonuclear leukocytes (PMN) were isolated from heparinized venous blood of healthy volunteer donors in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. PMNs were isolated under endotoxin-free conditions and examined for viability as previously described[40]. Prior to use in membrane damage and fluorescence assays, PMNs were gently washed and resuspended in DPBS. Supernatants were collected from *S. aureus* cultures grown to early-stationary (5 hours) and passed through 0.2 µM filters. Filtered supernatants were then diluted 1:25 in DPBS. Diluted supernatants (100 µL) were mixed with 100 µL of PMNs (1 x 10^6 cells) in a 96-well plate. Samples were incubated for 1-hour at 37°C followed by staining with propidium iodide (PI; ThermoFisher Scientific) as previously described[35]. Neutrophil membrane damage was analyzed using FACSCalibur Flow cytometer.

Pulmonary Surfactant Isolation

Pulmonary surfactant isolation was performed using an adaptation of the method described in Inselman et al.[41]. Briefly, murine lungs were homogenized in 3 mLs of ice-cold DPBS using a tissue grinder. Homogenate slurry was passed through a 70 µM cell strainer followed by centrifugation at 300 x G for 10 minutes at 4°C. The supernatant was collected and placed in microcentrifuge tubes for centrifuged at 18,000 x G for 35 minutes at 4°C. The resulting supernatant was aspirated and discarded leaving a pellet containing the functionally active fraction of surfactant[42]. The surfactant pellet was resuspended in 1 mL of DPBS at 60°C. For treatment with surfactant, mid-log USA300 cultures were pelleted and resuspended in 1 mL of active surfactant solution. For growth in surfactant, 1 mL of active surfactant was added to 4 mLs of TSB pre-warmed to 37°C. Overnight culture (50 µL) was used to inoculate the surfactant/TSB mixture. Samples were incubated at 37°C shaking at 250 rpm.

GFP Fluorescence Assay

Plasmid based USA300-*hlgA*-sGFP reporter strain was constructed as previously described[35]. Mid-log culture (1 mL) was pelleted and resuspended in the appropriate concentration of palmitate. PMNs (1 x 10^6 cells in 100 µL) were aliquoted into wells of a 96-well plate and mixed with 100 µL of palmitate treated bacteria, each treatment was performed in triplicate. PMN phagocytosis was synchronized by centrifugation at 1,500 RPM for 8 minutes at 10°C as previously described[40]. Temperature was maintained at 37°C and GFP fluorescence was detected by excitation at 485 nm and emission at 528 nm every 10-minutes for 19.5 hours (BioTek Cytation 5 imaging reader).

Free Fatty Acid Quantification

Free fatty acid (FFA) was quantified using an adaptation of the manufacturer's protocol (Abcam ab65341). Murine lungs were harvested, washed in ice-cold DPBS, and resuspended in 700 µL of chloroform/Triton x-100 (1% triton x-100). Lungs were minced with scissors and transferred to a lysing matrix D column (Mp Biomedicals). Samples were processed using a bead beater at 60M/s for 60 seconds. The resulting slurry was centrifuged at 1,500 rpm for 3 minutes. The supernatant was collected and placed in a clean 1.5 mL tube. Samples were processed as described in the manufacturer's protocol beginning with incubation on ice (protocol section 11.2.3).

Lipidomic analysis

Mock and IAV infected lungs were harvested 6-days post infection and snap frozen. Whole lungs were provided to Avanti Polar Lipids, Inc. (Alabaster, AL) and subjected to a modified Bligh Dyer extraction followed by LC-MS/MS analysis and separation by HILIC.

RESULTS AND DISCUSSION

Healthy lung environment suppresses *S. aureus* virulence expression

In a previous study using an *in vivo* model of secondary bacterial pneumonia, we demonstrated that *S. aureus* virulence production is delayed upon exposure to a healthy lung environment compared to lungs infected with IAV[15]. These data suggest that apart from exogenous damage, components of a healthy lung environment may suppress SaeR/S-dependent *S. aureus* virulence expression. To that end, we examined the

influence of IAV infected and mock infected lung homogenate on gene expression of gamma hemolysin component A (*hlgA*), a bicomponent leucocidin identified to be important in lung infections and up-regulated following IAV[15]. Mid-log cultures of USA300 were treated with homogenate slurries for 1-hour prior to RNA purification and Taqman™ qRT-PCR analysis. Transcript analysis revealed treatment with IAV infected homogenate led to a >4-fold increase in *hlgA* transcript abundance compared to treatment with mock infected lungs. Furthermore, expression of *hlgA* in USA300 treated with mock infected homogenate was reduced >2-fold relative to basal expression levels detected in USA300 suspended in DPBS (Figure 1) (Supplemental Table 1). These data suggest the healthy murine lung environment suppresses *S. aureus* virulence expression.

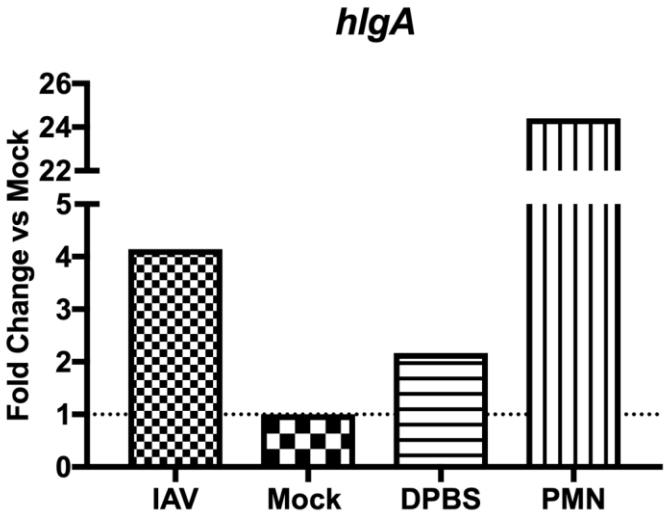


Figure 1. Healthy murine lung environment suppresses *hlgA* transcription. For IAV and mock treatments, mice were mock infected with DPBS or infected with IAV. 6-days post infection, mice were euthanized followed by harvest and homogenization of the lungs. Mid-exponential USA300 cultures were pelleted and resuspended in mock or IAV infected lung slurries. For DPBS and PMN treatments, aliquots of mid-exponential cultures were pelleted and resuspended in DPBS or PMNs (5×10^5 cells). Samples were incubated at 37°C for 1-hour with rocking followed by RNA purification and analysis by TaqMan™ qRT-PCR. Transcription was normalized to *gyrB* expression and calibrated to

expression in mock infection treatment. *Data represents 1 biological replicate containing 3 mice per infection group.

Active fraction of murine pulmonary surfactant suppresses *S. aureus* virulence production

S. aureus has the innate ability to sense and respond to its surrounding environment[34–36]. As microbial residents of the upper respiratory tract are trafficked into the lungs they immediately encounter the lipid rich complex pulmonary surfactant. While primarily studied for its role in lowering the surface tension at the air/liquid interface, pulmonary surfactant also acts as a first line of defense against potential pathogens as it forms a physical barrier between the underlying epithelia and organisms progressing down the respiratory tract[32,43]. In recent studies, treatment with Surfacten®, a modified pulmonary surfactant derived from bovine lungs, and free fatty acids demonstrated that host fatty acids induce transcriptional changes within *S. aureus*[23,28]. In addition, studies examining surfactant composition during bacterial pneumonia have noted changes in surfactant lipids during infection[33]. Taken together, we hypothesized that treatment of *S. aureus* with pulmonary surfactant decreases virulence gene production.

To initially test if pulmonary surfactant influences *S. aureus* virulence gene expression, we isolated murine pulmonary surfactant using previously established protocols and examined the influence of this lipoprotein complex on *hlgA* transcription[41]. USA300 cultures were treated with the active fraction of pulmonary surfactant for 1 hour followed by RNA extraction and Taqman™ qRT-PCR analysis. Treatment with murine pulmonary surfactant drastically reduced *hlgA* transcript abundance relative to basal expression levels (Figure 2) (Supplemental Table 1).

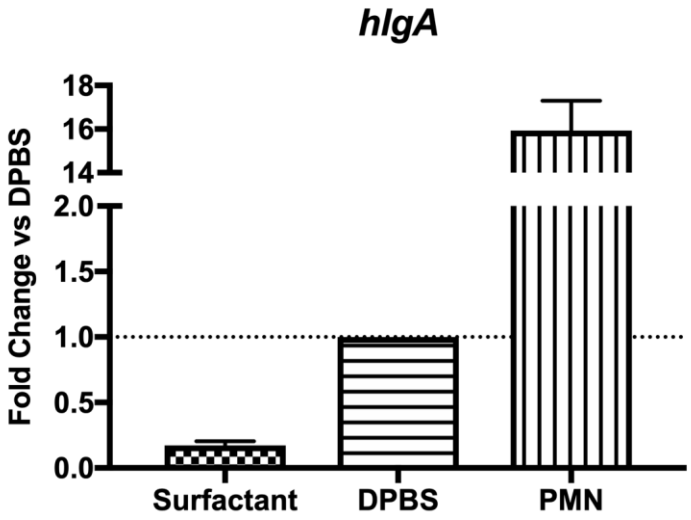


Figure 2. Treatment of *S. aureus* with murine surfactant suppresses *hlgA* transcription. USA300 was harvested at mid-exponential, pelleted, and resuspended in pulmonary surfactant, PBS, or PMNs. Samples were incubated with rocking at 37°C for 1-hour followed by RNA purification. Transcriptional analysis was performed by TaqMan™ qRT-PCR, samples were normalized to *gyrB* expression and calibrated to expression in PBS. *Data displayed is from 3 separate mice.

To confirm that the observed reductions in transcription were relevant towards toxin production, we examined if growth in pulmonary surfactant affected *S. aureus* cytotoxicity towards human polymorphonuclear cells (PMNs) (Figure 3). PMNs are the predominant recruited cell type and first line of defense against *S. aureus* infection. Upon phagocytosis by PMNs, *S. aureus* rapidly upregulates and array of secreted toxins that cause PMN lysis[35,37]. The outcome of infection can be determined in these initial encounters, thus the ability of pulmonary surfactant to suppress *S. aureus* toxin production may highlight a critical host defense strategy against *S. aureus* infection[37]. USA300 was grown in the presence of isolated murine pulmonary surfactant for 5 hours. Filtered and diluted supernatants were mixed with PMNs for 1 hour followed by staining with propidium iodide (PI). Flow cytometry analysis of PMNs treated with supernatants

from cultures supplemented with pulmonary surfactant exhibited reduced size and degranulation compared to PMNs treated with supernatants from USA300 cultures grown in TSB alone (Figure 3A). Supplementation of USA300 growth media with pulmonary surfactant significantly reduced the ability of supernatants to cause PMN membrane damage (Figure 3B). The addition of pulmonary surfactant to USA300 growth media had no effect on bacterial CFUs (data not shown). Taken together, these data indicate that pulmonary surfactant may participate in innate defenses by suppressing virulence production of *S. aureus* that has been trafficked from the upper respiratory tract to the lungs.

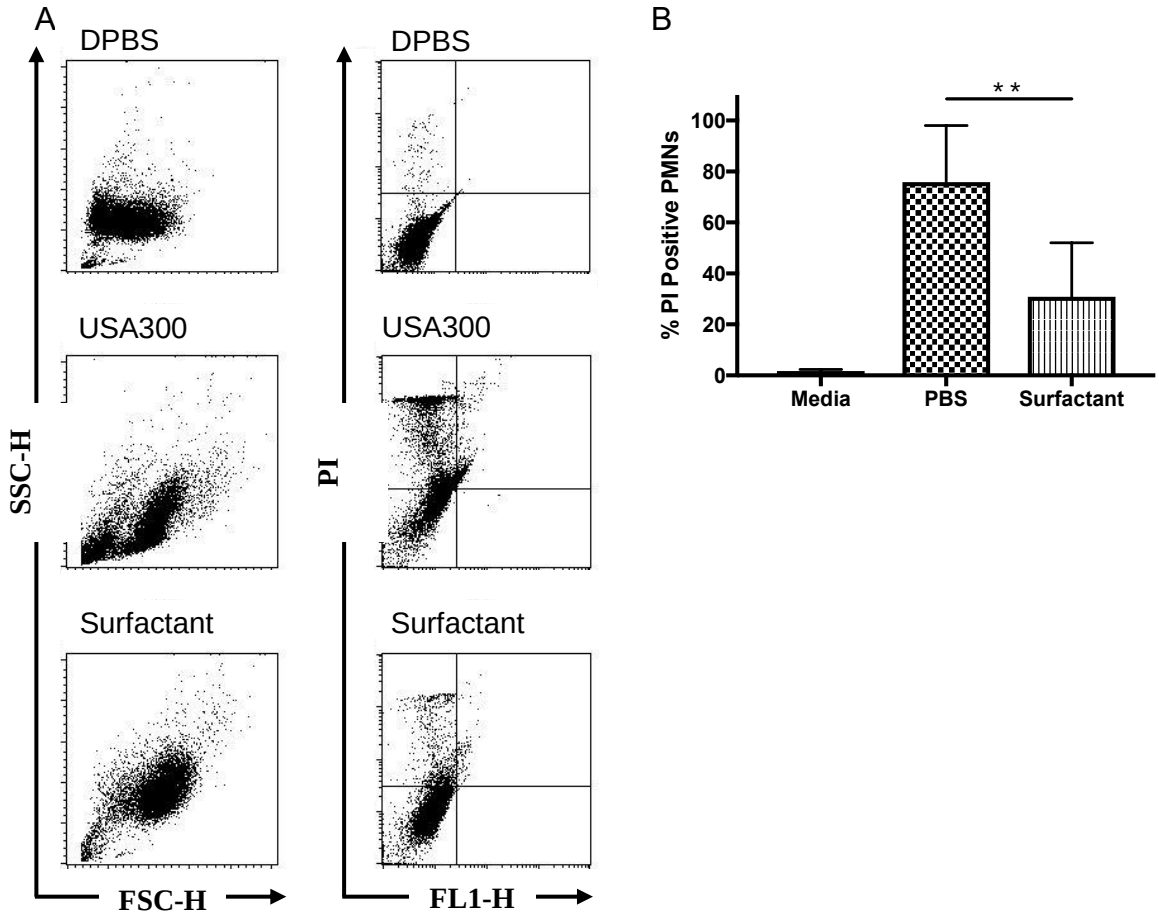


Figure 3. Murine surfactant suppresses *S. aureus* ability to lyse PMNs. USA300 cultures were grown with or without pulmonary surfactant to early stationary growth phase (5 hours). Supernatants were harvested, filter sterilized, and combined with PMNs (1 x 10^6 cells) at a final dilution of 1:50. After 1-hour incubation at 37°C, PMN plasma membrane permeability to propidium iodide was analyzed using flow cytometry. A) Representative flow cytometry plots of PMN membrane damage when exposed to USA300 supernatants from surfactant treated and untreated cultures. B) Proportion of PI positive PMNs after 1-hour incubation with culture supernatants. Data displayed is from 2 biological replicates containing surfactant from 4 mice per replicate. ** $P < 0.01$ as determined by Mann-Whitney Test.

Palmitate suppresses *S. aureus* virulence production

The innate immune properties of pulmonary surfactant have predominantly been attributed to the hydrophilic proteins SP-A and SP-D. These proteins belong to the family of collagen-containing C-type lectins and act as opsonins on invading bacteria [26,44–46]. Binding of SP-A and SP-D to *S. aureus* peptidoglycan has been demonstrated to increase uptake by neutrophils, yet phagocytosis by neutrophils is a potent stimulus for *S. aureus* virulence production as the phagosome products human neutrophil peptide-1 (HNP-1) and H_2O_2 have been demonstrated to directly activate SaeR/S (Appendix 1)[36,37,47]. In contrast, our data indicated the predominant innate contribution of pulmonary surfactant was the suppression of virulence production (Figures 1-3). Given recent studies demonstrating *S. aureus* gene regulation in response to fatty acids, we hypothesized that the lipid constituents of pulmonary surfactant suppress virulence production[23,28,29,48].

Phosphocholines are the most abundant lipids within pulmonary surfactant. While various forms of phosphocholines exist, DPPC is the dominant species within lungs[49]. The primary building block for the synthesis of DPPC is palmitic acid. Palmitic acid can be synthesized directly in the lungs or trafficked to lungs as triglycerols or diacylglycerols via the blood stream. Triglycerols and diacylglycerols are hydrolyzed by the enzyme lipoprotein lipase to produce the free fatty acid palmitic acid[25]. To examine the effects of palmitate on *S. aureus* virulence expression we developed a plasmid based USA300-$_{hlgA}$-sGFP reporter assay (Figure 4, Supplemental Figure 1). Expression of *hlgA* is directly regulated by *saeR/S*, thus to examine the influence of palmitate on *hlgA* expression cultures were resuspended in 0 µM - 180 µM palmitate and mixed with

PMNs, a well described stimuli of *saeR/S*[34–37]. The expression of *hlgA* was determined by GFP detection at 528 nm during incubation at 37°C for 19.5 hours (Figure 4A). Treatment with palmitate led to a concentration dependent inhibition of *hlgA* expression (Figure 4A and B). Analysis of total peak area revealed significant suppression of *hlgA* at 1.8 µM and 180 µM concentrations compared to no treatment with palmitate. Importantly, treatment with palmitate at all concentrations had no effect on bacterial survival (Figure 4C, Supplemental Figure 2). No differences in bacterial CFUs confirmed that the observed suppressive effect conferred by palmitate was due to changes in bacterial expression and not bacterial density (Figure 4C, Supplemental Figure 2A and B).

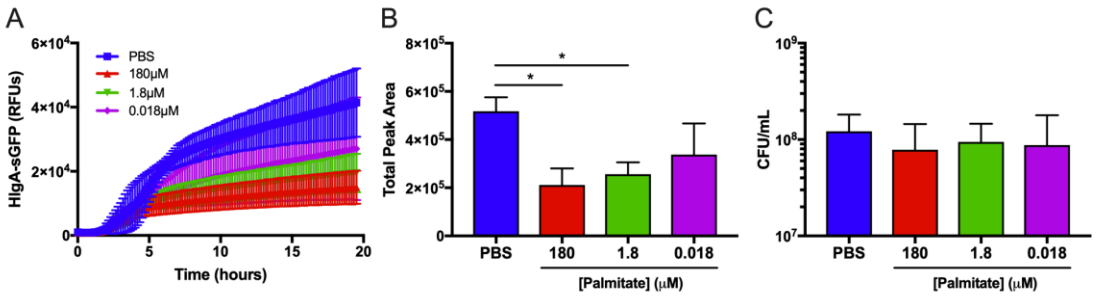


Figure 4. Palmitate suppresses *hlgA*-sGFP detection. A) Kinetic detection of GFP fluorescence in samples treated with palmitate. B) The total peak area of GFP fluorescence over the time course for experiments show in panel A. C) Bacterial concentrations after 19.5 hours of incubation with indicated concentrations of palmitate. Data shown is from 3 biological replicates, each containing 3 technical replicates. * $P < 0.05$ Tukey's multiple comparisons test.

To determine if treatment with palmitate conferred the same suppressive effect on toxin production and PMN cytolysis as observed with isolated surfactant, supernatants from USA300 cultured in the presence of palmitate were used to treat PMNs. Growth of USA300 in 180 µM palmitate mirrored the phenotypic trends observed by growth in the

presence of pulmonary surfactant (Figure 5). PMNs exposed to supernatants from cultures supplemented with palmitate resembled PMNs exposed to PBS in both size and granularity (Figure 5A). Growth media with or without palmitate had no effect on membrane permeability; however, membrane damage caused by supernatants from cultures grown in TSB only was significantly higher compared to supernatants from cultures supplemented with palmitate (Figure 5B). Differences in membrane damage between growth conditions was not due to bacterial density (Figure 5C). These data demonstrate the lipid component of pulmonary surfactant suppresses *S. aureus* toxicity.

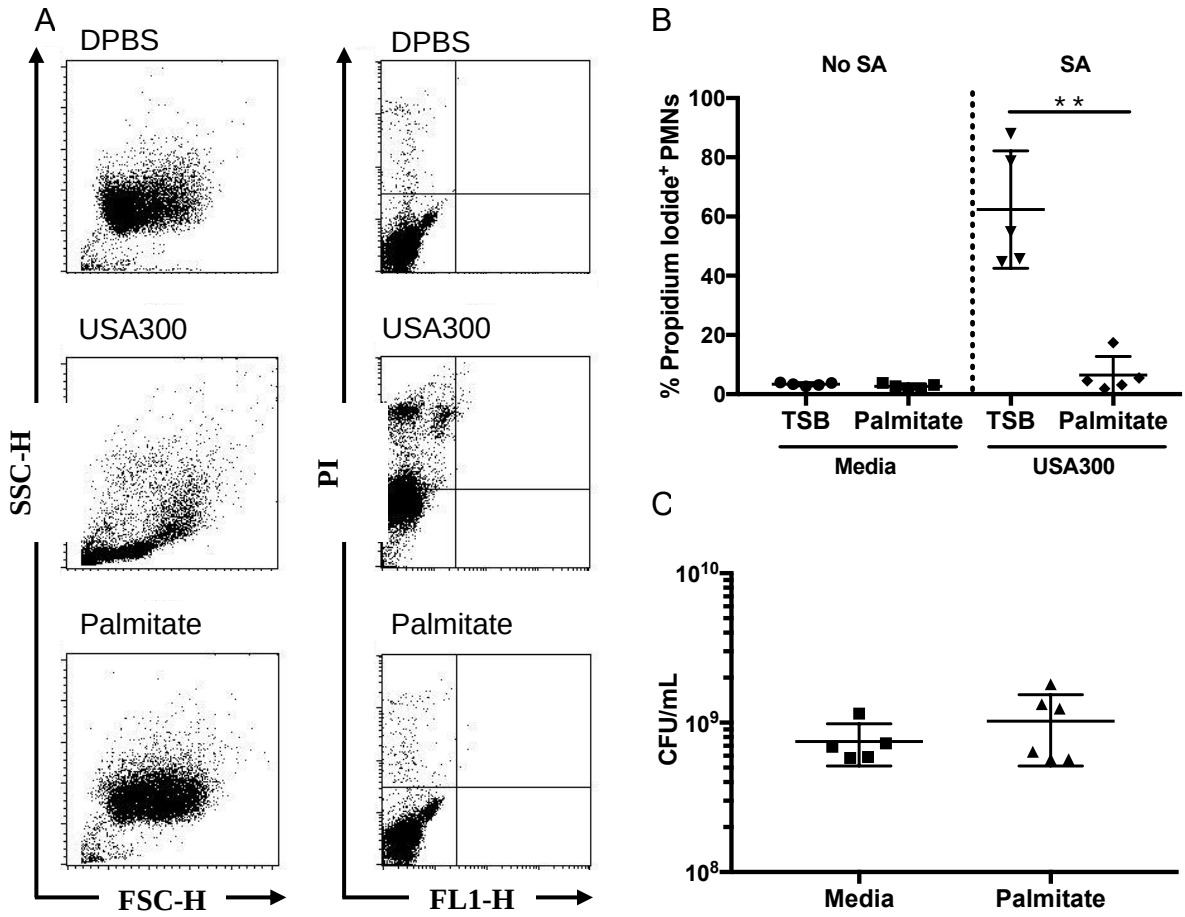


Figure 5. Palmitate significantly reduces *S. aureus* virulence expression. A) Representative flow cytometry plots of PMN membrane damage when exposed to USA300 supernatants from palmitate treated and untreated cultures. B) USA300 was sub-cultured with or without 180 µM of palmitate to early stationary growth phase. Filtered supernatants, media containing 180 µM palmitate, or media alone were combined with PMNs ($1x10^6$ cells) at a final dilution of 1:50. After 1 hour of incubation at 37°C, PMN membrane permeability was analyzed. C) Bacterial concentration of USA300 cultured with and without 180 µM palmitate at early stationary growth phase from experiments in panel B. **P < .01 as determined by Mann-Whitney Test.

Reduction of pulmonary surfactant fatty acid concentrations by IAV

The increased susceptibility to *S. aureus* pneumonia following antecedent IAV infection, together with our data demonstrating the lipid constituent of pulmonary surfactant suppresses virulence production, led to the hypothesis that the lipid concentrations within the lungs are reduced following infection with IAV. To test this hypothesis, mice were randomly sorted into mock and IAV treatment groups and randomly match-paired. Mice were then intratracheally infected with DPBS (mock) or IAV[39]. Six-days post infection mice were euthanized and the lungs were harvested. Free fatty acid (FFA) within the lungs was quantified. FFA concentration was normalized to lung weight. Collectively, mice infected with IAV had significantly less FFA compared to mock infected mice (Figure 6A). Similarly, matched-pairs analysis of FFA concentration between mock and IAV infected mice revealed a significant reduction of FFA in IAV infected mice compared to mock infected pairs (Figure 6B).

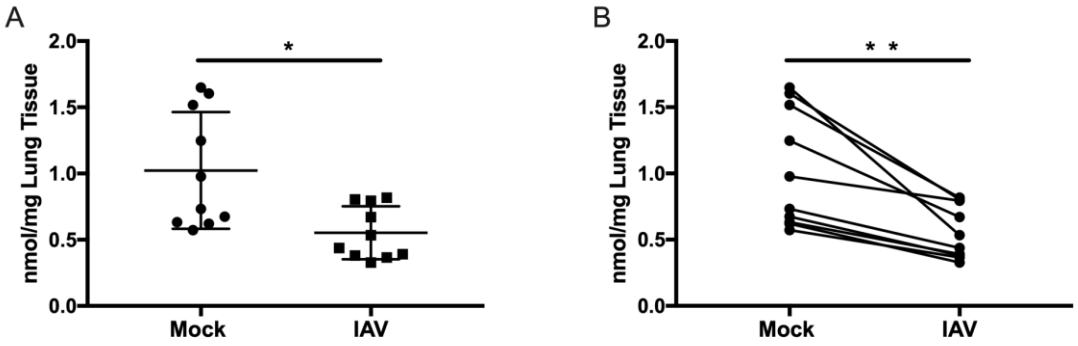


Figure 6. Infection with IAV leads to a decrease in the concentration of free fatty acids within the lungs. Male and female mice were randomly assigned and paired into mock infected and IAV treatment groups (n=10 mice per group). Mice were intratracheally mock infected with PBS or infected with IAV (100pfu/50µL). 6-days post infection, mice were euthanized and lungs were harvested. FFA was quantified and normalized to lung weight. A) FFA concentration in mock and IAV infected mice. * $P < .05$ as determined

by Mann-Whitney Test. B) Change in lung FFA concentration plotted according to matched-pairs. ** P < .001 Wilcoxon matched-pairs signed rank test.

The decrease in total lung FFA indicated infection with IAV reduced lipid concentrations within the lungs but were incomplete in identifying changes in lipid species that composed pulmonary surfactant. The alveolar surface is dominated by type I and type II pneumocytes. Type I pneumocytes are metabolically inactive cells that cover ~95% of the alveolar surface area whereas type II pneumocytes are metabolically active and cover the remaining ~5%[30,50]. Type II pneumocytes compose ~15% of the total cells within lungs and are responsible for synthesis and secretion of pulmonary surfactant[25,30,50]. In addition, evidence suggests type II pneumocytes serve as stem cells capable of differentiating into type I pneumocytes[30,50]. During lower respiratory tract infection, IAV preferentially infects type II pneumocytes causing changes in type II pneumocyte morphology, function, and surfactant production[30,31]. To further characterize these disruptions, we employed a lipidomic approach to identify the changes in specific lipid species following infection with IAV.

Lipidomic analysis (Avanti Polar Lipids) identified 1,081 unique lipid analytes (Supplemental Table 2). Analytes were analyzed according to fold-change in IAV infected lungs compared to mock infected lungs. Of the 1,081, 426 analytes were significantly changed: 384 analytes were significantly decreased while only 42 analytes were significantly increased (Figure 7A) (Supplemental Table 3) (Supplemental Table 4). Together phosphatidylcholines and phosphatidylglycerols compose ~90% of the total lipid constituents within pulmonary surfactant. Eighty-five unique phosphatidylcholine analytes were identified, the majority of these analytes were not significantly changed

(67.1%); however, 25.9% were significantly decreased and 7.1% were significantly increased (Figure 7B). Sixty-two phosphatidylglycerol analytes were identified, of which nearly 70% were significantly decreased and only ~5% were significantly increased (Figure 7C). Collectively, these data indicate pulmonary surfactant lipid production is significantly disrupted during lower respiratory tract infection by IAV.

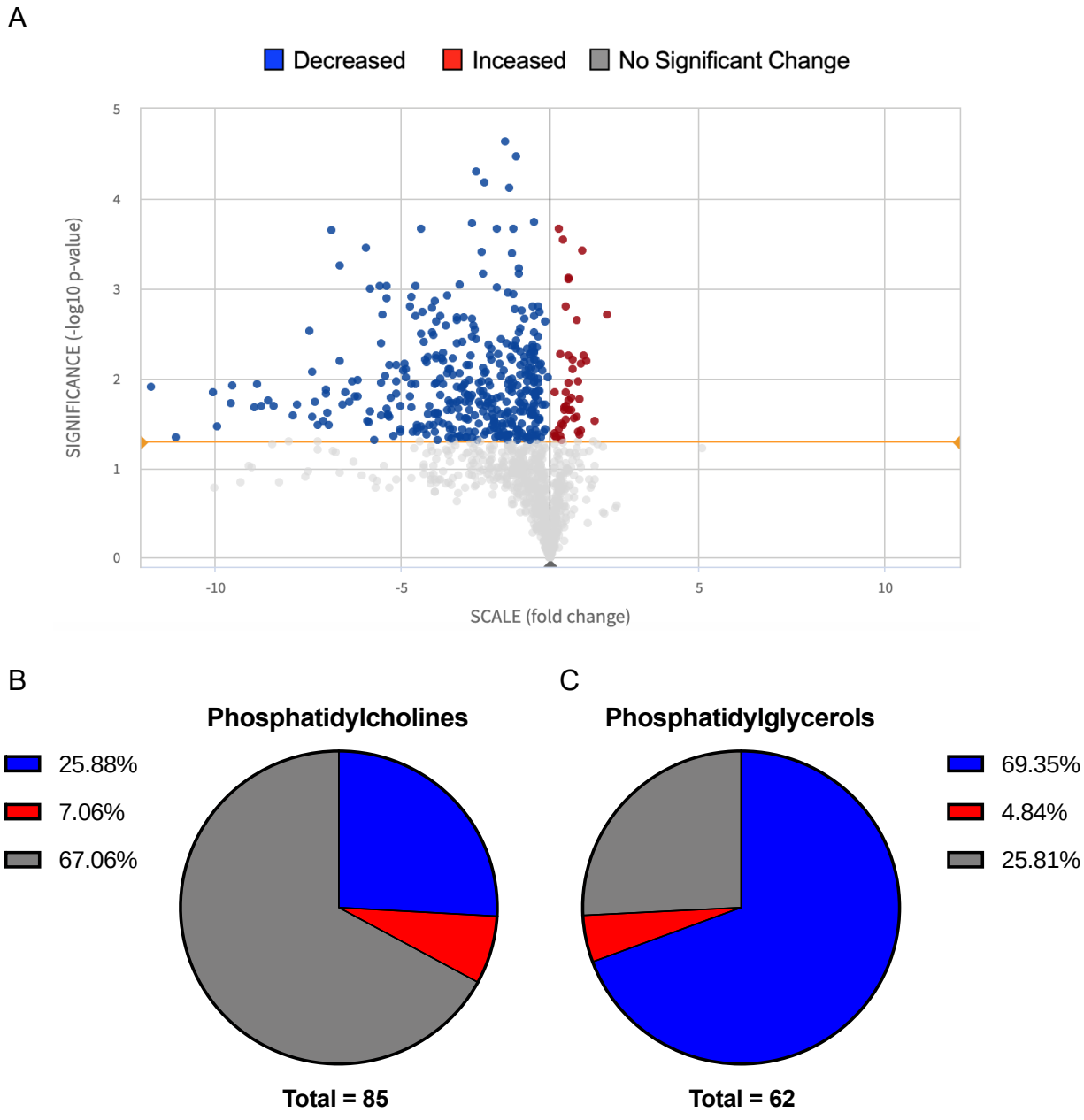


Figure 7. Lipodomic analysis of mock and IAV infected murine lungs. A) Volcano plot of all lipid analytes identified. 426 of 1,081 analytes were significantly different in IAV infected lungs compared to mock infected lungs. Plot displays the negative log10-transformed p-values from t-test value against fold-change of IAV compared to mock infected lungs. Non-significant changes in grey, significant decreases in blue, and significant increases in red. B and C Fold-change of dominant pulmonary surfactant lipid species. n=3 mice per treatment group. B) Phosphatidylcholines. C) Phosphatidylglycerols.

CONCLUSIONS

These studies provide a strong rational for the low incidence of *S. aureus* pneumonia in immunocompetent hosts. As *S. aureus* is trafficked from the upper respiratory tract it encounters the pulmonary surfactant coated epithelium of the lower respiratory tract. The association with pulmonary surfactant leads to suppression of *S. aureus* virulence production. Together with previous studies, this virulence suppression is likely mediated through reduced expression of *saeR/S*[15]. Suppression of *saeR/S* severely limits the *S. aureus* virulence arsenal and likely enables sufficient clearance by resident innate immune cells prior to the onset of infection.

Inasmuch as these data identify a unique role for pulmonary surfactant on *S. aureus* virulence regulation, several studies have reported treatment with palmitate and other host lipids increases *S. aureus* virulence[23,28]. These reports revealed host fatty acids induced expression of *essC*, an integral component of the *S. aureus* type VII secretion system (T7SS), and gamma hemolysin component C (*hlgC*)[23,28]. While expression of the T7SS has been associated with virulence since its initial identification, evidence suggests its influence on host disease is tissue specific[28,51]. In the only study implicating surfactant induction of T7SS and *hlgC* in pneumonia, investigators demonstrate treatment with a

bactericidal concentration of palmitate using *S. aureus* strain Newman (Supplemental Figure 2C and D)[23]. Strain Newman contains an amino acid substitution mutation in the histidine kinase domain of SaeS resulting in constitutive expression of the SaeR/S operon[52]. Importantly, T7SS and SaeR/S expression have been shown to be inversely related (Supplemental Figure 3)[53,54]. In addition, secondary bacterial pneumonia by *S. aureus* has been demonstrated to be dependent on *saeR/S* expression[15]. Furthermore, the authors were not able to demonstrate increased expression of *hlgC* by palmitate in USA300[23].

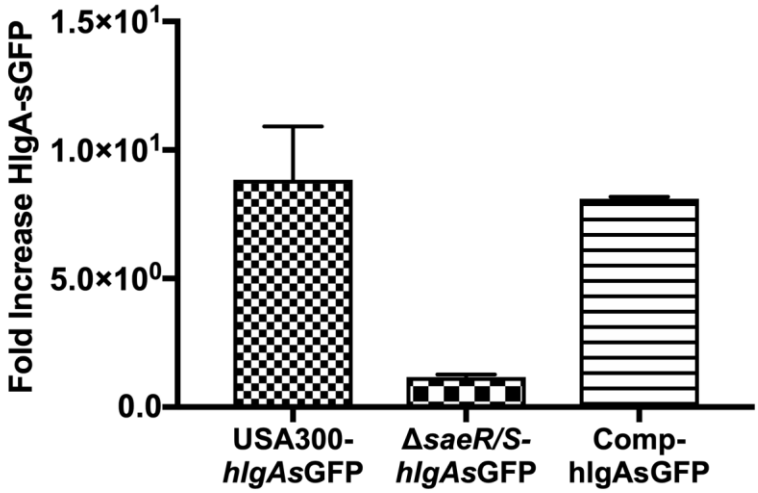
More recent characterizations have revealed upregulation of the T7SS aids in *S. aureus* persistence and nasal colonization as it protects against the antimicrobial effects of host fatty acids[48,55–57]. A study by Reddinger et al. demonstrated IAV infection promotes dispersion of *S. aureus* biofilms from the nares to the lungs[14]. Notably, *S. aureus* biofilm growth is also characterized by decreased expression of toxins directly regulated by *saeR/S*[58]. Thus, in addition to pulmonary surfactant disruption, infection by IAV may simultaneously aid in *S. aureus* dissemination and transition from commensal to pathogen through upregulation of *saeR/S*.

Our findings build upon previous studies that revealed infection with IAV leads to a rapid upregulation of *S. aureus* virulence genes[15]. Taken together, we propose a novel model of secondary bacterial pneumonia wherein infection with antecedent IAV drastically affects host pulmonary surfactant production and composition leading to a loss of virulence suppression and initiation of disease. Furthermore, these data imply that

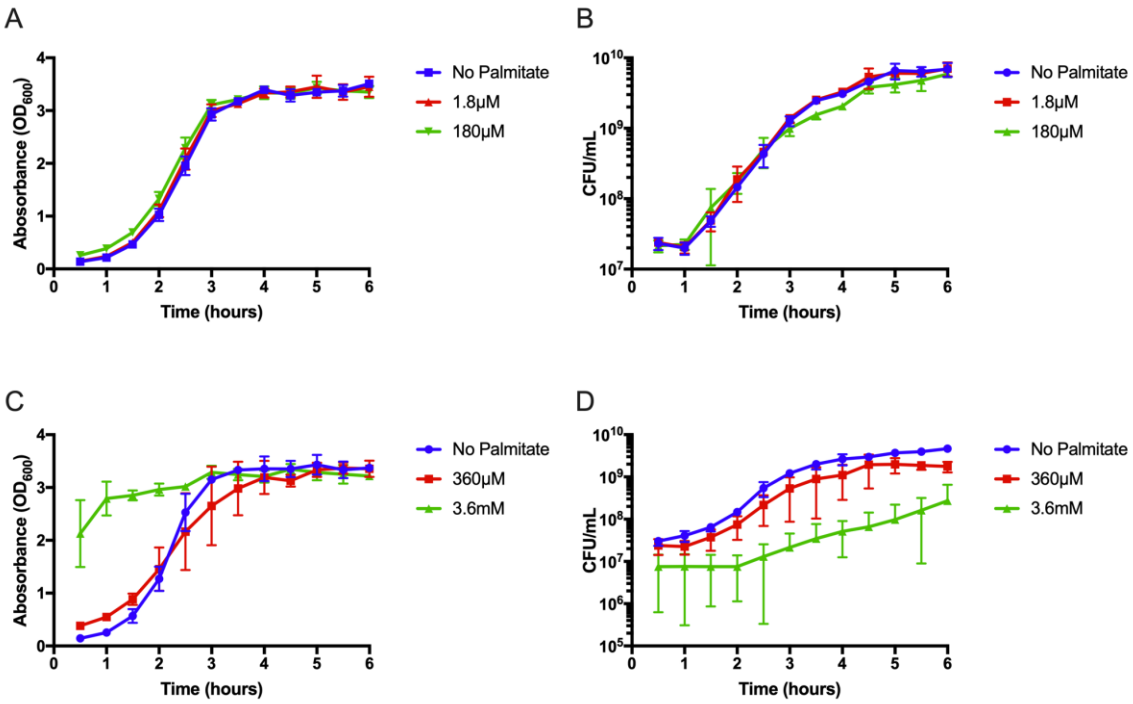
administration of pulmonary surfactant replacement therapies may significantly alleviate secondary bacterial pneumonia morbidity and mortality.

Future studies will further characterize bacterial interactions with pulmonary surfactant. Due the severity of infection outcomes, *S. aureus* has been the focus of this current study; however, the disruption of surfactant may have relevant consequences on virulence regulation of other common lung pathogens such as *S. pneumoniae*, *H. influenzae*, and *Streptococcus pyogenes*. In addition, future studies will investigate if treatment of IAV infection with surfactant replacement therapies may effectively combat secondary bacterial pneumonia.

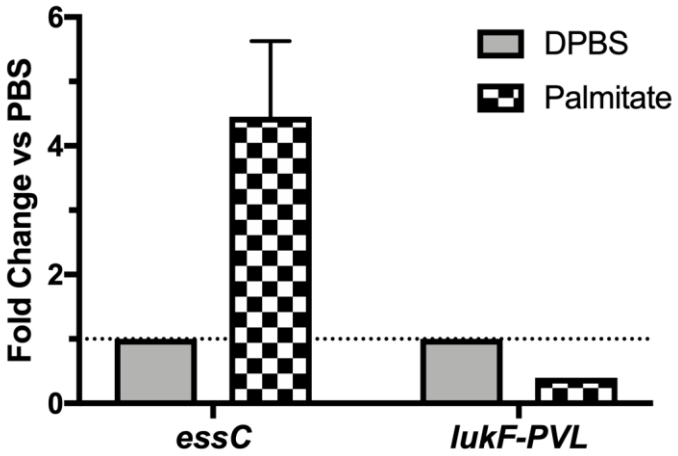
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Generation of USA300-hlgA-sGFP reporter assay. Relative fluorescence of HlgA-sGFP in USA300, USA300ΔsaeR/S (ΔsaeR/S), and USA300ΔsaeR/S complemented with a plasmid overexpressing the saeR/S (Comp) following 3-hour incubation with PMNs relative to incubation without PMNs (n=2).



Supplemental Figure 2. Growth of USA300 in palmitate. In vitro growth of USA300 in the presence of palmitate. A and B) TSB supplemented with 1.8 μM and 180 μM have no effect on *S. aureus* growth kinetics. C and D) TSB supplemented with 360 μM and 3.6 mM severely impact *S. aureus* growth kinetics.



Supplemental Figure 3. Inverse regulation of the T7SS and SaeR/S TCS. *S. aureus* was grown in 180 µM palmitate to mid-log. RNA was purified and transcriptional analysis was performed by Taqman™ qRT-PCR on T7SS component essC and saeR/S regulated virulence gene *lukF-PVL*. Samples were normalized to *gyrB* expression and calibrated to treatment with DPBS.

SUPPLEMENTAL TABLES

Supplemental Table 1. TaqMan™ qRT-PCR Primers and Probes

RT-PCR Primers and Probes	**Sequence**
essC fwd	5' - CGTGAACCGACGGATGATATT - 3'
essC rvs	5' - GCGGTATAATGGAACGCCATA - 3'
essC probe	5' - ACCGCCACAGCCAATACAGAAGAA - 3'
gyrB fwd	5’ - CAAATGATCACAGCTTTGGTACAG - 3’
gyrB rvs	5’ - CGGCATCAGTCATAATGACGAT - 3’
gyrB probe	5’ - AATCGGTGGCGACTTTGATCTAGCGAAAG - 3’
hlgA fwd	5’ - ACTTATTTGACAAGACCCAACTG - 3’
hlgA rvs	5’ - CCACTTTGAATTAAAGGAGGTAATTGAT - 3’
hlgA probe	5’ - CAGCAGCAAGAGACTATTTCGTCCCAG - 3’
lukF-PVL fwd	5’ - TTGCTTTTGCTATCCAATACAGTTG - 3’
lukF-PVL rvs	5’ - TCGGAATCTGATGTTGCAGTTG - 3’
lukF-PVL probe	5’ - TGCAGCTCAACATATCACACCTGTAAGT - 3’

Supplemental Table 2. Total lipid analytes identified

ANALYTE NAME	FOLD CHANGE	P-VALUE	Q-VALUE
CE(14:0)	-2.031064823	0.251377631	0.073359699
CE(16:0)	-1.687945913	0.256482192	0.074409076
CE(16:1)	-1.406815663	0.032106977	0.023527374
CE(18:0)	-1.050792719	0.650400634	0.158798794
CE(18:1)	1.391209701	0.064498164	0.032991066
CE(18:2)	1.145750055	0.236262685	0.069941953
CE(18:3)	-1.156261085	0.411563593	0.108463983
CE(20:0)	2.461295419	0.326649895	0.089514469
CE(20:1)	1.975100402	0.225340282	0.067766117
CE(20:2)	1.809309309	0.017344957	0.018516073
CE(20:3)	1.094873881	0.586924685	0.14473381
CE(20:4)	1.040003892	0.761229468	0.180670673
CE(20:5)	-1.387310606	0.070736679	0.034002819
CE(22:0)	-1.003424658	0.988773793	0.223091827
CE(22:1)	1.399026764	0.271325774	0.077890682
CE(22:2)	2.197080292	0.095035592	0.040633301
CE(22:4)	1.664588205	0.121532889	0.045580187
CE(22:5)	1.198480717	0.264425778	0.076354307
CE(22:6)	1.390362281	0.111569866	0.043601876

CE(24:0)	2.745454545	0.283043712	0.080504848
CE(24:1)	1.751572327	0.039798088	0.025963189
CE[mFA14:0]	-1.939324413	0.177363751	0.056143711
CE[mFA16:0]	-1.592414978	0.171291832	0.054786038
CE[mFA16:1]	-1.388106591	0.136816147	0.048335871
CE[mFA18:0]	-1.044294795	0.794365766	0.187094303
CE[mFA18:1]	1.415205258	0.028834538	0.022082322
CE[mFA18:2]	1.168589642	0.156993338	0.052105075
CE[mFA18:3]	-1.135670136	0.276593589	0.078943429
CE[mFA20:0]	2.429379831	0.314182837	0.087052264
CE[mFA20:1]	2.075845439	0.166576537	0.054079588
CE[mFA20:2]	1.869896769	0.000379717	0.005811341
CE[mFA20:3]	1.111635906	0.391019205	0.103905307
CE[mFA20:4]	1.047966954	0.77186952	0.182844029
CE[mFA20:5]	-1.359941842	0.138223065	0.048554672
CE[mFA22:0]	1.036210787	0.82941478	0.193136087
CE[mFA22:1]	1.464604047	0.101800471	0.041700466
CE[mFA22:2]	2.259613763	0.053464143	0.029708488
CE[mFA22:4]	1.693626389	0.069921161	0.033874935
CE[mFA22:5]	1.205393474	0.24581309	0.072076988
CE[mFA22:6]	1.407749474	0.103919858	0.042010353
CE[mFA24:0]	2.812978559	0.259196804	0.075020105

CE[mFA24:1]	1.772112204	0.05115127	0.028894106
CER(14:0)	-1.530232558	0.024242335	0.020472893
CER(16:0)	-1.141703038	0.037122725	0.025192958
CER(18:0)	-1.168630767	0.161110281	0.05304312
CER(18:1)	1.600660066	0.006236258	0.013797409
CER(20:0)	-1.295214106	0.028570116	0.021947982
CER(20:1)	-1.042253521	0.288255665	0.081237662
CER(22:0)	-1.16197686	0.056450814	0.030594716
CER(22:1)	1.128988149	0.159181541	0.052618867
CER(24:0)	-1.447686088	0.004159372	0.013018029
CER(24:1)	-1.172403116	0.040196114	0.026057034
CER(26:0)	-1.073979592	0.668722836	0.161792057
CER(26:1)	-1.282945736	0.08758127	0.038914
DAG(14:0_14:0)	-3.387096774	0.013035124	0.016829431
DAG(14:0_16:1)	-4.255390513	0.033110763	0.023558777
DAG(14:0_18:1)	-3.438911565	0.000917097	0.008544161
DAG(14:0_18:2)	-3.321307941	0.012200712	0.016248919
DAG(14:0_18:3)	-2.036616162	0.22987105	0.0686265
DAG(14:0_20:0)	-2.563953488	0.099673694	0.04114232
DAG(14:0_20:4)	-4.134715026	0.094490244	0.040603645
DAG(14:0_22:6)	-3.335832084	0.003924718	0.013018029
DAG(16:0_16:0)	-1.122501881	0.669221289	0.161792057

DAG(16:0_16:1)	-2.180481283	0.018548677	0.018541762
DAG(16:0_18:0)	-1.275718431	0.236831143	0.070026171
DAG(16:0_18:1)	-2.125059199	0.011750317	0.016008795
DAG(16:0_18:2)	-2.505719375	0.029765849	0.022446992
DAG(16:0_18:3)	-1.474528543	0.529862878	0.132787139
DAG(16:0_20:3)	-2.603862565	0.132255334	0.047541918
DAG(16:0_20:4)	-2.020376543	0.166028696	0.054079588
DAG(16:0_20:5)	-2.623578077	0.086233136	0.03864314
DAG(16:0_22:5)	-1.979436082	0.114769789	0.044221679
DAG(16:0_22:6)	-1.816332982	0.082242383	0.0376965
DAG(16:1_16:1)	-3.86655914	0.006489286	0.013915112
DAG(16:1_18:0)	-2.802158805	0.007548955	0.014430613
DAG(16:1_18:1)	-4.459478912	0.000218225	0.004543902
DAG(16:1_18:2)	-4.101070565	0.024967038	0.020632808
DAG(16:1_18:3)	-2.498063517	0.157702241	0.052270006
DAG(16:1_20:2)	-3.354251012	0.07592535	0.035853971
DAG(16:1_20:4)	-4.038197845	0.144653723	0.049959608
DAG(16:1_22:6)	-2.827176781	0.010793283	0.016001092
DAG(18:0_18:1)	-1.451771923	0.080979035	0.037395467
DAG(18:0_18:2)	-1.3560686	0.308036908	0.085638047
DAG(18:0_18:3)	1.23373494	0.784065386	0.184991741
DAG(18:0_22:6)	-1.122099328	0.524465131	0.131791424

DAG(18:1_18:1)	-1.768612276	0.041285701	0.026582053
DAG(18:1_18:2)	-1.853386143	0.16707887	0.054079588
DAG(18:1_20:1)	-2.260148148	0.125559781	0.045802741
DAG(18:1_20:2)	-1.847605893	0.198002724	0.060805553
DAG(18:1_20:3)	-2.43196005	0.128618414	0.046642504
DAG(18:1_20:4)	-1.864419364	0.192731129	0.059482978
DAG(18:1_20:5)	-2.432460733	0.097521218	0.041038277
DAG(18:1_22:4)	-1.06733394	0.798529549	0.187716792
DAG(18:1_22:5)	-1.558673469	0.168767727	0.054331069
DAG(18:1_22:6)	-1.622743378	0.184447761	0.057574996
DAG(18:2_18:3)	-3.458896521	0.122749998	0.045580187
DAG(18:2_20:3)	-2.699171447	0.116744892	0.044725075
DAG(18:2_20:4)	-2.181139215	0.153585305	0.051319324
DAG(18:2_20:5)	-2.908200734	0.096488506	0.040915921
DAG(18:2_22:4)	-1.088793631	0.585241842	0.14446329
DAG(18:2_22:5)	-1.809888846	0.076313687	0.035913586
DAG(18:2_22:6)	-1.917666192	0.098206379	0.041100407
DAG(20:0_20:0)	1.391566265	0.657287677	0.15968976
DAG[mFA16:0]	1.172970643	0.306377202	0.085456416
DAG[mFA16:1]	-1.625766135	0.021388773	0.019534834
DAG[mFA18:0]	1.367993598	0.033577366	0.023725142
DAG[mFA18:1]	1.015848184	0.905509034	0.207910358

DAG[mFA18:2]	-1.156482903	0.372321739	0.099580681
DAG[mFA18:3]	-1.174014586	0.661591346	0.16041925
DAG[mFA20:0]	2.029322153	0.41169328	0.108463983
DAG[mFA20:2]	-1.023384744	0.946660795	0.215552743
DAG[mFA20:3]	-1.223116971	0.591974576	0.145833264
DAG[mFA20:4]	1.012760844	0.972607776	0.220443076
DAG[mFA20:5]	-1.316195366	0.326811591	0.089514469
DAG[mFA22:4]	1.83294915	0.037900688	0.025461725
DAG[mFA22:5]	1.089466314	0.728279137	0.174191445
DAG[mFA22:6]	1.145921539	0.557575894	0.138465749
DCER(14:0)	1.041666667	0.873220335	0.202039522
DCER(16:0)	1.472556361	0.111541645	0.043601876
DCER(18:0)	1.227963526	0.227978377	0.068309553
DCER(18:1)	1.784615385	0.088433633	0.039011557
DCER(20:0)	1.146845915	0.348899921	0.094133086
DCER(20:1)	1.434782609	0.088062485	0.038962166
DCER(22:0)	1.348338692	0.125455587	0.045802741
DCER(22:1)	1.919786096	0.065945835	0.033120242
DCER(24:0)	1.009153953	0.955871867	0.217346023
DCER(24:1)	1.349523632	0.171223225	0.054786038
DCER(26:0)	1.177083333	0.36939913	0.099013784
DCER(26:1)	1.3125	0.404834264	0.107114674

GluCer(18:0_18:0)	1.051948052	0.811437643	0.190207234
GluCer(18:0_20:0)	-1.015544041	0.956299254	0.217346023
GluCer(18:0_22:0)	1.15212528	0.545394258	0.136402166
GluCer(18:0_24:0)	-1.057937428	0.826055099	0.193136087
GluCer(18:0_24:1)	1.308042489	0.167556243	0.054082265
GluCer(18:0_26:0)	-1.333333333	0.284627179	0.080759938
GluCer(18:0_26:1)	1	1	0.224792073
HCER(14:0)	1.416666667	0.020168929	0.019047384
HCER(16:0)	2.531440781	0.001989894	0.010138466
HCER(18:0)	1.045172219	0.828924588	0.193136087
HCER(18:1)	1.76	0.01089544	0.016001092
HCER(20:0)	-1.072219294	0.744391553	0.177528677
HCER(20:1)	1.055555556	0.759545974	0.180444784
HCER(22:0)	1.09801202	0.521137119	0.131133469
HCER(22:1)	1.322815534	0.139886602	0.048929933
HCER(24:0)	-1.094663532	0.56644809	0.140386276
HCER(24:1)	1.289372599	0.090207178	0.039638873
HCER(26:0)	-1.236009732	0.210389732	0.064051181
HCER(26:1)	1.027586207	0.883184195	0.203732917
HexCerd18:1_12:0	-1.285714286	0.183503419	0.05735282
LacCer(18:0_18:0)	1.576923077	0.33085884	0.090153332
LacCer(18:0_22:0)	2.119617225	0.152829948	0.051143784

LacCer(18:0_24:0)	1.314655172	0.145993765	0.050281579
LacCer(18:0_24:1)	1.9	0.005500792	0.013797409
LCER(16:0)	5.098894115	0.059444727	0.031867142
LCER(18:0)	1.656862745	0.286599829	0.081038793
LCER(20:0)	1.189814815	0.526291374	0.13202627
LCER(22:0)	1.641830065	0.208393683	0.063600541
LCER(24:0)	1.604069129	0.083554454	0.037875496
LCER(24:1)	2.164953718	0.05085666	0.028837064
LPC(14:0)	-2.809193122	0.000676797	0.008069895
LPC(16:0)	-1.413250662	0.02017938	0.019047384
LPC(16:1)	-1.494163424	0.026267274	0.021099115
LPC(18:0)	-1.196098142	0.124824926	0.045737653
LPC(18:1)	-1.112923932	0.272100804	0.077931726
LPC(18:2)	1.103076004	0.344955922	0.093273095
LPC(18:3)	-1.440251572	0.01094698	0.016001092
LPC(20:0)	-1.840953307	0.003132	0.011855319
LPC(20:1)	-1.389173789	0.015223911	0.017573441
LPC(20:2)	-1.158208955	0.24520081	0.071983048
LPC(20:3)	-1.812254192	0.007043879	0.01393494
LPC(20:4)	-1.067124857	0.576293121	0.142539718
LPC(20:5)	-1.526315789	0.036548065	0.02503511
LPC(22:4)	1.13026419	0.28861762	0.081246817

LPC(22:5)	-1.076114171	0.555075037	0.137983655
LPC(22:6)	1.131561892	0.431413605	0.112339239
LPC[mFA14:0]	-2.210423143	2.31468E-05	0.003707606
LPC[mFA16:0]	-1.115881706	0.002302357	0.010138466
LPC[mFA16:1]	-1.17140166	0.112654765	0.043817533
LPC[mFA18:0]	1.05697538	0.253308834	0.073661762
LPC[mFA18:1]	1.138514375	0.099566647	0.04114232
LPC[mFA18:2]	1.39550763	0.020994556	0.019317882
LPC[mFA18:3]	-1.137883875	0.177105767	0.056135905
LPC[mFA20:0]	-1.443999048	0.00517079	0.013797409
LPC[mFA20:1]	-1.095528879	0.229421666	0.0686265
LPC[mFA20:2]	1.092583164	0.318892889	0.087863686
LPC[mFA20:3]	-1.426623093	0.018872833	0.018541762
LPC[mFA20:4]	1.189215364	0.141420933	0.049299024
LPC[mFA20:5]	-1.208625449	0.170636246	0.054718296
LPC[mFA22:4]	1.429709424	0.001573961	0.010039792
LPC[mFA22:5]	1.178126861	0.149140144	0.050727583
LPC[mFA22:6]	1.428146803	0.014295931	0.017244915
LPE(16:0)	-1.657045641	0.008291573	0.014709902
LPE(16:1)	-1.731707317	0.020059385	0.019047384
LPE(18:0)	-1.148485785	0.151755347	0.051123495
LPE(18:1)	-1.122957584	0.326430034	0.089514469

LPE(18:2)	1.502767992	0.107377956	0.04270818
LPE(18:3)	-1.133333333	0.565374537	0.140261177
LPE(20:0)	-1.692307692	0.006651486	0.01393494
LPE(20:1)	-1.408695652	0.015116442	0.017573441
LPE(20:2)	1.18	0.203444549	0.062167033
LPE(20:3)	1.09787234	0.518749578	0.130799632
LPE(20:4)	1.523762376	0.152852565	0.051143784
LPE(20:5)	-1.3125	0.20017304	0.061395587
LPE(22:4)	1.579090909	0.098522466	0.041108858
LPE(22:5)	1.503480278	0.162582039	0.053432758
LPE(22:6)	1.796610169	0.132158711	0.047541918
LPE[mFA16:0]	-1.446173801	0.001581899	0.010039792
LPE[mFA16:1]	-1.506692564	0.007727127	0.014616362
LPE[mFA18:0]	1.005833482	0.914883381	0.209283311
LPE[mFA18:1]	1.019115172	0.593649064	0.146099821
LPE[mFA18:2]	1.718350274	0.026934084	0.021425361
LPE[mFA18:3]	1.04002836	0.808235431	0.189636874
LPE[mFA20:0]	-1.47875687	0.022360451	0.019865172
LPE[mFA20:1]	-1.225342338	0.006930003	0.01393494
LPE[mFA20:2]	1.352040743	0.000290499	0.005510478
LPE[mFA20:3]	1.258544855	0.06904883	0.033851347
LPE[mFA20:4]	1.739709669	0.060531276	0.032003418

LPE[mFA20:5]	-1.112162024	0.420210378	0.110236785
LPE[mFA22:4]	1.811870408	0.041843259	0.02667466
LPE[mFA22:5]	1.719719212	0.074300556	0.035303058
LPE[mFA22:6]	2.047551339	0.064817299	0.033024267
MAG[mFA16:0]	-1.182052301	0.386662546	0.102969532
MAG[mFA16:1]	1.415887416	0.356315896	0.095924014
MAG[mFA18:0]	1.185913868	0.371933175	0.099580681
MAG[mFA18:1]	1.271057796	0.3035927	0.085073886
MAG[mFA18:2]	1.034899924	0.904381104	0.207844902
MAG[mFA20:0]	-1.435064356	0.425026294	0.11102772
MAG[mFA20:1]	1.393012824	0.148785391	0.050676819
MAG[mFA20:2]	-1.056931488	0.873385379	0.202039522
MAG[mFA20:3]	-1.116558003	0.759505447	0.180444784
MAG[mFA20:4]	-1.761868336	0.104348027	0.0421083
MAG[mFA22:0]	-1.160741721	0.653575187	0.159135406
MAG[mFA22:1]	-1.120839747	0.768348599	0.182184986
MAG[mFA22:2]	-1.00251225	0.994579764	0.223777637
MAG[mFA22:3]	-1.079578427	0.795122838	0.187094303
MAG[mFA22:4]	-1.031534788	0.887832758	0.204422791
MAG[mFA22:5]	1.428466146	0.17314179	0.055163087
MAG[mFA22:6]	-2.212196782	0.167004653	0.054079588
MAG16:0	-1.618307427	0.055462332	0.030392976

MAG16:1	1.128797084	0.784686906	0.184991741
MAG18:0	-1.155416013	0.436174558	0.113237871
MAG18:1	-1.02792696	0.919056121	0.209848513
MAG18:2	-1.363540569	0.477130241	0.121548389
MAG20:0	-2.152610442	0.128072679	0.046512999
MAG20:1	1.04526749	0.876028738	0.202461082
MAG20:2	-1.343629344	0.485859103	0.123516856
MAG20:3	-1.394009217	0.461055548	0.118063209
MAG20:4	-2.614785992	0.061812243	0.032336354
MAG22:0	-1.656565657	0.304093472	0.08511749
MAG22:1	-1.65	0.103753847	0.042010353
MAG22:2	-1.43852459	0.240558408	0.070968094
MAG22:3	-1.55	0.048597866	0.028131651
MAG22:4	-1.377604167	0.227616715	0.068284157
MAG22:5	1.082882883	0.799339441	0.18772822
MAG22:6	-3.009966777	0.16272711	0.053432758
PC(14:0_14:0)	-4.303155202	0.006635483	0.01393494
PC(14:0_18:1)	-1.613005336	0.013416126	0.017054335
PC(14:0_18:2)	-1.359984374	0.114224306	0.044080376
PC(14:0_18:3)	-2.13671875	0.105173926	0.04217165
PC(14:0_20:3)	-1.852739726	0.211786044	0.064396773
PC(14:0_20:4)	-1.377036154	0.284923066	0.080759938

PC(14:0_22:4)	-1.312780269	0.166360477	0.054079588
PC(14:0_22:5)	-1.227066818	0.069598221	0.033851491
PC(14:0_22:6)	-1.24270073	0.393763637	0.104521952
PC(16:0_14:0)	-1.884311521	0.012168334	0.016248919
PC(16:0_16:0)	1.055142803	0.679007368	0.163836707
PC(16:0_16:1)	-1.37764518	0.07098675	0.034056639
PC(16:0_18:0)	-1.108366343	0.547862986	0.136880766
PC(16:0_18:1)	-1.313381425	0.011123456	0.016001092
PC(16:0_18:2)	-1.194557063	0.079327849	0.03697921
PC(16:0_18:3)	-1.936424392	0.001706212	0.010138466
PC(16:0_20:1)	-1.084581537	0.460231038	0.118063209
PC(16:0_20:2)	-1.05055114	0.330749868	0.090153332
PC(16:0_20:3)	-1.54495614	0.005479807	0.013797409
PC(16:0_20:4)	-1.274579667	0.00181499	0.010138466
PC(16:0_20:5)	-2.049062093	0.005732483	0.013797409
PC(16:0_22:4)	1.043156655	0.708908864	0.170218823
PC(16:0_22:5)	-1.165417178	0.365911514	0.098292643
PC(16:0_22:6)	-1.309495491	0.013977725	0.017244915
PC(16:1_18:1)	-1.075466777	0.413464933	0.108698478
PC(16:1_18:2)	-1.101077234	0.302672364	0.084912478
PC(18:0_14:0)	-1.680358992	0.018711753	0.018541762
PC(18:0_16:1)	-1.56190759	0.090702819	0.039639021

PC(18:0_18:0)	-1.317446152	0.010214752	0.016001092
PC(18:0_18:1)	-1.383178705	0.014355638	0.017244915
PC(18:0_18:2)	-1.057152272	0.409193853	0.10815213
PC(18:0_18:3)	-1.853523144	0.037172635	0.025192958
PC(18:0_20:0)	2.18690721	0.02942305	0.022393929
PC(18:0_20:1)	-1.386565011	0.08248951	0.037739625
PC(18:0_20:2)	-1.40731857	0.070392875	0.033903643
PC(18:0_20:3)	-1.446439754	0.060737177	0.032003418
PC(18:0_20:4)	-1.072187318	0.149831541	0.050834492
PC(18:0_20:5)	-1.763986186	0.032247981	0.023527374
PC(18:0_22:4)	-1.053651454	0.722060104	0.172871637
PC(18:0_22:5)	-1.05472444	0.776189852	0.183514875
PC(18:0_22:6)	-1.114856074	0.340259541	0.092306875
PC(18:1_16:1)	-1.082963672	0.389036067	0.103489848
PC(18:1_18:1)	1.493464871	0.000793293	0.008505374
PC(18:1_18:2)	1.518435068	0.000766254	0.008505374
PC(18:1_18:3)	-1.088537549	0.500793455	0.126790673
PC(18:1_20:1)	1.001129784	0.993995528	0.223777637
PC(18:1_20:2)	1.228200903	0.243473558	0.071561175
PC(18:1_20:3)	-1.07008301	0.457035272	0.117535327
PC(18:1_20:4)	1.130101057	0.040683783	0.026263075
PC(18:1_20:5)	-1.415425735	0.026051125	0.021062711

PC(18:1_22:4)	1.622462611	0.00797821	0.014616362
PC(18:1_22:5)	1.241350493	0.193373783	0.059552304
PC(18:1_22:6)	1.082161809	0.10211433	0.041759665
PC(18:2_16:1)	-1.008271104	0.91713829	0.209604692
PC(18:2_18:2)	1.242159713	0.080135981	0.037145272
PC(18:2_18:3)	-1.314137721	0.246234924	0.072114929
PC(18:2_20:1)	-1.002573605	0.986802081	0.223045222
PC(18:2_20:2)	1.329411765	0.192096608	0.059361439
PC(18:2_20:3)	-1.078592725	0.55412184	0.137885701
PC(18:2_20:4)	-1.082516192	0.170455821	0.054718296
PC(18:2_20:5)	-1.889920424	0.003789271	0.01297809
PC(18:2_22:4)	1.557491857	0.016701981	0.018006598
PC(18:2_22:5)	1.241416775	0.265168658	0.076389918
PC(18:2_22:6)	1.033275754	0.681942234	0.16422348
PC(20:0_18:1)	-1.442332474	0.078639031	0.036797232
PC(20:0_20:3)	-1.591478697	0.160235366	0.052896312
PC(20:0_20:4)	-1.407440953	0.006254566	0.013797409
PC(20:0_22:4)	-1.453273999	0.04390794	0.027166749
PC(20:0_22:5)	-1.430340557	0.096047081	0.040836056
PC(20:0_22:6)	-1.380828049	0.024200411	0.020472893
PC[mFA14:0]	-1.832145276	0.000594486	0.00771571
PC[mFA16:0]	1.037839713	0.215907845	0.065247802

PC[mFA16:1]	-1.031825378	0.394551122	0.104618371
PC[mFA18:0]	-1.056740813	0.422869725	0.110816541
PC[mFA18:1]	-1.004576178	0.939974431	0.214228082
PC[mFA18:2]	1.027398924	0.746191548	0.177786015
PC[mFA18:3]	-1.650530829	0.009211412	0.015348011
PC[mFA20:0]	1.190593744	0.073703592	0.035087022
PC[mFA20:1]	-1.026324246	0.773240713	0.182993057
PC[mFA20:2]	1.044656496	0.553272852	0.137885701
PC[mFA20:3]	-1.332534226	0.053494282	0.029708488
PC[mFA20:4]	-1.049059656	0.598474576	0.147140556
PC[mFA20:5]	-1.765027599	0.015777248	0.017683137
PC[mFA22:4]	1.18742531	0.176187315	0.055916662
PC[mFA22:5]	1.029847323	0.853859286	0.198453399
PC[mFA22:6]	-1.077514967	0.438267148	0.113643871
PE(14:0_14:0)-H	-7.509090909	0.109667181	0.043258009
PE(14:0_16:1)	-4.705882353	0.001250241	0.00934259
PE(14:0_18:1)	-2.776335497	6.61295E-05	0.003707606
PE(14:0_18:2)	-2.307291667	0.016019392	0.017683137
PE(14:0_20:4)	-1.68627451	0.090634723	0.039639021
PE(16:0_14:0)	-2.028290283	0.004447057	0.013529855
PE(16:0_16:0)	-1.382126804	0.079526246	0.037001747
PE(16:0_16:1)	-2.565025253	0.00466428	0.013529855

PE(16:0_18:1)	-1.313682734	0.003437752	0.012286074
PE(16:0_18:2)	-1.300430863	0.100945263	0.041487982
PE(16:0_18:3)	-1.950963222	0.00433805	0.013371872
PE(16:0_20:1)	-1.07827176	0.135584361	0.048107459
PE(16:0_20:2)	-1.00213858	0.97232482	0.220443076
PE(16:0_20:3)	-1.480545477	0.024690403	0.02061437
PE(16:0_20:4)	-1.191258207	0.104545013	0.0421083
PE(16:0_20:5)	-2.434324065	0.000970152	0.008544161
PE(16:0_22:4)	1.164343108	0.287515678	0.081121826
PE(16:0_22:5)	-1.002694481	0.971888031	0.220443076
PE(16:0_22:6)	1.085798128	0.412475676	0.108554135
PE(18:0_14:0)	-1.656626506	0.04664574	0.027452733
PE(18:0_16:0)	-1.222905282	0.091453435	0.03984476
PE(18:0_16:1)	-1.427170181	0.002048361	0.010138466
PE(18:0_18:0)	-1.082262372	0.457087567	0.117535327
PE(18:0_18:1)	-1.093500752	0.416098851	0.109274428
PE(18:0_18:2)	1.325131162	0.049562009	0.028489133
PE(18:0_18:3)	1.137931034	0.044195658	0.027166749
PE(18:0_20:1)	-1.286821705	0.230618936	0.068683473
PE(18:0_20:2)	-1.220742534	0.190393231	0.059016754
PE(18:0_20:3)	-1.225337647	0.227291074	0.068269519
PE(18:0_20:4)	1.151459012	0.346555527	0.093602979

PE(18:0_20:5)	-1.226102941	0.283610998	0.080573265
PE(18:0_22:4)	1.254681554	0.230330184	0.068680423
PE(18:0_22:5)	1.181871374	0.216961809	0.065406003
PE(18:0_22:6)	1.493597595	0.022802039	0.019908191
PE(18:1_16:1)	-1.544360342	0.006318461	0.013797409
PE(18:1_18:1)	1.139333153	0.151750917	0.051123495
PE(18:1_18:2)	1.162256317	0.286892761	0.081038793
PE(18:1_18:3)	-1.140472879	0.342056234	0.092590569
PE(18:1_20:1)	1.088948787	0.718093008	0.172104547
PE(18:1_20:2)	1.281211372	0.216667527	0.065397235
PE(18:1_20:3)	-1.116257947	0.423622304	0.110895911
PE(18:1_20:4)	1.056920684	0.687652898	0.165437147
PE(18:1_20:5)	-1.982142857	0.099922686	0.04114232
PE(18:1_22:4)	1.501511031	0.005541502	0.013797409
PE(18:1_22:5)	1.258559622	0.152606077	0.051143784
PE(18:1_22:6)	1.280382868	0.122878846	0.045580187
PE(18:2_16:1)	-1.744046827	0.124153281	0.045580187
PE(18:2_18:2)	1.586524823	0.02225146	0.019865172
PE(18:2_18:3)	-1.303724928	0.386320856	0.102969532
PE(18:2_20:1)	1.52699784	0.067046858	0.033301819
PE(18:2_20:2)	1.986111111	0.006322493	0.013797409
PE(18:2_20:3)	1.325782093	0.11355205	0.044056748

PE(18:2_20:4)	1.19313871	0.146927929	0.050322184
PE(18:2_22:4)	1.822311289	0.006900884	0.01393494
PE(18:2_22:5)	1.489940828	0.017871652	0.018541762
PE(18:2_22:6)	1.551171393	0.069860797	0.033874935
PE(O-16:0_16:0)	-1.368861024	0.041866322	0.02667466
PE(O-16:0_16:1)	-1.810055866	0.0235715	0.020182844
PE(O-16:0_18:0)	-1.14354067	0.471332655	0.120320055
PE(O-16:0_18:1)	-1.38613685	0.037996887	0.025461725
PE(O-16:0_18:2)	1.03301783	0.688509905	0.165481882
PE(O-16:0_20:1)	-1.6765286	0.060621668	0.032003418
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PE(O-16:0_20:5)	-1.34153264	0.180136039	0.056731787
PE(O-16:0_22:4)	-1.253369967	0.11404379	0.044079695
PE(O-16:0_22:5)	-1.381158917	0.016206671	0.017707216
PE(O-16:0_22:6)	-1.077787976	0.335135423	0.091117263
PE(O-18:0_16:0)	1.037037037	0.827628038	0.193136087
PE(O-18:0_16:1)	-1.23580786	0.305123648	0.085212397
PE(O-18:0_18:1)	1.073726827	0.620829003	0.152181421
PE(O-18:0_18:2)	1.328149002	0.032350612	0.023532628
PE(O-18:0_20:1)	-1.329004329	0.169876842	0.054616823

PE(O-18:0_20:2)	-1.498023715	0.367694471	0.098664111
PE(O-18:0_20:3)	-1.19245648	0.236261663	0.069941953
PE(O-18:0_20:4)	1.12475442	0.326012274	0.089514469
PE(O-18:0_20:5)	1.206967213	0.188503171	0.058544456
PE(O-18:0_22:4)	-1.258604008	0.117575479	0.044725075
PE(O-18:0_22:5)	-1.276350753	0.116976702	0.044725075
PE(O-18:0_22:6)	1.042840237	0.633839784	0.155216413
PE(P-14:0_18:1)	-1.635235732	0.065127112	0.033045564
PE(P-16:0_16:0)	-1.916087353	3.42705E-05	0.003707606
PE(P-16:0_16:1)	-2.074882014	7.51754E-05	0.003707606
PE(P-16:0_18:0)	-1.46006539	0.014405909	0.017244915
PE(P-16:0_18:1)	-1.485735907	0.00524555	0.013797409
PE(P-16:0_18:2)	-1.090091752	0.341053785	0.092420667
PE(P-16:0_18:3)	-1.514804202	0.051857889	0.029196336
PE(P-16:0_20:1)	-1.990936556	0.001151376	0.009158895
PE(P-16:0_20:2)	-1.641405388	0.013996741	0.017244915
PE(P-16:0_20:3)	-1.346382444	0.049038848	0.02832044
PE(P-16:0_20:4)	-1.031341565	0.869526613	0.201524972
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PE(P-16:0_22:6)	1.025337399	0.664035937	0.160853837

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PE(P-18:0_16:0)	-1.196724891	0.135827847	0.048124607
PE(P-18:0_16:1)	-1.269879518	0.167431339	0.054082265
PE(P-18:0_18:0)	-1.306306306	0.181027091	0.056794809
PE(P-18:0_18:1)	-1.518639634	0.088163672	0.038962166
PE(P-18:0_18:2)	1.124194185	0.304741911	0.085202281
PE(P-18:0_18:3)	1.157051282	0.292603746	0.082275003
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PE(P-18:0_20:3)	-1.269581364	0.076041616	0.035853971
PE(P-18:0_20:4)	-1.141186047	0.273006771	0.078100492
PE(P-18:0_20:5)	-1.195785777	0.250974229	0.073328753
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PE(P-18:0_22:6)	-1.036385815	0.652670171	0.159135406
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PE(P-18:1_18:1)	-1.29745383	0.317324147	0.087687454
PE(P-18:1_18:2)	-1.048645425	0.441179349	0.114174635
PE(P-18:1_18:3)	-1.3	0.240592341	0.070968094
PE(P-18:1_20:1)	-1.516050878	0.064583789	0.032991066

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PE(P-18:1_20:3)	-1.153572921	0.26486562	0.076389918
PE(P-18:1_20:4)	-1.027946681	0.829169013	0.193136087
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PE(P-18:1_22:4)	1.014316366	0.884249981	0.203788138
PE(P-18:1_22:5)	-1.01073358	0.912477539	0.209064567
PE(P-18:1_22:6)	1.225404853	0.036451004	0.02503511
PE(P-18:2_18:2)	1.342135043	0.052798772	0.02959094
PE(P-18:2_20:4)	1.079082536	0.482330591	0.122746368
PE(P-18:2_22:6)	1.272999252	0.044238156	0.027166749
PE[mFA14:0]	-2.150065659	0.004103705	0.013018029
PE[mFA16:0]	-1.113645524	0.019624954	0.018889656
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PE[mFA18:0]	1.141526067	0.014222038	0.017244915
PE[mFA18:1]	-1.002224653	0.879087219	0.202977703
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PE[mFA20:1]	-1.431589213	0.003070521	0.011830954
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PE[mFA20:3]	-1.127338109	0.060526382	0.032003418
PE[mFA20:4]	1.11997204	0.054859795	0.030129745
PE[mFA20:5]	-1.426040546	0.000184205	0.004543902

PE[mFA22:4]	1.035811669	0.45671248	0.117535327
PE[mFA22:5]	1.016780647	0.611396776	0.15001846
PE[mFA22:6]	1.269903312	0.00532098	0.013797409
PG(14:0_18:1)	-3.106666667	0.000190768	0.004543902
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PG(16:0_14:0)	-4.153613851	0.00306111	0.011830954
PG(16:0_16:0)	-1.614276389	0.010832691	0.016001092
PG(16:0_16:1)	-2.995537594	5.00614E-05	0.003707606
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PG(16:0_20:2)	-1.611050852	0.004685436	0.013529855
PG(16:0_20:3)	-3.281591713	0.011234588	0.016001092
PG(16:0_20:4)	-3.078972768	0.015392988	0.017573441
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PG(18:0_14:0)	-1.755015839	0.001784282	0.010138466
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PG(18:0_22:5)	-2.082685329	0.042964147	0.027096741
PG(18:0_22:6)	-1.809163788	0.009080585	0.015337289
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PG(18:1_18:1)	-1.447603056	0.00451348	0.013529855
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PG(18:1_18:3)	-2.436628644	0.000219454	0.004543902
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PG(18:1_22:5)	-2.011563877	0.012927813	0.01677873

PG(18:1_22:6)	-1.817868168	0.018024558	0.018541762
PG(18:2_16:1)	-1.435501066	0.004999873	0.013797409
PG(18:2_18:2)	-1.261197154	0.095505787	0.040676047
PG(18:2_20:2)	1.165850673	0.577375109	0.142664243
PG(18:2_20:3)	-1.566514922	0.139682856	0.048929933
PG(18:2_20:4)	1.140961098	0.516082061	0.130260223
PG(18:2_22:6)	-1.217985612	0.338494029	0.091929052
PG[mFA14:0]	-2.097751501	0.013416799	0.017054335
PG[mFA16:0]	-1.206691997	0.008260754	0.014709902
PG[mFA18:0]	1.137040688	0.084643832	0.038154493
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PG[mFA20:1]	-1.018236102	0.782676195	0.184871194
PG[mFA20:2]	1.388057812	0.022306508	0.019865172
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PG[mFA22:5]	-1.564401559	0.007540522	0.014430613
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PI(16:0_18:1)	-1.153603494	0.656682125	0.15968976
PI(18:0_18:0)	-2.009626147	0.006301813	0.013797409
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PI[mFA18:0]	-1.049206486	0.113640942	0.044056748
PI[mFA18:1]	1.13718923	0.271975523	0.077931726
PS(16:0_16:0)	1.135419771	0.236107593	0.069941953
PS(16:0_16:1)	-1.478536497	0.172603009	0.055062571
PS(16:0_18:0)	-1.250992141	0.075291062	0.035636359
PS(16:0_18:1)	1.484485082	0.103543536	0.042010353
PS(16:0_18:2)	1.632713249	0.027436542	0.021547027
PS(16:0_20:2)	1.241689128	0.567039837	0.140391835
PS(16:0_20:3)	-1.3078203	0.459983081	0.118063209
PS(16:0_20:4)	1.391184573	0.252492106	0.073510946
PS(16:0_22:4)	1.656194216	0.180694317	0.056783248
PS(16:0_22:5)	1.193851945	0.491035713	0.124576015
PS(16:0_22:6)	1.427419355	0.383617443	0.102490654
PS(18:0_16:1)	-1.304711218	0.125057183	0.045754769
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PS(18:0_18:1)	1.068237133	0.718158161	0.172104547

PS(18:0_18:2)	1.831777797	0.099457422	0.04114232
PS(18:0_18:3)	1.277799607	0.330272726	0.090153332
PS(18:0_20:1)	-1.213622944	0.519899255	0.130955614
PS(18:0_20:2)	1.036650775	0.913079341	0.209064567
PS(18:0_20:3)	-1.079318187	0.738287134	0.176414071
PS(18:0_20:4)	1.237829633	0.486598019	0.123577307
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PS(18:0_22:5)	1.13982159	0.648511462	0.158494468
PS(18:0_22:6)	1.42033168	0.311177475	0.086316537
PS(18:1_16:1)	-1.010261544	0.930453282	0.2122543
PS(18:1_18:1)	1.409414863	0.274386204	0.078404158
PS(18:1_18:2)	2.202777093	0.136618009	0.048335119
PS(18:1_20:1)	1.174629443	0.635016318	0.155350256
PS(18:1_20:2)	1.177462569	0.524820777	0.131791424
PS(18:1_20:3)	-1.295734931	0.31834629	0.087811196
PS(18:1_20:4)	1.369822785	0.42428197	0.110950817
PS(18:1_22:4)	2.049180328	0.134189334	0.047681087
PS(18:1_22:5)	1.011477231	0.984675193	0.222768673
PS(18:1_22:6)	1.136400161	0.661090516	0.16041925
PS(18:2_18:2)	1.732291825	0.002241869	0.010138466
PS(18:2_20:1)	1.195908246	0.468813152	0.119800903
PS(18:2_20:4)	1.378114028	0.178954573	0.056431769

PS(18:2_22:6)	1.322890819	0.271000405	0.07788795
PS(20:0_18:1)	1.040474642	0.829029923	0.193136087
PS[mFA16:0]	-1.038084001	0.677643052	0.163667658
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PS[mFA18:0]	-1.043975231	0.009821065	0.015829048
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PS[mFA18:2]	1.491307166	0.011212959	0.016001092
PS[mFA18:3]	1.129302406	0.73926737	0.176477294
PS[mFA20:0]	-1.158407618	0.229639531	0.0686265
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PS[mFA20:2]	-1.134423998	0.280948976	0.080001325
PS[mFA20:3]	-1.332035203	0.017795413	0.018541762
PS[mFA20:4]	1.018770784	0.755080383	0.179730197
PS[mFA22:4]	1.026225792	0.552742286	0.137885701
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SM(14:0)	-1.167489527	0.126271699	0.045994402
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SM(18:0)	-1.210023192	0.068598589	0.033830232
SM(18:1)	-1.050044328	0.653715287	0.159135406
SM(20:0)	-1.376026428	0.006246156	0.013797409
SM(20:1)	-1.411994389	0.00964419	0.015646233

SM(22:0)	-1.167125061	0.119608545	0.045030682
SM(22:1)	-1.084435554	0.467632083	0.119623054
SM(24:0)	-1.401221026	0.015876135	0.017683137
SM(24:1)	-1.356870257	0.004575696	0.013529855
SM(26:0)	-1.452799433	0.103581158	0.042010353
SM(26:1)	-1.371044844	0.008001759	0.014616362
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TAG[mFA16:0]	-1.244249425	0.095075868	0.040633301
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TAG[mFA17:0]	1.000949407	0.988817635	0.223091827
TAG[mFA18:0]	1.225783664	0.180759973	0.056783248
TAG[mFA18:1]	1.206299771	0.063449826	0.032801951
TAG[mFA18:2]	1.251304049	0.182747039	0.057188901
TAG[mFA18:3]	-1.871661572	0.151042556	0.051092766
TAG[mFA20:0]	1.175485637	0.358259627	0.09634211
TAG[mFA20:1]	1.274273079	0.124155521	0.045580187
TAG[mFA20:2]	1.5028463	0.21311431	0.064705861
TAG[mFA20:3]	1.386521657	0.449662235	0.115988823
TAG[mFA20:4]	1.108265674	0.496476913	0.125826999
TAG[mFA20:5]	-1.849878738	0.038248273	0.02556072
TAG[mFA22:4]	-1.102141729	0.609489833	0.149699508
TAG[mFA22:5]	-1.292124396	0.327062245	0.089514469

TAG[mFA22:6]	-1.230296169	0.425959146	0.111153658
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TAG40:0-FA16:0	-11.03798561	0.045643815	0.027166749
TAG42:0-FA14:0	-5.330520073	0.163464518	0.053485034
TAG42:0-FA16:0	-7.235274204	0.05049486	0.028837064
TAG42:1-FA14:0	-6.150641026	0.010548838	0.016001092
TAG42:1-FA16:0	-8.863929147	0.011729207	0.016008795
TAG42:1-FA16:1	-9.573070608	0.01878366	0.018541762
TAG42:1-FA18:1	-7.91972866	0.026403131	0.021139384
TAG42:2-FA18:2	-7.000234714	0.014996702	0.017573441
TAG44:0-FA14:0	-6.655391121	0.110991562	0.043601876
TAG44:0-FA16:0	-6.278672591	0.086274189	0.03864314
TAG44:0-FA18:0	-3.867027677	0.108608089	0.043074592
TAG44:1-FA14:0	-6.76797748	0.067499462	0.033424012
TAG44:1-FA16:0	-6.160933986	0.015704012	0.017683137
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TAG46:0-FA18:0	-3.634182306	0.154845435	0.051530911
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TAG46:4-FA18:2	-3.090401945	0.081388395	0.037444265
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TAG47:2-FA16:1	-4.626069595	0.011485113	0.016001092
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TAG47:2-FA18:2	-3.370146365	0.036872661	0.02518749
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TAG48:0-FA18:0	-4.103367297	0.132859483	0.047619561
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TAG48:4-FA16:0	-4.937969925	0.008157876	0.014683992
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TAG48:5-FA18:2	-3.096381225	0.12284086	0.045580187
TAG48:5-FA18:3	-4.572847682	0.035481147	0.024716217

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TAG52:8-FA18:2	-2.033466135	0.087336314	0.038875207
TAG53:0-FA16:0	-2.703433307	0.015826255	0.017683137
TAG53:1-FA16:0	-2.263168537	0.032225425	0.023527374
TAG53:1-FA17:0	-1.788517493	0.081591651	0.037467874
TAG53:1-FA18:0	-1.982659952	0.103102223	0.041954932
TAG53:1-FA18:1	-2.032468145	0.031873343	0.023527374
TAG53:2-FA16:0	-2.284927627	0.045545155	0.027166749
TAG53:2-FA17:0	-1.824799955	0.068399297	0.033801713

TAG53:2-FA18:1	-1.877796766	0.044491342	0.027166749
TAG53:2-FA18:2	-1.636041956	0.193438744	0.059552304
TAG53:3-FA16:0	-2.272307692	0.034274247	0.024079553
TAG53:3-FA17:0	-2.25883056	0.039601505	0.025903471
TAG53:3-FA18:2	-1.651867537	0.140429948	0.049050411
TAG53:4-FA16:0	-3.005472788	0.002843662	0.01149571
TAG53:4-FA17:0	-2.709993715	0.039505027	0.025903471
TAG53:4-FA18:2	-1.815437922	0.09082047	0.039639021
TAG53:4-FA18:3	-4.39008524	0.117532073	0.044725075
TAG53:4-FA20:4	-1.951892744	0.02254644	0.019865172
TAG53:5-FA20:4	-1.890200889	0.04025894	0.026057034
TAG53:6-FA20:4	-2.1294252	0.064618349	0.032991066
TAG54:0-FA16:0	-2.049848943	0.030333974	0.022787729
TAG54:0-FA18:0	-1.785840552	0.309423007	0.085926526
TAG54:1-FA16:0	-2.115081124	0.044555071	0.027166749
TAG54:1-FA18:0	-1.648684486	0.307037043	0.085456416
TAG54:1-FA18:1	-1.55218673	0.174178311	0.055350299
TAG54:1-FA20:0	-2.882647289	0.10962867	0.043258009
TAG54:1-FA20:1	-2.403331442	0.124210341	0.045580187
TAG54:2-FA16:0	-1.833158242	0.112273519	0.043799671
TAG54:2-FA18:0	-1.7353498	0.203106569	0.062140758
TAG54:2-FA18:1	-1.570648491	0.095362613	0.040676047

TAG54:2-FA18:2	-1.33091122	0.506794097	0.128178313
TAG54:2-FA20:0	-3.153961867	0.116482511	0.044725075
TAG54:2-FA20:1	-2.569626227	0.078303744	0.036710002
TAG54:2-FA20:2	-1.784127335	0.185666027	0.057808923
TAG54:3-FA16:0	-1.825127762	0.118633141	0.044868965
TAG54:3-FA16:1	-2.113835377	0.057091526	0.030874109
TAG54:3-FA18:0	-1.867097769	0.16710934	0.054079588
TAG54:3-FA18:1	-1.76091509	0.043196406	0.02710458
TAG54:3-FA18:2	-1.400415219	0.355930092	0.095924014
TAG54:3-FA18:3	-3.486467437	0.235980194	0.069941953
TAG54:3-FA20:1	-2.78864544	0.083782952	0.037909388
TAG54:3-FA20:2	-2.264496668	0.082832984	0.037826588
TAG54:3-FA20:3	-2.482280301	0.10981287	0.043258009
TAG54:4-FA16:0	-1.709690934	0.078258044	0.036710002
TAG54:4-FA16:1	-2.022828508	0.063440703	0.032801951
TAG54:4-FA18:0	-2.160087844	0.133855291	0.047681087
TAG54:4-FA18:1	-1.88097468	0.036411638	0.02503511
TAG54:4-FA18:2	-1.520613179	0.220356272	0.066348198
TAG54:4-FA18:3	-3.456319754	0.190792314	0.059032364
TAG54:4-FA20:1	-3.221841248	0.066837351	0.033296735
TAG54:4-FA20:2	-2.388270786	0.112431223	0.043799671
TAG54:4-FA20:3	-2.609007697	0.059079841	0.031740536

TAG54:4-FA20:4	-2.876848342	0.051203904	0.028894106
TAG54:4-FA22:4	-4.070441523	0.005554561	0.013797409
TAG54:5-FA16:0	-1.992523437	0.028170594	0.021865542
TAG54:5-FA16:1	-1.964507542	0.054202949	0.029902191
TAG54:5-FA18:0	-3.694247474	0.05069657	0.028837064
TAG54:5-FA18:1	-2.167409976	0.031760724	0.023527374
TAG54:5-FA18:2	-1.699141172	0.15201955	0.051142633
TAG54:5-FA18:3	-3.723588415	0.142402064	0.049528784
TAG54:5-FA20:2	-2.831675288	0.137632037	0.048416026
TAG54:5-FA20:3	-2.780107285	0.072393248	0.034529885
TAG54:5-FA20:4	-3.015358925	0.039233959	0.025899823
TAG54:5-FA20:5	-5.846865365	0.023498053	0.020182844
TAG54:5-FA22:4	-4.858128834	0.0098969	0.015847694
TAG54:5-FA22:5	-4.61952862	0.000954905	0.008544161
TAG54:6-FA16:0	-2.137977566	0.023445193	0.020182844
TAG54:6-FA16:1	-2.617834395	0.01225603	0.016248919
TAG54:6-FA18:1	-3.343997515	0.012583376	0.016516507
TAG54:6-FA18:2	-2.004253521	0.106746175	0.042525487
TAG54:6-FA18:3	-4.719400136	0.130680465	0.047280165
TAG54:6-FA20:3	-3.520444719	0.055730337	0.030472126
TAG54:6-FA20:4	-3.566321305	0.05085258	0.028837064
TAG54:6-FA20:5	-5.394449117	0.021909332	0.019863138

TAG54:6-FA22:5	-5.842228055	0.0010221	0.008691268
TAG54:6-FA22:6	-4.42035054	0.001849874	0.010138466
TAG54:7-FA16:1	-2.739726027	0.008609121	0.015056614
TAG54:7-FA18:1	-3.485354331	0.00210898	0.010138466
TAG54:7-FA18:2	-3.49381334	0.018338136	0.018541762
TAG54:7-FA18:3	-6.046404911	0.119163403	0.044965323
TAG54:7-FA20:4	-4.093697479	0.0390637	0.025899823
TAG54:7-FA20:5	-7.082015131	0.029662221	0.022437459
TAG54:7-FA22:5	-4.717377049	0.011447139	0.016001092
TAG54:7-FA22:6	-5.506119062	0.001937742	0.010138466
TAG54:8-FA18:2	-2.788091465	0.013751098	0.017244915
TAG54:8-FA18:3	-9.000357143	0.096732691	0.040915921
TAG54:8-FA20:4	-3.408250356	0.043761832	0.027166749
TAG54:8-FA20:5	-8.742474916	0.020616843	0.019119646
TAG54:8-FA22:6	-5.117870722	0.007056015	0.01393494
TAG55:1-FA16:0	-1.567158198	0.144120438	0.049850782
TAG55:1-FA18:1	-2.132906764	0.024572941	0.02061437
TAG55:2-FA18:1	-1.731419616	0.066516393	0.033271271
TAG55:2-FA18:2	-1.738258094	0.123908481	0.045580187
TAG55:3-FA18:1	-1.767475912	0.067117648	0.033301819
TAG55:3-FA18:2	-1.430668678	0.324669849	0.089355557
TAG55:4-FA18:1	-1.758595518	0.068731383	0.033830232

TAG55:4-FA18:2	-1.523011897	0.242888125	0.071505697
TAG55:5-FA18:1	-2.035140866	0.012101854	0.016248919
TAG55:5-FA18:2	-1.697373372	0.149947549	0.050834492
TAG55:5-FA20:4	-2.289685737	0.065833218	0.033120242
TAG55:7-FA22:6	-1.71050727	0.064005099	0.032991066
TAG56:1-FA16:0	-1.827039627	0.070385591	0.033903643
TAG56:1-FA18:1	-1.591217746	0.11770859	0.044725075
TAG56:10-FA18:2	-1.512012579	0.265960608	0.076528661
TAG56:2-FA16:0	-1.736512262	0.098075787	0.041100407
TAG56:2-FA18:0	-1.645335149	0.247459845	0.0723877
TAG56:2-FA20:0	-2.07574654	0.152347476	0.051143784
TAG56:2-FA20:1	-1.688125552	0.232864393	0.069268562
TAG56:3-FA16:0	-1.829131653	0.09781935	0.041093609
TAG56:3-FA18:0	-1.652112676	0.252396629	0.073510946
TAG56:3-FA18:1	-1.577183147	0.146903476	0.050322184
TAG56:3-FA18:2	-1.360711368	0.436242206	0.113237871
TAG56:3-FA20:0	-2.011877828	0.122656993	0.045580187
TAG56:3-FA20:1	-1.712710923	0.147555586	0.050397162
TAG56:3-FA20:2	-1.474313637	0.214069173	0.0648511
TAG56:4-FA16:0	-1.398083303	0.158147611	0.052347263
TAG56:4-FA18:0	-1.831435303	0.146585012	0.050322184
TAG56:4-FA18:1	-1.646927241	0.123466343	0.045580187

TAG56:4-FA18:2	-1.34036641	0.434938757	0.113137712
TAG56:4-FA20:1	-1.972875497	0.12084284	0.045426022
TAG56:4-FA20:2	-1.473381504	0.298364691	0.083799327
TAG56:4-FA20:3	-1.915777133	0.106394618	0.042454019
TAG56:4-FA20:4	-1.997233202	0.093862232	0.040526497
TAG56:4-FA22:4	-2.741169658	0.008768422	0.01512074
TAG56:5-FA16:0	-1.466845878	0.097051553	0.040980501
TAG56:5-FA18:0	-1.727687509	0.079841949	0.037078677
TAG56:5-FA18:1	-1.56729048	0.04571915	0.027166749
TAG56:5-FA18:2	-1.479263516	0.317542091	0.087687454
TAG56:5-FA20:1	-2.242238446	0.098782561	0.04114232
TAG56:5-FA20:2	-1.627040877	0.242994835	0.071505697
TAG56:5-FA20:3	-1.840343327	0.123053454	0.045580187
TAG56:5-FA20:4	-2.18733614	0.043455323	0.027166749
TAG56:5-FA22:4	-3.331952118	0.002071605	0.010138466
TAG56:5-FA22:5	-2.872236504	0.013881122	0.017244915
TAG56:6-FA16:0	-1.517162471	0.13980223	0.048929933
TAG56:6-FA18:0	-1.710214083	0.098335542	0.041100407
TAG56:6-FA18:1	-1.712474669	0.02356946	0.020182844
TAG56:6-FA18:2	-1.40403106	0.285541368	0.08084227
TAG56:6-FA18:3	-4.075215783	0.184707511	0.057583186
TAG56:6-FA20:2	-2.172380544	0.146854335	0.050322184

TAG56:6-FA20:3	-1.914918965	0.145482835	0.050175687
TAG56:6-FA20:4	-2.005146142	0.083406763	0.037875496
TAG56:6-FA20:5	-3.711737629	0.045161503	0.027166749
TAG56:6-FA22:4	-3.725908238	0.017912361	0.018541762
TAG56:6-FA22:5	-3.668896543	0.007821125	0.014616362
TAG56:6-FA22:6	-2.420711348	0.022847076	0.019908191
TAG56:7-FA16:0	-1.617361577	0.115511256	0.044437938
TAG56:7-FA16:1	-1.861154446	0.048487348	0.028131651
TAG56:7-FA18:0	-2.649284254	0.094004248	0.040526497
TAG56:7-FA18:1	-1.79656493	0.015082735	0.017573441
TAG56:7-FA18:2	-1.590128039	0.141541554	0.049299024
TAG56:7-FA18:3	-3.943676939	0.133150574	0.047619561
TAG56:7-FA20:3	-2.180879928	0.124050272	0.045580187
TAG56:7-FA20:4	-2.31065836	0.099937384	0.04114232
TAG56:7-FA20:5	-3.661945231	0.047293533	0.027767711
TAG56:7-FA22:4	-4.158375635	0.001657758	0.010138466
TAG56:7-FA22:5	-3.993817935	0.029563324	0.022431459
TAG56:7-FA22:6	-3.285452287	0.01842364	0.018541762
TAG56:8-FA16:0	-1.712954246	0.092636418	0.040218053
TAG56:8-FA16:1	-2.195394278	0.074445116	0.035303721
TAG56:8-FA18:1	-2.33095927	0.009516957	0.015645681
TAG56:8-FA18:2	-1.697299109	0.099521629	0.04114232

TAG56:8-FA18:3	-4.392624729	0.096685483	0.040915921
TAG56:8-FA20:4	-2.267611623	0.093367844	0.040401427
TAG56:8-FA20:5	-4.375672591	0.037187152	0.025192958
TAG56:8-FA22:5	-5.01156961	0.039137429	0.025899823
TAG56:8-FA22:6	-4.026838607	0.024621556	0.02061437
TAG56:9-FA18:3	-3.739012234	0.071817515	0.034388305
TAG56:9-FA20:4	-3.012541806	0.070358106	0.033903643
TAG56:9-FA20:5	-4.50812065	0.050567906	0.028837064
TAG56:9-FA22:6	-5.551448276	0.025834557	0.021025485
TAG57:10-FA22:6	-2.255555556	0.084111955	0.037988549
TAG57:2-FA18:1	-1.467130298	0.118132906	0.044748401
TAG57:3-FA18:2	-1.288085676	0.441240036	0.114174635
TAG58:10-FA18:2	-1.291412911	0.449384081	0.115988823
TAG58:10-FA20:4	-1.152209493	0.515719678	0.130260223
TAG58:10-FA20:5	-2.385899814	0.097351575	0.041036918
TAG58:10-FA22:5	-3.671787709	0.037414566	0.025277579
TAG58:10-FA22:6	-2.985198109	0.083188909	0.0378523
TAG58:2-FA18:1	-1.732400165	0.066679369	0.033285275
TAG58:3-FA18:1	-1.556361987	0.185965454	0.057829136
TAG58:5-FA18:1	-1.20685112	0.065433454	0.033053666
TAG58:6-FA16:0	-1.559618442	0.155748446	0.05176157
TAG58:6-FA18:0	-1.273821721	0.332524311	0.090507136

TAG58:6-FA18:1	-1.255689424	0.190502539	0.059016754
TAG58:6-FA20:4	-2.601355932	0.03775124	0.025435352
TAG58:6-FA22:4	-1.960412371	0.089057143	0.039216457
TAG58:6-FA22:5	-2.204954955	0.026045374	0.021062711
TAG58:7-FA16:0	-1.441090555	0.151393005	0.051123495
TAG58:7-FA18:0	-1.255924721	0.277381068	0.079076662
TAG58:7-FA18:1	-1.141928123	0.475007689	0.121132808
TAG58:7-FA18:2	-1.056882821	0.860952816	0.199913652
TAG58:7-FA20:4	-2.008617718	0.053502247	0.029708488
TAG58:7-FA22:4	-2.252507716	0.088082693	0.038962166
TAG58:7-FA22:5	-2.510121846	0.055999718	0.030505081
TAG58:7-FA22:6	-1.905758841	0.065545197	0.033053666
TAG58:8-FA18:1	-1.37378285	0.213327355	0.064705861
TAG58:8-FA18:2	-1.045979921	0.899805525	0.206986247
TAG58:8-FA20:3	-1.746929825	0.092021095	0.040021371
TAG58:8-FA20:4	-1.405452128	0.25959274	0.075046619
TAG58:8-FA22:5	-2.528831169	0.062905632	0.032657546
TAG58:8-FA22:6	-2.468954932	0.046219859	0.027302674
TAG58:9-FA18:1	-1.438647203	0.171754314	0.054862801
TAG58:9-FA18:2	-1.277651663	0.461003726	0.118063209
TAG58:9-FA20:4	-1.550302487	0.2151593	0.065101371
TAG58:9-FA22:5	-3.07540221	0.045551412	0.027166749

TAG58:9-FA22:6	-2.903819708	0.065065793	0.033045564
TAG60:10-FA22:5	-2.61414791	0.005561169	0.013797409
TAG60:10-FA22:6	-1.611539648	0.109001894	0.043145312
TAG60:11-FA22:5	-3.063157895	0.002597875	0.0108581
TAG60:11-FA22:6	-2.421279937	0.035082524	0.024507767
TAG60:12-FA22:6	-2.385835095	0.040096226	0.026057034

Supplemental Table 3. Analytes significantly reduced in IAV infected compared to mock infected lungs.

ANALYTE NAME	FOLD CHANGE	P-VALUE	Q-VALUE
PS[mFA18:0]	-1.043975231	0.00982107	0.01582905
PE[mFA16:0]	-1.113645524	0.01962495	0.01888966
LPC[mFA16:0]	-1.115881706	0.00230236	0.01013847
CER(16:0)	-1.141703038	0.03712273	0.02519296
CER(24:1)	-1.172403116	0.04019611	0.02605703
Ceramide	-1.199859736	0.01976315	0.01888966
PG[mFA16:0]	-1.206691997	0.00826075	0.0147099
LPE[mFA20:1]	-1.225342338	0.00693	0.01393494
PE(O-16:0_20:4)	-1.245634079	0.04628005	0.02730267
PC(16:0_20:4)	-1.274579667	0.00181499	0.01013847
PE(P-18:1_16:0)	-1.276977681	0.02819679	0.02186554
PG[mFA20:4]	-1.278149772	0.00723638	0.01405093
CER(20:0)	-1.295214106	0.02857012	0.02194798
PC(16:0_22:6)	-1.309495491	0.01397772	0.01724491
PC(16:0_18:1)	-1.313381425	0.01112346	0.01600109
PE(16:0_18:1)	-1.313682734	0.00343775	0.01228607
PE[mFA18:3]	-1.316337364	0.02928449	0.02235748
PC(18:0_18:0)	-1.317446152	0.01021475	0.01600109
PG[mFA20:3]	-1.320167453	0.00158782	0.01003979

PS[mFA20:3]	-1.332035203	0.01779541	0.01854176
PE(P-16:0_20:3)	-1.346382444	0.04903885	0.02832044
SM(24:1)	-1.356870257	0.0045757	0.01352986
PE(O-16:0_16:0)	-1.368861024	0.04186632	0.02667466
SM(26:1)	-1.371044844	0.00800176	0.01461636
SM(20:0)	-1.376026428	0.00624616	0.01379741
PC(20:0_22:6)	-1.380828049	0.02420041	0.02047289
PE(O-16:0_22:5)	-1.381158917	0.01620667	0.01770722
PC(18:0_18:1)	-1.383178705	0.01435564	0.01724491
PE(O-16:0_18:1)	-1.38613685	0.03799689	0.02546172
LPC(20:1)	-1.389173789	0.01522391	0.01757344
PG[mFA22:6]	-1.389442083	0.04445285	0.02716675
SM(24:0)	-1.401221026	0.01587613	0.01768314
CE(16:1)	-1.406815663	0.03210698	0.02352737
PC(20:0_20:4)	-1.407440953	0.00625457	0.01379741
LPE(20:1)	-1.408695652	0.01511644	0.01757344
SM(20:1)	-1.411994389	0.00964419	0.01564623
LPC(16:0)	-1.413250662	0.02017938	0.01904738
PC(18:1_20:5)	-1.415425735	0.02605112	0.02106271
PE[mFA20:5]	-1.426040546	0.00018421	0.0045439
LPC[mFA20:3]	-1.426623093	0.01887283	0.01854176
PE(18:0_16:1)	-1.427170181	0.00204836	0.01013847

PE[mFA20:1]	-1.431589213	0.00307052	0.01183095
PG(18:1_18:2)	-1.431666627	0.01130469	0.01600109
PG(18:2_16:1)	-1.435501066	0.00499987	0.01379741
LPC(18:3)	-1.440251572	0.01094698	0.01600109
LPC[mFA20:0]	-1.443999048	0.00517079	0.01379741
LPE[mFA16:0]	-1.446173801	0.0015819	0.01003979
PG(18:0_18:1)	-1.446625306	0.04774093	0.02796382
PG(18:1_18:1)	-1.447603056	0.00451348	0.01352986
CER(24:0)	-1.447686088	0.00415937	0.01301803
PC(20:0_22:4)	-1.453273999	0.04390794	0.02716675
PE(P-16:0_18:0)	-1.46006539	0.01440591	0.01724491
LPE[mFA20:0]	-1.47875687	0.02236045	0.01986517
PE(16:0_20:3)	-1.480545477	0.0246904	0.02061437
PE(P-16:0_18:1)	-1.485735907	0.00524555	0.01379741
LPC(16:1)	-1.494163424	0.02626727	0.02109912
PS[mFA16:1]	-1.497347454	0.02246182	0.01986517
LPE[mFA16:1]	-1.506692564	0.00772713	0.01461636
PG(18:1_16:1)	-1.507117438	0.0093398	0.01545748
PE(O-16:0_20:3)	-1.510920437	0.04150284	0.02665227
LPC(20:5)	-1.526315789	0.03654807	0.02503511
CER(14:0)	-1.530232558	0.02424234	0.02047289
PE(18:1_16:1)	-1.544360342	0.00631846	0.01379741

PC(16:0_20:3)	-1.54495614	0.00547981	0.01379741
MAG22:3	-1.55	0.04859787	0.02813165
PG[mFA22:5]	-1.564401559	0.00754052	0.01443061
TAG56:5-FA18:1	-1.56729048	0.04571915	0.02716675
PG(18:1_20:4)	-1.584179567	0.00903758	0.01533729
PE(P-18:0_20:2)	-1.594852941	0.04558001	0.02716675
PG(16:0_20:2)	-1.611050852	0.00468544	0.01352986
PC(14:0_18:1)	-1.613005336	0.01341613	0.01705434
PG(16:0_16:0)	-1.614276389	0.01083269	0.01600109
DAG[mFA16:1]	-1.625766135	0.02138877	0.01953483
PE(P-16:0_20:2)	-1.641405388	0.01399674	0.01724491
PC[mFA18:3]	-1.650530829	0.00921141	0.01534801
PE(18:0_14:0)	-1.656626506	0.04664574	0.02745273
LPE(16:0)	-1.657045641	0.00829157	0.0147099
PG(16:0_18:0)	-1.674655286	0.0058603	0.01379741
PC(18:0_14:0)	-1.680358992	0.01871175	0.01854176
PG(18:0_18:2)	-1.687624614	0.00218961	0.01013847
LPE(20:0)	-1.692307692	0.00665149	0.01393494
TAG56:6-FA18:1	-1.712474669	0.02356946	0.02018284
LPE(16:1)	-1.731707317	0.02005938	0.01904738
PG(18:0_14:0)	-1.755015839	0.00178428	0.01013847
TAG54:3-FA18:1	-1.76091509	0.04319641	0.02710458

PC(18:0_20:5)	-1.763986186	0.03224798	0.02352737
PC[mFA20:5]	-1.765027599	0.01577725	0.01768314
DAG(18:1_18:1)	-1.768612276	0.0412857	0.02658205
PE(P-18:0_20:1)	-1.785087719	0.00509616	0.01379741
TAG[mFA16:1]	-1.79505911	0.00275275	0.01131366
TAG56:7-FA18:1	-1.79656493	0.01508273	0.01757344
Diacylglycerol	-1.808652771	0.01899827	0.01859093
PG(18:0_22:6)	-1.809163788	0.00908059	0.01533729
PE(O-16:0_16:1)	-1.810055866	0.0235715	0.02018284
LPC(20:3)	-1.812254192	0.00704388	0.01393494
PG(18:1_22:6)	-1.817868168	0.01802456	0.01854176
PE[mFA16:1]	-1.828230453	0.00068723	0.00806989
PC[mFA14:0]	-1.832145276	0.00059449	0.00771571
LPC(20:0)	-1.840953307	0.003132	0.01185532
TAG[mFA20:5]	-1.849878738	0.03824827	0.02556072
PC(18:0_18:3)	-1.853523144	0.03717264	0.02519296
TAG56:7-FA16:1	-1.861154446	0.04848735	0.02813165
TAG53:2-FA18:1	-1.877796766	0.04449134	0.02716675
TAG54:4-FA18:1	-1.88097468	0.03641164	0.02503511
PC(16:0_14:0)	-1.884311521	0.01216833	0.01624892
PC(18:2_20:5)	-1.889920424	0.00378927	0.01297809
TAG53:5-FA20:4	-1.890200889	0.04025894	0.02605703

PE(P-16:0_16:0)	-1.916087353	3.4271E-05	0.00370761
PC(16:0_18:3)	-1.936424392	0.00170621	0.01013847
PE(16:0_18:3)	-1.950963222	0.00433805	0.01337187
TAG53:4-FA20:4	-1.951892744	0.02254644	0.01986517
PG(16:0_18:1)	-1.979903182	0.01136569	0.01600109
PG(18:0_16:1)	-1.980144404	0.00021662	0.0045439
PE(P-16:0_20:1)	-1.990936556	0.00115138	0.0091589
TAG54:5-FA16:0	-1.992523437	0.02817059	0.02186554
PI(18:0_18:0)	-2.009626147	0.00630181	0.01379741
PG(18:1_22:5)	-2.011563877	0.01292781	0.01677873
TAG51:3-FA18:2	-2.013466543	0.03331357	0.02360639
PG(18:0_20:4)	-2.021213397	0.0102926	0.01600109
PE(16:0_14:0)	-2.028290283	0.00444706	0.01352986
PE(P-16:0_20:5)	-2.031374502	0.00040062	0.00581134
TAG53:1-FA18:1	-2.032468145	0.03187334	0.02352737
PG(16:0_20:1)	-2.034646244	0.01417149	0.01724491
TAG55:5-FA18:1	-2.035140866	0.01210185	0.01624892
PC(16:0_20:5)	-2.049062093	0.00573248	0.01379741
TAG54:0-FA16:0	-2.049848943	0.03033397	0.02278773
PE(P-16:0_16:1)	-2.074882014	7.5175E-05	0.00370761
PG(16:0_18:2)	-2.081856933	0.01057727	0.01600109
PG(18:0_22:5)	-2.082685329	0.04296415	0.02709674

TAG52:3-FA18:2	-2.095597643	0.04213235	0.02667466
PG[mFA14:0]	-2.097751501	0.0134168	0.01705434
TAG51:2-FA16:0	-2.114593336	0.04822297	0.02804631
TAG54:1-FA16:0	-2.115081124	0.04455507	0.02716675
PG(18:0_20:3)	-2.11659919	0.00111196	0.00914017
DAG(16:0_18:1)	-2.125059199	0.01175032	0.01600879
TAG55:1-FA18:1	-2.132906764	0.02457294	0.02061437
TAG54:6-FA16:0	-2.137977566	0.02344519	0.02018284
PE[mFA14:0]	-2.150065659	0.00410371	0.01301803
TAG51:4-FA18:2	-2.158389574	0.04312982	0.02710458
TAG54:5-FA18:1	-2.167409976	0.03176072	0.02352737
DAG(16:0_16:1)	-2.180481283	0.01854868	0.01854176
TAG56:5-FA20:4	-2.18733614	0.04345532	0.02716675
TAG58:6-FA22:5	-2.204954955	0.02604537	0.02106271
LPC[mFA14:0]	-2.210423143	2.3147E-05	0.00370761
TAG51:2-FA18:1	-2.230208932	0.00810608	0.01468399
TAG53:3-FA17:0	-2.25883056	0.0396015	0.02590347
TAG53:1-FA16:0	-2.263168537	0.03222542	0.02352737
TAG53:3-FA16:0	-2.272307692	0.03427425	0.02407955
TAG53:2-FA16:0	-2.284927627	0.04554516	0.02716675
PG[mFA20:5]	-2.296887719	0.0037121	0.01290829
PE(14:0_18:2)	-2.307291667	0.01601939	0.01768314

TAG52:1-FA18:1	-2.314179349	0.02492059	0.02063281
TAG51:1-FA18:0	-2.320238264	0.04218673	0.02667466
TAG56:8-FA18:1	-2.33095927	0.00951696	0.01564568
TAG51:5-FA18:2	-2.355136591	0.04464035	0.02716675
TAG52:2-FA18:1	-2.369690385	0.00690716	0.01393494
TAG50:4-FA14:0	-2.372317746	0.043702	0.02716675
TAG51:1-FA18:1	-2.385061207	0.0054566	0.01379741
TAG60:12-FA22:6	-2.385835095	0.04009623	0.02605703
TAG52:3-FA16:1	-2.412445809	0.03258095	0.02355878
TAG56:6-FA22:6	-2.420711348	0.02284708	0.01990819
TAG60:11-FA22:6	-2.421279937	0.03508252	0.02450777
TAG52:2-FA16:1	-2.425004793	0.01667906	0.0180066
PE(16:0_20:5)	-2.434324065	0.00097015	0.00854416
PG(18:1_18:3)	-2.436628644	0.00021945	0.0045439
TAG58:8-FA22:6	-2.468954932	0.04621986	0.02730267
TAG52:4-FA18:2	-2.487622364	0.03060964	0.02287346
TAG52:7-FA16:0	-2.488471141	0.0359897	0.02499986
TAG52:4-FA16:0	-2.493269881	0.02795752	0.02181721
DAG(16:0_18:2)	-2.505719375	0.02976585	0.02244699
TAG52:4-FA16:1	-2.527379209	0.02501738	0.02063281
TAG49:0-FA18:0	-2.528424977	0.03304088	0.02355878
TAG51:0-FA16:0	-2.551880267	0.01878738	0.01854176

TAG52:5-FA14:0	-2.557857504	0.01727721	0.01851607
TAG52:3-FA18:1	-2.560528082	0.00621004	0.01379741
PE(16:0_16:1)	-2.565025253	0.00466428	0.01352986
TAG51:4-FA20:4	-2.572632944	0.02175261	0.01979382
TAG58:6-FA20:4	-2.601355932	0.03775124	0.02543535
TAG60:10-FA22:5	-2.61414791	0.00556117	0.01379741
TAG54:6-FA16:1	-2.617834395	0.01225603	0.01624892
TAG50:1-FA16:0	-2.657154039	0.01091268	0.01600109
TAG50:2-FA16:0	-2.662241724	0.01807181	0.01854176
TAG52:7-FA18:1	-2.665927568	0.00417047	0.01301803
TAG50:0-FA16:0	-2.675335428	0.02724613	0.02146585
TAG52:1-FA16:1	-2.697470623	0.01885891	0.01854176
TAG53:0-FA16:0	-2.703433307	0.01582626	0.01768314
TAG53:4-FA17:0	-2.709993715	0.03950503	0.02590347
TAG52:6-FA14:0	-2.718795532	0.02057536	0.01911965
TAG54:7-FA16:1	-2.739726027	0.00860912	0.01505661
TAG56:4-FA22:4	-2.741169658	0.00876842	0.01512074
TAG50:4-FA18:2	-2.750153946	0.04183952	0.02667466
PE(14:0_18:1)	-2.776335497	6.6129E-05	0.00370761
PG(18:0_18:3)	-2.785597381	0.01564664	0.01768314
TAG54:8-FA18:2	-2.788091465	0.0137511	0.01724491
DAG(16:1_18:0)	-2.802158805	0.00754896	0.01443061

LPC(14:0)	-2.809193122	0.0006768	0.00806989
TAG45:1-FA18:1	-2.813326282	0.02344641	0.02018284
PG[mFA18:3]	-2.827129194	0.00039622	0.00581134
DAG(16:1_22:6)	-2.827176781	0.01079328	0.01600109
TAG56:5-FA22:5	-2.872236504	0.01388112	0.01724491
TAG52:5-FA16:1	-2.907802911	0.01111472	0.01600109
TAG51:1-FA17:0	-2.913283164	0.01859798	0.01854176
TAG50:2-FA18:2	-2.919927494	0.03210771	0.02352737
TAG48:3-FA18:1	-2.948887485	0.04810464	0.02804631
PG(14:0_18:2)	-2.974683544	0.00371655	0.01290829
PG(16:0_16:1)	-2.995537594	5.0061E-05	0.00370761
TAG53:4-FA16:0	-3.005472788	0.00284366	0.01149571
TAG54:5-FA20:4	-3.015358925	0.03923396	0.02589982
TAG46:3-FA18:1	-3.030318735	0.02625021	0.02109912
TAG60:11-FA22:5	-3.063157895	0.00259787	0.0108581
TAG58:9-FA22:5	-3.07540221	0.04555141	0.02716675
PG(16:0_20:4)	-3.078972768	0.01539299	0.01757344
TAG52:6-FA18:1	-3.095522388	0.00337419	0.01223626
PG(18:1_20:5)	-3.098455598	0.00220842	0.01013847
PG(14:0_18:1)	-3.106666667	0.00019077	0.0045439
TAG51:3-FA16:1	-3.115471046	0.00551202	0.01379741
TAG47:1-FA18:1	-3.141104294	0.01672167	0.0180066

TAG51:4-FA16:1	-3.150360017	0.01823912	0.01854176
TAG49:3-FA18:2	-3.161987961	0.03948356	0.02590347
TAG51:2-FA16:1	-3.176074685	0.00706538	0.01393494
TAG50:3-FA18:1	-3.177095924	0.01058951	0.01600109
TAG51:2-FA17:0	-3.183564989	0.02273443	0.01990819
TAG49:2-FA18:1	-3.197022161	0.01531317	0.01757344
TAG46:1-FA18:0	-3.20035824	0.01527296	0.01757344
TAG49:1-FA14:0	-3.208168754	0.02841404	0.02189629
TAG52:4-FA18:0	-3.233471687	0.04526166	0.02716675
TAG52:5-FA18:2	-3.24673768	0.00712013	0.01393494
TAG50:3-FA18:2	-3.247348149	0.02833077	0.02189629
TAG47:2-FA14:0	-3.273051049	0.02772036	0.02170081
PG(16:0_20:3)	-3.281591713	0.01123459	0.01600109
TAG52:4-FA20:4	-3.282964946	0.04520646	0.02716675
TAG56:7-FA22:6	-3.285452287	0.01842364	0.01854176
TAG49:1-FA18:1	-3.28682348	0.01504457	0.01757344
DAG(14:0_18:2)	-3.321307941	0.01220071	0.01624892
TAG56:5-FA22:4	-3.331952118	0.00207161	0.01013847
DAG(14:0_22:6)	-3.335832084	0.00392472	0.01301803
TAG54:6-FA18:1	-3.343997515	0.01258338	0.01651651
TAG47:2-FA18:2	-3.370146365	0.03687266	0.02518749
DAG(14:0_14:0)	-3.387096774	0.01303512	0.01682943

TAG54:8-FA20:4	-3.408250356	0.04376183	0.02716675
TAG50:5-FA16:0	-3.423398329	0.00869897	0.01510661
TAG49:2-FA18:2	-3.426971501	0.04557322	0.02716675
TAG50:5-FA18:2	-3.434896552	0.02682662	0.02140894
TAG50:1-FA18:1	-3.437817284	0.01746412	0.01854176
DAG(14:0_18:1)	-3.438911565	0.0009171	0.00854416
TAG49:2-FA14:0	-3.449638396	0.02202026	0.01986517
TAG54:7-FA18:1	-3.485354331	0.00210898	0.01013847
TAG52:3-FA20:3	-3.489399111	0.04558341	0.02716675
TAG54:7-FA18:2	-3.49381334	0.01833814	0.01854176
PG(16:0_22:5)	-3.503757209	0.0126588	0.01651651
TAG52:4-FA18:1	-3.513803935	0.00227826	0.01013847
TAG52:6-FA18:2	-3.517510451	0.00412143	0.01301803
TAG49:2-FA17:0	-3.591770278	0.04359098	0.02716675
PG(16:0_22:6)	-3.595163241	0.03645689	0.02503511
TAG46:3-FA18:2	-3.600356765	0.01927925	0.01876673
TAG49:0-FA16:0	-3.61558239	0.00916616	0.01534801
TAG46:2-FA18:1	-3.629139834	0.00602547	0.01379741
TAG47:1-FA14:0	-3.647278811	0.02225222	0.01986517
TAG56:7-FA20:5	-3.661945231	0.04729353	0.02776771
TAG56:6-FA22:5	-3.668896543	0.00782112	0.01461636
TAG58:10-FA22:5	-3.671787709	0.03741457	0.02527758

TAG47:0-FA16:0	-3.702137959	0.01866333	0.01854176
TAG47:0-FA14:0	-3.707317073	0.03301991	0.02355878
TAG56:6-FA20:5	-3.711737629	0.0451615	0.02716675
TAG56:6-FA22:4	-3.725908238	0.01791236	0.01854176
TAG49:1-FA16:0	-3.732037185	0.00799363	0.01461636
TAG50:4-FA18:1	-3.749569365	0.00894111	0.01531146
TAG52:5-FA18:1	-3.770734648	0.00122137	0.00934259
TAG49:3-FA16:0	-3.773180685	0.02255603	0.01986517
TAG52:8-FA16:1	-3.785714286	0.0025553	0.0108581
TAG50:2-FA18:1	-3.863721847	0.01170899	0.01600879
DAG(16:1_16:1)	-3.86655914	0.00648929	0.01391511
TAG46:1-FA18:1	-3.867668894	0.03205006	0.02352737
PG(18:0_20:5)	-3.868735084	0.00526611	0.01379741
TAG46:3-FA16:0	-3.9125	0.00591218	0.01379741
TAG50:2-FA16:1	-3.942779965	0.00199897	0.01013847
TAG45:0-FA14:0	-3.952027027	0.01024157	0.01600109
TAG50:1-FA16:1	-3.95229364	0.0153349	0.01757344
TAG56:7-FA22:5	-3.993817935	0.02956332	0.02243146
TAG49:2-FA16:0	-4.001835176	0.02536343	0.02084847
TAG46:2-FA18:2	-4.02373014	0.0494251	0.02847681
TAG56:8-FA22:6	-4.026838607	0.02462156	0.02061437
TAG50:3-FA16:1	-4.048850863	0.00237172	0.01026068

TAG52:6-FA16:0	-4.049330085	0.00602708	0.01379741
TAG45:0-FA16:0	-4.055536569	0.01107539	0.01600109
TAG54:4-FA22:4	-4.070441523	0.00555456	0.01379741
TAG52:4-FA22:4	-4.090236686	0.00140467	0.0098968
TAG54:7-FA20:4	-4.093697479	0.0390637	0.02589982
DAG(16:1_18:2)	-4.101070565	0.02496704	0.02063281
TAG49:0-FA17:0	-4.105301987	0.01165216	0.01600879
TAG47:1-FA16:0	-4.107979237	0.02388336	0.02037911
TAG50:3-FA16:0	-4.114894153	0.00330943	0.01218053
TAG50:5-FA18:1	-4.116550117	0.03274594	0.02355878
PG(16:0_14:0)	-4.153613851	0.00306111	0.01183095
TAG56:7-FA22:4	-4.158375635	0.00165776	0.01013847
TAG46:2-FA16:0	-4.231652488	0.02026497	0.01904738
DAG(14:0_16:1)	-4.255390513	0.03311076	0.02355878
TAG52:5-FA16:0	-4.287657278	0.00576454	0.01379741
TAG44:1-FA18:1	-4.294276837	0.02474424	0.02061437
PC(14:0_14:0)	-4.303155202	0.00663548	0.01393494
PG(16:0_22:4)	-4.30764671	0.04217491	0.02667466
TAG44:2-FA18:2	-4.364154685	0.00617967	0.01379741
TAG56:8-FA20:5	-4.375672591	0.03718715	0.02519296
TAG51:3-FA17:0	-4.39703154	0.00396732	0.01301803
TAG54:6-FA22:6	-4.42035054	0.00184987	0.01013847

DAG(16:1_18:1)	-4.459478912	0.00021822	0.0045439
TAG48:1-FA18:1	-4.459629427	0.03494118	0.02447837
TAG50:5-FA14:0	-4.464773922	0.003173	0.01185532
TAG48:5-FA18:3	-4.572847682	0.03548115	0.02471622
TAG48:2-FA18:2	-4.581412665	0.03928084	0.02589982
TAG48:1-FA14:0	-4.592368032	0.03315085	0.02355878
TAG54:5-FA22:5	-4.61952862	0.0009549	0.00854416
TAG49:1-FA17:0	-4.625407925	0.00201964	0.01013847
TAG47:2-FA16:1	-4.626069595	0.01148511	0.01600109
TAG52:6-FA20:4	-4.668803419	0.03914761	0.02589982
PE(14:0_16:1)	-4.705882353	0.00125024	0.00934259
TAG54:7-FA22:5	-4.717377049	0.01144714	0.01600109
TAG48:3-FA18:2	-4.72414025	0.02116189	0.01939946
TAG48:2-FA18:1	-4.741222131	0.01595474	0.01768314
TAG50:4-FA16:0	-4.743077362	0.001574	0.01003979
TAG54:5-FA22:4	-4.858128834	0.0098969	0.01584769
PG(16:0_20:5)	-4.864240506	0.00785235	0.01461636
TAG47:0-FA17:0	-4.892782427	0.00678041	0.01393494
TAG48:4-FA16:0	-4.937969925	0.00815788	0.01468399
TAG48:2-FA14:0	-5.010522416	0.03627807	0.02503511
TAG56:8-FA22:5	-5.01156961	0.03913743	0.02589982
TAG49:3-FA16:1	-5.033220339	0.02062405	0.01911965

TAG54:8-FA22:6	-5.117870722	0.00705601	0.01393494
TAG44:3-FA18:2	-5.129470262	0.01068586	0.01600109
TAG49:2-FA16:1	-5.145809341	0.01501235	0.01757344
TAG46:2-FA14:0	-5.188625576	0.04421566	0.02716675
TAG46:3-FA16:1	-5.263689745	0.01622823	0.01770722
TAG44:2-FA18:1	-5.308274843	0.00707489	0.01393494
TAG48:0-FA16:0	-5.327509627	0.02714345	0.02145349
TAG48:2-FA16:0	-5.346423347	0.02562079	0.02092055
TAG52:5-FA20:4	-5.359627469	0.02551025	0.0208995
TAG54:6-FA20:5	-5.394449117	0.02190933	0.01986314
TAG52:6-FA22:6	-5.395558901	0.00128911	0.00934975
TAG52:6-FA16:1	-5.406804374	0.0009501	0.00854416
TAG44:2-FA16:0	-5.430396549	0.00960082	0.01564623
TAG54:7-FA22:6	-5.506119062	0.00193774	0.01013847
TAG49:1-FA16:1	-5.53032335	0.00410007	0.01301803
TAG47:1-FA16:1	-5.545873321	0.01123758	0.01600109
TAG56:9-FA22:6	-5.551448276	0.02583456	0.02102548
TAG52:7-FA22:6	-5.565726084	0.00093318	0.00854416
TAG49:3-FA18:3	-5.736343367	0.04816546	0.02804631
TAG54:6-FA22:5	-5.842228055	0.0010221	0.00869127
TAG54:5-FA20:5	-5.846865365	0.02349805	0.02018284
TAG50:4-FA20:4	-5.855838539	0.03148114	0.02345363

TAG46:3-FA14:0	-5.89434638	0.0304025	0.02278773
TAG50:4-FA16:1	-5.936870316	0.00035695	0.00581134
TAG42:1-FA14:0	-6.150641026	0.01054884	0.01600109
TAG44:1-FA16:0	-6.160933986	0.01570401	0.01768314
TAG50:5-FA20:4	-6.239694656	0.01606274	0.01768314
PG(16:0_18:3)	-6.30675619	0.01085524	0.01600109
TAG48:3-FA14:0	-6.37220302	0.01826106	0.01854176
TAG48:1-FA16:0	-6.513460755	0.01418833	0.01724491
TAG48:3-FA16:0	-6.581516409	0.01975181	0.01888966
TAG47:1-FA17:0	-6.647717485	0.00640621	0.01385747
TAG50:5-FA16:1	-6.658653846	0.00054697	0.0074934
TAG52:5-FA22:5	-6.86541555	0.00022112	0.0045439
TAG46:1-FA14:0	-6.951323097	0.0327573	0.02355878
TAG46:3-FA18:3	-6.988031915	0.02415214	0.02047289
TAG42:2-FA18:2	-7.000234714	0.0149967	0.01757344
TAG48:4-FA14:0	-7.029458854	0.01317424	0.01692046
TAG54:7-FA20:5	-7.082015131	0.02966222	0.02243746
TAG52:5-FA20:5	-7.231508165	0.03282016	0.02355878
TAG44:2-FA16:1	-7.318868531	0.01847946	0.01854176
TAG46:1-FA16:0	-7.378448585	0.02706551	0.02145349
TAG48:3-FA16:1	-7.389536378	0.00857034	0.01505661
TAG48:4-FA16:1	-7.478683967	0.00293559	0.01167593

TAG48:4-FA18:3	-7.780284302	0.01937405	0.01876673
TAG42:1-FA18:1	-7.91972866	0.02640313	0.02113938
TAG48:3-FA18:3	-8.423799796	0.02031438	0.01904738
TAG48:1-FA16:1	-8.56681192	0.01767224	0.01854176
TAG54:8-FA20:5	-8.742474916	0.02061684	0.01911965
TAG42:1-FA16:0	-8.863929147	0.01172921	0.01600879
TAG46:1-FA16:1	-8.938771779	0.02089053	0.01929416
TAG48:2-FA16:1	-9.547740951	0.01223955	0.01624892
TAG42:1-FA16:1	-9.573070608	0.01878366	0.01854176
TAG52:7-FA20:5	-9.94655704	0.03403507	0.02397984
TAG46:2-FA16:1	-10.06031671	0.01437511	0.01724491
TAG40:0-FA16:0	-11.03798561	0.04564382	0.02716675
TAG52:6-FA20:5	-11.72357724	0.01261312	0.01651651

Supplemental Table 4. Analytes significantly increased in IAV infected compared to mock infected lungs.

ANALYTE NAME	FOLD CHANGE	P-VALUE	Q-VALUE
HCER(16:0)	2.531440781	0.00198989	0.01013847
PC(18:0_20:0)	2.18690721	0.02942305	0.02239393
PE(18:2_20:2)	1.986111111	0.00632249	0.01379741
LacCer(18:0_24:1)	1.9	0.00550079	0.01379741
CE[mFA20:2]	1.869896769	0.00037972	0.00581134
DAG[mFA22:4]	1.83294915	0.03790069	0.02546172
PE(18:2_22:4)	1.822311289	0.00690088	0.01393494
LPE[mFA22:4]	1.811870408	0.04184326	0.02667466
CE(20:2)	1.809309309	0.01734496	0.01851607
HCER(18:1)	1.76	0.01089544	0.01600109
CE(24:1)	1.751572327	0.03979809	0.02596319
PS(18:2_18:2)	1.732291825	0.00224187	0.01013847
LPE[mFA18:2]	1.718350274	0.02693408	0.02142536
PS(16:0_18:2)	1.632713249	0.02743654	0.02154703
PC(18:1_22:4)	1.622462611	0.00797821	0.01461636
CER(18:1)	1.600660066	0.00623626	0.01379741
PE(18:2_18:2)	1.586524823	0.02225146	0.01986517
PC(18:2_22:4)	1.557491857	0.01670198	0.0180066
PC(18:1_18:2)	1.518435068	0.00076625	0.00850537

PE(18:1_22:4)	1.501511031	0.0055415	0.01379741
PE(18:0_22:6)	1.493597595	0.02280204	0.01990819
PC(18:1_18:1)	1.493464871	0.00079329	0.00850537
PS[mFA18:2]	1.491307166	0.01121296	0.01600109
PE(18:2_22:5)	1.489940828	0.01787165	0.01854176
LPC[mFA22:4]	1.429709424	0.00157396	0.01003979
LPC[mFA22:6]	1.428146803	0.01429593	0.01724491
HCER(14:0)	1.416666667	0.02016893	0.01904738
CE[mFA18:1]	1.415205258	0.02883454	0.02208232
LPC[mFA18:2]	1.39550763	0.02099456	0.01931788
PG[mFA20:2]	1.388057812	0.02230651	0.01986517
DAG[mFA18:0]	1.367993598	0.03357737	0.02372514
LPE[mFA20:2]	1.352040743	0.0002905	0.00551048
PE(O-18:0_18:2)	1.328149002	0.03235061	0.02353263
PE(18:0_18:2)	1.325131162	0.04956201	0.02848913
PE(P-18:2_22:6)	1.272999252	0.04423816	0.02716675
PE[mFA22:6]	1.269903312	0.00532098	0.01379741
PG[mFA18:1]	1.258108906	0.0002138	0.0045439
PE(P-18:1_22:6)	1.225404853	0.036451	0.02503511
PG[mFA18:2]	1.170059484	0.04603807	0.02729049
PE[mFA18:0]	1.141526067	0.01422204	0.01724491
PE(18:0_18:3)	1.137931034	0.04419566	0.02716675

PC(18:1_20:4)	1.130101057	0.04068378	0.02626308

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CHAPTER FIVE

CONCLUSIONS AND FUTURE STUDIES

Conclusions

Secondary bacterial pneumonia by *S. aureus* following influenza A infection continues to be a global health threat. Despite advances in influenza vaccine efficacy and progress towards development of a universal influenza vaccine, seasonal influenza burdens have remained relatively constant throughout the previous decade[1,2]. Similarly, though progress has been made identifying the contributing factors that increase host susceptibly to secondary bacterial pneumonia, no findings have led to productive treatments capable of alleviating the increased morbidity and mortality associated with these infections[3–7]. Furthermore, the decreasing effectiveness of antibiotics due to the high prevalence of multi-drug resistant strains necessitates efforts to identify alternative treatment strategies. To that end, these studies provide a refined method and model to characterize the increased pathogenesis of *S. aureus* pneumonia following IAV infection and lend insights into clinically actionable therapeutic approaches.

Beginning in chapter 2, we describe a method of murine lower respiratory tract infection that enables precise delivery and recovery of pathogens from the lungs. Previous methods of infection were not optimized to analyze the contribution of bacterial virulence gene expression to disease and suffered from inconsistencies in pathogen doses between infections. The deficiencies of previous infection models may, in part, contribute to the heavy immuno-centric bias found within co-infection literature.

Using the methods described in chapter 2, we investigated the impact of an antecedent IAV infection on *S. aureus* pathogenesis during secondary bacterial pneumonia. In these studies mice were infected with IAV and challenged at six-days post-IAV infection with USA300 or an isogenic gene deletion mutant, USA300Δ*saeR/S*. Co-infection with USA300 but not USA300Δ*saeR/S* caused significant morbidity and mortality in infected mice, indicating a crucial role for *saeR/S* in *S. aureus* secondary pneumonia. Analysis of bacterial transcript abundance from infected mice demonstrated an increase in *saeR*-target genes in co-infected mice compared to mice infected with USA300 alone. Further study demonstrated SaeR/S regulated transcripts were rapidly up-regulated during co-infection with IAV, suggesting changes in the lung environment during the antecedent infection led to increased expression of *saeR/S*.

The findings in chapter 2 were extended in chapter 3 by examining the contribution of the lung environment on *S. aureus* pathogenesis. These studies revealed that the lipid components of pulmonary surfactant from healthy mice suppressed *S. aureus* virulence expression and secreted toxin production. Upon infection with IAV, the predominant lipid species composing pulmonary surfactant were significantly reduced.

Collectively, these data provide a novel model of secondary bacterial pneumonia wherein infection by IAV reduces pulmonary surfactant lipid concentrations resulting in a loss of toxin suppression and onset of *S. aureus* pneumonia through upregulation of the SaeR/S TCS (Figure 1). In addition, these data provide substantial rationale for the low incidence of *S. aureus* lung infections in immunocompetent hosts.

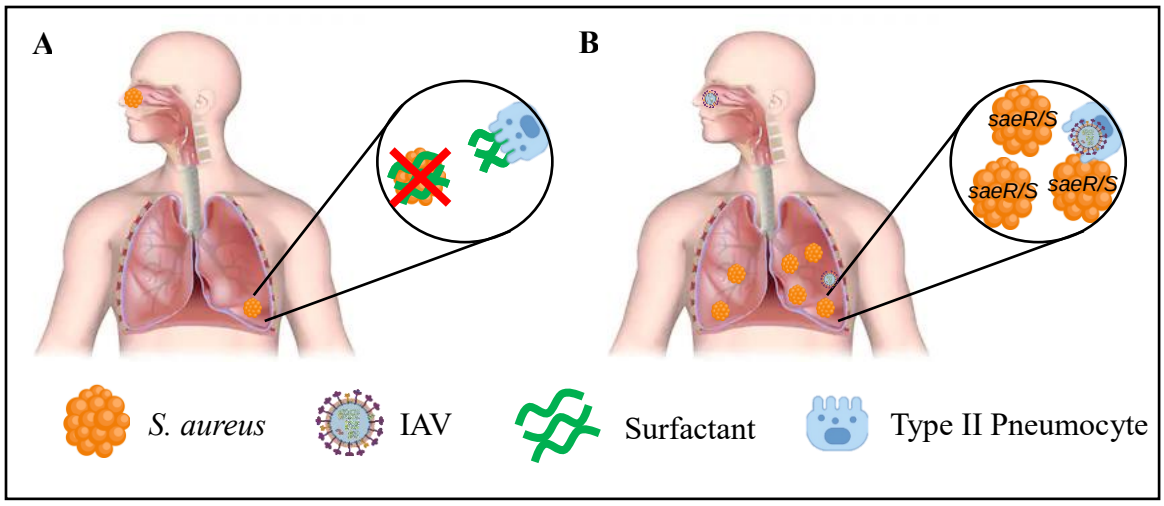


Figure 1. Model of increased susceptibility to secondary bacterial pneumonia by *S. aureus*. *S. aureus* frequently encounters the lung environment. A) In healthy hosts, pulmonary surfactant suppresses virulence production and *S. aureus* is cleared by innate immune defenses. B) Infection with IAV disrupts type II pneumocyte pulmonary surfactant production causing a loss of virulence suppression and upregulation of saeR/S.

Future Studies

The decreased pathogenesis of *S. aureus* in the presence of pulmonary surfactant represents an unexplored avenue for therapeutic intervention. Natural and Semi-synthetic pulmonary surfactants are currently in use to treat respiratory distress syndrome, a disorder resulting from insufficient surfactant production[8,9]. The effects of surfactant replacement therapy have not been tested within the context of an IAV infection or *S. aureus* pneumonia; however, the data herein suggest surfactant replacement therapy may be a viable treatment strategy to protect against severe disease outcomes.

In order to test if exogenous pulmonary surfactant can rescue the increased morbidity and mortality associated with secondary bacterial pneumonia, the following experiments are necessary:

1. The direct effects of exogenous pulmonary surfactant on *S. aureus* virulence gene expression will need to be confirmed. *S. aureus* will be grown in the presence of commercially available surfactant replacements and virulence expression will be determine by Quantigene 2.0 assays as performed in chapter 3.
2. The timing of surfactant disruption must be determined. Mice are reported to be most susceptible to bacterial challenge at days 6 - 7 post IAV infection, however pulmonary surfactant levels may be disrupted soon after IAV infection[10,11]. To determine the timing of surfactant disruption, we will infect mice with IAV and measure pulmonary surfactant concentrations on days 0 – 6 post infection. Due to the variability between mice, it will be critical to include sufficient replicates.
3. The timing for productive exogenous surfactant treatment will need to be determined. It is unknown if treatment with surfactant during IAV infection will alleviate the susceptibility to secondary bacterial infection or if treatment with surfactant during co-infection is sufficient to quell infection onset. To determine the most productive treatment strategy data from experiment 2 will be utilized to identify when surfactant replacement therapy can be administered (based on the timing of surfactant reduction). We anticipate at least 3 experimental groups: 1) Mice will be infected with IAV. Mice will be treated with surfactant on days 0 - 6 post infection. On day 6, mice will be challenged with *S. aureus* and susceptibility to secondary infection will be determined using a morbidity and mortality scale. 2) Mice will be infected with IAV and challenged with *S. aureus* on day 6-post IAV infection. Exogenous surfactant will be administered directly after *S. aureus*

challenge. Susceptibility to infection will be determined as mentioned above. 3) Mice will be infected with IAV. Mice will be treated with surfactant on days 0 - 6 post infection. On day 6, mice will be challenged with *S. aureus* followed by immediate treatment with surfactant. Susceptibility to infection will be determined as mentioned above.

If it is determined that pulmonary surfactant replacement therapy represents a novel and productive treatment strategy against secondary bacterial infections, we will investigate whether these findings can be extended to other common etiological agents of bacterial pneumonia. Specifically, our efforts will be directed towards the effects of pulmonary surfactant and its components on *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Haemophilus influenzae.*

We will carry on.

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APPENDICES

APPENDIX A

CO-AUTHORED PUBLICATIONS

frontiers in Cellular and Infection Microbiology

REVIEW
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Epic Immune Battles of History: Neutrophils vs. *Staphylococcus aureus*

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Neutrophils are the most abundant leukocytes in human blood and the first line of defense after bacteria have breached the epithelial barriers. After migration to a site of infection, neutrophils engage and expose invading microorganisms to antimicrobial peptides and proteins, as well as reactive oxygen species, as part of their bactericidal arsenal. Ideally, neutrophils ingest bacteria to prevent damage to surrounding cells and tissues, kill invading microorganisms with antimicrobial mechanisms, undergo programmed cell death to minimize inflammation, and are cleared away by macrophages. *Staphylococcus aureus* (*S. aureus*) is a prevalent Gram-positive bacterium that is a common commensal and causes a wide range of diseases from skin infections to endocarditis. Since its discovery, *S. aureus* has been a formidable neutrophil foe that has challenged the efficacy of this professional assassin. Indeed, proper clearance of *S. aureus* by neutrophils is essential to positive infection outcome, and *S. aureus* has developed mechanisms to evade neutrophil killing. Herein, we will review mechanisms used by *S. aureus* to modulate and evade neutrophil bactericidal mechanisms including priming, activation, chemotaxis, production of reactive oxygen species, and resolution of infection. We will also highlight how *S. aureus* uses sensory/regulatory systems to tailor production of virulence factors specifically to the triggering signal, e.g., neutrophils and defensins. To conclude, we will provide an overview of therapeutic approaches that may potentially enhance neutrophil antimicrobial functions.

Keywords: host-pathogen interactions, innate immunity, immune evasion, host defense, phagocytosis, chemotaxis, *Staphylococcus aureus*

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INTRODUCTION

Polymorphonuclear leukocytes (PMNs or neutrophils) are the first line of defense against bacterial pathogens that have breached epithelial barriers. Within minutes of bacterial invasion, neutrophils respond to soluble factors including chemokines and cytokines and are recruited to the site of infection where they ingest microbes. Subsequently, neutrophils expose microorganisms to antimicrobial proteins, peptides, and reactive oxygen species to kill the invading pathogen. This is a delicate process that must eliminate the pathogen while controlling excessive inflammation. Concurrently, neutrophils secrete cytokines and chemokines to continue to recruit neutrophils

and enhance other host responses to infection. Finally, neutrophil death is essential for proper resolution of infection and must be regulated to minimize bystander damage while continuing to signal if more immune response is needed or if tissue repair should begin. These potent mechanisms are effective at eliminating most fungal and bacterial microorganisms. However, successful pathogens have developed strategies to disrupt various neutrophil functions to cause infection.

S. aureus is a highly-adaptable Gram-positive pathogen estimated to colonize 50–60% of the population (Wertheim et al., 2005; Gorwitz et al., 2008). It is also a leading cause of infections ranging from superficial skin abscesses to life-threatening diseases, including septicemia and necrotizing pneumonia (Klevens et al., 2007; Kobayashi et al., 2015). The ability of *S. aureus* to cause human disease is based in part on its ability to evade the innate immune response, thereby circumventing rapid elimination. Many factors contribute to *S. aureus* pathogenesis. These include production of numerous toxins, such as the barrel forming two-component toxins capable of directly lysing host immune cells (Menestrina et al., 2003), and tissue destroying enzymes including protease, lipase, and hyaluronidase, as well as many surface proteins and adhesins linked to virulence (Lowy, 1998). In this review, we will focus on evasion strategies used by *S. aureus* to disrupt neutrophil functions essential for bacterial clearance. First, we will highlight virulence factors produced by *S. aureus* to alter neutrophil priming, activation, chemotaxis, and adhesion. Then, we will discuss strategies used by *S. aureus* to subvert neutrophil killing by antimicrobial peptides and proteins and reactive oxygen species. Additionally, we will examine recent literature investigating mechanisms used by *S. aureus* to modulate neutrophil cell death programs. Finally, we will highlight the reciprocal communication between *S. aureus* and the neutrophil emphasizing sensing and adaptive responses used by *S. aureus* to recognize and respond to neutrophil challenge. The review will conclude with an overview of potential therapeutic approaches aimed at disrupting bacterial sensing and signaling to decrease production of virulence factors during neutrophil interaction and discuss putative immunotherapies to boost immune responses to *S. aureus* while limiting inflammatory damage caused by neutrophils.

STRATEGIES USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL PRIMING, ACTIVATION, CHEMOTAXIS AND ADHESION

Neutrophils are initially recruited to a site of infection by following chemokine gradients in a process termed chemotaxis. Taking cues from activated endothelium, neutrophils slow their movement through blood vessels by selectin-mediated tethering to the endothelium followed by complete movement arrest through interaction with integrins on the endothelium. Extravasation from the blood vessels through the endothelial barrier is required for neutrophils to access interstitial fluid and migrate via a chemotactic gradient to the site of infection where ingestion of bacteria can take place. For detailed reviews of neutrophil chemotaxis, adhesion to the epithelium, and transmigration, please refer to (Kolaczkowska and Kubes, 2013; de Oliveira et al., 2016). Herein, we will focus on virulence factors produced by *S. aureus* to inhibit specific neutrophil receptors from binding host and bacterial derived ligands, which results in impaired neutrophil priming, activation, chemotaxis, and adhesion to the endothelium.

NEUTROPHIL PRIMING: A POTENTIAL TARGET OF *S. AUREUS*?

Priming refers to the ability of a primary agonist to enhance a neutrophil's response to a secondary stimulus (Swain et al., 2002). There are many known neutrophil priming agents including: complement components C3a and C5a (Skjeflo et al., 2014), interferon-γ (IFN-γ) (Edwards et al., 1988), interleukin-8 (IL-8) (Mitchell et al., 2003), and tumor-necrosis factor-α (TNF-α; Rainard et al., 2000). Bacterial derived products such as N-formyl methionyl peptide, formyl-methionyl-leucyl phenylalanine (fMLF), peptidoglycan, and *S. aureus* cytolytic toxins also demonstrate an ability to prime neutrophils (Elbim et al., 1994; El-Benna et al., 2008; Clarke et al., 2010; Malachowa et al., 2012). The ability of these agents to prime neutrophils is typically not universal in that concentration and neutrophil response can vary drastically (Swain et al., 2002). Primed neutrophil responses influence many neutrophil functions including increases in adhesion, phagocytosis, superoxide production, and degranulation (Ellis and Beaman, 2004). It can also influence neutrophil apoptosis (Wright et al., 2013). Thus, priming can set the stage for subsequent neutrophil-pathogen interactions and influences outcome of this interaction.

Not much is known about the impact of *S. aureus* on neutrophil priming. Earlier studies investigated priming of neutrophils with conditioned medium from peripheral blood mononuclear cells (PBMC) challenged with killed *S. aureus* (Ferrante et al., 1989; Bates et al., 1991). These studies demonstrated increased neutrophil staphylocidal activity following priming with conditioned media from PBMCs challenged with killed *S. aureus* vs. priming with medium from unstimulated PBMCs. This enhanced bactericidal activity was shown to be dependent on TNF-α produced by PBMCs in response to killed *S. aureus* (Ferrante et al., 1989, 1993; Bates et al., 1991). These studies suggest that the response of PBMCs and resident cells to *S. aureus* may strongly impact the outcome of neutrophil-*S. aureus* interactions. Supporting this idea are studies demonstrating that *S. aureus* promotes production of IFN-γ and this production has been linked to poorer outcome of infection in mouse models (Watkins et al., 2011, 2013). IFN-γ impacts neutrophil function in many ways, including priming of oxidative burst and degranulation mechanisms (Ellis and Beaman, 2004). Using a murine wound model of *S. aureus* infection, IFN-γ-mediated CXC chemokine production by T cells promoted a robust recruitment of neutrophils that resulted in elevated *S. aureus* burdens at the infectious foci (McLoughlin et al., 2008). Subsequent studies using a mouse peritonitis model

also demonstrated IFN-γ was associated with higher bacterial burdens but that the source of IFN-γ was neutrophils (Watkins et al., 2013). Potentially, priming by IFN-γ causes overactivation and inflammation in neutrophils making them less effective at clearing *S. aureus*. The role of IFN-γ and neutrophil bactericidal activity against *S. aureus* warrants further investigation especially considering that IFN-γ is used to treat patients with chronic granulomatous disease (Gallin et al., 1991). CGD patients receiving recombinant IFN-γ show a decrease in *S. aureus* infections (Gallin et al., 1991). Furthermore, *in vitro* studies have demonstrated that priming of human neutrophils with IFN-γ increased bactericidal activity against *S. aureus* (Edwards et al., 1988). Thus, more studies are needed to determine under what conditions IFN-γ promotes an effective neutrophil response against clinically relevant strains of *S. aureus*. Clearly, source, timing and amount of IFN-γ play an important role in outcome of *S. aureus*-neutrophil interactions.

S. aureus produces hemolysins, and bi-component leukocidins that directly impact immune cell function (Seilie and Bubeck Wardenburg, 2017). Many of these toxins act by forming pores in the membrane of immune cells causing lysis. Among these, the bi-component toxins leukotoxin GH (LukGH, also known as LukAB) and Panton-Valentine leukocidin (PVL) have the ability to cause neutrophil pore-formation when present at high concentrations. However, at sublytic concentrations, these factors can promote enhanced binding, uptake and killing of *S. aureus* through priming of neutrophils (Graves et al., 2012). This is also consistent with reports that have demonstrated the ability of PVL to enhance neutrophil superoxide, granule exocytosis, and release of leukotriene B_4 and IL-8 (König et al., 1995; Colin and Monteil, 2003). In addition, low doses of alpha-toxin, a single component toxin encoded by *hla*, has been shown to promote transcriptional activity leading to production of inflammatory mediators that can prime neutrophils including IL-8 (Dragneva et al., 2001).

As mentioned above, most of the studies thus far have not directly investigated how modulation of known neutrophil priming agents (such as cytokines and chemokines) by *S. aureus* actually influences neutrophil function. Also, the impact of *S. aureus* toxins and immunomodulatory proteins on neutrophil priming has not been thoroughly investigated. Taken together, targeting neutrophil priming may be very important for *S. aureus* to gain an edge on evading the neutrophil. However, additional studies are needed to understand the impact of *S. aureus* on neutrophil priming.

KEY FACTORS USED BY *S. AUREUS* TO DISRUPT NEUTROPHIL ACTIVATION, CHEMOTAXIS AND ADHESION

In contrast to our understanding of how *S. aureus* may impact priming, there is an abundance of data that highlight how specific *S. aureus* factors impact neutrophil activation, chemotaxis and adhesion. In this section, we highlight individual virulence factors that have been studied for their ability to disrupt these key mechanisms (**Figure 1**).

CHIPS

The chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14.1-kDa exoprotein that inhibits neutrophil migration and activation (De Haas et al., 2004). CHIPS prevents neutrophils from responding to both host- and bacteria-derived chemoattractants. Neutrophil activation by host-derived C5a is inhibited by CHIPS binding to the C5a receptor (C5aR). Similarly, neutrophil detection of bacteria-derived formylated peptides is inhibited by CHIPS binding to the formyl peptide receptor (FPR). CHIPS shows high affinity to both C5aR and FPR with K_D-values of 1.1 ± 0.2 and 35.4 ± 7.7 nM, respectively; the affinity of C5aR and FPR for their natural ligands is within the CHIPS binding affinity to C5aR and FPR (Falk et al., 1982; Huey and Hugli, 1985; Postma et al., 2004). While C5aR and FPR are G-protein coupled receptors (GPCRs), the active blocking domain in CHIPS is distinct for inhibiting C5aR and FPR (Haas et al., 2005). By using FITC-labeled CHIPS, Postma et al. demonstrated that CHIPS is not internalized following binding to neutrophil C5aR and FPR and its activity is ATP and cell-signaling independent (Postma et al., 2004). It has also been demonstrated that CHIPS binds to C5aR and FPR extracellularly and does not act as an agonist since receptor binding does not induce calcium mobilization (De Haas et al., 2004). It was further demonstrated that intravenously administered CHIPS was able to inhibit mouse neutrophil influx following intraperitoneal administration of C5a despite a 30-fold lower affinity of CHIPS for mouse C5aR (De Haas et al., 2004). Finally, neutralizing antibodies to CHIPS have been observed in human sera isolated from both normal donor controls and *S. aureus* infected samples, implying CHIPS plays an active role during staphylococcal infection (Wright et al., 2007).

FLIPR AND FLIPR-LIKE

Neutrophils express formyl peptide receptor-like-1 (FPRL1). This receptor is activated by diverse peptides and proteins including: the synthetic peptides Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm), and L-conformer WKYMVM, lipoxin A_4, the T21/DP107 leucine zipper-like domain of the HIV-1 envelope protein gp41, serum amyloid A, the mitochondrial peptide fragment MYFINILTL, the antimicrobial peptide LL-37, and prion peptide fragments (Fiore et al., 1994; Su et al., 1999a,b; Chiang et al., 2000; De Yang et al., 2000; Christophe et al., 2001; Le et al., 2001). Activation of FPRL1 on neutrophils leads to intracellular calcium mobilization and chemotaxis, while superoxide production through FPRL1 is ligand dependent (Bae et al., 2003). *S. aureus* produces two proteins that bind FPRL1 and inhibit its activation. FPRL1 inhibitory protein (FLIPr) is a secreted protein that binds and antagonizes both FPRL1 and FPR, but FPRL1 antagonism by FLIPr is much stronger than on FPR (Prat et al., 2006). Similarly, a second FPRL1 antagonist termed FLIPr-like shares 73% overall homology with FLIPr (Prat et al., 2009). FLIPr and FLIPr-like show similar inhibitory activity toward FPRL1 following stimulation with the FPRL1 agonist MMK-1; however, FLIPr-like antagonism toward FPR is 100-fold stronger than that of FLIPr following stimulation with fMLF (Prat et al., 2009). As expected, neutrophil exposure to purified

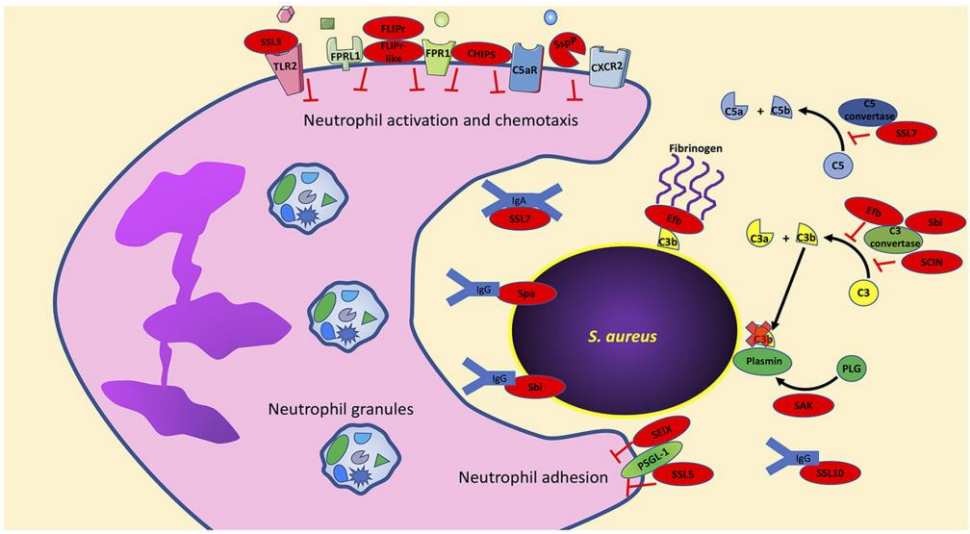


FIGURE 1 | *S. aureus* has an arsenal of virulence factors to inhibit neutrophil activation, chemotaxis, and phagocytosis. Multiple virulence factors produced by *S. aureus* target key host effector proteins, for example, Efb, Sbi, and SCIN target the complement protein C3 convertase to prevent formation of C3a and C3b. Further studies are needed to determine if the production of these virulence factors are truly redundant or if they have multiple host targets as is the case with Sbi, which also targets immunoglobulins. Bacterial components indicated in red. PLG, plasminogen; SAK, staphylokinase; SspP, staphopain A.

FLIPr and FLIPr-like completely inhibits neutrophil chemotaxis toward the FPRL1 agonist MMK-1 (Prat et al., 2006, 2009).

FLIPr and FLIPr-like have also been shown to bind different FcγR isoforms to block IgG binding (Stemerding et al., 2013). Stemerding et al. showed that FLIPr preferentially binds to FcγR class II receptors while FLIPr-like can bind to FcγR cla ss I, II, and III receptors. As expected, neutrophil pretreatment with FLIPr or FLIPr-like significantly reduced phagocytosis of *S. aureus* opsonized with purified IgG (Stemerding et al., 2013). However, it should be noted that phagocytic inhibition of neutrophils toward *S. aureus* opsonized with human serum by FLIPr and FLIPr-like was only observed at low serum concentrations (<1% serum). At higher serum concentrations (1–10% serum), FLIPr and FLIPr-like do not inhibit neutrophil-mediated phagocytosis of opsonized *S. aureus*. Under these higher serum conditions, neutrophil phagocytosis of opsonized *S. aureus* is likely mediated by complement receptors, which are not antagonized by FLIPr and FLIPr-like proteins. It follows that experiments using neutrophils treated with FLIPr and FLIPr-like had reduced uptake of *S. aureus* only when opsonized with complement inactivated serum thus showing the specific inhibition of FLIPr and FLIPr-like toward serum immunoglobulins (Stemerding et al., 2013).

STAPHOPAIN A

The CXCR2 chemokine receptor (also known as IL8RB) is highly expressed on human neutrophils and has high specificity for the potent neutrophil chemoattractants CXCL1, CXCL2, and CXCL7, as well as CXCL8 (IL-8, which can also bind to CXCR1) (Yoshimura et al., 1987; Baggiolini et al., 1989; Ben-Baruch et al., 1997; Ritzman et al., 2010). *S. aureus* infection induces CXCL1, CXCL2, CXCL8 production resulting in neutrophil recruitment (Mempel et al., 2003; Sasaki et al., 2003; Olaru and Jensen, 2010). To prevent neutrophil recruitment induced by CXCR2 ligands, *S. aureus* secretes the cysteine protease staphopain A which cleaves the N-terminus of CXCR2 required for ligand binding (Laarman et al., 2012). Neutrophils pretreated with staphopain A blocked calcium mobilization following stimulation with CXCL1 and CXCL7. Importantly, these chemokines only bind CXCR2 (and not CXCR1). In contrast, staphopain A did not inhibit neutrophil calcium mobilization following stimulation with CXCL8 since it can also signal through CXCR1. The impacts of staphopain A *in vivo* are lacking since staphopain A is specific for human CXCR2 and does not cleave murine CXCR2 (Laarman et al., 2012).

SEIX AND SSL5

Neutrophils express P-selectin glycoprotein ligand 1 (PSGL-1) on the cellular surface to bind P- and E-selectin on activated endothelial cells or platelets and L-selectin expressed on leukocytes (Moore et al., 1995; Guyer et al., 1996; Hidalgo et al., 2007; Huo and Xia, 2009; Stadtmann et al., 2013). PSGL-1 binding to selectins tethers neutrophils to activated endothelial cells and is a first step in the process of transmigration. *S. aureus* secretes two proteins that inhibit PSGL-1 binding to

selectins. The staphylococcal enterotoxin-like toxin X (SElX) is a highly conserved superantigen that binds glycosylated PSGL-1 on neutrophils and inhibits binding to P-selectin (Wilson et al., 2011; Fevre et al., 2014). Similarly, the staphylococcal superantigen-like 5 (SSL5) protein directly binds PSGL-1 on neutrophils inhibiting rolling on endothelial cells (Bestebroer et al., 2007). In addition to binding PSGL-1, SSL5 also binds to other GPCRs but only inhibits the ligands that require the N-terminal domain of their respective receptors for activation (Bestebroer et al., 2009). Thus, SSL5 pretreatment of neutrophils inhibited activation induced by C3a, C5a, CXCL1, and CXCL8 (Bestebroer et al., 2009).

SSL3

Toll-like receptor 2 (TLR2) recognizes staphylococcal peptidoglycan-associated lipoproteins (Fournier and Philpott, 2005; Kurokawa et al., 2009). TLR2 can also discriminate between diacylated and triacylated lipoproteins by associating with TLR6 or TLR1, respectively (Takeuchi et al., 2001, 2002). The recognition of staphylococcal lipoproteins such as the staphylococcal iron transporter C (SitC) are exclusively dependent on TLR2 to induce immune activation (Stoll et al., 2005; Kurokawa et al., 2009; Müller et al., 2010). The importance of TLR2 during *S. aureus* infection is highlighted by $TLR2^{-/-}$ mice that show increased susceptibility and nasal colonization to *S. aureus* compared to wild-type mice (Takeuchi et al., 2000; González-Zorn et al., 2005; Hoebe et al., 2005). In addition, $TLR2^{-/-}$ mice infected with *S. aureus* show alterations in cytokines that modulate neutrophil function including TNF-α and IL-1β (Knuefermann et al., 2004). Engagement of TLR2 influences many neutrophil functions including: adhesion molecule expression, reactive oxygen species production (following stimulation with fMLF), and modulates CXCL8, and chemokine receptor expression (Sabroe et al., 2003). *S. aureus* produces the staphylococcal superantigen-like 3 protein that binds TLR2 to inhibit activation in neutrophils and other cell types expressing TLR2 (Bardoel et al., 2012; Yokoyama et al., 2012). Crystal structures showed that SSL3 binding to TLR2 reduced the lipopeptide binding pocket by ∼50%, which inhibited binding of the TLR2 agonist Pam_2CSK_4 (Koymans et al., 2015). The same study demonstrated SSL3 can bind a preformed TLR2- Pam_2CSK_4 complex and this blocked TLR2-TLR1 and TLR2-TLR6 heterodimerization thereby inhibiting downstream signaling.

STRATEGIES USED BY *S. AUREUS* TO INHIBIT NEUTROPHIL PHAGOCYTOSIS

Phagocytosis is a process by which neutrophils, and other phagocytes, ingest particles from their extracellular environment including bacteria and host cells. In neutrophils, this sequestration results in the formation of an intracellular compartment termed the phagosome following invagination of the cellular membrane. Neutrophil granules fuse with the phagosome to release antimicrobial peptides and proteins, as well as produce reactive oxygen species to kill invading microorganisms. Neutrophils bind to pathogen-associated molecular patterns on the *S. aureus* surface to initiate the phagocytic process. Furthermore, binding of immunoglobulins to *S. aureus* and complement activation enhances phagocytosis, as well as engages different neutrophil receptors resulting in branching downstream signaling. *S. aureus* employs strategies and produces a wide range of virulence factors to disrupt neutrophil phagocytosis. Herein, we will highlight select strategies used by *S. aureus* to disrupt neutrophil phagocytosis. Since a complete review on the strategies used by *S. aureus* to avoid phagocytosis is outside the scope of this publication, we recommend previous detailed reviews focusing on immunoglobulin and complement evasion by *S. aureus* to inhibit phagocytosis (Lambris et al., 2008; Serruto et al., 2010; van Kesse et al., 2014).

CAPSULE SYNTHESIS

The production of capsular polysaccharide by *S. aureus* has been proposed as an antiphagocytic evasion strategy but its actual role in inhibiting neutrophil phagocytosis remains controversial. Indeed, previous studies have shown that *S. aureus* strains producing high levels of capsular polysaccharide withstand neutrophil killing better than microencapsulated strains (Xu et al., 1992; Thakker et al., 1998). However, it should be noted that under these experimental conditions neutrophil killing of *S. aureus* was correlated to the ability of neutrophils to associate with bacteria without clearly showing ingestion. Furthermore, the experimental conditions used suspended instead of adherent neutrophils which can greatly influence neutrophil ingestion of *S. aureus* (Lu et al., 2014). Also, an intraperitoneal *S. aureus* infection model showed no differences in neutrophil intracellular staphylococcal survival between a highly and microencapsulated strain (Gresham et al., 2000). Notably, the predominant clinical isolate USA300 is unencapsulated, further questioning the role of capsule synthesis in *S. aureus* pathogenicity (Montgomery et al., 2008; Carrel et al., 2015).

COMPLEMENT INHIBITION

Human pathogens have developed strategies to evade complement, thus inhibiting immune recognition, cytokine production, and neutrophil uptake (Lambris et al., 2008; Serruto et al., 2010). *S. aureus* produces several virulence factors that target different machinery in the complement system. The staphylococcal complement inhibitor (SCIN), is a 9.8-kDa exoprotein that specifically binds and inhibits human C3 convertases (Rooijakkers et al., 2005a, 2006). SCIN binding to C3 convertases does not prevent C3 binding but completely inhibits generation of C3b and thus opsonization of *S. aureus* by C3b (Rooijakkers et al., 2005a; Ricklin et al., 2009; Garcia et al., 2010). In addition, SCIN stabilizes C3 convertases by competing with factor H which accelerates decay of C3 convertases (Ricklin et al., 2009). *In vitro*, recombinant SCIN can significantly inhibit neutrophil phagocytosis of human serum opsonized *S. aureus* resulting in increased bacterial survival (Rooijakkers et al.,

2005a). *In vivo* studies with SCIN are lacking since it is human specific, but SCIN is immunogenic since a limited study with 80 healthy people and 20 individuals with recurring staphylococcal infections showed all produced antibodies to SCIN (Rooijakkers et al., 2005a).

S. aureus is also equipped with virulence factors that target complement without direct binding to C3 convertase. Staphylokinase is a secreted protein that binds human plasminogen converting it into its active form plasmin (Parry et al., 2000; Mölkänen et al., 2002; Rooijakkers et al., 2005b). Plasmin, a serine protease, is bound to the *S. aureus* surface and degrades C3 convertase-dependent C3b to prevent deposition on the bacterial surface. Human neutrophils show decreased phagocytic activity toward human serum opsonized *S. aureus* (depleted of IgG and IgM to rule out immunoglobulin mediated phagocytosis) and pretreated with recombinant staphylokinase (Rooijakkers et al., 2005b). In addition, *S. aureus* secretes the extracellular fibrinogen-binding (Efb) protein that binds to C3 to prevent cleavage to C3b and can also directly bind C3b deposited on the extracellular bacterial membrane (Lee et al., 2004; Ko et al., 2013). As its name implies, the extracellular fibrinogen-binding protein also contains a fibrinogen binding domain that recruits fibrinogen and inhibits neutrophil phagocytosis (Ko et al., 2013). Complement receptor recognition of C3b is blocked by a thick layer of fibrinogen linked by Efb on the bacterial surface (Ko et al., 2013). *Ex vivo* studies with human blood exposed to Efb and *S. aureus* showed a significant reduction in neutrophil phagocytosis compared to *S. aureus* alone. Similarly, supernatants from wild-type *S. aureus* reduced neutrophil phagocytosis of opsonized *S. aureus* compared to supernatants from Efb-deficient *S. aureus* in the presence of fibrinogen. Finally, neutrophils from mice intraperitoneally infected with GFP-expressing wild-type *S. aureus* or an Efb-deficient mutant showed significantly higher ingestion of the Efb mutant compared to the wild-type (Ko et al., 2013). While this observation suggests that sufficient levels of Efb are produced by *S. aureus* to have an *in vivo* effect, it should be noted that intraperitoneal injection was performed with *S. aureus* grown to late exponential phase without washing culture toxins. Thus, increased and unknown levels of Efb were likely present from the beginning giving an advantage to *S. aureus* to prevent neutrophil phagocytosis.

INHIBITION OF IMMUNOGLOBULIN RECOGNITION

Complement and immunoglobulins function as opsonins to enhance *S. aureus* recognition and ingestion by neutrophils. While *S. aureus* produces virulence factors that inhibit complement activation and deposition on the bacterial surface, the efficacy of these virulence factors *in vivo* is controversial since immunoglobulin opsonization plays a redundant role in opsonophagocytosis. In fact, opsonophagocytosis assays investigating the role of *S. aureus* virulence factors that disrupt complement must be done in the absence of immunoglobulins to observe an inhibitory effect on phagocytosis because *S. aureus* produces virulence factors that inhibit opsonization by immunoglobulins. Protein A, encoded by the *spa* gene, is one of the better characterized virulence factors produced by *S. aureus* that inhibits neutrophil phagocytosis. Protein A is a 42-kDa secreted and membrane bound protein that binds the constant Fcγ region of IgG thus preventing proper engagement of Fcγ receptors on neutrophils and antigen recognition (Forsgren and Sjöquist, 1966; Kronvall et al., 1970; Sjödahl, 1977). Indeed, early studies clearly showed that purified protein A inhibited neutrophil phagocytosis of *S. aureus* independent of capsule polysaccharide on the bacterial surface (Dossett et al., 1969; King and Wilkinson, 1981). More recent studies have demonstrated protein A induces B cell proliferation and production of V_H3 serum IgG and IgM (Pauli et al., 2014; Kim et al., 2016). Although abundant, these antibodies do not provide protection against *S. aureus*. Generation of protein A variants that were unable to bind immunoglobulin induce a protective antibody response presumably via allowing Fc mediated uptake of opsonized *S. aureus* (Falugi et al., 2013). These data suggest that mechanisms of neutrophil recognition and uptake of *S. aureus* are likely critical to resolution of infection.

S. aureus produces a second binding protein of immunoglobulin (termed Sbi) that also binds IgG to inhibit neutrophil phagocytosis (Jacobsson and Frykberg, 1995; Zhang et al., 1998, 1999; Smith et al., 2011). Human neutrophils can phagocytose significantly more opsonized Sbi-deficient *S. aureus* compared to wild-type, and Sbi plays a protective role in *ex vivo* whole human blood killing assays (Smith et al., 2011). Interestingly, Sbi binds to lipoteichoic acid in the bacterial cell surface to remain anchored, while it can also exist in a secreted form that binds complement protein C3 to induce its degradation away from the bacterial surface (Burman et al., 2008; Upadhyay et al., 2008). Sbi also plays another immunomodulatory role that was demonstrated to impact neutrophil function. In a mouse model of peritonitis Sbi induced production of IL-6 and CXCL-1, which resulted in neutrophil recruitment and subsequently an exacerbated inflammatory response (Gonzalez et al., 2015).

In addition to Sbi, *S. aureus* secretes other proteins that bind both immunoglobulins and complement proteins to disrupt neutrophil phagocytosis. The 23-kDa staphylococcal superantigen-like 7 (SSL7) exoprotein binds to human IgA with high affinity (K_D of 1.1 nM) and inhibits FcαR binding on the neutrophil surface (Langley et al., 2005). Using an *ex vivo* whole blood infection model it was demonstrated that binding of IgA by SSL7 reduced neutrophil phagocytosis of *S. aureus* (Bestebroer et al., 2010). SSL7 also binds to the C5 complement protein (K_D of 18 nM) and inhibits C5a generation in an IgA binding-dependent manner, as well as formation of the C5b-9 membrane attack complex (Langley et al., 2005; Bestebroer et al., 2010; Laursen et al., 2010; Lorenz et al., 2013). *In vivo* mouse studies showed that administration of purified SSL7 inhibited neutrophil recruitment following exposure to heat-killed *S. aureus*. Thus, SSL7 serves diverse functions that include inhibiting neutrophil phagocytosis, recruitment, and complement activation (Bestebroer et al., 2010; Lorenz et al., 2013). Another staphylococcal superantigen-like protein termed SSL10 also binds to IgG (K_D of 220 nM) and to the complement protein C1q (Itoh et al., 2010). SSL10 significantly

inhibited neutrophil ingestion of IgG-opsonized *S. aureus* by blocking binding of FcγR (Patel et al., 2010).

Taken together, it is clear that *S. aureus* produces virulence factors that decrease neutrophil phagocytosis via production of physical barriers (capsule polysaccharide), inhibition of complement activation leading to decreased deposition of opsonins, and disruption of immunoglobulin binding to bacterial antigens. However, the efficacy of these virulence factors has been difficult to determine. Neutrophil-mediated phagocytosis of *S. aureus* is efficient in the absence of opsonins. *In vitro* studies have shown that adherent neutrophils rapidly ingest both opsonized and unopsonized *S. aureus* (Lu et al., 2014). Thus, neutrophils contain pattern-recognition receptors that are sufficient for phagocytosis of *S. aureus* independent of opsonins. Clearly, the redundancy in neutrophil receptor-mediated phagocytosis mechanisms compounds studies and strongly suggests the combination of *S. aureus* virulence factors are needed to thwart the innate ability of neutrophils to recognize this pathogen.

EVASION STRATEGIES USED BY *S. AUREUS* TO SURVIVE NEUTROPHIL KILLING

S. aureus is clearly decorated to avoid ingestion by neutrophils. Additionally, *S. aureus* is loaded with mechanisms to disarm potent neutrophil bactericidal mechanisms. Soon after initiation of the phagocytic process, and possibly before complete sealing of the phagosome, neutrophil granules mobilize to fuse and release antimicrobial agents into the phagosome containing *S. aureus* (Flannagan et al., 2009). Following phagocytosis, neutrophils expose *S. aureus* to antimicrobial peptides, proteins, and reactive oxygen species to degrade essential bacterial proteins and disrupt homeostasis resulting in bacterial death (**Figure 2**). In this section, we will highlight strategies used by *S. aureus* to avoid oxidative and non-oxidative mechanisms of neutrophil killing. Although we separate *S. aureus* evasion of oxidative and non-oxidative killing, it is important to remember that these mechanisms likely have synergistic antimicrobial activity in a physiological setting.

S. AUREUS EVASION OF NON-OXIDATIVE KILLING

The *dlt* operon in *S. aureus*, containing genes *dltABCD* is involved in activation and transfer of D-alanine, and increases tolerance to cationic antimicrobial peptides (AMPs), including human neutrophil peptide-1 (HNP-1, also known as alpha-defensin), by incorporation of positively charged D-alanine into teichoic acids (Peschel et al., 1999; Collins et al., 2002). Teichoic acids are highly negatively charged by deprotonized phosphate groups that electrostatically interact with cationic peptides. Thus, a *S. aureus dlt* mutant strain retains a negatively charged teichoic acid backbone and is significantly more susceptible to killing by cationic peptides (Peschel et al., 1999). *In vitro*, *S. aureus*

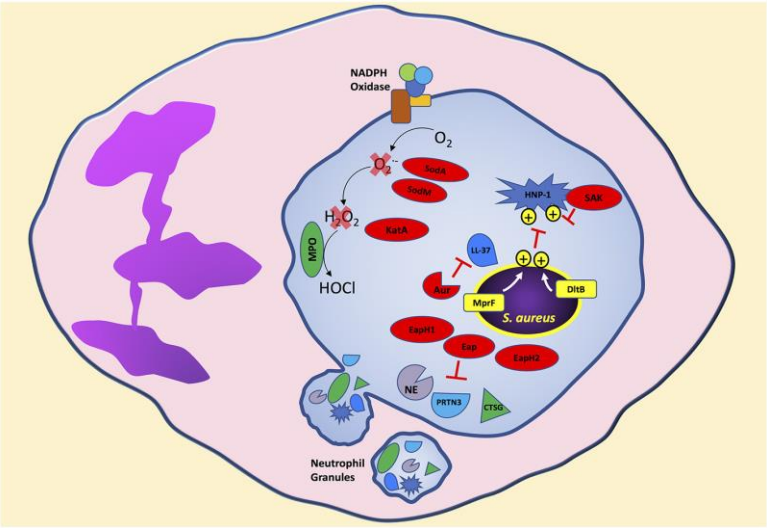


FIGURE 2 | *S. aureus* produces virulence factors that target different neutrophil bactericidal mechanisms following phagocytosis. Cationic antimicrobial peptides are ineffective toward *S. aureus* due to the presence of positive charges on the bacterial surface transferred by MprF and DltB. *S. aureus* secretes virulence factors that degrade antimicrobial proteins and enzymes released into the neutrophil phagosome. In addition, neutrophil reactive oxygen species production is decreased by *S. aureus* virulence factors that degrade intermediate reactive oxygen species like superoxide and hydrogen peroxide to reduce the formation of the highly bactericidal chemical agent hypochlorous acid. Bacterial components indicated in red. Aur, aureolysin; PRTN3, proteinase 3; CTSG, cathepsin G; SAK, staphylokinase.

dlt mutants are highly susceptible to neutrophil non-oxidative killing mechanisms whereas wild-type *S. aureus* is resistant and mainly susceptible to neutrophil oxidative killing (Collins et al., 2002). The importance of the *dlt* operon to increase *S. aureus* survival was observed in an *in vivo* mouse model of sepsis, which showed increased host mortality infected with wild-type *S. aureus* compared to the non-lethal *S. aureus dlt* mutant (Collins et al., 2002). Similarly, the *mprF* gene in *S. aureus* encoding lysylphosphatidylglycerol (LPG) synthetase confers resistance to cationic AMPs by transferring the positively charged L-lysine to the negatively charged lipid phosphatidylglycerol creating LPG (Peschel et al., 2001; Oku et al., 2004; Ernst et al., 2009). Electrostatic repulsion between cationic AMPs and the attached lysine prevents binding of cationic AMPs and disruption of the bacterial membrane. Wild-type *S. aureus* is more resistant to killing by human neutrophils compared to a *S. aureus mprF* mutant (Peschel et al., 2001). Thus, *S. aureus* modifies its cell surface as an evasion strategy by increasing electrostatic repulsion of neutrophil antimicrobial peptides leading to decreased bacterial killing.

In addition to cell membrane modifications to decrease efficacy of antimicrobial peptides by electrostatic repulsion, *S. aureus* produces proteins that directly bind, inhibit, and degrade antimicrobial peptides. Staphylokinase, which activates plasminogen to the active serine protease plasmin leading to degradation of IgG and C3b (Rooijakkers et al., 2005b), also binds HNP-1 and inhibits its bactericidal activity (Jin et al., 2004). The cathelicidin LL-37, which has potent staphylococcal bactericidal activity, is degraded by the *S. aureus* secreted metalloprotenaise aureolysin (Sieprawska-Lupa et al., 2004). Thus, *S. aureus* strains producing aureolysin are significantly more resistant to LL-37 than aureolysin-negative strains. Furthermore, the extracellular adherence protein (Eap) and the Eap-homologs 1 and 2 (EapH1 and EapH2) are neutrophil serine protease inhibitors (Harraghy et al., 2005). Purified Eap, EapH1, or EapH2 inhibit the activity of neutrophil elastase, proteinase 3, and cathepsin G which are found in neutrophil azurophilic granules and are bactericidal to *S. aureus* (Papayannopoulos et al., 2010; Stapels et al., 2014).

S. AUREUS EVASION OF OXIDATIVE KILLING

Neutrophil activation leads to assembly of the NADPH oxidase resulting in production of reactive oxygen species (ROS; DeLeo and Quinn, 1996; DeLeo et al., 1999; Bréchard and Tschirhart, 2008; Nunes et al., 2013). Assembly of the membrane and cytoplasmic NADPH oxidase components results in electrons shuttled from NADPH to oxygen producing superoxide. Spontaneous and myeloperoxidase (MPO) catalyzed dismutation of superoxide produces hydrogen peroxide, which in the presence of MPO and chloride results in the highly bactericidal agent hypochlorous acid/hypochlorite anion (Kettle et al., 2007; Klebanoff et al., 2013). Neutrophil ingestion exposes *S. aureus* to concentrated ROS in the phagosome resulting in oxidation and chlorination of bacterial and host proteins (Green et al., 2014). Inhibition of neutrophil ROS production by diphenyleneiodonium significantly increases *S. aureus* survival following neutrophil phagocytosis even with functioning non-oxidative killing mechanisms (Hampton and Winterbourn, 1995; Hampton et al., 1996). Individuals with chronic granulomatous disease carry mutations in components of NADPH oxidase preventing formation of superoxide and hydrogen peroxide and suffer from recurring bacterial infections including *S. aureus* (Lekstrom-Himes and Gallin, 2000; Assari, 2006). Thus, uninhibited neutrophil ROS production is essential for *S. aureus* clearance.

Bacterial pathogens have developed strategies to inhibit ROS killing (Flannagan et al., 2009). *S. aureus* produces several enzymes in response to neutrophils to degrade and detoxify ROS. The ROS chain of production begins with the formation of superoxide from oxygen. *S. aureus* produces two superoxide dismutases, encoded by *sodA* and *sodM*, that convert superoxide into hydrogen peroxide and molecular oxygen (Karavolos et al., 2003). Superoxide dismutase activity assessed on nondenaturing polyacrylamide gel electrophoresis from the *S. aureus* cytoplasm shows three distinct bands consisting of SodA and SodM homodimers, and a SodA-SodM hybrid (Clements et al., 1999; Valderas and Hart, 2001). Wild-type *S. aureus* is more resistant to superoxide stress compared to isogenic *sodA,* or *sodM,* or *sodAsodM*-deficient strains (Karavolos et al., 2003). In a mouse subcutaneous model of infection, significantly higher wild-type *S. aureus* colony forming units were recovered from the site of infection compared to single or double *sod* mutant strains suggesting a role for superoxide dismutase in virulence (Karavolos et al., 2003). The expression of *sodA* and *sodM* is increased under oxidative stress and regulated by the transcriptional regulator SarA (Ballal and Manna, 2009). Interestingly, *S. aureus* uses multiple regulatory systems to respond to oxidative stress. KatA, the only catalase encoded by *S. aureus* that degrades hydrogen peroxide to water and oxygen, is co-regulated by the ferric uptake repressor (Fur) and the peroxide response regulator (Horsburgh et al., 2001b). As expected, *katA* deficient *S. aureus* is more sensitive to killing by hydrogen peroxide compared to wild-type *S. aureus* (Horsburgh et al., 2001a). Furthermore, the alkyl hydroperoxide reductase (AhpC) increases *S. aureus* resistance to the organic-hydroperoxide cumene hydroperoxide and its absence increases *S. aureus* resistance to hydrogen peroxide by a compensatory increase in *katA* expression (Cosgrove et al., 2007). However, the significance of KatA in surviving neutrophil ROS production is unclear since studies have shown no significant differences in neutrophil killing of wild-type *S. aureus* compared to an isogenic *katA* deficient strain (Cosgrove et al., 2007).

Superoxide dismutase and catalase protect *S. aureus* from ROS through enzymatic degradation. *S. aureus* also produces virulence factors that inhibit ROS killing by different mechanisms. The iconic yellow-golden pigment of *S. aureus* is a result of the antioxidant carotenoid named staphyloxanthin and synthesized by genes encoding *crtM* and *crtN* (Wieland et al., 1994; Pelz et al., 2005). The production of staphyloxanthin increases *S. aureus* resistance to killing by hydrogen peroxide and singlet oxygen (Liu et al., 2005). In

addition, staphyloxanthin increases *S. aureus* survival during exposure to neutrophil ROS; in contrast, staphyloxanthin does not confer protection during exposure to neutrophils from patients with chronic granulomatous disease or neutrophils pretreated with diphenyleneiodonium which have deficiencies in ROS production (Liu et al., 2005). Following exposure to neutrophil ROS, surviving bacteria undergo repair of oxidized proteins to maintain homeostasis. *S. aureus* encodes four methionine sulfoxide reductases that play a role in reducing oxidized methionine residues (Singh, 2003; Schöneich, 2005; Singh et al., 2015). Deletion of the methionine sulfoxide reductases *msrA1* and *msrB* increases *S. aureus* susceptibility to exogenous hydrogen peroxide and hypochlorous acid. Furthermore, a *msrA1* and *msrB* double mutant strain of *S. aureus* is more susceptible to neutrophil killing compared to wild-type (Pang et al., 2014). Finally, the SaeR/S TCS also plays a role in regulating virulence factors that decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis (Guerra et al., 2016). The SaeR/S-regulated virulence factors that decrease neutrophil ROS remain unknown and are an active area of research, but are independent of superoxide dismutase and catalase activity since their expression is not regulated by SaeR/S (Rogasch et al., 2006; Voyich et al., 2009; Nygaard et al., 2010; Sun et al., 2010).

Human neutrophils extrude DNA decorated with antimicrobial proteins termed neutrophil extracellular traps (NETs) to ensnare and kill bacteria as a terminal cell fate pathway that has been named NETosis and is dependent on ROS (Brinkmann et al., 2004; Fuchs et al., 2007; Galluzzi et al., 2012). Neutrophil exposure to *S. aureus* is a potent inducer of NETs (Pilsczek et al., 2010). However, *S. aureus* escapes NETs by secreting nuclease (*nuc*), an SaeR/S-regulated factor (Berends et al., 2010; Olson et al., 2013). Furthermore, the nuclease products from DNA degradation 2′-deoxyadenosine-3′-monophosphate and 2′-deoxyadenosine-5′-monophosphate are converted by the *S. aureus* exoprotein adenosine synthase (*adsA*) into 2′-deoxyadenosine (dAdo), which induces caspase-3-mediated apoptosis in macrophages (Thammavongsa et al., 2013). A murine intravenous infection model showed macrophages are unable to diffuse into kidney abscesses containing neutrophils and wild-type *S. aureus*; however, infection with a *nuc* or *adsA* deficient *S. aureus* strain allowed macrophages to efficiently infiltrate the neutrophil abscess (Thammavongsa et al., 2013).

S. AUREUS MODULATION OF NEUTROPHIL FATE

Neutrophils undergo an apoptotic differentiation program in response to bacterial pathogens to limit host damage caused by a prolonged inflammatory response (Kobayashi et al., 2003). For excellent reviews on the mechanisms of neutrophil apoptosis and how bacterial pathogens modulate neutrophil fate, we recommend (Kennedy and Deleo, 2009; Rigby and DeLeo, 2012; Kobayashi et al., 2017). *S. aureus* produces pore-forming toxins that lyse neutrophils, other leukocytes, and red blood cells and this lytic activity promotes an intense inflammatory response. While these toxins clearly play a role in lysing neutrophils and other immune cells, their role if any beyond neutrophil cytolysis is unknown. Notable exceptions include Panton-Valentine Leukocidin (PVL) and LukGH which have been shown to prime neutrophils at sublytic concentrations and promote neutrophil extracellular trap formation, respectively (Graves et al., 2012; Malachowa et al., 2013). In addition, neutrophils exposed to *S. aureus* have been shown to undergo cell death with intact phagosomes undermining the role of cytolytic toxins in *S. aureus* escape from neutrophils (Kobayashi et al., 2010). For reviews on the lytic properties of these toxins and their targets, please refer to (DuMont and Torres, 2014; Reyes-Robles and Torres, 2016). Herein, we will highlight recent studies suggesting *S. aureus* modulates neutrophil fate to disrupt proper neutrophil clearance following phagocytosis of *S. aureus* independent of lytic activity.

Following phagocytosis, neutrophils are ingested by macrophages in a process called efferocytosis to remove spent neutrophils and limit inflammation (Martin et al., 2014). *S. aureus* inhibits macrophage efferocytosis of *S. aureus* laden neutrophils by upregulating the "don't eat me" signal CD47 (Greenlee-Wacker et al., 2014). Interestingly, macrophage production of cytokines that modulate neutrophil fate was altered and inflammasome activation was reduced in response to neutrophils harboring *S. aureus* compared to *S. aureus* alone. *In vitro*, human neutrophils containing *S. aureus* do not undergo the classical apoptotic death pathway as they fail to activate caspase-3, as well as caspase-2, -8, and -9. Instead, neutrophil death in this *in vitro* model in response to *S. aureus* is dependent on receptor-interacting protein 1 (RIP-1) which is a hallmark of programmed cell death or necroptosis. Studies by Zurek et al. showed that the SaeR/S TCS plays a role in modulating neutrophil fate by inhibiting IL-8 production and NF-κB activation (Greenlee-Wacker et al., 2014; Zurek et al., 2015). Programmed neutrophil death in response to wild-type *S. aureus* was accelerated compared to exposure to a *saeR/S* deletion mutant.

In addition, ROS production is implicated in programmed neutrophil death leading to NET formation (NETosis) and as discussed above *S. aureus* inhibits neutrophil ROS production (Fuchs et al., 2007; Akong-Moore et al., 2012). Interestingly, Pilsczek et al. described a NET forming mechanism that did not require neutrophil lysis and is ROS independent. Instead, DNA-containing vesicles budding from the nuclear membrane are released while maintaining neutrophil plasma membrane integrity (Pilsczek et al., 2010). Thus, these anuclear neutrophils may still retain phagocytic and chemotactic activity, as previously observed (Malawista et al., 1989). Further studies are needed to determine if inhibition of neutrophil ROS production by *S. aureus* modulates NETosis and to better elucidate ROS independent and dependent NET formation and NETosis. However, it is clear that *S. aureus* alters the neutrophil death program through a variety of mechanisms that impact not only cellular signaling by the neutrophil but clearance via efferocytosis.

THE OTHER SIDE OF THE EQUATION: THE ABILITY OF *S. AUREUS* TO SENSE NEUTROPHILS

As reviewed above, the neutrophil has many mechanisms to sense and respond to *S. aureus* and likewise individual *S. aureus* factors have evolved to disrupt every step of the neutrophil response to the invading pathogen. However, the ability of *S. aureus* to sense the host has been underestimated. The *S. aureus* genome consists of 16 two-component systems (TCS) that sense environmental stimuli and regulate gene expression accordingly (Kawada-Matsuo et al., 2011). Of these 16 TCSs, SaeR/S is recognized as a major contributor to *S. aureus* pathogenesis and neutrophil evasion (Voyich et al., 2009; **Figure 3**). Geiger et al. was the first to demonstrate that the TCS SaeR/S contained an upstream promoter element that could be activated by human neutrophil products H_2O_2 and alpha-defensin (Geiger et al., 2008). SaeR/S target genes are differentially regulated in response to whole blood, neutrophils and neutrophil components (H_2O_2, alpha-defensin, and calprotectin; Voyich et al., 2005; Palazzolo-Ballance et al., 2007; Malachowa et al., 2011; Flack et al., 2014; Zurek et al., 2014; Cho et al., 2015). The nine amino acid extracellular loop of SaeS plays an important role in sensing different neutrophil-derived stimuli (Flack et al., 2014; Liu et al., 2015). Highly conserved residues M31, W32, and F33 on SaeS are essential to the appropriate activation of *sae*-target genes. Residue M31 is essential to the activation of *sae* regulon and plays an important role in sensing neutrophil-derived stimuli including HNP-1. Strains with mutations in aromatic residues W32 and F33 have disrupted normal basal signaling of SaeS in the absence of inducing signals, yet both mutant kinases have appropriate activation of effector genes following exposure to neutrophil-derived stimuli (Flack et al., 2014). This posits that response regulators turn-on only subsets of genes based on the host stimulus. Such observations support a unique hypothesis that emerging strains of bacteria are not more virulent because they harbor new virulence factors but are better able to sense and respond to their human hosts.

SaeR/S is the best studied for its role in sensing the neutrophil but additional studies are needed to understand just how *S. aureus* senses the neutrophil *in vivo*. Certainly other regulatory systems play a role either directly or potentially by responding to the activation of SaeR/S, however, these studies are currently limited. Considering HNP-1 is recognized early following phagocytosis, the recognition of HNP-1 is likely very important for *S. aureus* adaptation to the neutrophil environment. Interestingly, the recognition of HNP-1 by SaeS has not been demonstrated to influence the genes in *S. aureus* directly correlated with combating the antimicrobial capacity of antimicrobial peptides [e.g., *dlt* and *mprF* are regulated by the GraRS two-component system (Yang et al., 2012)] but instead

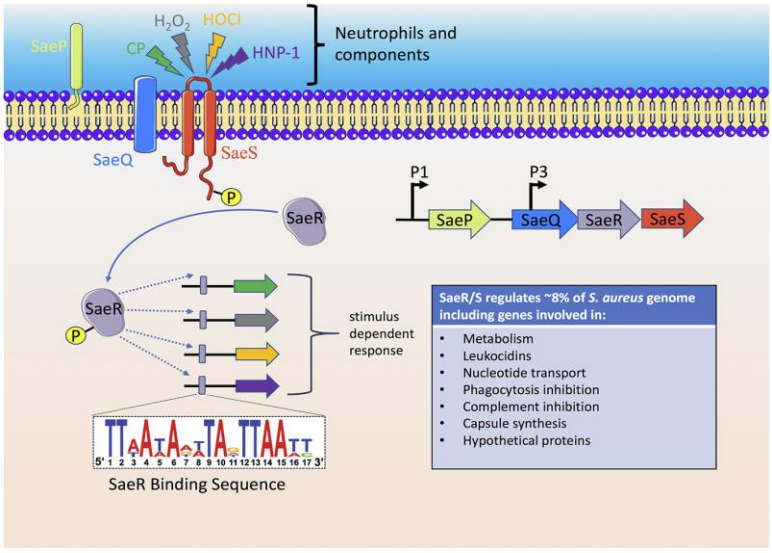


FIGURE 3 | The *S. aureus* SaeR/S two-component system senses neutrophils and neutrophil components to activate an anti-host immune response. Activation of the SaeS histidine kinase results in autophosphorylation which is subsequently transferred to the SaeR response regulator. A promoter binding region recognized by phosphorylated SaeR (SaeR binding sequence) initiates gene transcription of SaeR/S-regulated virulence factors that target neutrophil antimicrobial mechanisms, cell fate and cellular signaling. The immediate genes transcribed by SaeR/S are dependent on the stimulus, i.e., the SaeR/S transcriptional profile is dynamic and specific to the stimulus. Since bacterial sensory-regulatory systems activate multiple virulence factors, therapeutic approaches to inhibit bacterial sensing, and activation is an active area of research. CP, calprotectin.

appears to ready *S. aureus* for combating neutrophil reactive oxygen species and for producing cytolytic toxins. Additionally, the GraRS system recognizes cationic peptides including LL-37, and polymyxin B but does not recognize HNP-1 implying a complex interplay of sensory systems to combat the neutrophil (Yang et al., 2012). Another interesting two-component gene regulatory system is WalK/R. Studies have demonstrated this system is essential for *S. aureus* viability (Dubrac and Msadek, 2004; Dubrac et al., 2007). However, studies using a strain of *S. aureus* with a constitutively active WalR response regulator demonstrated constitutive expression caused up-regulation of virulence genes that are known to be SaeR-regulated including *coa*, *hla hlgA*, *hlgB*, *hlgC*, and *sbi* (Delauné et al., 2012). Although the exact mechanism of communication between WalR and SaeR/S is unknown data suggest WalR influences the SaeR/S system indirectly. This is inferred since deletion of *saeR/S* in a strain that constitutively expressed WalR showed the impact of WalR on the virulence factors during growth *in vitro* was through SaeR/S (Delauné et al., 2012). However, in mouse models of infection constitutive expression of WalR in *S. aureus* caused increased neutrophil recruitment and enhanced bacterial clearance compared to wild-type *S. aureus*. Constitutive expression of WalR decreasing virulence *in vivo* was independent of SaeR/S and linked to WalR dependent peptidoglycan release promoting an inflammatory environment that recruited neutrophils (Delaune et al). Currently the stimulus recognized by WalK is unknown. Understanding what stimulus(i) activates this system may reveal how WalK/R and SaeR/S regulation of virulence are linked. Additionally, the fatty acid kinase VfrB, that lacks any traditional DNA-binding domain, has been shown to influence gene expression of well-defined SaeR/S-dependent genes (Bose et al., 2014; Krute et al., 2017). However, when a *vfrB* mutant strain was exposed to the known SaeS-activation stimulus HNP-1, this kinase no longer impacted virulence genes regulated by SaeR/S (Krute et al., 2017). These data further emphasize the importance of defining virulence regulation in physiologically relevant environments and highlight the complexity of virulence gene regulation in *S. aureus*.

THE CHALLENGE OF INVESTIGATING NEUTROPHIL—*S. AUREUS* INTERACTIONS *IN VIVO*

Investigating the neutrophil-*S. aureus* interactions *in vivo* has been challenging. The relevance of using the murine immune system to mimic interactions between *S. aureus* and the human immune system is heavily debated (Buer and Balling, 2003; Kim et al., 2014; Montgomery et al., 2014; Reizner et al., 2014; Tseng et al., 2015). Interestingly, though HNP-1 is recognized as the most prominent trigger of *saeR/S*, murine neutrophils are deficient in its production (Inaba et al., 2010). Not only do they differ in the production of HNP-1 but notable differences are observed in chemokine production, cell trafficking, and susceptibility to secreted toxins (Johnston et al., 1990; Singer and Sansonetti, 2004; Parker and Prince, 2012; Tseng et al., 2015). Often the importance of single *S. aureus* virulence genes is highlighted in models of infection; however, canonical *S. aureus* toxins such as HlgA, HlgC, and LukF-PVL have extremely limited lytic activity on murine cells (Liu, 2009; Baba-Moussa et al., 2011; Parker and Prince, 2012; Vandenesch et al., 2012). While deficiencies in the murine model are apparent it remains the most prominent and cost effective method to explore relationships *in vivo* between *S. aureus* and the host immune system. To that end, advances are being made toward creating more effective murine models that retain benefits such as availability of working with immune-deficient lines while displaying similar disease characteristics as observed in human hosts (Montgomery et al., 2014; Tseng et al., 2015). Tseng et al. developed a "humanized" murine model wherein NOD/SCID/IL2ry^{null} mice are engrafted with human CD34^{+} umbilical cord blood cells leading to the production of human immune cells within mice (Tseng et al., 2015). These mice demonstrated susceptibility to *S. aureus* infections at concentrations similar to what is observed in humans as well as increased susceptibility to LukF-PVL. In future efforts, humanized murine models such as this may be key to understanding how *S. aureus* regulates its pathogenesis in the context of a more relevant host environment and may be critical to advance research into effective therapeutics.

POTENTIAL OF THERAPEUTICS TO AUGMENT NEUTROPHIL FUNCTION AGAINST *S. AUREUS*

Vaccine development to prevent or reduce *S. aureus* infections has not been successful (Fowler and Proctor, 2014). Antibody based vaccines targeting individual virulence factors have not conferred significant protection against *S. aureus*, and in some cases have even increased mortality (Fowler et al., 2013). As discussed above, *S. aureus* produces virulence factors that target specific neutrophil receptors to inhibit neutrophil functions. However, *S. aureus* virulence factors show redundancy in the neutrophil functions they inhibit. For example, multiple virulence factors target the complement pathway and immunoglobulin recognition to inhibit neutrophil phagocytosis and neutralizing one of these virulence factors is not likely to have a significant effect *in vivo*. Thus, vaccine approaches that simultaneously target multiple *S. aureus* virulence factors are needed and encouragingly some are in the pipeline (Torre et al., 2015; Frenck et al., 2016).

Targeting *S. aureus* sensory/regulatory systems has great potential for therapeutic and vaccine development. Theoretically, chemical inhibitors and neutralizing antibodies of sensory/regulatory systems could inhibit *S. aureus* from sensing its environment and producing virulence factors that disrupt host antimicrobial functions. The SaeR/S TCS is an attractive candidate for vaccine development since SaeR/S-regulated factors disrupt key neutrophil functions that include production of ROS, cytokine expression, and cell fate (Watkins et al., 2011, 2013; Nygaard et al., 2013; Zurek et al., 2014; Guerra et al., 2016). Many of the best studied immunomodulatory factors and cytolytic proteins that target neutrophil function are regulated by SaeR/S including Sbi, Efb, HlgABC, Hla, LukG/H (LukA/B),

and PVL (Nygaard et al., 2010). Thus, inhibiting SaeS from sensing its environment has the potential to prevent expression of multiple virulence factors in response to host signals. In this line of thinking, targeting *S. aureus* quorum sensing via inhibition of Agr has been proposed as a therapeutic approach to inhibit production of virulence of factors (Clatworthy et al., 2007; Park et al., 2007; Cegelski et al., 2008; Kaufmann et al., 2008; Njoroge and Sperandio, 2009; Rutherford and Bassler, 2012). For a review on the *S. aureus* Agr regulatory system please refer to (Painter et al., 2014). In brief, findings have identified synthetic peptides, small molecules, and antibodies that disrupt *S. aureus* Agr-dependent quorum sensing either by blocking the sensor histidine kinase AgrC from recognizing its cognate signal autoinducing peptide (AIP), by inhibiting the AgrA response regulator from binding DNA, or by antibody interference of AIP (Park et al., 2007; Kaufmann et al., 2008; Kirchdoerfer et al., 2011; Tal-Gan et al., 2013; Broderick et al., 2014; Murray et al., 2014; Sully et al., 2014). Using this approach, reductions in mouse dermonecrosis following infection with *S. aureus*, increased bacterial clearance, and reduced hemolytic activity have been reported (Tal-Gan et al., 2013; Sully et al., 2014). In addition, the development of materials with quorum sensing inhibitors to apply during a skin infection is an active area of research and showing promising results (Broderick et al., 2014; Kratochvil et al., 2017). However, studies are needed to fully elucidate the effects of inhibiting sensory/regulatory systems since this can also increase the production of negatively regulated virulence factors and the impact on cross talk between regulatory systems that might result in compensation by one system at the loss of another, is not known.

Modulation of the inflammatory response may be another attractive target for therapeutics that might increase the likelihood of an effective neutrophil response. However, additional research is needed to identify key inflammatory mediators that resolve *S. aureus* infection and to understand those that exacerbate disease. Studies have identified IL-17 as a key determinant of proper host defense during *S. aureus* cutaneous infection (Cho et al., 2010). Murphy et al. identified γδ T cells as the major source of IL-17 during peritonitis caused by *S. aureus* and identified a subset of memory γδ T cells that enhanced IL-17 production to protect the host during subsequent *S. aureus* infection (Murphy et al., 2014). Thus, immunotherapies that enhance host bactericidal functions by neutralizing detrimental inflammatory responses, increasing beneficial cytokines at the site of infection, or stimulating the expansion of protective T cell subpopulations in response to *S. aureus* show therapeutic potential. Further research is also needed to understand site-specific cytokine requirements to mount an effective host response to *S. aureus* infection.

Lastly, as discussed above, the use of mouse models has undeniably increased our understanding of *S. aureus*-host interactions. However, due to the specificity of many of the *S. aureus* virulence factors toward human cells, improved models of disease are needed like the "humanized" mouse as discussed above. With improved models and advances in understanding neutrophil-*S. aureus* interactions, perhaps future reviews will be able to document neutrophils as the clear winner in this epic immune battle.

In conclusion, the ability of *S. aureus* to survive a neutrophil encounter is thought to contribute significantly to the virulence of this pathogen. This is exemplified by the observed increase in susceptibility to *S. aureus* infections in individuals suffering from defects that alter normal neutrophil function, such as chronic granulomatous disease, leukocyte adhesion deficiency, and neutropenia (Bodey et al., 1966; Pincus et al., 1976; Dale et al., 1979; Lekstrom-Himes and Gallin, 2000). It is clear that neutrophil antimicrobial activity is essential to eliminate *S. aureus*. However, studies have also demonstrated that increased neutrophil numbers at the site of infection can exacerbate disease (Gresham et al., 2000; McLoughlin et al., 2008) and that modulation of the neutrophil inflammatory response can significantly impact outcome of infection (Watkins et al., 2013; Zurek et al., 2015). Clearly more studies are needed to define the neutrophil responses that resolve a *S. aureus* infection. Finding the right balance of controlling inflammation while maintaining an effective neutrophil antimicrobial response will be key to the design of an effective therapeutic.

AUTHOR CONTRIBUTIONS

FG and JV wrote, edited, prepared manuscript. TB, DP, and ES wrote sections and contributed equally.

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Aspartic Acid Residue 51 of SaeR Is Essential for *Staphylococcus aureus* Virulence

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Staphylococcus aureus is a common Gram-positive bacteria that is a major cause of human morbidity and mortality. The SaeR/S two-component sensory system of *S. aureus* is important for virulence gene transcription and pathogenesis. However, the influence of SaeR phosphorylation on virulence gene transcription is not clear. To determine the importance of potential SaeR phosphorylation sites for *S. aureus* virulence, we generated genomic alanine substitutions at conserved aspartic acid residues in the receiver domain of the SaeR response regulator in clinically significant *S. aureus* pulsed-field gel electrophoresis (PFGE) type USA300. Transcriptional analysis demonstrated a dramatic reduction in the transcript abundance of various toxins, adhesins, and immunomodulatory proteins for SaeR with an aspartic acid to alanine substitution at residue 51. These findings corresponded to a significant decrease in cytotoxicity against human erythrocytes and polymorphonuclear leukocytes, the ability to block human myeloperoxidase activity, and pathogenesis during murine soft-tissue infection. Analysis of SaeR sequences from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. Collectively, these results demonstrate that aspartic acid residue 51 of SaeR is essential for *S. aureus* virulence and underscore a conserved target for novel antimicrobial strategies that treat infection caused by this pathogen.

Keywords: *Staphylococcus aureus*, *saeR/S*, virulence, pathogenesis, two-components system, neutrophil, toxin, transcription

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can cause a wide range of disease in both humans and animals (Nygaard et al., 2008). The diverse pathogenesis of *S. aureus* can be attributed to the expression of an extensive array virulence factors that are often redundant in function (Guerra et al., 2017). Expression of these virulence genes *in vivo* is thought to be primarily dictated by the concerted influence of two-component sensory systems that recognize environmental signals and alter gene expression accordingly. The *S. aureus* genome encodes 16 two-component systems that have been identified by sequence analysis (Cheung et al., 2004). Of these, the SaeR/S two-component system has been shown to be an important mediator of *S. aureus* virulence by transcriptionally upregulating numerous adhesins, toxins, and immunomodulatory

proteins that interact directly with host components to advance pathogenesis (Giraudo et al., 1997; Goerke et al., 2001; Voyich et al., 2009; Nygaard et al., 2010; Borgogna et al., 2018). The mechanisms used by this two-component system to recognize host-specific cues and alter gene transcription in response are not completely understood.

Typical two-component systems are minimally composed of a transmembrane histidine kinase sensor and cognate intracellular response regulator (Groisman, 2016). Recognition of environmental stimulus activates the histidine kinase sensor, inducing autophosphorylation at a histidine residue on the intracellular domain. This phosphate group is then transferred to an aspartic acid residue within the receiver domain of the cognate response regulator. In general, the phosphorylated response regulator then binds to promoter regions within the bacterial genome to mediate gene transcription and promote survival. However, some response regulators such as RcsB from *Escherichia coli* (Pannen et al., 2016) and SreR from *Xanthomonas campestris* (Wang et al., 2014) can regulate gene transcription without being phosphorylated. For the SaeR/S two-component system, the need to artificially phosphorylate recombinant SaeR for the *in vitro* DNA binding activity of this response regulator has not been consistent between studies (Nygaard et al., 2010; Sun et al., 2010). It has also been suggested that different levels of SaeR phosphorylation correspond to the upregulation of distinct groups of SaeR/S regulated genes (Mainiero et al., 2010).

To clarify the importance of potential SaeR phosphorylation sites for mediating *S. aureus* pathogenesis, we have generated individual single amino acid substitutions at conserved aspartic acid residues of SaeR in the genome of *S. aureus* PFGE-type USA300. In this report, we assess the significance of these residues for mediating the virulence of a clinically relevant MRSA strain using both *in vitro* and *in vivo* models of infection. Results from this study underscore the importance of SaeR aspartic acid residue 51 for virulence gene transcription and demonstrate that the substitution of this single amino acid in the USA300 genome can attenuate pathogenesis of this clinically significant MRSA strain.

MATERIALS AND METHODS

Bacteria Strains and Culture Conditions

Staphylococcus aureus PFGE-type USA300 strain LAC has been described previously (Diep et al., 2006) and the USA300 isogenic deletion mutant of *saeR/S* (USA300Δ*saeR/S*) was generated in previous studies (Nygaard et al., 2010). Bacteria were cultured in an Excella E24 rotary incubator (New Brunswick) at 250 rpm and 37°C. Unless noted otherwise, overnight bacteria cultures grown in 20 mL of tryptic soy broth (TSB; EMD Millipore) were used to start subcultures in 20 mL TSB containing 0.5% glucose (1:100 dilution). Optical density at 600 nm (OD_{600}) was measured using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific) and colony forming units (CFUs) were determined by plating diluted samples on tryptic soy agar (TSA; EMD Millipore).

DNA Sequence Alignment

DNA sequence alignment of SaeR homologs was performed using Clustal Omega[1] with protein sequences obtained from the NCBI protein data base using the following accession numbers: ABD22784 for USA300, ATX72322 for USA400, EOR90509 for USA100, WP_076742615.1 for MT-0541, AAW53763 for *Staphylococcus epidermidis* RP62A, Q8CQ17 for *Staphylococcus epidermidis* 12228, WP_049307325 for *Staphylococcus capitis*, WP_029378577 for *Staphylococcus xylosus*, PCQ20359 for *Klebsiella pneumonia*, NP_346930 for *Clostridium acetobutylicum*, ACJ50526 for *Escherichia coli,* and NP_388082 for *Bacillus subtilis*.

Generation of USA300 Genomic Mutations

Allelic exchange with plasmid pKOR1 was used to impart genomic mutations in USA300 as previously described (Bae and Schneewind, 2006). All primers used to generate and sequence USA300 mutants are listed in **Supplementary Table S1**. Primers containing *attb* sites were used to amplify staphylococcal peroxidase inhibitor (*spn*) and saeR from the USA300 genome for BP Clonase II (ThermoFisher Scientific) mediated insertion into pKOR1. Spn-check-fwd and rvs primers were used to verify the loss of *spn*. Site-directed mutagenesis was performed on pKOR1-*saeR* using saeR-D46A, saeR-D51A, or saeR-D61A primers and PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Inc.) following the manufacture's protocol and as previously performed (Ran et al., 2010). The *saePQRS* operon in generated *saeR* point mutants was PCR amplified using saePQRS_fwd and rvs primers and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit) using saeR-seq and saePQRS-seq primers. PCR amplification was performed using saeR-EcoRI-fwd and saeR-XhoI-rvs and cloned into pEPSA5 as previously described (Flack et al., 2014) to generate the complementary plasmid that expresses wt SaeR, pEPSA5-*saeR*.

Whole Genome Sequencing and *saeR* Sequence Analysis

Wild-type USA300 and its SaeR mutants were subject to whole genome sequencing. Briefly, genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI, United States) following by treatment with 20 μg/ml lysostaphin. The DNA library was sequenced on an Illumina NextSeq platform (Illumina, San Diego, CA, United States) with 2 × 150 bp paired-end reads. The reads were mapped against the published NC_007793 (*S. aureus* USA300_FPR3757) genome using BWA (Li and Durbin, 2009) and Samtools (Li et al., 2009), and the SNPs and InDels were examined using freebayes[2]. SNPs and InDels were further annotated using snpEff (Cingolani et al., 2012).

[1]https://www.ebi.ac.uk/Tools/msa/clustalo/

[2]https://github.com/ekg/freebayes

Blast analysis of the USA300 *saeR* (locus_tag SAUSA300_0691) sequence against more than 8,000 *S. aureus* draft genomes downloaded from the NCBI FTP site[3] (dated as June 1, 2018) were extracted, translated into amino acids and aligned using Geneious 11.1.

Relative Quantitative Real Time RT-PCR

Staphylococcus aureus transcription analysis using relative quantitative real time RT-PCR was performed as previously described (Voyich et al., 2005, 2009; Nygaard et al., 2010). Briefly, subcultured strains were harvested at mid-exponential (ME; OD_{600} = 1.5) or early stationary (ES; OD_{600} = 3.0) growth phases, mechanically disrupted using a FastPrep FP120 cell disrupter (ThermoFisher Scientific), and RNA purified with an RNeasy Kit (Qiagen). TaqMan real-time RT-PCR was performed using previously published primer and probe sets (Nygaard et al., 2010).

Hemolysis Assays

Heparinized venous blood from healthy donors was collected in accordance with the protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All donors provided written informed consent to participate in the study. We adopted protocol described by others (Young et al., 1986) to quantify hemolysis of human blood by *S. aureus* extracellular proteins. Briefly, freshly drawn human blood was washed three times with 10 times the volume of sterile DPBS then resuspended at a final dilution of 1:200 with sterile DPBS. Sterile-filtered (0.22 um) *S. aureus* supernatants from 6-h subcultures grown in TSB were combined with washed diluted blood in individual wells of a 96 well plate on ice at a ratio of 1:1. TSB alone and TSB + 0.5% Triton X-100 were also used as negative and positive controls for hemolysis. Samples were then placed in a SpectraMax 190 microplate reader (Molecular Devices) heated to 37°C and absorbance at 630 nm was measured after 6 min of incubation. Percent hemolysis was determined using the following formula: $\%\text{Hemolysis} = (\text{Absorbance}_{Experimental} - \text{Absorbance}_{\text{TSBcontrol}})/(\text{Absorbance}_{\text{Triton X}} - \text{Absorbance}_{\text{TSBcontrol}}) \times 100$.

Human Myeloperoxidase Activity Assays

To obtain extracellular proteins from USA300 strains, supernatant from *S. aureus* sub-cultured for 5 h was sterile-filtered (0.22 um) and stored at −80°C. For human myeloperoxidase (MPO) activity assays, 0.5 ug of recombinant human MPO (R&D Systems) in 50 uL of DPBS was mixed with 50 uL of sterile-filtered *S. aureus* supernatant for 30 min at room temperature. The MPO-supernatant solution was then exposed to 150 uL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences) and incubated at 37°C. The oxidation of TMB catalyzed by human MPO was quantified by measuring the OD_{650} every minute for 30 min using a SpectraMax Paradigm microplate reader (Molecular Devices). The MPO inhibitor sodium azide was used at a concentration of 1 mM.

[3]http://ftp.ncbi.nih.gov

Human PMN Plasma Membrane Integrity Assays

Human polymorphonuclear leukocytes (neutrophils or PMNs) were isolated under endotoxin-free conditions (<25.0 pg/ml) using freshly drawn heparinized venous blood from healthy donors with written informed consent as previously described (Voyich et al., 2005; Nygaard et al., 2013). Cell viability and purity of preparations were assessed using a FACSCalibur Flow cytometer (BD Biosciences) and only preparations containing ≥ 98% viable PMNs were used. Assays intoxicating PMNs with extracellular *S. aureus* proteins were performed as previously described (Nygaard et al., 2012; Flack et al., 2014). Briefly, supernatant from *S. aureus* subcultured for 5 h in TSB was sterile-filtered (0.22 um) and diluted by 1:10 with TSB. To intoxicate PMNs, 20 ul of diluted *S. aureus* supernatant was combined with 100 uL Roswell Park Memorial Institute (RPMI) 1640 Medium (Corning Cellgro) with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Corning Cellgro) containing $1x10^6$ purified human PMNs in a serum coated well of a 96 well plate. Samples were incubated at 37°C for 90 min then stained with propidium iodide (PI; ThermoFisher Scientific) following the manufactures protocol and then analyzed with a FACSCalibur Flow cytometer. Triton-X 100 (0.5%) was used as a positive control for causing PMN plasma membrane permeability. The following formula was used to determine % propidium iodide$^+$: $\%\text{Propidium Iodide}^+ = (\text{Mean PI signal}_{Experimental} - \text{Mean PI signal}_{\text{untreated}})/(\text{Mean PI signal}_{\text{Triton}-X} - \text{Mean PI signal}_{\text{untreated}})$.

Assays measuring human PMN plasma membrane permeability following the phagocytosis of live *S. aureus* were performed as previously described (Voyich et al., 2009; Nygaard et al., 2012; Flack et al., 2014). Briefly, subcultured *S. aureus* was harvested at ME growth by centrifugation (5,000 × g, 5 min, 4°C), washed with DPBS, then opsonized with 50% normal human serum for 15 min at 37°C. Opsonized bacteria were washed with DPBS and then 2×10^7 CFU in 100 uL of DPBS was combined with 100 uL of RPMI/H containing 1×10^6 purified human PMNs in a serum coated well of 96 well plate. Phagocytosis was synchronized by centrifugation (524 × g, 8°C, 8 min) in an Allegra X-15R centrifuge (Beckman Coulter) and samples were incubated at 37°C for 90 min. Following incubation, human PMNs were stained with PI and analyzed using flow cytometry as described above.

Murine Model of Soft-Tissue Infection

All animal studies conformed to National Institute of Health guidelines and were approved by the Animal Care and Use Committee at Montana State University-Bozeman. Female BALB/C mice (8–10 weeks old) with an average weight of 22 g were purchased from Animal Resource Facility at Montana State University (Bozeman, MT, United States). The murine model of soft-tissue infection was performed as previously described (Voyich et al., 2006; Nygaard et al., 2010; Malachowa et al., 2013).

Briefly, 12-week-old BALB/C mice were shaved and hair completely removed with Nair^{TM} treatment. Two days later, shaved mice (5 per group) were subcutaneously inoculated with 1×10^7 CFUs of *S. aureus* in 100 uL DPBS. Mice were weighed and abscess size measured at indicated times post-inoculation. The area of soft-tissue infections was determined using the following formula as previously published (Malachowa et al., 2013): Area = π (Length/2) $\times$ Width/2.

RESULTS

The Generation of Point Mutations in the USA300 Genome That Confer Aspartic Acid to Alanine Substitutions Within the Receiver Domain of SaeR

Analysis of the three-dimensional crystal structure of the two-component response regulator PhoB of *Escherichia coli* (Solà et al., 1999) indicates that the homologous *S. aureus* response regulator SaeR is phosphorylated on a conserved aspartic acid at residue 51 (**Figure 1A**). Aspartic acid residue 46 is also highly conserved in the OmpR family of proteins, suggesting this residue might be important for SaeR function. Analysis of the SaeR sequences extracted from over 8,000 draft *S. aureus* genomes revealed that aspartic acid residue 51 is 100% conserved. The biological significance of this conservation is heightened by the identification of 50 unique SaeR protein sequences identified from the draft genomes (**Supplementary Figure S1**).

To determine if these aspartic acid residues in the receiver domain of SaeR are important for *S. aureus* pathogenesis, we used allelic recombination to induce substitutions of specific nucleic acids within the *S. aureus* genome as previously described by others (Bae and Schneewind, 2006; Villaruz et al., 2009; Mairpady Shambat et al., 2016). Using this technique, we generated USA300 strains with an aspartic acid to alanine substitution at the conserved SaeR residue 51 (USA300Δ*saeR*-D51A) or the proximal conserved residue 46 (USA300Δ*saeR*-D46A). In addition, we generated an aspartic acid to alanine substitution at the less maintained residue 61 of SaeR (USA300Δ*saeR*-D61A) to serve as a control for this study. Primary DNA sequencing analysis and additional whole genome sequencing analysis verified the targeted amino acid substitutions that were generated in the three mutants and showed that there were no additional SNPs or InDels when compared to the wild-type strain. As with previously generated isogenic deletion mutants of *saeR/S* in *S. aureus* (Voyich et al., 2009; Nygaard et al., 2010),

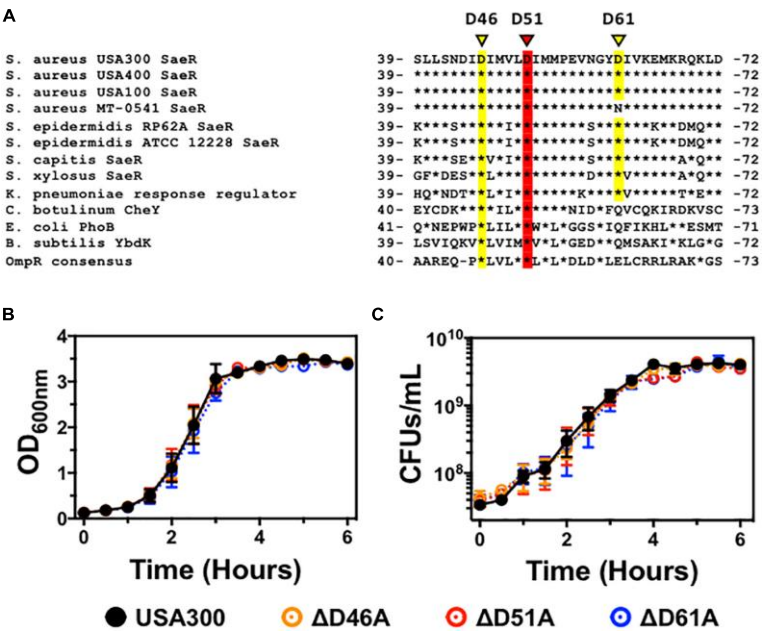


FIGURE 1 | The generation of USA300 genomic point mutations in specific aspartic acid residues of *saeR*. **(A)** DNA sequence alignment of *saeR* homologues from different bacteria highlighting the putative phosphorylation site at conserved aspartic acid residue 51 (red) as well as proximal aspartic acid residues 46 and 61 (yellow). *In vitro* growth as measured by **(B)** absorbance at 600 nm, and **(C)** colony forming units (CFU) per mL of USA300, USA300 with an aspartic acid to alanine point mutation at SaeR residue 46 (ΔD46A), at residue 51 (ΔD51A), or at residue 61 (ΔD61A). Data in panels **(B,C)** are presented as the mean ± SEM of three independent experiments.

no differences during *in vitro* growth could be detected in these SaeR point mutants relative to the parental USA300 strain (**Figures 1B,C**).

Substitution of SaeR Aspartic Acid Residue 51 With Alanine Substantially Decreases the Transcript Abundance of USA300 Virulence Genes

The SaeR/S two-component system plays an essential role during pathogenesis by upregulating *S. aureus* virulence gene transcription (Giraudo et al., 1997; Goerke et al., 2001; Voyich et al., 2009; Nygaard et al., 2010). It is thought that this process requires the activation of SaeR via phosphorylation at an aspartic acid residue within the receiver domain of this response regulator. Indeed, others have indicated that aspartic acid residue 51 of SaeR is necessary for the transcription of α-hemolysin (Hla) using an artificial plasmid overexpression system in *S. aureus* strain Newman lacking wild-type *saeR/S* (Mainiero et al., 2010). However, the transcription of *saeR* that is under a positive feedback loop by the SaeR/S two-component system was actually enhanced in the absence of aspartic acid 51 while no difference in SaeR/S regulated coagulase A transcription could be demonstrated (Mainiero et al., 2010). This study suggested that different levels of SaeR phosphorylation corresponds to the up-regulation of specific sets of virulence genes, prompting us to also examine the influence of the highly conserved SaeR aspartic acid residue 46 and partially conserved aspartic acid residue 61 on SaeR/S-mediated virulence gene transcription.

To resolve the importance of these SaeR aspartic acid residues for virulence gene expression in clinically relevant MRSA, we examined the transcript abundance of numerous adhesins, toxins, and immunomodulatory proteins in the USA300 aspartic acid point mutant strains using relative quantitative real time RT-PCR (**Figure 2**). Compared to USA300, we observed substantial decreases in the abundance of transcripts encoding various adhesins, toxins, and immunomodulatory proteins in a USA300 isogenic deletion mutant of *saeR/S* (USA300Δ*saeR/S*) relative to the USA300 wt (**Figures 2A,B**). The virulence gene transcription profile for USA300Δ*saeR*-D51A was almost identical to that of USA300Δ*saeR/S*. In contrast, the transcript abundance of virulence genes in USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A was comparable to the USA300Δ parental wt (**Figures 2A,B**). Reintroduction of wild-type SaeR to USA300Δ*saeR*-D51A rescued defects in gene transcription (**Supplementary Figure S2**), supporting results indicating aspartic acid residue 51 of SaeR is required for

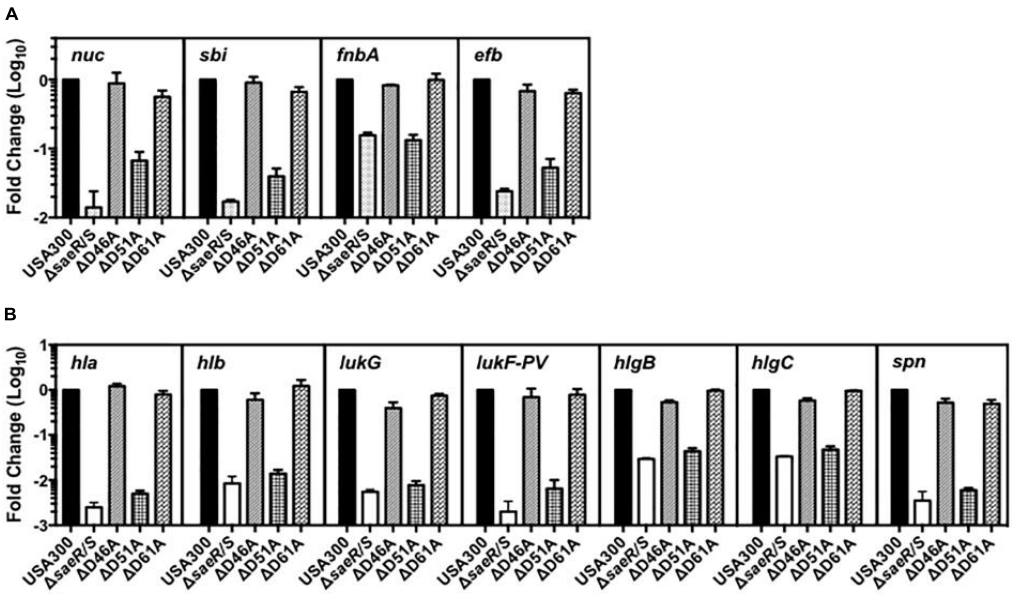


FIGURE 2 | Aspartic acid residue 51 of SaeR is important for the transcription of numerous virulence genes in USA300. Taqman© RT-PCR analysis of USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), and USA300 genomic point mutants that confer aspartic acid to alanine substitutions at SaeR residue 46 (ΔD46A), 51 (ΔD51A), or 61 (ΔD61A) during growth *in vitro*. Transcriptional analysis was performed at **(A)** mid-exponential growth for nuclease (*nuc*), the second binder of IgG (*sbi*), fibronectin-binding protein A (*fnbA*), and the extracellular fibrinogen-binding protein (*efb*) or at **(B)** early-stationary growth for γ-hemolysin (*hla*), γ-hemolysin (*hlb*), leukocidin subunit G (*lukG*), the Panton-Valentine leukocidin subunit F (*lukF-PV*), γ-hemolysin component B (*hlgB*), γ-hemolysin component C (*hlgC*), and the staphylococcal peroxidase inhibitor (*spn*). All panels show the mean ± SEM of at least two separate experiments and are presented as fold change relative to USA300 wt.

the upregulation of virulence gene expression by this response regulator.

Aspartic Acid Residue 51 of SaeR Is Essential for the SaeR/S Mediated Hemolysis of Human Erythrocytes by USA300

Staphylococcus aureus expresses numerous hemolysins under direct transcriptional regulation by the SaeR/S two-component system (Voyich et al., 2009; Nygaard et al., 2010) that lyse mammalian erythrocytes including Hla, Hlb, and HlgA/B (Salgado-Pabón et al., 2014; Spaan et al., 2015). Real time RT-PCR analysis indicated aspartic acid residue 51 of SaeR is essential for the transcriptional upregulation of these hemolysins (**Figure 2**). To determine the importance of this putative SaeR phosphorylation site for hemolysis caused by *S. aureus*, we first qualitatively assessed the ability of USA300, USA300Δ*saeR/S*, USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A to lyse human red blood cells during growth on agar (**Figure 3A**). The zone of hemolysis generated by USA300Δ*saeR/S* on agar was decreased relative to USA300 (**Figure 3A**), corresponding to observations in this study (**Figure 2**) and in previously published reports (Voyich et al., 2009; Nygaard et al., 2010) that demonstrate a decrease in transcript abundance of various *S. aureus* hemolysins when this two-component system is absent. A reduced hemolysis of human erythrocytes during growth on agar of USA300Δ*saeR*-D51A paralleled that of USA300Δ*saeR/S*, supporting the notion that aspartic acid residue 51 is essential for SaeR/S activity. In contrast, the zone of hemolysis for USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A was indistinguishable from that caused by USA300.

To quantify the hemolysis caused by aspartic acid point mutants of SaeR, we measured the turbidity of human erythrocytes in solution after being combined with extracellular proteins produced by these strains. Corresponding to hemolysis during growth on agar, significantly less human red blood cells were lysed by filtered supernatants from USA300Δ*saeR*/S or USA300Δ*saeR*-D51A relative to the USA300 parental strain (**Figure 3B**). In addition, extracellular proteins produced by USA300Δ*saeR*-D46A generated significantly less hemolysis than USA300. In support of these findings, the reintroduction of wild-type SaeR to USA300Δ*saeR*-D46A or USA300Δ*saeR*-D51A

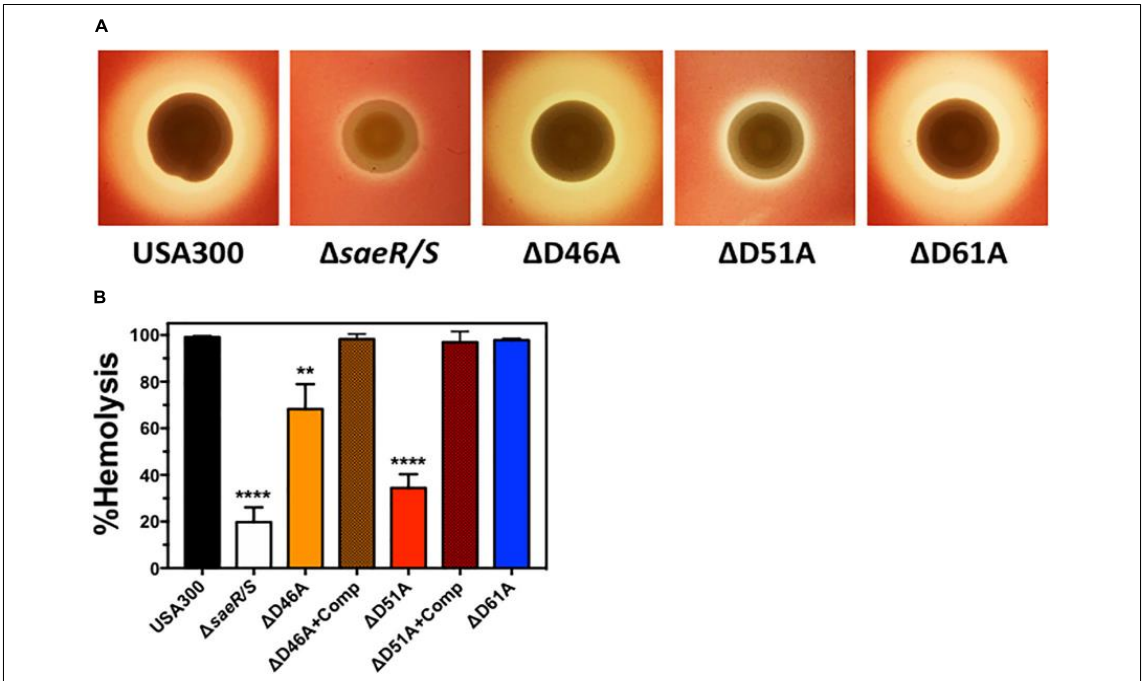


FIGURE 3 | Aspartic acid residues 46 and 51 of SaeR are important for the hemolysis of human erythrocytes by USA300. **(A)** Zone of hemolysis caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 (ΔD46A), 51 (ΔD51A), or 61 (ΔD61A) during growth on agar containing 2.5% human red blood cells. **(B)** Percent hemolysis of human red blood cells in solution exposed to 6 h supernatants from USA300, USA300Δ*saeR/S*, ΔD46A, ΔD51A, ΔD61A or ΔD46A and ΔD51A complemented with a plasmid expressing wt SaeR (ΔD46A+Comp or ΔD51A+comp, respectively). All panels represent at least 3 separate experiments. Panels **(B,C)** show the mean ± SEM with $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

rescued the hemolytic activity of supernatants from these strains to levels observed for USA300. Taken together, these findings show that aspartic acid residue 51 of SaeR is necessary for causing the hemolysis of human erythrocytes that is facilitated by the SaeR/S two-component system. In addition, SaeR aspartic acid residue 46 appears to play a significant but less important role than residue 51 mediating hemolysis caused by USA300.

Inhibition of Human MPO Activity by USA300 Extracellular Proteins Requires Aspartic Acid Residue 51 of SaeR

Recently published findings demonstrate that *S. aureus* prevents the generation of reactive oxygen species by human polymorphonuclear leukocytes (neutrophils or PMNs) via SPIN, a protein under strong SaeR/S regulation that binds directly to the active site of human MPO and inhibits peroxidase activity of this enzyme (Guerra et al., 2016; de Jong et al., 2017). To determine if the putative phosphorylation site at aspartic acid residue 51 of SaeR is required for the inhibition of MPO activity by USA300, we assessed the activity of human MPO in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of SPIN in USA300 (USA300Δ*spn*), USA300Δ*saeR/S,* USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A (**Figure 4**). Congruent with previously published findings (de Jong et al., 2017), a strong inhibition of human MPO activity was observed in the presence of the MPO inhibitor azide or extracellular proteins produced by USA300 but not extracellular proteins produced by USA300Δ*saeR/S* or by USA300Δ*spn* (**Figures 4A,B**). As with USA300Δ*saeR/S* and USA300Δ*spn*, extracellular proteins produced by USA300Δ*saeR*-D51A did not inhibit human MPO activity (**Figures 4A,B**). Reintroduction of wild-type SaeR to USA300Δ*saeR*-D51A rescued the inhibition of human MPO

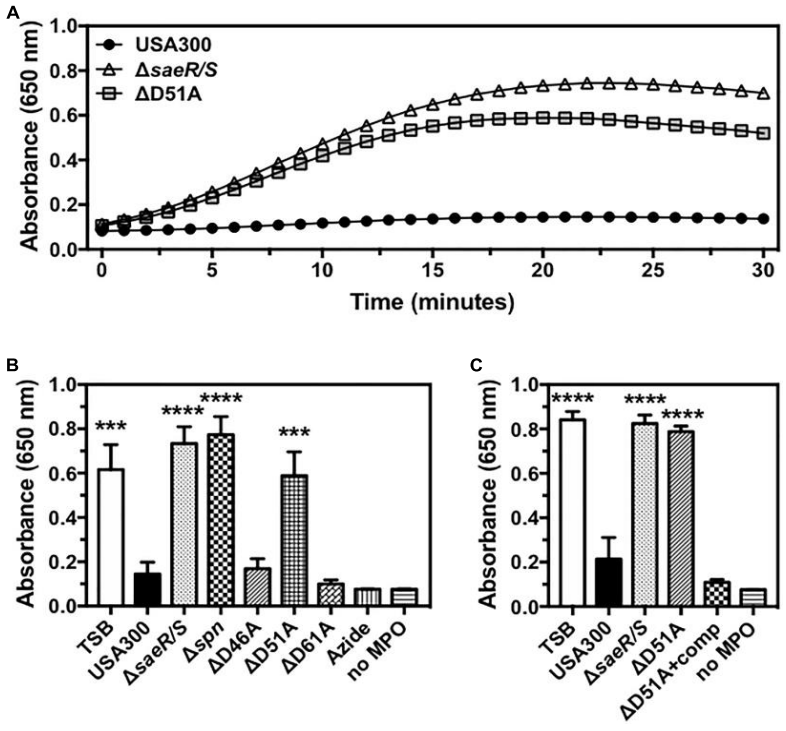


FIGURE 4 | Aspartic acid residue 51 of SaeR is essential for the inhibition of human myeloperoxidase activity by USA300. **(A)** Human myeloperoxidase (MPO) activity in the presence of extracellular proteins produced by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (Δ*saeR/S*), or an aspartic acid to alanine substitution at residue 51 of SaeR in USA300 (ΔD51A) as measured by absorbance at 650 nm following exposure to 3,3′,5,5′-Tetramethylbenzidine (TMB) and hydrogen peroxide. **(B)** The activity of human MPO in the presence of extracellular proteins produced by USA300, Δ*saeR/S,* an isogenic deletion mutant of SPIN in USA300 (Δ*spn*), ΔD51A, or USA300 with an aspartic acid to alanine substitution at residue 46 (ΔD46A) or 61 (ΔD61A) of SaeR. Peroxidase activity in the presence of the MPO inhibitor sodium azide (Azide) or samples lacking MPO (no MPO) were included as negative controls. **(C)** Experiments in panel **(B)** were repeated using a D51A point mutant of *saeR* complemented with a plasmid expressing wt SaeR (ΔD51A+comp). All panels show the mean ± SEM of at least three independent experiments. For panels **(B,C)**, absorbance is shown at 20 min with $^{***}P < 0.001$ and $^{****}P < 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparisons test.

activity by extracellular proteins produced from this strain (**Figure 4C**). In contrast, supernatant from USA300Δ*saeR*-D46A and USA300Δ*saeR*-D61A blocked the activity of human MPO.

Human PMN Plasma Membrane Permeability Caused by USA300 Requires Aspartic Acid at Residue 51 of SaeR

Previous studies have demonstrated that the SaeR/S two-component system upregulates *S. aureus* toxins that target human PMNs (Gauduchon et al., 2001; Voyich et al., 2009; Nygaard et al., 2010; Ventura et al., 2010; DuMont et al., 2011; Malachowa et al., 2011). To assess the importance of aspartic acid residues within the receiver domain of SaeR for mediating toxicity against PMNs, we first examined the ability of extracellular proteins produced by D46A, D51A, and D61A point mutants of SaeR to cause human PMN plasma membrane permeability as measured by propidium iodide staining (**Figure 5A**). No plasma membrane permeability was observed for PMNs exposed to extracellular proteins produced by USA300Δ*saeR*-D51A, indicating aspartic acid residue 51 of SaeR is necessary for the toxicity of USA300 against human PMNs. A significant reduction in PMN plasma membrane permeability was also observed for extracellular proteins produced by USA300Δ*saeR*-D46A, though this difference was less than that observed for USA300Δ*saeR*-D51A. In support of these findings, complementation of USA300Δ*saeR*-D46A, or USA300Δ*saeR*-D51A with wild-type SaeR rescued the lytic capacity of extracellular proteins produced by these strains against human PMNs. Corresponding to the toxicity of extracellular proteins produced by these strains, significantly less PMN plasma membrane permeability was observed following phagocytosis of live USA300Δ*saeR/S*, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D46A relative to USA300 (**Figure 5B**). These results demonstrate that SaeR aspartic acid residue 51 and, to a lesser extent, aspartic acid residue 46 are important for activity of this two-component system.

Aspartic Acid Residue 51 of SaeR Is Essential for the Pathogenesis of USA300 During Murine Soft-Tissue Infections

Previously published findings demonstrate the upregulation of virulence gene transcription *in vivo* by the SaeR/S two-component system is essential for the pathogenesis of *S. aureus* during both systemic and localized infection (Voyich et al., 2009; Nygaard et al., 2010). Transcriptional analysis of USA300Δ*saeR*-D51A in this report (**Figure 2**) indicates aspartic acid residue 51 of SaeR is required for upregulating *S. aureus* virulence gene transcription mediated by SaeR/S *in vitro*. To determine if potential SaeR phosphorylation sites are important for pathogenesis *in vivo*, we assessed the virulence of USA300, USA300Δ*saeR/S,* USA300Δ*saeR*-D46A, USA300Δ*saeR*-D51A, and USA300Δ*saeR*-D61A during a murine model of soft-tissue infection (**Figure 6**). In a manner indistinguishable from USA300Δ*saeR/S*, soft-tissue infections caused by USA300Δ*saeR*-D51A were significantly smaller than infections caused by USA300 (**Figure 6A**) and did not exhibit the open dermonecrotic lesions characteristic of USA300 pathogenesis (**Figure 6B**) that is attributed to the high expression of Hla by this strain (Kennedy et al., 2010). In addition, a significant decrease in the weight of mice following infection with USA300 was not observed following inoculation with USA300Δ*saeR/S* or USA300Δ*saeR*-D51A (**Figure 6C**). These findings could not be explained by differences in inoculum

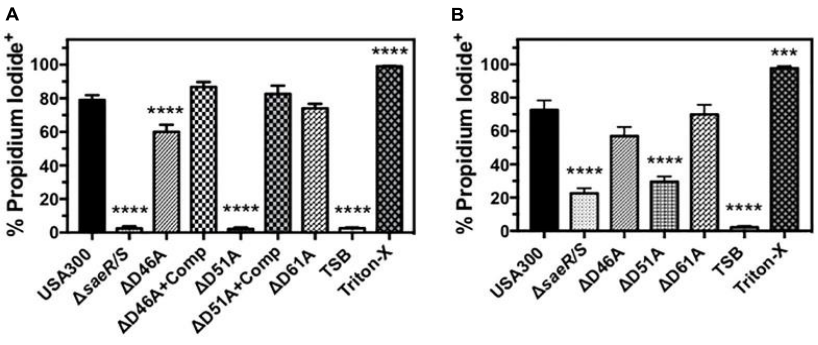


FIGURE 5 | Human PMN plasma membrane permeability caused by USA300 is significantly influenced by aspartic acid residues 46 and 51 of SaeR. **(A)** Flow cytometry was used to assess the percentage of purified human PMNs permeable to propidium iodide 90 min after exposure to 5 h supernatants from USA300, an isogenic deletion mutant of *saeR/S* in USA300 (USA300Δ*saeR/S*), or USA300 expressing SaeR with aspartic acid to alanine substitutions at residue 46 (ΔD46A), 51 (ΔD51A), 61 (ΔD61A), and ΔD46A or ΔD51A complemented with plasmids expressing wt SaeR (ΔD46A+Comp or ΔD51A+comp, respectively). **(B)** The percentage of propidium iodide positive human PMNs 90 min after the phagocytosis of live *Staphylococcus aureus* at a ratio of 20 colony forming units per PMN. For these experiments, PMNs were also treated with tryptic soy broth (TSB) alone or TSB with 0.5% Triton X-100 (Triton-X). All panels show the mean ± SEM of at least 5 independent experiments. ***$P \leq 0.001$ and ****$P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

concentration given to mice for each *S. aureus* strain tested (**Figure 6D**). As opposed to USA300Δ*saeR*-D51A, no differences could be distinguished between soft-tissue infections caused by USA300Δ*saeR*-D46A or USA300Δ*saeR*-D61A relative to USA300 (**Figures 6A–C**). These results demonstrate that USA300 pathogenesis during murine soft-tissue infection is dependent upon aspartic acid residue 51 of SaeR while aspartic acid residues 46 and 61 are not essential for causing disease.

DISCUSSION

Bacterial pathogens must recognize environmental cues and respond appropriately to cause disease. For *S. aureus*, the SaeR/S two-component system plays an important role during pathogenesis by sensing host-specific signals and up-regulating virulence gene expression in response (Geiger et al., 2008; Zurek et al., 2014). In this report, we show that the substitution of the putative SaeR phosphorylation site at aspartic acid residue 51 completely ameliorates SaeR/S-mediated virulence. Specifically, we observed a dramatic decrease in the transcript abundance of various hemolysins and leukocidins in USA300 lacking aspartic acid residue 51 of SaeR. These findings correspond to a decrease in the ability of this strain to lyse human erythrocytes and PMNs. SaeR aspartic acid residue 51 was also shown to be important for the transcription of the human MPO inhibitor SPIN and USA300 lacking SaeR aspartic acid residue 51 did not inhibit human MPO activity. Moreover, we show that the virulence of USA300Δ*saeR*-D51A during murine models of soft-tissue infection paralleled the transcript abundance of Hla in this strain, consistent with previous findings demonstrating this toxin is a major *S. aureus* virulence determinant in this model (Kennedy et al., 2010). Collectively, these findings indicate that SaeR residue 51 is essential for *S. aureus* evasion of innate immunity and support other studies that suggest only phosphorylated SaeR has DNA binding activity (Sun et al., 2010).

We also observed a more subtle decrease in the toxicity of USA300 against human erythrocytes and PMNs in the absence of the conserved aspartic acid residue 46 of SaeR. It is not clear if substitution of this residue simply perturbs the receiver domain structure of SaeR to diminish activity or if aspartic acid residue 46 plays a more direct role in the phosphorylation state of this response regulator. Regardless, a reduction in the lytic capacity of USA300Δ*saeR*-D46A suggests this conserved residue also influences SaeR function and will be further examined in future studies.

This study demonstrates that the substitution of a single amino acid residue in the SaeR response regulator of USA300 renders this highly pathogenic strain avirulent, highlighting the critical importance of two-component sensory systems for bacterial pathogenesis. Indeed, others have shown that point mutations in the dimerization interface of the histidine kinase sensor AgrC (Mairpady Shambat et al., 2016) or in the P2 promoter region of the Agr two-component system (Villaruz et al., 2009) have a profound effect on the lytic capacity and colonization potential of *S. aureus* while a point mutation in the histidine kinase sensor LiaS of *Streptococcus pyogenes* contributes to the carrier phenotype of this bacterium (Flores et al., 2017). Interestingly, no natural mutations of aspartic acid residue 51 could be identified in over 8,000 *S. aureus* genomes, suggesting this putative phosphorylation site is imperative for the fitness of this pathogen. Taken together, these studies indicate that novel therapeutic strategies that inhibit the activation of this highly conserved response

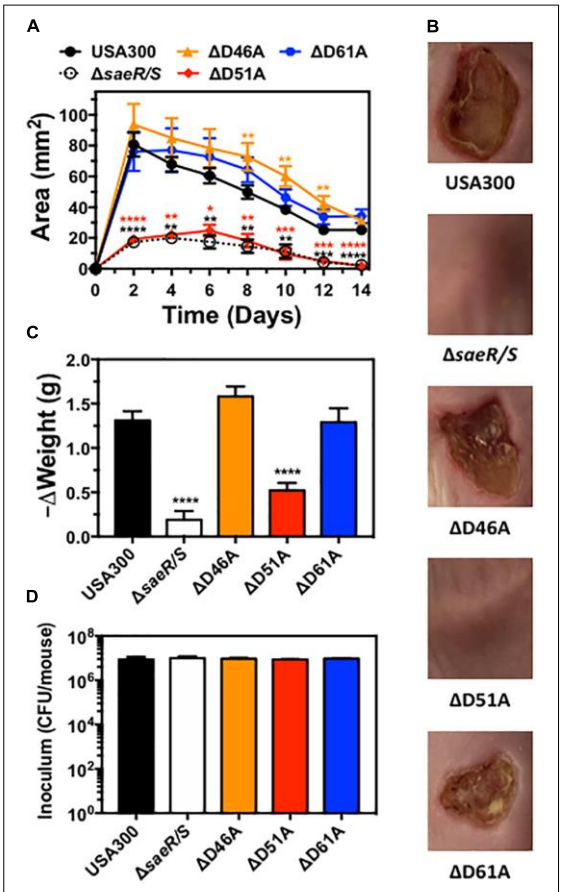


FIGURE 6 | Aspartic acid residue 51 of SaeR significantly influences the pathogenesis of murine soft-tissue infections caused by USA300. **(A)** Area of soft-tissue infection caused by USA300, an isogenic deletion mutant of *saeR/S* in USA300 (Δ*saeR/S*), or USA300 with an aspartic acid to alanine substitution at SaeR residue 46 (ΔD46A), 51 (ΔD51A) or 61 (ΔD61A). **(B)** Representative images of soft-tissue infections from experiments in panel **(A)** on day 6 post-inoculation. **(C)** Change in the weight of mice during experiments in panel **(A)** between days 0 and 2 post-inoculation. **(D)** Inoculum of each strain given to mice in panels **(A-C)**. Panels **(A)**, **(C)**, and **(D)** show the mean ± SEM of two independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ relative to USA300 as determined by one-way ANOVA with Dunnett's multiple comparison test.

regulator would impede the expression of multiple virulence factors and effectively block different aspects of pathogenesis resulting in clearance of *S. aureus* by innate immune mechanisms.

AUTHOR CONTRIBUTIONS

TN and JV contributed to the conception and design of this study. TN, TB, ES, FG, JD, MC, KP, LC, and BK performed the experiments and data analysis. TN and JV wrote and prepared the manuscript for submission. All authors read and approved this manuscript.

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SUPPLEMENTARY MATERIAL

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AUTHOR CONTRIBUTIONS

TN and JV contributed to the conception and design of this study. TN, TB, ES, FG, JD, MC, KP, LC, and BK performed the experiments and data analysis. TN and JV wrote and prepared the manuscript for submission. All authors read and approved this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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