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THE *STAPHYLOCOCCI* AND STAPHYLOCOCCAL PATHOGENESIS

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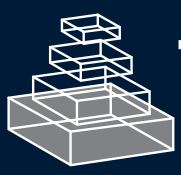

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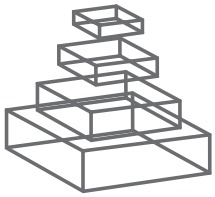

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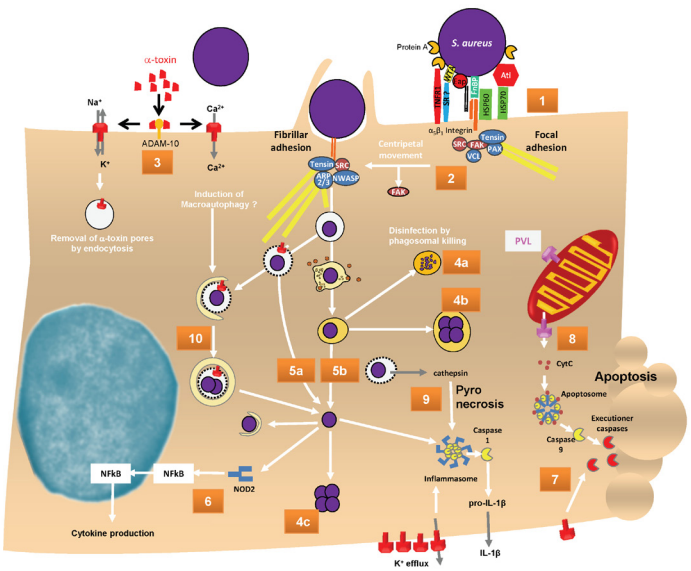
THE *STAPHYLOCOCCI* AND STAPHYLOCOCCAL PATHOGENESIS

Hosted By:
David Heinrichs, University of Western Ontario, Canada
Martin J. McGavin, University of Western Ontario, Canada



Members of the genus *Staphylococcus* play important roles in disease causation in humans and animals. Over the past decade, the completed sequencing of many staphylococcal genomes has contributed to a surge in the number of publications, which have promoted a tremendous advance in our knowledge of these important pathogens. Significant developments include the emergence of new and highly virulent strains of *S. aureus*, advances in tracking the evolution of human and animal adapted strains, a heightened appreciation of the role of mobile genetic elements in antibiotic resistance and pathogenesis, and important insights into staphylococcal physiology, immune evasion strategies, and cell surface proteins, as well as significant advances in vaccine development and therapeutics. This Research Topic will focus attention on the latest developments in these areas as they pertain to *S. aureus* and members of the coagulase-negative *Staphylococci*, and will also strive to identify areas of future development.

Table of Contents

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EDITORIAL
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The staphylococci and staphylococcal pathogenesis

Martin J. McGavin* and David E. Heinrichs*

Department of Microbiology and Immunology, Western University, London, ON, Canada
**Correspondence: mmcgavin@uwo.ca; deh@uwo.ca*

Over the past 30 years, the scientific literature has been rife with articles that have chronicled the enduring threat and changing nature of *Staphylococcus aureus* as a leading cause of infectious morbidity and mortality to humans (Sheagren, 1984; Archer, 1998; Lowy, 1998; Otto, 2010). The most current surveys indicate that *S. aureus* is responsible for almost 500,000 hospitalizations and 30,000 deaths annually in the USA (Klein et al., 2007). At the turn of the twentieth century, the major threat was hospital-associated strains of methicillin resistant *S. aureus* (HA-MRSA) that are resistant to multiple antibiotics, and reports that some had acquired high level vancomycin resistance (Pearson, 2002; Weigel et al., 2003) seemed to assure the inevitable proliferation of strains that are resistant to all antibiotics. Although this crisis has not yet materialized, we have instead experienced the sudden emergence and global dissemination of hyper-virulent strains of community acquired MRSA (CA-MRSA). Another unexpected threat was the emergence of CA-MRSA in animal adapted strains of *S. aureus*, and their zoonotic transmission to humans. Conversely, some livestock-associated strains have undergone a recent evolutionary transition from human to animal hosts. Amidst this backdrop of newly emergent strains, common clonal types of HA-MRSA and methicillin susceptible *S. aureus* (MSSA) persist in their threat to health care, and attempts to lessen the impact through vaccine development have thus far been unsuccessful.

In this Special Topics issue, recent advances in Staphylococcal research are captured in a collection of research, review, opinion, and methods articles, which we have assigned to the general themes of vaccine development, virulence, and immune evasion, metabolic activity in response to host environment, methods development, and comparative genomics and genome evolution.

VACCINE DEVELOPMENT

On the front lines of vaccine development, an opinion article from Bagnoli et al. (2012) at Novartis offers plausible explanations for the vaccine failures that have plagued human clinical trials, and offer optimism that success can be achieved with an appropriate multivalent vaccine that stimulates cellular immunity and opsonophagocytosis, while inhibiting bacterial viability and/or toxicity. Pancari et al. (2012) outline the vaccine strategy and progress at Merck Research labs, defining the mechanistic basis of protection offered by a vaccine that targets the IsdB protein, which is expressed *in vivo* under iron-limited growth conditions.

METABOLIC ACTIVITY IN RESPONSE TO HOST ENVIRONMENT

The host environment imposes many stresses on bacteria, among them nutrient limitation and oxidative stress imparted by host phagocytes. Proteins involved in iron acquisition are attractive vaccine components, because their expression is promoted by growth in the iron-restricted host environment. Sheldon and Heinrichs (2012) review the biogenesis, substrate specificity, and impact on host innate immune response, of cell envelope lipoproteins that are up-regulated in response to low iron. Gaupp et al. (2012) summarize the cellular targets of oxidative stress, mechanisms employed to sense oxidative stress and damage, oxidative stress protection and repair mechanisms, and regulation of the oxidative stress response. Original research by Fuller et al. (2011) defines the role of a lactate-quinone oxidoreductase (Lqo) in countering oxidative stress imposed by host phagocyte derived nitric oxide, and show that this cytoplasmic protein is essential for virulence in a murine model of sepsis, particularly in association with myocarditis.

VIRULENCE STUDIES

Although most manifestations of *S. aureus* disease are attributed to its growth as an "extracellular" pathogen, it is gaining new respect for its ability to survive within host cells as a facultative intracellular pathogen. Fraunholz and Sinha (2012) present a guide to recent developments in this emerging field of study, summarizing the variety of intracellular fates of *S. aureus*, including artwork that appears on the cover of this issue. Original research by Spaulding et al. (2012) shows that major clonal types of *S. aureus* known for causing hospital and community acquired infections, exhibit distinct differences in their capacity for cardiotropic versus lethal sepsis infections, and that production of superantigen toxins and cytolysins account for some of these differences. Expanding on the theme that some strains specialize in certain types of infection, Anderson et al. (2012) describe an *ex vivo* porcine vaginal mucosa model to evaluate the contribution of cytolysins in biofilm formation and tissue damage caused by *S. aureus* strains associated with menstrual toxic shock syndrome. Xu and McCormick (2012) review molecular biology of staphylococcal superantigen toxins, including toxic shock syndrome toxin TSST, and offer a unique perspective on superantigen toxins, which includes a proposed role in colonization, in addition to disease progression. Vandenesch et al. review developments in understanding the potentially redundant arsenal of membrane-damaging virulence factors at the disposal of *S. aureus*, and their interplay with the host immune system (Vandenesch et al., 2012). Hanke and Kielian (2012) have authored a review that summarizes the immune defenses that staphylococcal biofilms evade, and place specific emphasis on the idea that staphylococcal biofilms skew the host immune response away from a proinflammatory bactericidal phenotype toward an anti-inflammatory, pro-fibrotic response that favors bacterial persistence.

GENE REGULATION

An emerging theme in regulation of gene expression by microbial pathogens is that of regulatory RNA's and control of RNA stability. Morrison et al. (2012) extend this theme, identifying a new role for the SarA transcriptional regulator of *S. aureus*, in binding to and altering the mRNA turnover properties of target transcripts. Cue et al. (2012)

review how various regulatory factors affect production of poly *N*-acetyl glucosamine, an intercellular polysaccharide adhesin that contributes to biofilm related infections in both *S. aureus* and *S. epidermidis*.

METHODS

An increasing appreciation of the role of biofilms in persistent infection and immune evasion highlights the importance of standard techniques to evaluate biofilm structure and genetics of biofilm formation. Walker and Horswill (2012) describe a simple method for quantitative evaluation of *S. aureus* biofilms on glass coverslips coated with human plasma. Our current and future knowledge of gene function could not be achieved without the less heralded development of efficient techniques for construction of gene deletion mutations. Monk and Foster (2012) review the restriction-modification barrier in Staphylococci, and how knowledge of these systems can be used to develop plasmid vectors that can be used to manipulate previously un-transformable strains.

COMPARATIVE GENOMICS AND EVOLUTION

A comparative genomics evaluation by Ben Zakour et al. (2012) identifies new directions for research into pathogenesis and patho-adaptation of the *S. intermedius* group of animal pathogens, revealing extensive differences in the accessory genome content between closely related species that nevertheless inhabit distinct host niches. Research by McGavin et al. (2012) reveal three distinct clades within *S. aureus* clonal complex CC30, of which Clade 3 comprised of hospital-associated strains exhibit numerous features that are consistent with evolution through niche adaptation mechanisms. Finally, McCarthy et al. (2012) have analyzed 79 sequenced *S. aureus* genomes to assess the role of bacteriophage in dissemination of toxins and immune evasion genes, finding that horizontal transfer is restricted by bacteriophage family, and lineage of the host bacterium.

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OPINION ARTICLE
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Inferring reasons for the failure of *Staphylococcus aureus* vaccines in clinical trials

Fabio Bagnoli*, Sylvie Bertholet and Guido Grandi

Novartis Vaccines and Diagnostics, Siena, Italy
**Correspondence: fabio.bagnoli@novartis.com*

Multidrug resistant *Staphylococcus aureus* strains are emerging and current antibiotics are not efficacious against such strains. Therefore, there is an urgent need to develop vaccines to target this pathogen. However, both active and passive immunization strategies have thus far failed to show efficacy in humans. There are several potential reasons behind the disappointing results of clinical trials, however, we believe that just a few of them, which are common to all the trials, determined their downfall. First of all, preclinical results obtained with antigens tested in clinical trials were likely overestimated by vaccine manufacturers. Furthermore, vaccines tested in humans to date, since they all targeted single antigens, were probably disproportionate to the complex pathogenic mechanisms of the bacterium. In addition, the lack of known correlates of protection in humans has severely limited the ability to interpret both preclinical and clinical data. Finally, the vaccines did not contain new generation adjuvants, which may be critical in augmenting antibody production and steering the T-cell response toward the proper profile of cytokine production.

ANTIGENS TESTED IN CLINICAL TRIALS GENERATE ONLY PARTIAL PROTECTION IN ANIMAL MODELS

> We often read in recent publications the statement: "the vaccine failed in clinical trials despite being protective against staphylococcal infection in animal models."
>
> We think that this parallel should not be made.

Staphylococcus aureus capsular polysaccharides type 5 and 8 (CP5 and 8) conjugated with recombinant *Pseudomonas aeruginosa* exoprotein A were included in the first vaccine tested in humans, StaphVax (by Nabi Biopharmaceutical), which failed in phase III clinical trial in 2005. No consistent protective efficacy appears to be associated with CP8 immunization (Tuchscherr et al., 2008; Cook et al., 2009), and this may have significantly contributed to the clinical failure of StaphVax. Indeed, CP8 is expressed by approximately 40% of the circulating strains and therefore the poor protective efficacy associated with this antigen could have significantly affected the trials. On the other hand, although vaccination with CP5 has been shown to be efficacious in animal models of staphylococcal infection, *S. aureus* USA300, one of the most important clones worldwide, has recently been found to elaborate no detectable capsule because of a point mutation in the promoter region of *cap*-5, necessary for CP5 biosynthesis (Montgomery et al., 2008). Furthermore, the role of CPs in *S. aureus* virulence is not clear and their expression is limited to the stationary phase (O'Riordan and Lee, 2004; Schaffer and Lee, 2008).

Given all these variables, we think that the only approach to reliably measure vaccine efficacy in animal models is to perform challenge experiments with a large collection of strains representative of the circulating staphylococcal clones, and use different models with different routes of infection as well as low and large bacterial inocula. By doing that, vaccine efficacy will be tested in a number of different conditions, some of which may be more favorable than others. Ideally, a vaccine should consistently protect in all conditions.

A second failure occurred with an active immunization strategy based on a single surface staphylococcal protein, IsdB (Kuklin et al., 2006; Spellberg and Daum, 2011). The phase II/III vaccine clinical trial was interrupted, however, the precise reason for its premature interruption is not yet completely understood (Spellberg and Daum, 2011). IsdB is a protein which mediates iron uptake from hemoglobin. The protein shows several favorable characteristics as a vaccine candidate. Indeed, IsdB is conserved and expressed throughout different *S. aureus* strains and is an important virulence factor (Kuklin et al., 2006; Kim et al., 2010). However, as an antigen, the protein generated only partial protection in lethal infection mouse models (Kuklin et al., 2006; Stranger-Jones et al., 2006).

On top of active immunization, a number of passive approaches have also been undertaken: Veronate, based on polyclonal antibodies against the *S. aureus* surface protein ClfA; Altastaph, containing CP5 and CP8 antibodies purified from subjects vaccinated with StaphVax; Tefibazumab, monoclonal antibodies against ClfA; and Aurograb, single chain antibodies against an ABC transporter of the pathogen. They all failed to show efficacy against *S. aureus* infection in humans (Schaffer and Lee, 2008; Ohlsen and Lorenz, 2010; Otto, 2010).

The vaccines tested so far in humans differ quite substantially in the nature of the immunogen used, the immunization approach and the target population. However, there is an obvious commonality between them: they all target a single component of the pathogen. Since *S. aureus* expresses a plethora of toxins and immune evasion factors, efficacy of monovalent vaccines is likely to be insufficient in humans and this is reflected by partial protection achieved in animal models.

VACCINES SO FAR TESTED IN CLINICAL TRIALS ARE NOT THE BEST AVAILABLE OPTION

We strongly believe that a multivalent vaccine is needed against *S. aureus*. Indeed, it has already been demonstrated that protein combinations can generate additive protection (Stranger-Jones et al., 2006; Kim et al., 2011). In particular a combination of four surface proteins (IsdB, IsdA, SdrD, and SdrE) was shown to protect mice from lethal infection with *S. aureus* Newman strain with greater efficacy than any of the single components (Stranger-Jones et al.,

2006). Importantly, in this model IsdB, the candidate tested by Merck in clinical trials, failed to generate protective immunity. The additive efficacy may be due to the different functions played by the four antigens in *S. aureus* virulence. This hypothesis is supported by the observation that a *S. aureus* SrtA knockout mutant, which lacks all the LPXTG-containing proteins (including IsdB, IsdA, SdrD, and SdrE), is less virulent than any mutant of individual LPXTG proteins (Cheng et al., 2009). Stranger-Jones et al. (2006) also tested the protective efficacy of several other *S. aureus* antigens in a murine renal abscess model in terms of bacterial load reduction. ClfA, the target of two clinical trials based on passive immunization, was found to be inferior to IsdB. Therefore, it is conceivable to assume that strategies targeting ClfA only, may not be sufficiently protective. In a more recent paper two protein combinations (ClfA, FnBPB, and SdrD) and (ClfA, FnBPB, SdrD, and a non-toxigenic form of protein A, SpAKKAA) were shown to be highly protective in two different animal models of staphylococcal infection (Kim et al., 2011). Interestingly, the four-component combination elicited greater protection than the trivalent vaccine. Similar results were observed with passive immunization studies, such as the antibody combination of ClfA and FnBPA (Arrecubieta et al., 2008). Our internal research with several antigens known in the literature as well as novel candidates confirms that, in general, combinations generate greater protection than single antigens (unpublished data).

PROTECTIVE MECHANISMS AGAINST *S. AUREUS* REMAIN TO BE ELUCIDATED

The lack of correlates of protection for *S. aureus* is one of the major challenges for vaccine design and development. The failures of passive immunization strategies in clinical trials, advocate against an important role for antibodies in mediating protection against *S. aureus*. However, we believe that both preclinical and clinical data suggest that humoral responses play, at least, a partial role in protecting against the pathogen. Passive transfer of antibodies raised against different staphylococcal antigens (e.g., Hla, IsdA, IsdB) in animal models, confers protection against the infection (Bubeck Wardenburg and Schneewind, 2008; Kim et al., 2010). On the other hand, *in vitro* assays have shown that antibodies can have a direct role in inhibiting the function of virulence factors and toxins. For example, antibodies can neutralize the toxicity of Hla or interfere with heme-iron scavenging mediated by IsdB and IsdA (Bubeck Wardenburg and Schneewind, 2008; Kim et al., 2010). Moreover, antibodies against several antigens have been shown to mediate opsonophagocytosis (Stranger-Jones et al., 2006). From the clinical point of view, the scenario is much less clear. It is very well know that most individuals have circulating antibodies to *S. aureus* (Dryla et al., 2005; Clarke et al., 2006; Verkaik et al., 2009), however it is not known if they confer any protective immunity (Hermos et al., 2010). In our mind, an indication that antibodies do play, at least, a partial role in protecting humans against severe staphylococcal infections, comes from the observation that colonized patients present milder disease outcomes as compared to non-colonized patients (Wertheim et al., 2004). Indeed, colonized subjects have been shown to have higher antibodies titers against several staphylococcal antigens (Verkaik et al., 2009). Finally, sera from vaccines of StaphVax trials were found to mediate opsonophagocytosis and human sera of volunteers immunized with CP5 to confer passive protection in a murine infection model (Fattom et al., 1996, 2004). As already discussed for active vaccination strategies, failure of trials conducted with passive immunization may be due to the insufficient efficacy achieved targeting single antigens. In addition, immune evasion mechanisms deployed by *S. aureus*, may abolish or dampen anti-*S. aureus* humoral responses in humans (Serruto et al., 2010a; Kim et al., 2012).

S. AUREUS IMMUNE EVASION FACTORS MAY REPRESENT A MAJOR CHALLENGE IN DEVELOPING EFFICACIOUS VACCINES

S. aureus expresses a plethora of factors which, in different manners, inhibit the host immune response against the pathogen. Among them, the IgG-binding proteins SpA and Sbi, and complement-binding factors have been shown to efficiently interfere with antibody-mediated protection mechanisms (Serruto et al., 2010a; Kim et al., 2012). A critical aspect of some of these factors is the human specificity. For example SCIN, CHIPS, and SAK, which directly or indirectly inhibit C3 convertase, C5a receptor, and C3b, respectively, are all human specific (Rooijakkers et al., 2005; Serruto et al., 2010b). It is therefore likely that the contribution of these factors in evading the host immune response is underestimated in animal models. Therefore, protective efficacy of vaccines tested in animals may generally be greater than in humans. Development of improved animal models, such as humanized mice, and reliable surrogates of protection, such as the opsonophagocytosis assay, is another critical aspect that the scientific community needs to address to increase the likelihood of success of *S. aureus* vaccines. On the other hand, the presence of such mechanisms indicates that antibody-mediated protection is potentially important against *S. aureus*. Therefore, to develop efficacious vaccines we need to understand how to avoid or compensate for the detrimental effect of complement evasion factors on the host immune response.

CELL-MEDIATED IMMUNITY MAY BE A KEY ELEMENT OF *S. AUREUS* VACCINES

Patients with disruption of anatomical barriers, and those with quantitative and qualitative T-cell or neutrophil disorders are definitively at increased risk of developing staphylococcal infections (Spellberg and Daum, 2011).

These observations were recapitulated in animal models demonstrating that B cell-deficient mice were no more susceptible to systemic infection caused by *S. aureus* compared with wild-type mice (Spellberg et al., 2008), while Tcell-deficient (Spellberg et al., 2008), IFN-γ-deficient (Spellberg et al., 2008; Lin et al., 2009), TNF-deficient (Hultgren et al., 1998), and dual IL-17A/F-deficient (Ishigame et al., 2009) mice were hypersusceptible to *S. aureus* infection. Furthermore, Th17 cells were necessary for vaccine-induced protection against *S. aureus* infection by enhancing neutrophil recruitment to sites of infection, and killing of the bacteria (Lin et al., 2009). Superoxide-deficient mice (a model of chronic granulomatous disease) were also more susceptible to infection. Altogether, these data indicate that protection against *S. aureus* infections requires intact phagocytic function and is markedly enhanced by

Th1/Th17 adaptive immunity, suggesting that cell-mediated focused vaccines could effectively protect against *S. aureus* infections.

ADJUVANTS MAY BE IMPORTANT TO STEER THE RIGHT RESPONSE AGAINST *S. AUREUS*

Traditionally, adjuvants have been used to increase antibody-mediated responses. Aluminum hydroxyde (alum), oil-in-water emulsions (e.g., MF59 and AS03), have been shown to increase antibody titers, effectiveness, and even functionality (Galli et al., 2009; Jones, 2009; Dormitzer et al., 2011). However, the important role of adjuvants in stimulating T-cell responses is also becoming clear. Recently, the response generated by MF59 was demonstrated to be skewed toward Th1 by the addition of E6020, a synthetic analog of MPL (monophosphoryl lipid A; Baudner et al., 2009; Dormitzer et al., 2011). In another study, IC31, a KLK peptide plus non-CpG oligonucleotide, was demonstrated to, conversely to alum, induce strong Th1 and Th17 responses (Kamath et al., 2008). Therefore, on top of the proper antigen combination, design of adjuvant formulations inducing higher and more functional antibodies, and stimulating T-cell-mediated immunity will certainly be another critical area of investigation.

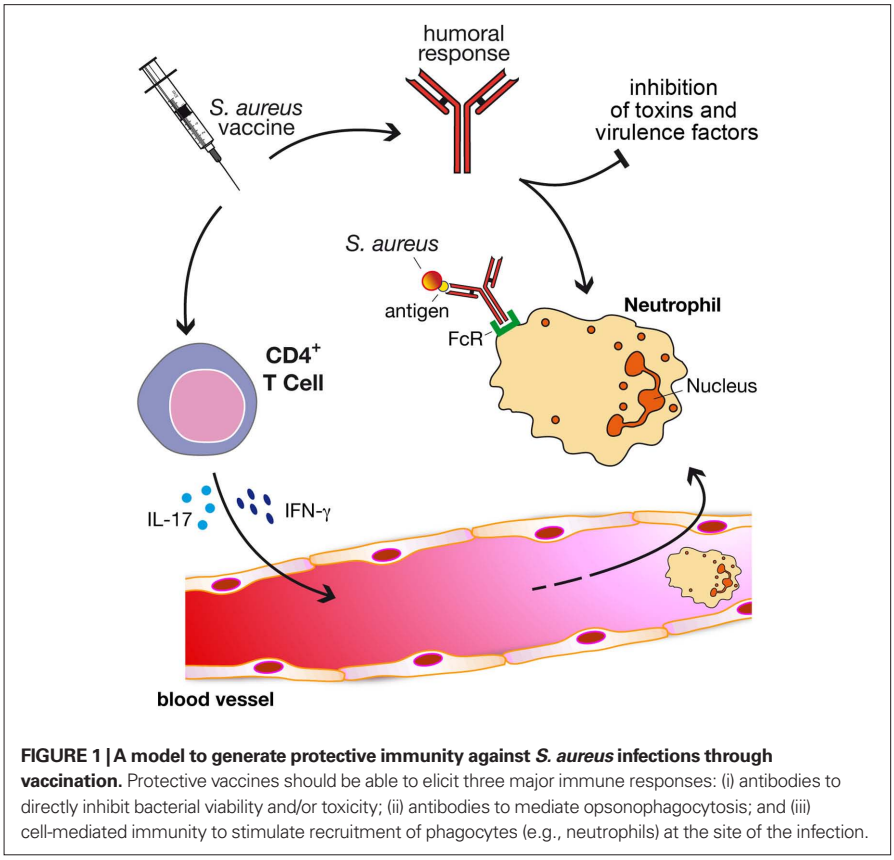

FIGURE 1 | A model to generate protective immunity against *S. aureus* infections through vaccination. Protective vaccines should be able to elicit three major immune responses: (i) antibodies to directly inhibit bacterial viability and/or toxicity; (ii) antibodies to mediate opsonophagocytosis; and (iii) cell-mediated immunity to stimulate recruitment of phagocytes (e.g., neutrophils) at the site of the infection.

CONCLUSION

In our opinion, the spreading fear that an efficacious vaccine against *S. aureus* is not feasible, is not justified. Doubts expressed by several scientists are obviously due to the numerous failures in clinical trials and to the lack of known correlates of protection in humans (Patti, 2011; Proctor, 2012; Spellberg and Daum, 2011). Furthermore, concerns are also based on the observation that failed vaccines were assumed to be efficacious in animal models. However, vaccines tested in humans so far generated only partial protection against staphylococcal infection in animal models. All vaccines tested in clinical trials targeted a single *S. aureus* component. Studies using antigen combinations have shown greater efficacy than single antigen vaccines in animal models. Therefore, multivalent vaccines will likely work better in humans as well. At the same time, we should be cautious in making direct correlations between animal studies and clinical trials. Indeed, although several animal infection models have been established for assessing preclinical protective efficacy of vaccines, they may not optimally resemble natural infections in humans.

Accumulating literature is unraveling *S. aureus* pathogenic and immune evasion mechanisms. It will be important to use this information to target critical factors involved in these processes with next generation vaccines and to develop improved animal models and surrogates of protection. Although the nature of protective immunity against the pathogen in humans is not known, animal studies as well as clinical observations indicate that both humoral as well as cell-mediated immunity are involved.

On the basis of that, we propose a model (**Figure 1**) in which vaccine efficacy is gained through three major immune responses: (i) antibodies to directly inhibit bacterial viability and/or toxicity; (ii) antibodies to mediate opsonophagocytosis; and (iii) cell-mediated immunity to stimulate recruitment of phagocytes at the site of the infection. It is very likely that only a combination of staphylococcal antigens with different properties and functions formulated with adjuvants able to elicit a potent antibody production, but also the proper cellular response, will satisfy the three criteria.

In conclusion, we are confident that by performing extensive preclinical work, using different animal models, readouts, and challenge strains, *S. aureus* vaccines with much greater chances of success in clinical trials can be developed.

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Characterization of the mechanism of protection mediated by CS-D7, a monoclonal antibody to *Staphylococcus aureus* iron regulated surface determinant B (IsdB)

Gregory Pancari[1], Hongxia Fan[1], Sharon Smith[1], Amita Joshi[1], Robin Haimbach[1], Desmond Clark[1], Yingzhe Li[2], Jin Hua[2], Troy McKelvey[2], Yangsi Ou[2], James Drummond[2], Leslie Cope[1], Donna Montgomery[2] and Tessie McNeely[1]*

[1] *Department of Vaccine Basic Research, Merck Research Labs, Merck/MSD, West Point, PA, USA*
[2] *Department of Biologics Research, Merck Research Labs, Merck/MSD, West Point, PA, USA*

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Olaf Schneewind, University of Chicago, USA
Jean C. Lee, Brigham and Women's Hospital, USA
Robert S. Daum, University of Chicago, USA

****Correspondence:***
Tessie McNeely, Department of Vaccine Basic Research, Merck Research Labs, Merck/MSD, WP26-253, West Point, PA 19486, USA.
e-mail: tessie_mcneely@merck.com

We previously reported the development of a human monoclonal antibody (CS-D7, IgG_1) with specificity and affinity for the iron regulated surface determinant B (IsdB) of *Staphylococcus aureus*. CS-D7 mediates opsonophagocytic killing *in vitro* and protection in a murine sepsis model. In light of recent data indicating that IsdB specific T cells (CD4+, Th17), not Ab, mediate protection after vaccination with IsdB, it is important to investigate the mechanism of protection mediated by CS-D7. The mAb was examined to determine if it blocked heme binding to IsdB *in vitro*. The mAb was not found to have heme blocking activity, nor did it prevent bacterial growth under *in vivo* conditions, in an implanted growth chamber. To assess the role of the mAb Fc a point mutation was introduced at aa 297 (CS-D7·N297A). This point mutation removes Fc effector functions. *In vitro* analysis of the mutein confirmed that it lacked measurable binding to FcγR, and that it did not fix complement. The mutein had dramatically reduced *in vitro* opsonic OP activity compared to CS-D7. Nonetheless, the mutein conferred protection equivalent to the wild type mAb in the murine sepsis model. Both wild type and mutein mAbs were efficacious in FcγR deletion mice (including both $Fc\gamma RII^{-/-}$ mice and $Fc\gamma RIII^{-/-}$ mice), indicating that these receptors were not essential for mAb mediated protection *in vivo*. Protection mediated by CS-D7 was lost in Balb/c mice depleted of C3 with cobra venom factor (CFV), was lost in mice depleted of superoxide dismutase (SOD) in P47phox deletion mice, and as previously reported, was absent in SCID mice (Joshi et al., 2012). Enhanced clearance of *S. aureus* in the liver of CS-D7 treated mice and enhanced production of IFN-γ, but not of IL17, may play a role in the mechanism of protection mediated by the mAb. CS-D7 apparently mediates survival in challenged mice through a mechanism involving complement, phagocytes, and lymphocytes, but which does not depend on interaction with FcγR, or on blocking heme uptake.

Keywords: iron regulated surface determinant B (IsdB), *Staphylococcus aureus*, vaccination, passive immunization, opsonophagocytosis

INTRODUCTION

Staphylococcus aureus is a significant cause of hospital acquired bloodstream and catheter infections (Thwaites et al., 2011), and is a leading cause of endocarditis, osteomyelitis, and skin and soft tissue infections (Lowy, 1998a). Although it has been extensively investigated, natural protective immunity against *S. aureus* is poorly understood. Acute infection with *S. aureus* does not prevent re-infection with this bacteria (Lee, 1996). Preclinical and clinical data indicate that immunization with intact bacteria induces high serum antibody immune titers to staphylococcus, but does not confer protection against *S. aureus* infection (Lee, 1996; Schaffer and Lee, 2008). *In vivo* bacterial clearance is currently believed to be facilitated by antibody (Ab) and complement mediated uptake and killing by phagocytes (Peterson et al., 1978; Leijh et al., 1981; Verbrugh et al., 1982; Gregory et al., 1996; Verdrengh and Tarkowski, 1997; Cunnion et al., 2004). *S. aureus* is a commensal species that colonizes people transiently or permanently, and therefore, individuals have

Abbreviations: IsdB, iron regulated surface determinant B; OP, opsonophagocytic uptake activity; CVF, cobra venom factor; IC, immune complexes; ECD, extracellular domains.

antibodies to *S. aureus*. Iron regulated surface determinant B (IsdB) is an antigen expressed on the cell surface of *S. aureus* in iron limited environments, with a MW of approximately 72 kD. Its function is to capture and import heme iron from hemoglobin (Mazmanian et al., 2003). Due to the low iron environment of mammalian blood and tissue, IsdB is upregulated during infection *in vivo* (Brown et al., 2009). Humans, as well as mammals examined to date, have pre-existing antibody titers to IsdB (Lowy, 1998b), but it is unknown whether these pre-existing titers offer protection.

We previously reported the development of a fully human monoclonal antibody (CS-D7, IgG_1) specific for IsdB of *Staphylococcus aureus* (Ebert et al., 2010). The mAb was isolated from the Cambridge Antibody Technology (CAT) scFv antibody library and has high affinity and specificity for IsdB. It recognizes a conformational epitope spanning amino acids 50–285 of the antigen. As reported, this mAb had functional activity *in vitro* (opsonophagocytic killing activity) and significantly enhanced survival in the murine sepsis model in Balb/c mice. In recent work, Joshi and co-authors demonstrated that T cells not B cells were the critical effector cells conferring enhanced survival following *S. aureus* challenge in the Balb/c murine sepsis model (Joshi et al., 2012). Using IsdB specific lymphocytes adoptively transferred from wild type into SCID mice, enhanced survival was determined to be mediated entirely by IsdB specific CD4+ T cells, with B cells and Ab playing no measurable role. This observation, that IsdB specific Ab does not play a critical role in the model, is in apparent disagreement with previously published data indicating that enhanced survival in the murine sepsis model correlates with anti-IsdB Ab titers (Kuklin et al., 2006) and with data indicating that Ab can confer enhanced survival in the same Balb/c sepsis model (Brown et al., 2009; Kim et al., 2010). We, therefore, sought to investigate the protective mechanism of mAb CS-D7 in the murine sepsis model. Since this model is routinely used to verify the efficacy of vaccine antigens targeting *S. aureus* (Fattom et al., 1996; Kuklin et al., 2006; Stranger-Jones et al., 2006; Vernachio et al., 2006; Spellberg et al., 2008), it is important to define the meaning of immune mediated enhanced survival in this important animal model.

We found that although CS-D7 mediates enhanced survival in the murine sepsis model, that activity was most likely not dependent on direct inhibition of bacterial growth or survival, or on prevention of the physiological function of IsdB. We investigated the role of the mAb Fc and found that a mutein lacking Fc function conferred equivalent survival to the wild type mAb in the murine sepsis model. This was a surprising finding, as the mechanism of antigen specific mAb mediated bacterial clearance is thought to rely on engagement of the receptors for complement and Fcγ by C3b or iC3b and Ab bound to the bacteria surface. The engagement of both the Fcγ receptor and the CR1 receptor, leads to synergy of bacteria uptake and killing by effector cells (Philippe, 2004; Roozendaal and Carroll, 2006). We investigated the role of other components necessary for bacterial clearance *in vivo*, and found that complement and phagocytes were necessary for protection mediated by CS-D7 in Balb/c mice. CS-D7 mediated survival in the sepsis model is dependent on the presence of lymphocytes (Joshi et al., 2012), therefore, lymphocytes may contribute essential cytokines for stimulation of a strong phagocyte killing response. It was determined that CS-D7 may enhance survival through a non-classical Ab mediated uptake/killing mechanism. A possible mechanism will be discussed.

MATERIALS AND METHODS

RECOMBINANT PROTEINS

Recombinant IsdB was prepared as previously described (Kuklin et al., 2006). Heme-free IsdB was prepared by isolation from recombinant heme containing IsdB following peak separation on a cation binding gel resin. Separation of the two fractions of IsdB was readily made by following the absorbance at 280 nm for the presence of protein, and at 400 nm for the presence of iron containing IsdB. The extracellular domains (ECDs) of FcγRIIA and FcγRIIIA were expressed recombinantly in *Pichia pastoris* at Merck/MSD (Rahway, NJ) (Li et al., 2006). After filtration, the FcγR's were isolated by Ni^{2+} affinity columns and further purified by hydrophobic interaction chromatography. SDS-PAGE electrophoresis indicated the proteins were of the correct molecular weight and the ability of the FcγR ECD's to bind mAbs was confirmed by surface plasmon resonance.

To construct the antibody Fc variant CS-D7· N297A, mutagenic primers were designed and synthesized. Mutant clones were generated through PCR and transformation steps by using QuikChange Lightning Site-Directed Mutagenesis kit and QuikChange Lightning Multi Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA). Commercially available antibodies were acquired and used as supplied by the manufacturer. Additional antibodies were produced by transient transfection in CHO or HEK cells (Sino Biological, Beijing, China) (Ebert et al., 2010). Antibodies were stored in 6% sucrose, 100 mM arginine, 100 mM histidine, pH 6.0.

BACTERIA

The following strains were used in this investigation: *S. aureus* Becker (obtained from Chia Lee, University of Arkansas, MSSA, *spa* type 715, CC45), SA025 (clinical isolate from pancreatic cyst, MRSA, SCC*mec* IV, *spa* type 785, CC22), and 8325-4 *spa* minus (obtained from Intercell AG). Bacteria were grown on tryptic soy agar (TSA), or tryptic soy broth (TSB) overnight, pelleted and stored as frozen 15% glycerol stocks. Alternatively, bacteria were passaged two to three times to stationary phase in iron restricted medium (<0.1 mg/L), Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Grand island, NY), pelleted and stored frozen in 15% glycerol. For use in experiments, bacteria were thawed, pelleted, and resuspended in the appropriate buffer or medium.

MICE

Balb/c, P47 $phox^{-/-}$ (B6.129S2-$Ncf1^{tm1shl}$ N14) missing a key component of the superoxide generating system, and FcγRII$^{-/-}$ (Fcgr2b; C129S4(B6)-$Fcgr2b^{tm1Ttk}$/cAnNTac N12; Balb/c background) missing the FcγRIIγ protein; and FcγRI$^{-/-}$ (Fcer1g; C.129P2(B6)-$Fcerlg^{tm1Rav}$N12; Balb/c background) missing the γ chain subunit of the Fc receptors, were obtained from Taconic

(Hudson, NY), and housed in micro-isolator cages during experiments.

GROWTH OF *S. aureus* IN CHAMBERS IMPLANTED IN RATS

In vivo growth of *S. aureus* Becker was investigated using growth chambers implanted sub-dermally on the back of rats ($n = 12$). The growth chambers were made from sterile dialysis tubing, with a MW cutoff of 50 kDa, and with a volume of 1 mL. To investigate whether mAb could inhibit the survival and growth of *S. aureus* Becker, 1.4×10^6 CFU bacteria (from TSA culture) with or without 1 mg of mAb, was added to growth chambers. It was previously determined that IsdB specific mAb binding to 5×10^6 CFU of IsdB expressing *S. aureus* Becker is saturated at approximately 5 μg (Brown et al., 2009). Therefore, the bacteria were exposed to a large excess of antibody during growth in the chamber. At designated times, rats ($n = 3$–4) were sacrificed and growth chambers removed aseptically. Bacteria were harvested and colony counts (CFU/mL) were obtained through serial dilution of the bacterial culture followed by plating on TSA overnight at 37°C.

HEME BINDING UPTAKE ASSAY

See Supplemental Data.

IgG/Fcγ RECEPTOR BINDING ASSAY

FcγR ELISA binding assays were performed as previously described (Shields et al., 2001) with minor modifications. Human FcγRIIA ECD (1 μg/mL) or human FcγRIIIA ECD (2 μg/mL) diluted in PBS was coated overnight at 4°C in white 96-well Maxisorp plates (Nunc, Rochester, NY). The plates were washed three times (BioTek ELX405) with PBS (phosphate buffered saline) containing 0.05% Tween20. Serial dilutions of the test antibodies in blocking buffer [2% BSA (Jackson ImmunoResearch Laboratories, West Grove, PA) in SuperBlock T20 (Pierce, Rockford, IL)] were added for 90 min with rocking. After washing, bound antibodies were labeled with either 0.1 μg/mL (for FcγRIIA) or 1 μg/mL (for FcγRIIIA) of biotinylated anti-κ light chain $F(ab')_2$ (Southern Biotech, Birmingham, AL) followed by 100 ng/mL of Streptavidin-Europium (PerkinElmer, Waltham, MA). Time-resolved fluorescence was measured by a Victor3 spectrophotometer (Perkin-Elmer, CityplaceWaltham, StateMA) and the relative fluorescence was plotted versus the log of the antibody concentration.

COMPLEMENT C3b DEPOSITION ASSAY

An *in vitro* cell free C3b deposition assay was developed to measure functional complement activation dependent on IgG-C1q binding. High binding microtiter plates were coated with mAb in PBS (12.5 μg/mL) and incubated at 4°C overnight. Plates were blocked with 0.1% BSA in PBS for 2 h at room temperature, and washed with PBS containing Ca^{2+} and Mg^{2+}. C1q (Calbiochem, EMD Chemicals, USA) was serially diluted into C1q depleted human serum (Quidel). The mix was added to the mAb coated microtiter plate and incubated 30 min at room temperature. C3b was detected by addition of sheep antihuman C3b-FITC (Accurate Chemical and Scientific Corp., Westbury, NY) to each well for 1 h at room temperature. Assay readout was measured at 535 nm.

OPSONOPHAGOCYTIC (OP) UPTAKE ASSAY

The OP assay has been described previously (Cope et al., 2008). This assay measures Ab and complement mediated opsonophagocytosis (uptake) by HL60 phagocytes, but not killing by the phagocytes. Components for the assay were dimethylformamide (DMF) differentiated HL60 cells, baby pig sera as a source of complement, 5′-6′-FAM-SE fluorescently labeled, iron starved *S. aureus*. In this flow cytometry based assay, non-fluorescent HL60 cells were examined for the presence (via phagocytosis) of opsonized fluorescently labeled *S. aureus* 8325-4 (*spa* minus), to determine OP activity of antibodies. The bacteria strain used in the assay was chosen to avoid non-specific binding of antibody to the bacteria surface through binding to Protein A. During assay development, it was determined that a *spa* positive strain (i.e., Becker) was found to have equivalent activity to the 8325-4 strain, however for simplification of the assay and interpretation of results, strain 8325-4 was routinely used. Briefly, 2.4×10^6 iron restricted bacteria were combined with antibodies. After a short incubation of 30 min, complement was added to the mixture and incubated for an additional 30 min at 37°C. Following this incubation the bacteria were washed and labeled with FAM-SE. Then 2×10^5 HL60 cells were mixed with the bacteria at a ratio of 8 bacteria to 1 HL60 cell. The reaction mix was incubated at 37°C, 170 RPM for 30 min. The HL60 phagocytosis of bacteria was stopped by addition of cold PBS. The number of HL60 cells containing engulfed bacteria was measured on a FACSCalibur (Becton Dickinson).

PASSIVE IMMUNIZATION AND CHALLENGE MODELS

All animal work was performed in accordance with the Merck Research Laboratories Institutional Animal Care and Use committee guidelines. Antibodies were evaluated for efficacy using passive immunization of mice in the murine sepsis model (Brown et al., 2009). In preliminary experiments, *S. aureus* SA025 (from TSA culture) was titrated via i.v. injection to determine the LD_{80} dose for Balb/c mice. Next, mAb CS-D7 was dose titrated via i.p. injection prior to i.v. challenge, to achieve approximately 80% survival at day 4 post challenge (data not shown). The optimal bacterial challenge inoculum was determined to be approximately $2–3 \times 10^8$ CFU and the optimal antibody dose was 400 μg. The final model used was as follows; 400 μg of antibody per mouse ($n = 5$) was injected via the i.p. route 2 h prior to challenge of bacteria strain SA025 (from TSA culture, $2–3 \times 10^8$ CFU) via tail vein injection. Mice were monitored for survival for 96 h. Monitoring of survival for 96 h post challenge was chosen because that time period reflected the largest differences between experimental and control groups, and few mice died thereafter. Data from three or more experiments were pooled for survival analysis. To deplete mice of C3, mice were treated with CVF (Cunnion et al., 2004). The amount of CVF was titrated to give essentially no C3 in the murine sera. C3 was measured using a murine ELISA for complement factor 3 (ALPCO™ Diagnostics, Salem, NH).

MURINE CYTOKINE AND CFU MEASUREMENT

Mice ($n = 20$–24 per group) were injected i.p. with mAb followed by i.v. *S. aureus* challenge as described above. Subgroups

of 5–6 mice were sacrificed at designated time points and blood and tissue collected. Organs were placed in 2 mL of cold PBS. Blood and tissue reserved for cytokine measurement were immediately frozen by placing on dry ice. Tissues were stored at −80°C until use, at which time they were removed, homogenized and evaluated for cytokines. Murine cytokines were measured using kits from MSD (Meso Scale Discovery, Inc., Gaithersburg, MD), as per the manufacturer's instructions. Tissues reserved for CFU measurement were placed on wet ice and stored at 4°C until homogenized (in a total of 5 mL PBS) and 50 μL of homogenate plated for CFU evaluation. As a control, survival of 10 mice was observed for the duration of the experiment to ensure that the cytokine and/or CFU data reflected conditions of efficacy of the antibody. The experiments were performed two or more times with representative data listed.

STATISTICAL METHODS

For comparison of survival in the murine challenge experiments, results of individual survival experiments of mice were pooled and curves were analyzed using the Prism® software (Prism for Windows, version 5.01, GraphPad Software, Inc., La Jolla, CA), and choosing the Log rank, Mantel Cox test statistical method for testing significance. Comparisons of CFU/mL were made using the two tailed unpaired *T* test with Prism® software. Comparisons of cytokines/mL were made using the two tailed *T* test with Windows, version 5.01, Excel® software.

RESULTS

mAb CS-D7 DOES NOT DIRECTLY INHIBIT GROWTH OF *S. aureus* IN IMPLANTED CHAMBERS *In vivo*

It was previously reported that CS-D7 enhanced survival in the murine lethal challenge model. To investigate the mechanism of this activity, the potential for direct inhibition of growth of *S. aureus* by the antibody was evaluated. Growth chambers implanted sub-dermally on the back of rats were used to measure outgrowth of the bacteria under conditions that induced expression of the antigen, that is, under iron restricted, but nutrient rich, *in vivo* conditions. This method allowed quantitative and qualitative analysis of the resulting bacteria. In preliminary experiments, it was determined that *S. aureus* Becker (starting concentration of 1×10^6 CFU) reached stationary phase at approximately 10–12 h (at a concentration of $4–6 \times 10^8$ CFU), in these chambers (Supplemental Data, **Figure A1**). It was also demonstrated that there was rapid expression of IsdB by the bacteria, and >95% of the bacteria expressed the antigen at 8 h after implantation in the chamber (as demonstrated by flow cytometry, Supplemental Data, **Figure A1**).

To investigate whether CS-D7 inhibited the survival and growth of *S. aureus* under these conditions, bacteria plus an excess of either mAb CS-D7 or 20C2HA (non-specific isotype matched control mAb) were added to growth chambers (**Figure 1**). At several time points (3–6 h), it was observed that the bacteria were aggregated in the presence of mAb CS-D7, but not the presence of control mAb 20C2HA. This most likely represents cross linking of bacteria due to CS-D7 binding to IsdB expressed on the bacteria surface. Aggregation may have contributed to an apparently lower CFU/mL at the 6 h time point, due to the difficulty of making a single CFU suspension from aggregated bacteria for serial dilution and plating. By 24 h, there were equivalent numbers of CFU in all three growth chamber conditions. Therefore, although CS-D7 bound to *S. aureus*, this binding did not significantly inhibit survival or division of the bacteria. Binding of CS-D7 did lead to transient bacteria aggregation.

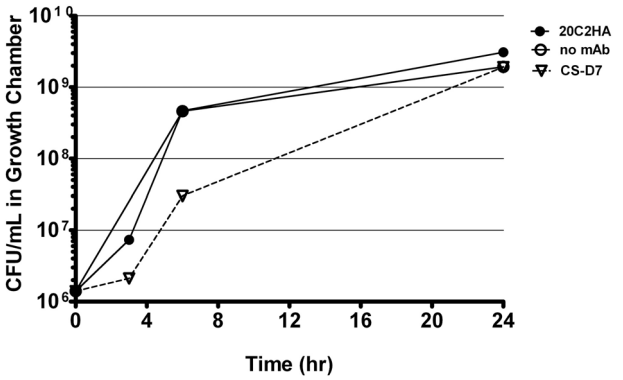

FIGURE 1 | *S. aureus* growth in the presence of IsdB specific mAb or isotype control mAb. Growth chambers containing 1×10^6 CFU Becker and 1 mg of mAb CS-D7 (inverted triangle), or 20C2HA, (closed circles), or no mAb (open circles) were evaluated for outgrowth of bacteria during a 24 h period. At designated times, a subset of rats were euthanized, chambers removed and bacteria were quantified by serial dilution on TSA.

mAb CS-D7 DOES NOT MEASURABLY INHIBIT HEME UPTAKE FROM HEMOGLOBIN *In vitro*

IsdB is a bacterial surface protein which functions to capture heme, a source of iron, from hemoglobin for import into the bacteria (Mazmanian et al., 2003). An antibody which could block this function of the antigen might possess enhanced efficacy against the bacteria (Kim et al., 2010). It is known that CS-D7 can bind equivalently to both heme free and heme replete IsdB (unpublished data), indicating that the presence of heme does not adversely affect the epitope which CS-D7 recognizes. To investigate potential mAb interference of heme uptake, an assay was developed to determine if CS-D7 binding to heme free IsdB would prevent heme transfer from hemoglobin to the antigen (See Supplemental Data and Supplemental **Table A1**). For this evaluation, CS-D7 and control mAb 20C2HA were incubated separately with IsdB$^-$ (heme free IsdB) and the complexes were exposed to human hemoglobin immobilized on Sepharose®. Equal amounts of protein samples were combined with the hemoglobin resin (containing a 1:1 molar ratio of antibody to antigen) in each case. As indicated in **Table 1**, the IsdB$^-$ in samples 1 and 2 bound heme after interaction with the hemoglobin-Sepharose®. In addition to CS-D7, a second IsdB specific mAb was evaluated, mAb 2H2 (Brown et al., 2009). The sample containing 2H2 plus IsdB$^-$, and the sample containing CS-D7 plus IsdB$^-$, had equivalent A_{400}/A_{280} ratios after exposure to hemoglobin (0.24 and 0.22 respectively), as the sample containing the non-specific control mAb 20C2HA plus IsdB$^-$ (0.23). This indicated that the presence of the two test mAbs did not prevent IsdB$^-$ from binding heme, under these *in vitro* conditions. This result did

Table 1 | Heme transfer from hemoglobin to heme minus IsdB (IsdB$^-$) in the presence of mAbs.

Sample number	Antibody added to IsdB$^-$	Absorbance A_{400} (iron)/A_{280} (protein); *(ratio)*	
		Starting sample (Antibody + IsdB$^-$)	Final, total column fractions (Antibody + IsdB$^-$ + heme)
1.	20C2HA	0.05/2.97 *(0.02)*	0.80/3.55 *(0.23)*
2.	CS-D7	0.03/2.64 *(0.01)*	0.64/2.89 *(0.22)*
3.	2H2	0.06/2.84 *(0.02)*	0.68/2.86 *(0.24)*

not rule out the possibility that CS-D7 and/or 2H2 caused the heme iron to bind at a position other than the NEAT domain (Grigg et al., 2007) of the antigen. Exposure of the hemoglobin-Sepharose® to antibody alone did not result in binding of heme to the mAb.

FUNCTIONALLY Fc DEFECTIVE MUTEIN CS-D7·N297A WAS PREPARED FOR EVALUATION OF mAb OP ACTIVITY

Based on the above results, the mechanism of protection mediated by CS-D7 may not involve direct growth suppression of the bacteria, or inhibition of the physiological function of IsdB. Therefore, experiments were devised to determine if CS-D7 enhanced uptake and killing of *S. aureus in vivo*. To this end, an Fc mutant of CS-D7 (IgG_1 isotype) was made. A point mutation was introduced which converted amino acid 297 from asparagine to alanine. Due to this mutation, the Fc cannot be glycosylated and therefore, loses its binding activity for Fcγ receptors and C1q (Lund et al., 1996). To verify that the mutein no longer bound to Fc, an *in vitro* ELISA to measure Fc receptor binding was employed, as described in Methods. Results from this assay indicated that while CS-D7 had robust binding to the Fc receptors tested, CS-D7·N297A had undetectable binding to either of the two receptors FcγRIIa or FcγRIIIa (**Figures 2A,B**).

To verify that the mutein CS-D7·N297A had lost its ability to fix complement, an assay to measure C3b deposition was developed. This assay measured the generation of C3b in C1q replenished human serum, when incubated with mAb bound to a microtiter plate surface. When CS-D7 was evaluated for complement activation in this assay, robust generation of C3b was observed (**Figure 3**), which was similar in quantity to the C3b generated in the presence of licensed mAb Rituxan© (Genentech) (Zhou et al., 2008; Pawluczkowycz et al., 2009). However, in the presence of mutein CS-D7·N297A, generation of C3b was not observed. This indicated that the classical pathway of complement fixation, starting with binding of C1q to the Fc of the mAb, was not activated by CS-D7·N297A.

OP activity was assessed in a third *in vitro* evaluation of functionality of CS-D7·N297A since the first two assays used to evaluate CS-D7·N297A were not cell based assays. Therefore, the mutein was compared to wild type CS-D7 in an OP assay, in the presence of HL60 phagocytic cells and fluorescently labeled *S. aureus*, as described in Methods (**Figure 4**). In this assay, there was a background level of approximately 20% fluorescent HL60 in the absence of antibody. The wild type CS-D7 had robust OP activity. In the presence of the maximal amount of 10 μg CS-D7mAb/mL, there was an increase of 50% of HL60 containing

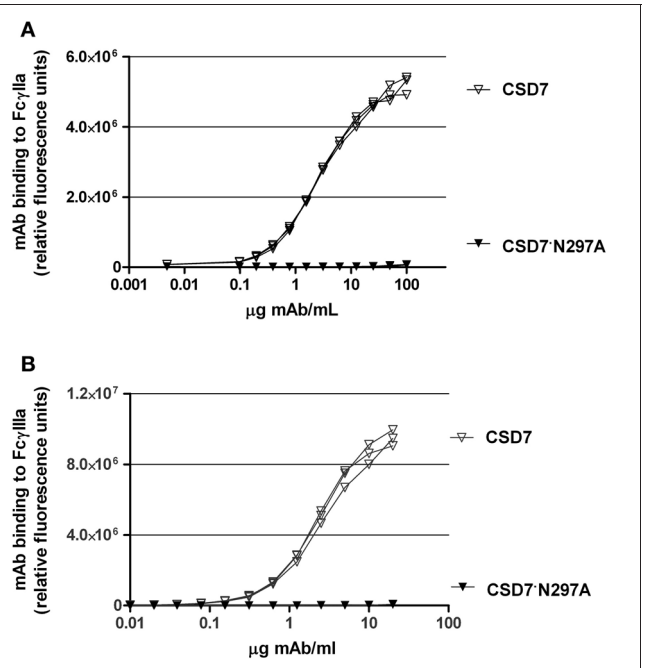

FIGURE 2 | CS-D7 or CS-D7·N297A mAb binding to FcγRIIa or FcγRIIIa. Binding of mAbs to **(A)** FcγIIa or **(B)** FcγIIIa was measured as described in Methods. Data shown are individual curves for triplet determinations.

phagocytosed bacteria, above background levels. At the same concentration, only 10% of HL60 in the presence of the isotype control mAb, and 0% of HL60 in the presence of the mutein contained phagocytosed bacteria above the background level. Both the isotype control and the mutein raised levels of uptake to approximately 20% above background at a 10-fold higher concentration of antibody, but further increases in concentration did not increase uptake above that level. Therefore, in agreement with the analysis above, the mutein had dramatically less functional activity than the wild type mAb.

CS-D7 AND MUTEIN CS-D7·N297A CONFER EQUIVALENT PROTECTION IN THE MURINE SEPSIS MODEL

After establishing that the mutein had significantly reduced Fc functionality, the mutein and wild type mAbs were compared for efficacy in the murine sepsis model. Balb/c mice were passively immunized with 400 μg of CS-D7, or CS-D7·N297A, or the isotype control 20C2HA. Mice ($n = 5$) were challenged via the tail vein with *S. aureus* SA025, and were monitored for

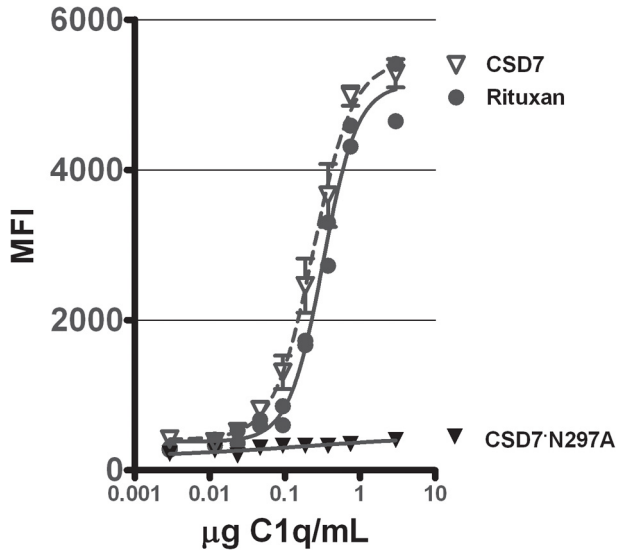

FIGURE 3 | Stimulation of complement C3b formation in human plasma by mAbs CS-D7, CS-D7·N297A, or Rituxan©. Microtiter plates coated with one of three mAbs were exposed to C1q serially diluted into C1q deficient human sera. C3b generation was quantitated as described in Methods. Data shown are the mean ± s.e. $n = 3$.

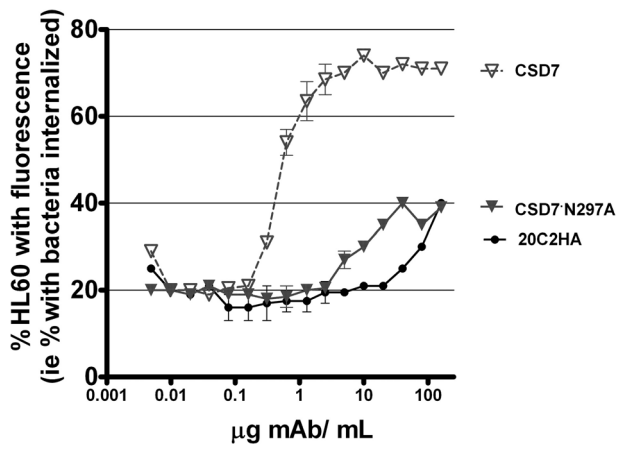

FIGURE 4 | OP activity mediated by mAbs CS-D7, CS-D7·N297A, or isotype control 20C2HA. OP activity was measured by combining differentiated HL60 with fluorescently labeled opsonized *S. aureus*, incubating the mixture, and measuring HL60 with engulfed bacteria, as described in the Methods section. Data shown are the mean ± s.e. $n = 2$.

survival for 96 h (**Table 2**). The experiment was repeated several times and data were pooled for analysis. Unexpectedly, CS-D7 and the mutein had equivalent activity in this model ($P = 0.4852$), and were significantly superior to the isotype control mAb ($P < 0.001$).

CS-D7 AND CS-D7·N297A CONFER EQUIVALENT PROTECTION IN FcR$^{-/-}$ MICE

Since the mutein was equally active in the murine sepsis model to the wild type CS-D7, it appeared that binding to Fcγ receptors and ability to fix complement were less important for the mechanism of protection in this model. To further determine whether binding to Fc receptors was non-essential for protection, mice genetically deficient for either the activating receptor (Fcγ IIII) or the inhibitory receptor (Fcγ RII) were investigated in the murine sepsis model. Mice ($n = 5$) were passively immunized i.p. with mAb prior to receiving a lethal dose of *S. aureus* via the tail vein. The experiment was repeated four to five times and data were combined for analysis. In these experiments, the wild type and the mutein had equivalent activity (**Tables 3** and **4**), which was significantly higher than for the control mAb 20C2HA. MAb mediated survival was lower in these deletion murine strains than in the wild type Balb/c mice. This is most likely due to increased sensitivity of the mice to *S. aureus* challenge.

NEITHER CS-D7 NOR CS-D7·N297A CONFER PROTECTION IN THE ABSENCE OF AN INTACT COMPLEMENT SYSTEM, OR IN THE ABSENCE OF EFFECTOR CELLS WITH FUNCTIONING OXIDATIVE BURST

To investigate whether the mechanism of CS-D7 efficacy in the murine sepsis model depended on phagocytes and complement, experiments were conducted to remove these components from mice prior to challenge. In the first case, Balb/c mice were depleted of C3 using CVF (Cunnion et al., 2004). CVF activates the alternative pathway and quickly depletes the host of C3, although not the components of the classical pathway. Mice were passively immunized and challenged as above. In mice depleted of C3, the mAbs were not protective **(Table 5)**. To effectively remove neutrophils and other phagocytes with an oxidative burst mechanism from playing a role in mAb mediated survival, P47 phox$^{-/-}$ mice lacking a functional oxidative burst mechanism were employed. These mice were passively immunized and challenged, but they were not significantly protected by the mAbs (**Table 6**).

mAb CS-D7 SIGNIFICANTLY REDUCES CFU BURDEN IN LIVER, BUT NOT IN OTHER ORGANS

To determine if CS-D7 enhanced bacteria clearance in the murine model, Balb/c mice were passively immunized with CS-D7 or isotype control mAb 20C2HA, or PBS alone, and challenged as above. At designated time points, mice were sacrificed and blood and organs were harvested. Due to increasing disease from *S. aureus* challenge in control mice, mice were not evaluated after 48 h post challenge for CFU burden. Survival was observed until 96 h post challenge in a subgroup of 10 mice (survival rate for CS-D7, 72%; 20C2HA, 28%; PBS, 39%). As shown in **Figure 5**, there were no significant differences in the blood burden (CFU/0.01mL) of the animals, nor in the bacteria burden in the filter organs, with the exception of a significant reduction in the liver of CS-D7 immunized animals at 1 h ($P = 0.007$) and 4 h post challenge ($P = 0.015$). There was a trend of CS-D7 mediating faster clearance in other organs, for example in the kidneys, but this difference did not reach statistical significance ($P > 0.05$). Interestingly there was an apparent higher level of CFU recovered from the spleens of animals injected with CS-D7 than Ab control animals at 48 h post challenge, however this difference was not statistically significant, and was no different from the PBS control animals.

Table 2 | Comparison of mAb efficacy in the murine sepsis model.

mAb	Number of Balb/c mice surviving Hours post challenge					Total (%)	*P*
	0	24	48	72	96		
CS-D7	20	20	20	17	14	4 (70%)	*<0.001
CS-D7·N297A	20	20	20	15	12	12 (60%)	†0.4852
20C2HA	30	28	21	8	4	4 (13%)	

*Comparison of survival with CS-D7 versus survival with isotype control 20C2HA.

†Comparison of survival with CS-D7·N297A versus survival with CS-D7.

Table 3 | Passive immunization and challenge of FcγRIII$^{-/-}$ mice.

mAb	Number of FcγIII$^{-/-}$ mice surviving Hours post challenge					Total (%)	*P*
	0	24	48	72	96		
CS-D7	20	20	20	8	7	7 (35%)	*0.002
CS-D7·N297A	20	20	18	7	6	6 (30%)	†0.517
20C2HA	20	20	13	3	1	1 (5%)	

*Comparison of survival with CS-D7 versus survival with isotype control 20C2HA.

†Comparison of survival with CS-D7 ·N297A versus survival with CS-D7.

Table 4 | Passive immunization and challenge of FcγII$^{-/-}$ mice.

mAb	Number of FcγII$^{-/-}$ mice surviving Hours post challenge					Total (%)	*P*
	0	24	48	72	96		
CS-D7	20	19	16	16	11	11 (55%)	*0.01
CS-D7·N297A	20	16	14	11	9	9 (45%)	†0.34
20C2HA	20	14	9	6	5	5 (25%)	

*Comparison of survival with CS-D7 versus survival with isotype control 20C2HA.

†Comparison of survival with CS-D7·N297A versus survival with CS-D7.

Table 5 | Passive immunization and challenge of complement deficient mice.

mAb	Number of complement deficient mice surviving Hours post challenge					Total (%)	*P*
	0	24	48	72	96		
CS-D7	25	15	6	6	2	2 (8%)	*0.097
CS-D7·N297A	25	11	7	5	0	0 (0%)	†0.33
20C2HA	25	9	5	3	0	0 (0%)	

*Comparison of survival with CS-D7 versus survival with isotype control 20C2HA.

†Comparison of survival with CS-D7·N297A versus survival with CS-D7.

CS-D7 MEDIATED ENHANCED SURVIVAL MAY AFFECT cytokine LEVELS POST CHALLENGE IN THE BLOOD AND THE ORGANS

CS-D7 mediated efficacy may be reflected in a modulation of cytokine levels. To investigate this possibility, Balb/c mice were passively immunized and challenged as above. At designated time points, groups of mice were sacrificed and blood and tissue harvested. A selection of cytokines, representing T helper subsets, or having pro-inflammatory properties, were evaluated. Cytokines INFγ (Th1), IL17 (Th17), IL5 (Th2), IL6 (pro-inflammatory mediator), and KCGRO (pro-inflammatory chemokine) were evaluated post challenge. IL17 and IL5 concentrations did not change over time for both the blood and the organs, and were equivalent between CS-D7 treated mice and 20C2HA treated mice (data not shown). Of the cytokines IFN-γ, IL6, and KCGRO, there was a trend toward higher IFN-γ in mice treated with CS-D7. In the sera at 24 and 48 h post challenge ($P = 0.03$) (**Table 7**),

Table 6 | Passive immunization and challenge of P47 phox$^{-/-}$ (neutrophil impaired) mice.

mAb	Number of P47 phox$^{-/-}$ mice surviving Hours post challenge					Total (%)	*P*
	0	24	48	72	96		
CS-D7	10	10	6	3	1	1 (10%)	*0.95
CS-D7·N297A	10	10	4	3	2	2 (20%)	†0.95
20C2HA	10	10	4	3	2	2 (20%)	

*Comparison of survival with CS-D7 versus survival with isotype control 20C2HA.

†Comparison of survival with CS-D7·N297A versus survival with CS-D7.

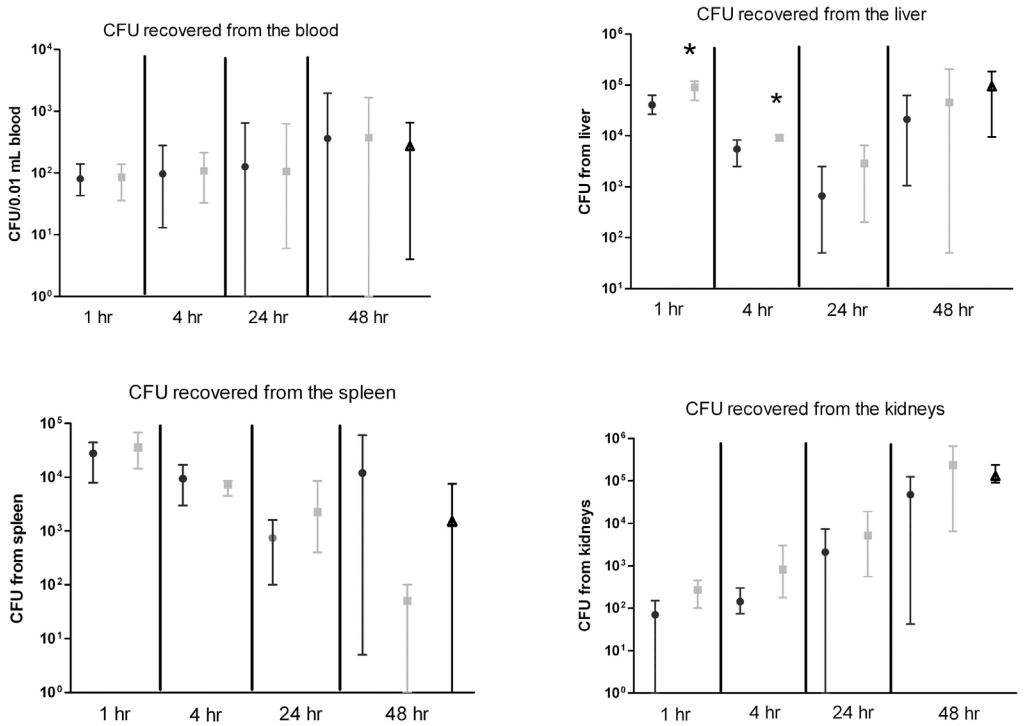

FIGURE 5 | Bacteria burden in murine tissue post immunization and challenge with *S. aureus*. Balb/c mice were passively immunized with CS-D7 (circles) or isotype control 20C2HA (squares) or PBS alone (triangles), and 2 h later were challenged via the tail vein with *S. aureus* SA025. At designated time points, subsets of animals were sacrificed and the blood and organs were harvested. Tissue bacterial burden was evaluated by homogenization of each organ in a total of 5 mL PBS and plating of 50 μL homogenate on TSA. The CFU count per 50 μL is plotted. For blood CFU determination, 10 μL of undiluted blood was plated. Data shown are means and ranges from mice ($n = 4$) in two experiments.

and in the kidney ($P = 0.05$), liver ($P = 0.008$), and spleen ($P = 0.08$) at 24 h post challenge (**Table 8**), IFN-γ levels were higher in CS-D7 treated mice. There was also a trend toward lower IL6 levels, which was observed at 4 h ($P = 0.005$), 24 h ($P = 0.0004$), and 48 h in the sera, and at 1 h in the kidney ($P = 0.011$). At 24 h post challenge, KCGRO was reduced in the blood of CS-D7 treated mice. However, there was no trend with this cytokine at other times or in other organs.

DISCUSSION

Clearance of bacteria from the blood stream is of vital importance in host defense against disseminated infection. Bacteria removal and killing occurs after opsonization with antigen specific antibody and/or complement. Both the innate (e.g., complement) and the adaptive (e.g., antibody) arms of the immune system cooperate in this function, indicating its evolutionary importance. A central method of bacterial removal from the bloodstream is mediated through binding to CR1 on erythrocytes (Edberg et al., 1992; Lindorfer et al., 2001a,b). Complement (C3b) opsonized *S. aureus* is carried to the liver by binding to CR1 on erythrocytes, where bacteria are taken up and removed by phagocytic cells of the reticuloendothelial system. Kupffer cells express CRIg, which is believed to be the capture receptor of complement opsonized bacteria (Helmy et al., 2006). Opsonized bacteria may be passed from erythrocytes, or captured directly from blood, by Kupffer cells. CRIg binds both C3b and iC3b. Neutrophils are called to the liver by chemokines and cytokines such as IL8 and IL17. Upon arrival at sites of bacterial residence,

Table 7 | Serum cytokine levels after passive immunization with one of two mAbs and challenge with *S. aureus* SA025.

Cytokine (Time post challenge, h)	pg cytokine/mL serum mean (std dev), $n = 5$	
	CS-D7	**20C2HA**
IFN-γ (0)	0 (0)*	0 (0)*
(4)	3 (3)	11 (12)
(24)	79 (40)	42 (24)
(48)	54 (24)†	22 (16)
IL6 (0)	0 (0)*	1 (6)*
(4)	114 (53)‡	209 (38)
(24)	73 (27)§	195 (50)
(48)	295 (157)	412 (343)
KCGRO (0)	20 (18)*	48 (36)*
(4)	760 (57)	634 (163)
(24)	365 (127)**	570 (169)
(48)	574 (182)	426 (180)

*$P < 0.001$ comparing cytokine concentration at time 0 to time points 4, 24, and 48 h.

†$P = 0.03$ comparing IFN-γ concentration in CS-D7 treated versus 20C2HA treated animals at 48 h.

‡$P = 0.005$ comparing IL6 concentration in CS-D7 treated versus 20C2HA treated animals at 4 h post challenge.

§$P = 0.0004$ comparing IL6 concentration in CS-D7 treated versus 20C2HA treated animals at 24 h post challenge.

**$P = 0.04$ comparing KCGRO concentration at time 24 h in CS-D7 treated versus 20C2HA treated animals.

neutrophils may release DNA nets to capture bacteria (Hickey and Kubes, 2009; Pilsczek et al., 2010). In the presence of antigen specific Ab, bacteria may be phagocytosed by different sets of effector cells, such as neutrophils and tissue macrophages, expressing both the FcγR and complement receptors. Binding of the two types of receptors is synergistic and greatly facilitates uptake and killing (Liszewski and Atkinson, 1993). Therefore, the presence of antigen specific Ab is a great advantage to the host for pathogen clearance. CS-D7 has high specificity and affinity for its target, *S. aureus* IsdB. IsdB is surface expressed in iron restricted environments, such as found in the human host, and as such has been proposed as a vaccine target (Kuklin et al., 2006; Stranger-Jones et al., 2006). Passively administered anti-IsdB Ab has demonstrated efficacy in rodent models (Ebert et al., 2010; Kim et al., 2010), however, in active immunization with IsdB, Ab response is not involved in protection in the murine sepsis model (Joshi et al., 2012). Therefore, it was of importance to determine the mechanism of protection mediated by the mAb CS-D7.

In an effort to determine if CS-D7 mediates protection by interfering with bacteria growth, *S. aureus* was cultured under *in vivo* conditions in the presence of CS-D7. The expression of IsdB is highly upregulated under this optimal *in vivo* environment with more than 50% of cells expressing antigen by 4 h, and essentially 100% of cells expressing the antigen by 8 h post implantation into rats. We found that during growth in implanted chambers, in which bacterial and host proteases may be present in high concentrations, the antigen remained available for antibody binding for the length of observation, 48 h. Expression of IsdB, in the presence of excess CS-D7, did not impact outgrowth of bacteria, implying that IsdB was not necessary for outgrowth under these conditions, or that the mAb binding to IsdB did not interfere with growth. However, the presence of CS-D7 did lead to transient aggregation of the bacteria under those conditions. It is possible that CS-D7 could mediate protection by interfering with heme uptake by the bacteria. The CS-D7 binding region on IsdB (aa 50–285) spans the domain 1 NEAT motif (aa 140–269) (Ebert et al., 2010). MAb binding to IsdB did not appear to interfere with heme transfer from human hemoglobin to IsdB *in vitro*. Since heme binding occurs through the domain 2 NEAT motif (aa 337–462), this may not be surprising (Muryoi et al., 2008). Kim and co-authors recently published data indicating that rabbit polyclonal antibodies to IsdB inhibited hemoglobin binding to IsdB. Their observations may relate to the use of polyclonal antibodies which would bind multiple epitopes on IsdB, whereas, CS-D7 recognizes a single epitope which may not obstruct hemoglobin binding.

Next, the functional activity of CS-D7 Fc was examined. Based on the current understanding of bacteria clearance, it was expected that CS-D7 would mediate protection through binding to its cognate antigen, inducing complement fixation through the classical pathway, and enhancing uptake and killing by phagocytes. To test this hypothesis, the mAb was mutated with a point mutation at aa 297. As expected, after mutation, the mutein did not mediate binding to FcγR nor did it fix complement in human serum (as measured by C3b production), whereas the wild type mAb did possess these functions. Activity in the OP assay was also dramatically reduced, essentially equivalent to the isotype control mAb. The mutein was then compared to the wild type mAb for protective efficacy. Unexpectedly, the mutein was as effective as the wild type mAb at enhancing survival in the murine sepsis model.

Further testing was performed to explore the mechanism of action of CS-D7 and the mutein. To confirm that Fc binding to FcγR was not critical for mAb efficacy, passive immunization experiments were conducted in FcγR deficient mice. In both FcγII$^{-/-}$ or FcγRIII$^{-/-}$ mice, both mAbs conferred equivalent protection, which was significantly more than the isotype control mAb. Overall survival in these deletion mice was lower than for wild type Balb/c mice. This may be due to defects in multiple cell types in the deletion mice (e.g., NK, macrophages, neutrophils, mast cells, and basophils in the FcγRIII$^{-/-}$), amplifying the effects of the challenge dose. Based on the challenge experiments, mAb binding to either the stimulatory or the inhibitory FcγR was not essential for *in vivo* efficacy. It was demonstrated that mutein CS-D7.N297A did not fix C3b *in vitro*, in the presence of C1q replenished human sera. To confirm that activation of the classical pathway was not necessary for *in vivo* mAb efficacy, mouse complement component C3 was depleted using CVF (Liszewski and Atkinson, 1993). It was observed that in the absence of C3, neither CS-D7 nor CS-D7.N297A enhanced survival in the murine sepsis model. Therefore, C3 was a critical component for mAb mediated survival in this model. Since CVF also depletes the host of the alternative complement components, the presence of that pathway may also be important in this model.

Table 8 | Tissue homogenate cytokine levels after passive immunization with one of two mAbs and challenge with *S. aureus* SA025.

Cytokine; (Time post challenge, h)	pg cytokine/mL tissue homogenate mean (std dev), $n = 5$					
	CS-D7			20C2HA		
	Liver	Spleen	Kidney	Liver	Spleen	Kidney
IFN-γ(0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (1)
(1)	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
(2)	31 (18)	6 (5)	0 (0)	15 (9)	13 (9)	0 (0)
(4)	20 (17)	28 (4)*	7 (13)	21 (13)	90 (29)	4 (5)
(24)	22 (12)*	23 (14)*	8 (6)*	3 (3)	9 (7)	1 (1)
(48)	7 (5)	3 (2)	1 (2)	2 (3)	1 (1)	1 (1)
IL6 (0)	0 (1)	0 (0)	1 (2)	0 (1)	0 (0)	1 (2)
(1)	3 (7)	5 (6)	2 (5)†	25 (20)	11 (8)	41(6)
(2)	19 (17)	3 (3)	0 (0)	9 (6)	4 (5)	0 (0)
(4)	5 (3)	25 (17)	15 (19)	8 (11)	26 (8)	1 (1)
(24)	7 (16)	2 (2)	10 (19)	2 (3)	0 (0)	19 (13)
(48)	16 (6)	1 (2)	190(148)	8 (8)	1(1)	140 (197)
KCGRO (0)	6 (3)	2 (1)	1 (2)	6 (3)	2 (1)	1 (2)
(1)	263 (127)	285 (105)	84 (24)	679 (296)	389 (218)	135 (95)
(2)	403 (122)	159 (80)	35 (8)	367 (206)	201 (84)	44 (11)
(4)	271 (144)	338 (75)	132 (70)	345 (78)	309 (185)	123 (71)
(24)	131 (58)	114 (68)	90 (55)	204 (87)	120 (52)	72 (19)
(48)	463 (208)	192 (85)	137 (106)	283 (203)	192 (120)	210 (297)

** Comparison of IFN-γ concentrations between mice treated with CS-D7 and mice treated with 20C2HA; Spleen at 4 h: $P = 0.002$; Spleen at 24 h: $P = 0.076$; Liver at 24 h: $P = 0.008$; Kidney at 24 h: 0.05.*

† Comparison of IL6 concentrations between mice treated with CS-D7 and mice treated with 20C2HA; Kidney at 1 h: $P = 0.011$.

Excess CS-D7, but not the isotype control, led to transient aggregation of *S. aureus* in the *in vivo* growth chamber, in parallel to an increase in IsdB expression. The aggregates are equivalent to IC. It is known that IC formation stimulates the activation of C3 to C3b and iC3b. In fact, one of the main functions of C3b is to bind to IC and reduce deposits of IC aggregates in small blood vessels (Liszewski and Atkinson, 1993). Therefore, CS-D7 may stimulate formation of C3b and iC3b through cross linking of the bacteria, as opposed to stimulation of the classical complement cascade. However, mere binding of a mAb to IsdB does not necessarily yield efficacy, as demonstrated previously for certain murine IsdB-specific mAbs (Brown et al., 2009). Therefore, the potential to bind IsdB in such a way as to produce cross linking of bacteria and complement activation may be important for efficacy.

Phagocytic cells were essential for mAb efficacy *in vivo*. When phagocytes (neutrophils and macrophages) were rendered nonfunctional by effectively deleting the oxidative burst mechanism (in P47 phox$^{-/-}$ mice), efficacy of the mAbs was abrogated. To further investigate bacteria clearance in the presence of CS-D7, blood and organ bacteria burden were measured post immunization and challenge. Differences in bacterial burden between CS-D7 treated and isotype control treated mice were observed in the liver, at 1 and 4 h post challenge. The liver is the primary organ responsible for clearance of blood born *S. aureus*, therefore, this observation suggests CS-D7 efficacy involves enhanced bacteria killing in the liver. Lack of more obvious differences in bacteria burden in other tissue may have several explanations. The *S. aureus* inoculum used to challenge mice in the sepsis model does not have IsdB expressed on its surface, due to culture of the bacteria on TSA. The expression of IsdB occurs over the course of several hours *in vivo*. Thus, antibody binding to IsdB on the bacteria takes place over several hours (Ebert et al., 2010). Additionally, a large challenge dose was necessary to produce sepsis in this model, and individual mice clear bacteria at different rates. Therefore, it may be difficult to capture a time at which expression of IsdB, leading to mAb mediated clearance of the bacteria, can be clearly observed; i.e., at any time, there may be only subtle differences in bacterial burden between CS-D7 and 20C2HA immunized animals. This subtle difference can be observed in the data shown in **Figure 5**, for example in the kidney. However differences in the CFU burden in these filter organs were not statistically significant. Lastly, although CFU measurements indicate similar numbers of viable bacteria, some or most of these may be sequestered in phagocytic cells in CS-D7 treated animals, potentially rendering them less able to cause pathologic sequelae leading to death of the animal.

Previously it was demonstrated that although CS-D7 confers enhanced survival in Balb/c mice, SCID mice (on a CB-17 background), lacking lymphocytes, were not protected after passive transfer of mAb (Joshi et al., 2012). There is increasing evidence that T cells play an active role in IgG mediated efficacy against pathogens such as *Cryptococcus neoformans*, *Francisella tularensis*, and *Pseudomonas aeruginosa* (Casadevall and Pirofski, 2003).

T cell secretion of certain cytokines or chemokines may be essential to IgG mediated protection. IL17 is known to stimulate neutrophils, which is important for neutrophil opsonophagocytosis and killing. It has been demonstrated that IL17 is critical for protection from *S. aureus* infection in non-immunized mice, as well as for IsdB immunized mice (Ishigame et al., 2009; Cho et al., 2010; Joshi et al., 2012). Therefore, the concentrations of several cytokines including IL17 were evaluated in the blood and organs of passively immunized and challenged mice. In mice treated with CS-D7 there was a higher level of IFN-γ in the sera and organs, observed at 24 h post challenge. T cells are the major source for IFN-γ, thus confirming the observed essential need for T cells in this model. IFN-γ is a potent activator of phagocytic cells, especially macrophages, inducing increased microbicidal activity. This activity could explain the observed enhanced clearance of *S. aureus* in the liver. An additional possibility for the mechanism of CS-D7 would be to prevent or damp down a cytokine storm post challenge. In that case, pro-inflammatory cytokines should be decreased in blood and organs. Lower levels of IL6, a potent pro-inflammatory cytokine, were measured in the sera and kidneys. The observed reduction of IL6 levels support the existence of a reduced pro-inflammatory or acute phase response in the CS-D7 treated mice. There was a single time point at which reduced KCGRO was observed (at 24 h in the sera), but at all other time points levels in sera and tissues were not significantly different between CS-D7 treated animals and controls. This most likely reflects the importance for neutrophil recruitment and function in this model, in the presence of either CS-D7 or control mAb. Importantly, IL17 levels were not measurably stimulated in either the blood or tissues. Therefore, the role of IL17 is either not important, or is yet undefined for mAb mediated protection in this model. Additionally, the Th2 cytokine IL5 was not measurably stimulated in these animals.

In summary, we have demonstrated that CS-D7 mediated protection in the murine sepsis model is dependent on complement, phagocytes and lymphocytes, but was not dependent on Fc functionality, classical complement activation, or direct inhibition of bacterial growth. The mechanism of CS-D7 mediated protection in this model may be triggered by cross linking, or aggregation, of *S. aureus in vivo*. Although this mechanism may not seem relevant to physiological infections, it may play a previously un-appreciated role in bacterial clearance under certain circumstances. When bacteria are localized to a site in which replication leads to large numbers, Ab may bind and aggregate the cells, leading to enhanced complement deposition and uptake by neutrophils or tissue macrophages. Additionally, during certain bloodstream infections, bacteria cross-linked by Ab (IC) may become aggregated and enhance the deposition of complement. Therefore, IsdB specific Ab may play a role in the murine sepsis model, and perhaps in human infection, by enhancing complement fixation on invading *S. aureus* through a non-classical mechanism. Importantly, *in vitro* assays designed to evaluate the supposed function of Abs may be inadequate or even misleading for predicting *in vivo* efficacy. Therefore, careful investigation of the mechanism of efficacy should be performed in order to design appropriate *in vitro* assays for testing Ab potency.

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APPENDIX

HEME BINDING UPTAKE ASSAY

An assay was developed to assess whether IsdB specific mAb CS-D7 could block IsdB from binding to immobilized heme (containing iron). Reagents used in the binding assay included IsdB prepared free of heme ($IsdB^-$), or containing heme, ($IsdB^+$), human hemoglobin prepared from fresh erythrocytes, hemoglobin:Sepharose® (hemoglobin was cross-linked to activated Sepharose® after preparation from fresh human packed red blood cells), test mAb and isotype matched negative control mAb 20C2HA. The antibody and $IsdB^-$ were combined, allowing the antibody to bind to the antigen. This combination was then exposed to hemoglobin (via passage over a hemoglobin:Sepharose® column) to determine whether the test antibody could prevent heme transfer from the hemoglobin to the $IsdB^-$ antigen. The $IsdB^-$ or $IsdB^-$/antibody passing through the hemoglobin column was evaluated for acquisition of heme (iron) by monitoring the antigen complex at 400 nm (the peak of absorbance for iron, no absorbance by protein). Column fractions were also monitored for protein content by measuring absorbance at 280 nm (no iron absorption at this wavelength). The assay procedure was as follows: $IsdB^-$ was combined in a 1:1 molar ratio with one of the monoclonal antibodies. After a short incubation, the antigen/antibody complex was applied to the hemoglobin-Sepharose column. Column flow through was collected and monitored for absorbance at 400 nm, and at 280 nm.

In preliminary experiments, $IsdB^-$ was evaluated for acquisition of heme iron from human hemoglobin in the absence of Ab. $IsdB^-$ was passed over a hemoglobin:Sepharose® column (sample 1, **Table A1**). The absorbance at 400 nm indicated that the initially heme free $IsdB^-$ contained little iron until the protein was exposed to immobilized hemoglobin. It was observed that although a portion of the $IsdB^-$ passed through the column (not binding to the matrix), a portion bound tightly to the column (sample 1, **Table A1**). In further tests, it was determined that 8M urea was required to completely elute the sample from the hemoglobin-Sepharose®. Removal of hemoglobin from the gel matrix by 8M urea was minimal (as determined by SDS PAGE). Urea did completely remove the starting sample (as demonstrated by SDS PAGE). Both the $IsdB^-$ flowing through the column, and the urea eluted $IsdB^-$ demonstrated an increase in absorbance at 400 nm, indicating acquired iron. Absorbance at A_{400} for both flow through and elution fractions was essentially at the background level if mAb CS-D7 was applied to the column in the absence of $IsdB^-$.

A fully heme loaded $IsdB^+$ sample was also applied to the hemoglobin column (sample 2, **Table A1**), and it was observed that this material consisted of two fractions also, one which bound tightly to the column, and one which did not bind. These data indicated that the heme loaded form of IsdB behaved similarly to the heme free antigen with regard to interaction with the immobilized hemoglobin, as did the heme free antigen. For simplification purposes, the two fractions were combined (i.e., for analysis of results, the flow through was combined with the urea eluted sample, see text and **Table 1**).

Table A1 | IsdB interaction with immobilized human hemoglobin.

No.	Sample	Starting sample A400/A280 (ratio)	Flow through A400/A280	Eluted material A400/A280	Total, post column fractions A400/280 (ratio)
1.	$IsdB^-$	0.06/0.82(0.07)	0.35/0.43	0.49/0.42	0.84/0.85(0.99)
2.	$IsdB^+$	0.82/0.95(0.87)	0.59/0.50	0.58/0.38	1.18/0.88(1.34)

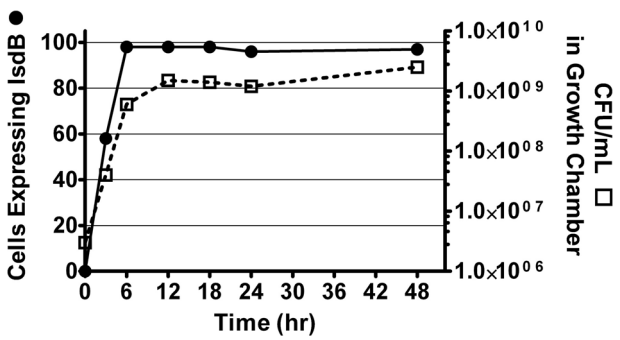

FIGURE A1 | *S. aureus* cultured in growth chambers *in vivo*. *S. aureus* Becker was placed in growth chambers made from dialysis tubing aseptically placed under the skin of rats. At designated times, rats were sacrificed and growth chambers removed for quantitation of CFU (squares) by serial dilution and plating on TSA, and for quantitation of surface expression of IsdB (circles) by flow cytometry (% of cells positive for IsdB).

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The iron-regulated staphylococcal lipoproteins

Jessica R. Sheldon and David E. Heinrichs*

Department of Microbiology and Immunology, Western University, London, ON, Canada

Edited by:
Martin J. McGavin, University of Western Ontario, Canada

Reviewed by:
Fabio Bagnoli, Novartis Vaccines, Italy
John Helmann, Cornell University, USA

****Correspondence:***
David E. Heinrichs, Department of Microbiology and Immunology, Western University, Dental Sciences Building, Room 3014, London, N6A 5C1 ON, Canada.
e-mail: deh@uwo.ca

Lipoproteins fulfill diverse roles in antibiotic resistance, adhesion, protein secretion, signaling and sensing, and many also serve as the substrate binding protein (SBP) partner to ABC transporters for the acquisition of a diverse array of nutrients including peptides, sugars, and scarcely abundant metals. In the staphylococci, the iron-regulated SBPs are significantly upregulated during iron starvation and function to sequester and deliver iron into the bacterial cell, enabling staphylococci to circumvent iron restriction imposed by the host environment. Accordingly, this subset of lipoproteins has been implicated in staphylococcal pathogenesis and virulence. Lipoproteins also activate the host innate immune response, triggered through Toll-like receptor-2 (TLR2) and, notably, the iron-regulated subset of lipoproteins are particularly immunogenic. In this review, we discuss the iron-regulated staphylococcal lipoproteins with regard to their biogenesis, substrate specificity, and impact on the host innate immune response.

Keywords: *Staphylococcus*, lipoproteins, iron-regulated, iron acquisition, substrate binding protein, TLR2, Fur

INTRODUCTION

The staphylococci are a diverse group of Gram-positive, catalase-positive facultative anaerobes, consisting of approximately 36 species hosted largely by the human body (Kloos and Bannerman, 1994). Historically, the staphylococci have been broadly divided into two groups based on their coagulase activity with *Staphylococcus aureus* representing the most notable, coagulase-positive pathogen of the genus. *S. aureus* is often regarded as the leading cause of infections of the bloodstream, skin, soft tissue, and lower respiratory system (Moet et al., 2007). In contrast, coagulase-negative staphylococci (CoNS) were largely considered to be harmless commensals of the skin and mucous membranes until recent years, when the opportunistically pathogenic *S. epidermidis* has emerged as the most frequent cause of device-associated nosocomial infections (reviewed in Otto, 2012). The appearance of vancomycin-resistance in clinical isolates of both *S. aureus* and CoNS, in addition to the increasing prevalence of community-associated methicillin-resistant *S. aureus* (CA-MRSA), highlights the continued need to develop novel strategies to combat these global pathogens (Srinivasan et al., 2002; Vandenesch et al., 2003).

The success of the staphylococci has been attributed, in part, to their ability to acquire iron from the host. In contrast to its relative abundance in nature, iron represents a severely growth-limiting nutrient *in vivo*. Indeed, while the solubility for ferric iron is often cited as 10^{-18} M, an adjusted calculation suggests that the actual concentration is closer to 10^{-9}–10^{-10} M at neutral pH in aerobic environments, based on $Fe(OH)_2^+$ being the primary species instead of $Fe(OH)_3$ (Ratledge and Dover, 2000). The revised value is still orders of magnitude lower than required to support microbial growth and additionally, iron within the host is further sequestered in glycoproteins such as transferrin and lactoferrin, bound within storage proteins such as ferritin and hemosiderin, or complexed with heme in the form of hemoglobin and myoglobin (Ratledge, 2007). Sequestration of iron functions both in preventing the catalysis of reactions generating damaging free radicals and in providing nutritional immunity against bacterial infection (Schaible and Kaufmann, 2004).

To circumvent the aforementioned restrictions, the staphylococci have evolved a plethora of mechanisms to acquire iron from the host, including the elaboration of multiple siderophores, utilization of xenosiderophores, acquisition of iron from heme and hemoproteins, and the uptake of inorganic free iron (Beasley and Heinrichs, 2010; Haley and Skaar, 2012; Hammer and Skaar, 2011). Each of these systems employs an iron-regulated membrane protein, almost always of the *A*TP-*b*inding *c*assette (ABC) transporter superfamily, for import of iron or complexed-iron across the membrane. In addition to the required ABC-type membrane permease and ATPase (Davidson et al., 2008), these iron-regulated ABC transporters employ a high-affinity membrane-anchored lipoprotein, i.e., a protein bearing an N-terminal, covalently linked lipid (Hutchings et al., 2009), for the specific recognition and binding of the iron substrate; these lipoproteins are analogous to the periplasmic substrate binding proteins (SBPs) of Gram-negative bacteria. Acylation of the hydrophilic protein promotes localization of the lipid into the phospholipid bilayer, anchoring the protein in close proximity to the membrane where it is positioned, once bound by substrate, to interact with its cognate ABC transporter to facilitate translocation of substrate into the cell. Whereas many other functions have been attributed to lipoproteins in bacteria, including sensing and signaling, protein secretion, antibiotic resistance, adhesion and the uptake of many nutrients in addition to iron (Sutcliffe and Russell, 1995), herein we focus attention solely on the iron-regulated lipoproteins (IRLPs) expressed by the staphylococci, given their important roles in staphylococcal pathogenesis and immune stimulation. Of the approximately 50 total lipoproteins encoded by *S. aureus*, the species in which most of the work has been performed, studies from several laboratories have identified, to date, a total of 9 IRLPs—SirA, HtsA, SstD, FhuD1, FhuD2,

IsdE, FepA, SitC, and Opp1A. These proteins will be the subject of this review.

CONTROL OF IRON-REGULATED GENE EXPRESSION

Fur

The *f*erric *u*ptake *r*egulator (Fur) is a homodimeric metalloprotein that functions as a transcriptional regulator of iron homeostasis in many bacteria. When complexed with iron, Fur regulates the transcription of genes by binding a 19-bp inverted repeat sequence (5′-GATAATGATAATCATTATC-3′), known as the Fur box, within the operator/promoter region (Escolar et al., 1999; Baichoo and Helmann, 2002). The Fur protein mainly functions as a repressor, mediating global suppression of iron-responsive genes in response to iron-replete conditions (Xiong et al., 2000; Hantke, 2001; Baichoo et al., 2002). Among the Fur-repressed genes are those involved in iron acquisition; Fur-boxes controlling expression of staphylococcal IRLPs are shown in **Figure 1A**. Binding of Fur to the Fur box sequences is dependent upon concomitant Fe^{2+} binding to Fur and, together, the competent DNA-binding complex blocks association of RNA polymerase with promoters, thereby inhibiting transcription. Under iron restriction, intracellular Fe^{2+} depletion results in dissociation of Fur from DNA, allowing transcription to proceed.

SirR/MntR

The notable exception to Fur regulation of the IRLPs is the *s*taphylococcal *i*ron *t*ransporter, SitABC/MntABC. SitABC and SirR were initially discovered in *S. epidermidis* (Cockayne et al., 1998), and in *Bacillus subtilis* where the SitABC and SirR homologues were named MntABCD and MntR, respectively, based on work of Helmann and colleagues in confirming their role in manganese uptake and homeostasis (Que and Helmann, 2000; Glasfeld et al., 2003; Lee and Helmann, 2007). The homologous region in *S. aureus* was likewise identified and referred to as MntABC and MntR (Horsburgh et al., 2002). Expression of *sitABC*/*mntABC* is controlled by the diphtheria toxin repressor (DtxR)-like homologue, SirR/MntR (Hill et al., 1998; Horsburgh et al., 2002; Ando et al., 2003). Transcription of *sitABC* is repressed by SirR in the presence of either Mn^{2+} or Fe^{2+} in *S. epidermidis* (Hill et al., 1998), although there is some contention over whether both Mn^{2+} or Fe^{2+}, or just Mn^{2+} controls expression in *S. aureus* (Horsburgh et al., 2002; Ando et al., 2003). Like with Fur, transcription is blocked in the presence of these divalent metal ions because the metal ions facilitate the binding of SirR/MntR to a conserved SirR (or MntR) box (**Figure 1A**), a region of dyad symmetry in the promoter/operator region of *sitABC/mntABC* (Hill et al., 1998; Ando et al., 2003). Repression of transcription is relieved when the metal ion concentration is depleted.

LIPOPROTEIN BIOGENESIS

A detailed and comprehensive analysis of lipoprotein biogenesis is outside the scope of this review, but we refer the reader to a recent review, and references therein, for an excellent summary of lipoprotein biogenesis in bacterial pathogens (Kovacs-Simon et al., 2011). Bacterial preprolipoproteins bear both an N-terminal signal peptide, characteristic of secreted proteins, and, within the C-region of the signal peptide, a conserved sequence $[L/V/I]_{-3} - [A/S/T/V/I]_{-2} - [G/A/S]_{-1} - C_{+1}$ which is referred to as the lipobox motif (see **Figure 1B** for lipobox motif of *S. aureus* IRLPs) (Kovacs-Simon et al., 2011). In the first biogenesis reaction, the lipoprotein diacylglyceryl transferase (Lgt) covalently links a diacylglycerol moiety to the thiol group on the side chain of the essential +1 cysteine of the lipobox. The subsequent reaction involves the cleavage of the signal peptide from the diacylated prolipoprotein by lipoprotein signal peptidase (Lsp; alternatively called signal peptidase II), an activity that appears contingent upon lipidation by Lgt in Gram-negative bacteria (Tokunaga et al., 1982), but is not a prerequisite in Gram-positive bacteria (Baumgartner et al., 2007). In a third reaction, highly conserved among Gram-negative bacteria and the Actinomycetes, the enzyme lipoprotein *N*-acyl transferase (Lnt) catalyzes the addition of a third fatty acid to the free amino group of the cysteine, resulting in a triacylated protein. With few exceptions, the *lgt*, *lsp*, and *lnt* genes are indispensible in Gram-negative bacteria, yet in contrast, these

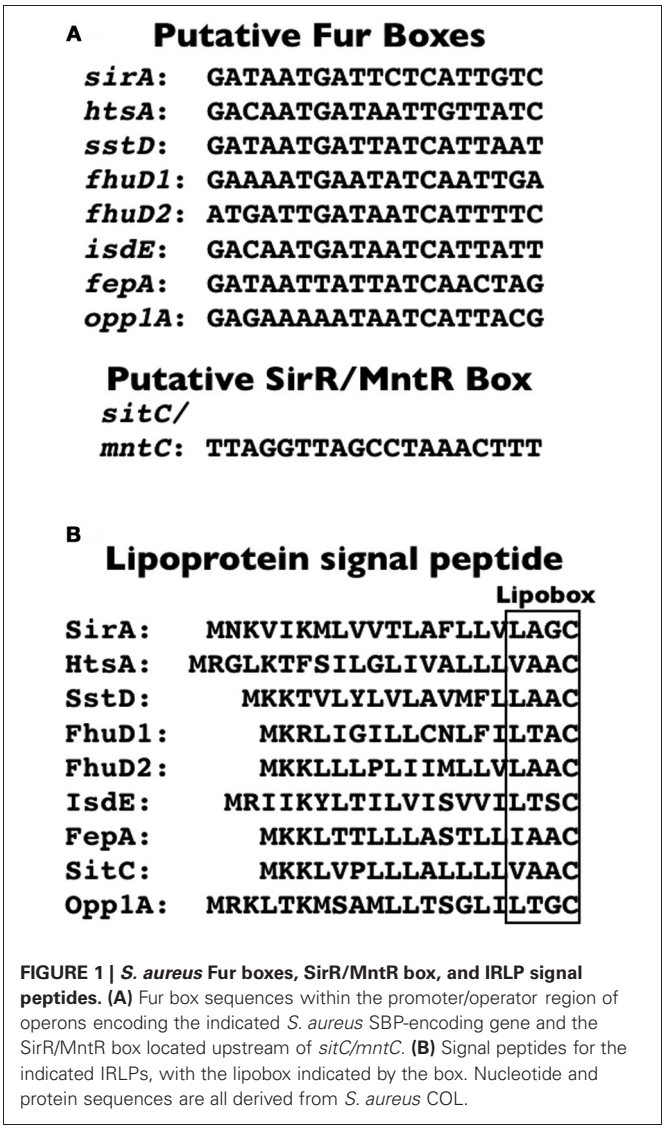

FIGURE 1 | *S. aureus* Fur boxes, SirR/MntR box, and IRLP signal peptides. (A) Fur box sequences within the promoter/operator region of operons encoding the indicated *S. aureus* SBP-encoding gene and the SirR/MntR box located upstream of *sitC/mntC*. **(B)** Signal peptides for the indicated IRLPs, with the lipobox indicated by the box. Nucleotide and protein sequences are all derived from *S. aureus* COL.

genes are dispensable in Gram-positive bacteria (reviewed in Kovacs-Simon et al., 2011).

Given the apparent lack of *lnt* homologues within Firmicute genomes, it was presumed that lipoproteins within this phylum were diacylated, a notion supported by the identification of diacylated lipoproteins among the staphylococci (Tawaratsumida et al., 2009). In contradistinction, SitC (MntC), the predominant lipoprotein of *S. aureus* and SBP for SitABC (MntABC), was found to be triacylated in multiple *S. aureus* strains and in *S. epidermidis* (Kurokawa et al., 2009; Asanuma et al., 2011). Furthermore, four other major staphylococcal lipoproteins were found to be triacylated (Asanuma et al., 2011), including the same lipoprotein earlier identified to be diacylated by Tawaratsumida et al. (2009). The discrepancies between these studies could be attributed to differences in culture conditions and preparation of the lipoproteins, given that Asanuma et al. noted a minor presence of diacylated SitC (MntC) lipopeptides in cultures grown at elevated temperatures (Asanuma et al., 2011). The notion of global *N*-acylation among the Firmicutes is further bolstered by a recent report of triacylated lipoproteins among the class Mollicute in *Acholeplasma laidlawii*, despite the lack of a recognizable Lnt homolog in this bacterium (Serebryakova et al., 2011). Consequently, the nature by, and degree to which, staphylococcal lipoproteins are *N*-acylated remains unknown, and it is possible that an as-yet unidentified enzyme, with no similarity to Gram-negative Lnt enzymes, exists within the Firmicutes that is responsible for this elusive activity.

THE STAPHYLOCOCCAL IRLPs: FUNCTIONS IN IRON ACQUISITION

The thus-far identified nine IRLPs in the staphylococci are involved in, or at least implicated in, the uptake of iron through either siderophore (**Figure 2A**) or non-siderophore (**Figure 2B**) based systems. Together, these systems engender staphylococci with versatility in that they are able to recognize and utilize a broad range of iron substrates. Unsurprisingly, the common function of these IRLP is reflected in their largely conserved overall protein structure.

As a result of a recent revisitation of the classification scheme to segregate SBPs based upon structural characteristics (Berntsson et al., 2010), the majority of the staphylococcal IRLPs, with the exception of FepA and Opp1A (discussed below), fall into cluster A, which consists of class III SBPs associated with ABC transporters. These proteins have an approximately 20 residue long α-helical backbone that joins two independently folded domains, each of which consists of central β-strands surrounded by α-helices; substrate is bound into the groove formed between the two domains (**Figure 3A**). Relative to members of other SBP clusters, hinge motion, upon binding of the substrate, between the two domains of cluster A proteins is restricted by the helical spine. Docking of the cluster A SBPs with their cognate membrane ABC-type permeases is facilitated by salt-bridges formed between conserved glutamic acid residues on the lobes of the SBP (**Figure 3A**) and patches of positive charge on the exterior surface of the permease, formed by three conserved arginine/lysine residues (Borths et al., 2002).

HtsA AND SirA—RECEPTORS FOR THE FERRATED STAPHYLOFERRIN SIDEROPHORES

Siderophores are secreted, low-molecular-weight molecules that have high-affinity for ferric iron. Among members of the staphylococci, two siderophores may be produced, staphyloferrin A (SA) and staphyloferrin B (SB) (Konetschny-Rapp et al., 1990; Meiwes et al., 1990; Drechsel et al., 1993; Haag et al., 1994). Enzymes for the synthesis of SA and SB are encoded from the *sfaABCD* and *sbnABCDEFGHI* loci, respectively (Dale et al., 2004a,b; Beasley et al., 2009; Cheung et al., 2009; Cotton et al., 2009) (**Figure 2A**). Adjacent to each of these biosynthetic loci, but transcribed separately, are operons encoding the requisite ABC-transporters for uptake of Fe-SA and Fe-SB complexes (Dale et al., 2004a,b; Beasley et al., 2009, 2011; Beasley and Heinrichs, 2010). The transport systems for the two siderophores have been shown to be non-interchangeable, where HtsABC specifically uptakes Fe-SA and SirABC specifically uptakes Fe-SB (Beasley et al., 2009). While each of the transporter-encoding operons codes for a SBP (HtsA and SirA) and a heterodimeric permease component (HtsBC and SirBC), both lack a gene encoding the ATPase component of the ABC transporter. Instead, at least in *S. aureus*, FhuC, expressed from the *fhuCBG* operon (**Figure 2A**) serves as the ATPase for import of both Fe-SA and Fe-SB (Speziali et al., 2006; Beasley et al., 2009). The notion of a common ATPase between multiple iron acquisition pathways is not unprecedented; the YusV ATPase in *B. subtilis* acts in the uptake of siderophores through both *feuABC* and *yfiYZyfhA* (Ollinger et al., 2006). Currently available genomic sequences reveal that while the *hts-sfa* locus is found in all staphylococcal genomes (incl. *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *S. saprophyticus*, *S. haemolyticus*, *S. pseudintermedius*, *S. warneri*, *S. capitis*, *S. caprae*, *S. hominis*, *S. carnosus* and *S. xylosus*), the *sir-sbn* locus is only found in the genomes of *S. aureus* and *S. pseudintermedius*. Curiously, however, SB was identified in the supernatants of several members of CoNS, and was first identified in coagulase-variable *S. hyicus* (Konetschny-Rapp et al., 1990; Meiwes et al., 1990; Drechsel et al., 1993); the reason for the discrepancy between these findings and the available genome information remains unknown. Genomes of *S. lugdunensis*, although possessing *htsABC*, carry a deletion of *sfaA* and *sfaD*, suggesting that this species may utilize Fe-SA as an iron source, but likely does not synthesize either staphyloferrin molecule.

Given that both SA and SB are highly hydrophilic, α-hydroxycarboxylate type siderophores (Beasley and Heinrichs, 2010), highly specific binding to their cognate receptors would be necessary to maintain the aforementioned uptake specificity. Without the specific recognition and binding of Fe-SA and Fe-SB to HtsA and SirA, respectively, one might anticipate that transporters for these structurally similar siderophores would be interchangeable. Recent structural characterizations of both HtsA and SirA have shed significant light on the reason for this specificity. HtsA and SirA both adopt the bilobed, α-helical backboned structure typical of the cluster A SBPs (see above), and their binding pockets are shallow and basic to accommodate the negatively charged siderophores, yet few residues within the substrate binding pocket are conserved between the two lipoproteins (Grigg et al., 2010a,b). HtsA coordinates Fe-SA through H-bonding between six arginine residues, directed into

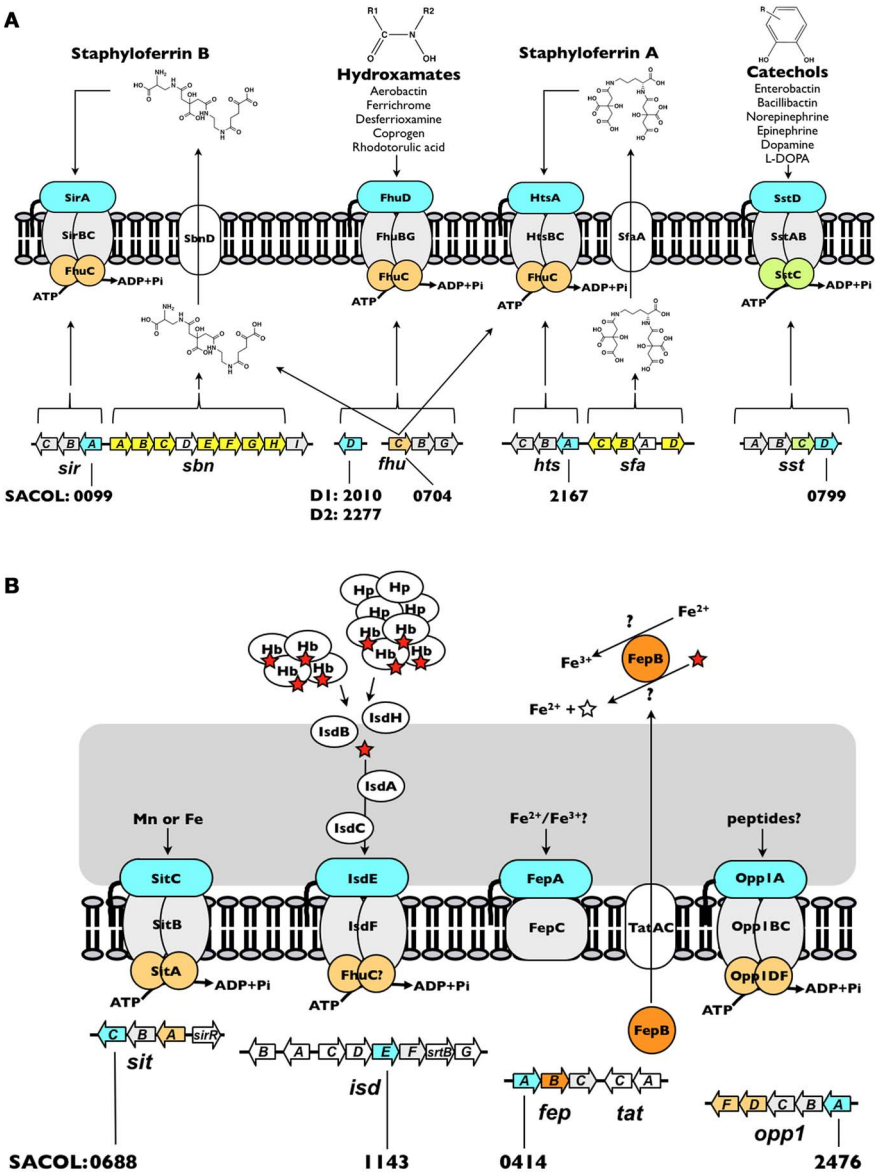

FIGURE 2 | Iron acquisition systems in *S. aureus*.
(A) Siderophore-dependent iron uptake systems.
(B) Siderophore-independent iron uptake systems. Each system is discussed in the text. The open reading frame accession number, based on *S. aureus* COL genome, for each of the IRLPs (blue) is indicated. The directionality indicated for each of the genes is as in the genome. The red star represents heme and the open star represents protoporphyrin IX. Hb, hemoglobin; Hp, haptoglobin.

the binding pocket, and oxygen atoms in SA (**Figure 3B**) (Grigg et al., 2010b). Conserved residue R125 also anchors Fe-SB in SirA (Grigg et al., 2010a), however the remaining coordinating residues are unique (**Figure 3C**). The number of coordinating arginine residues is a reflection of the net negative charge on the associated siderophore, where up to six are required to neutralize the –5 net charge on SA, and three are required for the –3 net charge of SB. Furthermore, while siderophore binding to HtsA and SirA induces negligible hinge movement along the α-helix, significant and specific conformational changes occur within the C-terminal domain of both (Grigg et al., 2010a,b). The unique coordinating residues, in addition to the distinct conformational changes, likely maintain the specificity and affinity of these receptors for their cognate siderophores, and it is unsurprising that their ligand affinities fall within the nanomolar range (Grigg et al., 2010a,b)

Inactivation of any of the individual biosynthetic or transport loci for SA or SB yields an unimpressive, if any, phenotype when examining for defects in iron-restricted growth (Beasley et al., 2009, 2011). However, the combined inactivation of either *sbn* together with *sfa* or *sir* together with *hts* yields mutants with severe growth defects in serum (Beasley et al., 2009, 2011). Notably, the inactivation of multiple transport systems in *S. aureus* results in decreased bacterial fitness during infection,

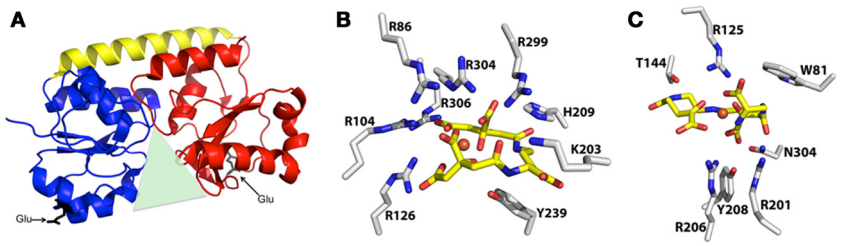

FIGURE 3 | Representative structure of cluster A SBPs, and structures of protein-bound Fe-SA and Fe-SB. (A) Representative ribbon diagram to highlight some of the key characteristics of the cluster A SBPs, including the helical spine (yellow) connecting the alpha/beta N-terminal (blue) and C-terminal (red) lobes that surround the binding pocket (green triangle), and the two conserved glutamic acid residues (black) that interact with the membrane permease. **(B)** Structure of iron-bound staphyloferrin A with coordinating residues from the *S. aureus* HtsA SBP (PDB 3LI2). **(C)** Structure of iron-bound staphyloferrin B with coordinating residues from the *S. aureus* SirA SBP (PDB 3MWF).

relative to inactivation of the corresponding biosynthetic loci (Beasley et al., 2011), likely due to the continued production of siderophores even in the absence of their cognate receptors, which would enhance iron-starvation *in vivo* (Beasley et al., 2011). Impairment of receptor function, therefore, represents a potential avenue for therapeutic intervention (see below).

SstD—RECEPTOR FOR FERRATED CATECHOLS/CATECHOLAMINES

Catechol siderophores, typified by the well-studied enterobactin (or enterochelin), bacillibactin, vibriobactin and salmochelin, are representative of a major class of bacterial siderophores (for a review, see Miethke and Marahiel, 2007). Additionally, the ability of catecholamine stress hormones to stimulate growth of bacteria, including members of CoNS, in the presence of serum (i.e., transferrin) has been studied over the past decade (Freestone et al., 2000, 2008; Neal et al., 2001). Indeed, recent work illuminated how hormones such as epinephrine and norepinephrine are capable of reducing transferrin-bound ferric iron to ferrous iron and thereby liberating iron from this central component of innate immunity (Sandrini et al., 2010). While catechol siderophores have not been identified in culture supernatants of staphylococci, staphylococci are capable of using both catechols and catecholamines as an iron source via the SstABCD ABC transporter (Beasley et al., 2011). The locus (**Figure 2A**), initially identified through screens for iron-regulated staphylococcal antigens (Morrissey et al., 2000), is highly conserved among *S. aureus* strains and present in the majority of CoNS. The function of this transporter remained uncharacterized until recently, when *sst* mutations were characterized in staphyloferrin-deficient strains (Beasley et al., 2011). The *sst* locus is required for growth promotion by catechol siderophores and catecholamine-liberated transferrin iron, and contributes to *in vivo* colonization of the murine liver and kidneys. With the exception of the colonization of murine hearts, these *in vivo* and *in vitro* effects were otherwise masked in strains synthesizing the staphyloferrins (Beasley et al., 2011).

SstD specifically binds both ferrated catechol siderophores and catecholamine stress hormones with micromolar and submicromolar affinities (Beasley et al., 2011). The reduced affinity of SstD for its ligands, relative to SirA and HtsA, is likely a trade-off in favor of an enhanced range of potential substrates. The closest homologs to SstD include the YclQ protein from *B. subtilis* and the CeuE protein from *Campylobacter jejuni.* Structural information exists for these proteins in complex with their ligands; YclQ binds the "stealth" siderophore petrobactin (Zawadzka et al., 2009), while CeuE binds iron complexed by the enterobactin analog, mecam (Muller et al., 2006). Some conservation in the binding mechanism of these proteins is indicated by shared binding pocket residues between SstD, CeuE, and YclQ, which in CeuE interact with the mecam substrate. Crystallographic information for liganded SstD unfortunately remains lacking, but this information would afford insight into the mechanism by which this receptor binds a diverse range of catechol ligands.

FhuD1 AND FhuD2—RECEPTORS FOR FERRIC-HYDROXAMATE SIDEROPHORES

While the staphylococci have not been demonstrated to synthesize hydroxamate-type siderophores, they are known to be able to utilize them as iron sources. These include aerobactin (produced by some enterobacteriaceae), ferrichrome (produced by the basidiomycete fungus *Ustilago sphaerogena*), coprogen (produced by *Neurospora crassa*), and desferrioxamine B, produced by *Streptomyces pilosus*; the mesylate salt of desferrioxamine B is used clinically under the name Desferal™ (Brock and Ng, 1983; Sebulsky et al., 2000; Sebulsky and Heinrichs, 2001). Uptake of multiple hydroxamate-type siderophores was similarly demonstrated for *B. subtilis* (Schneider and Hantke, 1993), and a homologous system identified in the *S. aureus* (Sebulsky et al., 2000). Ferric-hydroxamate uptake is achieved through the concerted effort of the FhuC ATPase, FhuBG heterodimeric permease, and the independently transcribed substrate-binding lipoproteins, FhuD1 and FhuD2 (**Figure 2A**) (Sebulsky et al., 2000; Sebulsky and Heinrichs, 2001). All species of staphylococci, with the possible exception of *S. hominis* and *S. xylosus*, possess the Fhu uptake system.

While many hydroxamate ligands are common between FhuD1 and FhuD2, the two lipoproteins do possess some unique substrate specificities with FhuD2 exhibiting both a wider range of ligands and greater substrate affinity than FhuD1 (Sebulsky et al., 2003, 2004). While FhuD1 appears to be a less effective

Fe^{3+}-siderophore SBP than FhuD2, it is certainly possible that the substrates and/or conditions under which FhuD1 are optimal are not defined. While the structures of FhuD1 and FhuD2 are not yet available, structures have been determined for *E. coli* FhuD bound to several hydroxamate ligands (Clarke et al., 2000, 2002). In contrast to the binding pocket of proteins such as SirA and HtsA, which harbor key charged residues that interact with the siderophore molecule, the structures of *E. coli* FhuD show that the binding of ligands is largely dependent upon hydrophobic contacts; accommodation of several different hydroxamate substrates occurs only through subtle rearrangements of the FhuD protein side-chains.

IsdE—A HEME RECEPTOR

isdE homologues have been identified in several Gram-positive pathogens including *S. aureus*, *S. lugdunensis, Bacillus anthracis, Clostridium tetani,* and *Listeria monocytogenes* (Skaar and Schneewind, 2004; Heilbronner et al., 2011). The *i*ron-regulated *s*urface *d*eterminant pathway (Isd) represents the predominant system for iron acquisition from heme and hemoproteins in *S. aureus* (Mazmanian et al., 2002, 2003). The proposed architectural arrangement of the nine Isd proteins, facilitated by anchoring to the cell wall through sortase A activity (for IsdA, IsdB, and IsdH), and *isd* locus-encoded sortase B activity (for IsdC), allows for heme, extracted from hemoglobin or haptoglobin-hemoglobin, at the cell surface to be shuttled proximal to the membrane where it is bound by the SBP IsdE, and transported into the cytoplasm through the ABC permease IsdF (**Figure 2B**) (Mazmanian et al., 2003; Skaar and Schneewind, 2004; Torres et al., 2006; Muryoi et al., 2008; Zhu et al., 2008; Grigg et al., 2010c). Once in the cytoplasm, heme may be degraded by IsdG and IsdI, releasing iron (Skaar et al., 2004a,b; Lee et al., 2008).

As a member of the cluster A SBPs (see above), IsdE possesses the characteristics depicted in **Figure 3A** but, unlike SirA and HtsA, bears a deep hydrophobic groove between the two lobes comprising a heme binding pocket (Grigg et al., 2007). A single intermediate or high-spin ferric or, preferentially, low-spin ferrous heme molecule is coordinated by the axial ligands H229 and M78 (Grigg et al., 2007; Pluym et al., 2007). As with several of the other iron ABC transporters in the staphylococci, the *isdEF* genes are not genetically linked with a gene encoding the obligatory ATPase for the transporter. It is possible that the promiscuous FhuC drives transport through IsdEF.

In vitro experiments using purified proteins demonstrated that IsdE could receive heme directly from IsdC (depicted in **Figure 2B**), as part of a larger study that provided support to the notion that the Isd proteins act together as a heme shuttle system within the bacterial cell wall and membrane (Muryoi et al., 2008). Moreover, growth impairment has been demonstrated for Isd mutants cultured on hemoglobin as a sole iron source (Torres et al., 2006; Pishchany et al., 2009, 2010); subsequent to hemoglobin binding at the cell surface and heme extraction, heme is likely never free from a protein and is shuttled through the wall and membrane via direct Isd-Isd protein contacts. On the contrary, it should be noted, that an *isdE* mutation had less of an impact when cultured in the presence of heme as a sole iron source (Grigg et al., 2007). The lack of a marked phenotype for the *isdE* mutant on heme raises the likely possibility that when presented with free heme, a growth-supporting amount of heme makes its way through the cell wall, bypassing the Isd proteins, and is taken into the cell via non-specific mechanisms or by secondary heme transporters, a notion currently under investigation.

FepA—AN IRON RECEPTOR

The translocation of staphylococcal preprolipoproteins was initially thought to occur exclusively through the dominant secretory pathway, Sec (Driessen and Nouwen, 2008; Natale et al., 2008). However, the *t*win-*a*rginine *t*ranslocation pathway (Tat) is another means of lipoprotein translocation across the membrane in a number of Gram-positive, high GC bacteria (McDonough et al., 2005; Widdick et al., 2006, 2011; Thompson et al., 2010), and was recently identified in some (incl. *S. aureus*, *S. haemolyticus* and *S. carnosus*), but not all, staphylococcal genomes. Prefolded proteins bearing a twin arginine motif in their signal peptide are secreted by Tat (Meissner et al., 2007; Biswas et al., 2009), however the role of Tat in the staphylococci appears limited both in the apparent lack of complete Tat homologues in many CoNS, such as *S. epidermidis* and *S. saprophyticus,* and in identified substrates (Yamada et al., 2007; Biswas et al., 2009). Currently, the sole identified substrate of Tat is the iron-dependent DyP-family peroxidase, FepB (Biswas et al., 2009).

Dye-decolorizing (DyP)-type peroxidases form a unique heme peroxidase family (for a review, see Sugano, 2009), and members of the family have been reported to have several different substrates (Letoffe et al., 2009; Ahmad et al., 2011; Liers et al., 2011). Insofar as iron uptake is concerned, two Dyp paralogs in *E. coli*, namely YfeX and EfeB (cytoplasmic and periplasmic enzymes, respectively), were reported to have deferrochelatase activity on heme (Letoffe et al., 2009). Deferrochelation would provide bacteria with an iron source in the absence of dedicated heme uptake systems, however this is currently a subject of some debate. A recent study has argued that the *E. coli* YfeX protein is not a heme deferrochelatase but rather is a peroxidase that oxidizes porphyrinogens to porphyrins (Dailey et al., 2011). Should DyP-type peroxidases bear deferrochelatase activity, it is possible that, like YfeX and EfeB, staphylococcal FepB could also serve to release ferrous iron from heme.

In addition to FepB, the *fepABC* operon, located adjacent to *tatAC*, encodes an iron permease (FepC) and a putative iron-binding lipoprotein (FepA) (**Figure 2B**). The exact function of *S. aureus fepABC* in iron acquisition is still under investigation, and the substrate specificity of FepA has not yet been determined. Free ferrous iron released through the potential deferrocheletase activity of FepB could serve as a substrate for FepAC, similar to *E. coli* ferrous iron-specific *efeUOB*, which has been shown to be induced by growth in aerobic, low pH and low iron conditions (Grosse et al., 2006; Cao et al., 2007). Alternatively, *fepABC* may function in elemental iron uptake in a manner analogous to the Ftr1p/Fet3p ferric iron permease in *Saccharomyces cerevisiae,* where Fe^{2+} is oxidized to Fe^{3+} by the multicopper oxidase, Fet3p, and is subsequently transported into the cell by Ftr1p (Kwok et al., 2006). A similar mechanism was first proposed in *B. subtilis* for *ywbLMN,* a *fepABC* homologue, and, accordingly, the *ywbLMN*

operon was shown essential to the normal growth of *B. subtilis* in iron-restricted media (Ollinger et al., 2006; van der Ploeg et al., 2011). Regardless, the presence of *fep* operon homologs in many different bacteria, including both Gram-positive and Gram-negative species, suggests a conserved and important functionality. Indeed, *S. aureus fep-tat* mutants are impaired in inorganic iron uptake and virulence in a murine renal abscess model (Biswas et al., 2009).

Although no structure of a staphylococcal FepA protein has yet been solved, homology searches and structural modeling indicate that it is rather unique among the IRLPs. The closest homologs to FepA are members of the imelysin-like superfamily (Xu et al., 2011). The canonical imelysin fold is all-helical, comprising two similar four-helix bundle domains with a predicted functional site at the domain interface (Xu et al., 2011). Specifically, a conserved GxHxxE motif, also present in staphylococcal FepA proteins, is located at the open end of the binding cleft; this motif has been implicated in the coordination of divalent metal cations (Rajasekaran et al., 2010a,b; Xu et al., 2011). With well over 100 members, the imelysin-like proteins are widely distributed in bacteria and are virtually always located next to a gene encoding a DyP-type peroxidase. Although biochemical evidence is still lacking, this reinforces the assumption that the FepABC-like systems are involved in iron uptake, where the peroxidase potentially converts ferrous iron to ferric iron, which is the ligand for the FepA-like binding proteins.

Opp1A

Despite being annotated as an oligopeptide permease, *opp1ABCDF* has no defined role in nitrogen metabolism (Hiron et al., 2007). However, Opp1A, the SBP of this complex, was shown to be significantly upregulated during iron-starvation, both by our laboratory (unpublished data) and others (Hempel et al., 2011), consistent with the identification of a putative Fur box upstream of Opp1A (**Figure 1A**). The role of oligopeptide permeases in metal ion and heme acquisition is not unprecedented; *S. aureus* Opp2BCDF and the orphan SBP Opp5A were recently renamed NikBCDE and NikA due to their role in nickel acquisition (Hiron et al., 2010), whereas in *E. coli* a di-peptide permease is involved in the uptake of both nickel and heme (DppA) (Letoffe et al., 2006, 2008; Shepherd et al., 2007). A potential role for Opp1A in iron acquisition is currently under investigation.

The *opp1ABCDF* operon encodes a prototypical oligopeptide permease: two homologous permease proteins (OppB and OppC) and two ATP-binding proteins (OppD and OppF), in addition to the peptide/SBP (**Figure 2B**). Like FepA, Opp1A is not a member of the cluster A SBPs, but instead is a member of cluster C which includes oligopeptide, di-peptide and nickel binding proteins (Berntsson et al., 2010). Cluster C members are characterized by a larger size (approx. 50–70 kDa) than the cluster A proteins (which are typically in the 30–35 kDa range), a large binding cavity, an extra domain that augments the binding cavity - in some cases to accommodate larger peptide ligands, and ligand binding via a "Venus flytrap" mechanism (Berntsson et al., 2009, 2010). The latter point indicates that these proteins capture their substrates through significant inter-domain hinge movement, unlike the relative lack of inter-domain movement afforded by the helical spine of the cluster A SBPs (see above).

According to Hiron et al. (Hiron et al., 2007), *S. aureus* possesses two additional oligopeptide permeases (*opp3* and *opp4*) and one di/tri-peptide permease (*dtpT*), in addition to *opp1* and *nikA/nikBCDE*. While *opp3* and *dtpT* are involved in nitrogen metabolism (Hiron et al., 2007; Borezee-Durant et al., 2009), the roles of *opp1* and *opp4* are unknown and none of the lipoproteins have been investigated to determine if, like *E. coli* NikA, they are capable of binding multiple substrates. Although the role of *opp1* is unknown, it has been implicated, along with *opp2* and *dtpT,* in staphylococcal infectivity and survival through signature tagged-mutagenesis studies (Mei et al., 1997; Coulter et al., 1998). The exact mechanism by which these permeases contribute to virulence, however, is not known.

SitC/MntC—RECEPTORS FOR IRON OR MANGANESE

SitC, the lipoprotein component of SitABC (**Figure 2B**), was first described as an immunogenic iron-repressible cytoplasmic membrane protein (IRMP) in *S. epidermidis* (Cockayne et al., 1998). The 32-kDa *S. epidermidis* SitC protein was found both to be expressed by *S. epidermidis* and to react strongly with antibodies in pooled human peritoneal dialysate (HPD) (Williams et al., 1988; Smith et al., 1991; Wilcox et al., 1991; Modun et al., 1992). HPD, the byproduct of continuous ambulatory peritoneal dialysis (CAPD) for renal failure, represents a severely iron-restricted environment (Wallaeys et al., 1986; Williams et al., 1988). Expression of SitC by *S. epidermidis* and *S. aureus* isolates known to cause peritonitis in CAPD patients highlighted a potential role for SitC in iron acquisition, and consequently *in vivo* survival of the staphylococci.

Sequence analysis of SitC revealed that it bears homology both to proteins involved in bacterial adhesion as well as to metal binding proteins (Cockayne et al., 1998). Two of the closest structural homologs to SitC are cluster A SBPs: the zinc-binding TroA protein from *Treponema pallidum* (PDB 1TOA) and the manganese-binding MntC protein from Synechocystis sp. (PDB 1XVL). Given this, and that SitC is lipid tethered to the cell membrane with distribution throughout the cell wall and minimal surface exposure (Smith et al., 1991; Wilcox et al., 1991; Cockayne et al., 1998), it is unlikely to play a role in bacterial adhesion, although involvement in adhesion has not been conclusively disproven. Greater surface exposure would be expected to promote adhesion, which is seen with the adhesins to which SitC was initially likened, but not for SitC itself (Jenkinson, 1992; Sutcliffe et al., 1993; Cockayne et al., 1998). The relatively porous nature of the cell wall, however, would render SitC accessible to metal cations, such as Fe^{2+}, Mn^{2+} or Zn^{2+}, as well as to antibodies. Additionally, the extracellular release of lipoproteins during iron starvation, such as SitC, would further contribute to immunogenicity (Cockayne et al., 1998). Despite a proposed role in divalent metal uptake, the fact that expression of *sitC* is repressed in the presence of excess Mn^{2+} or Fe^{2+} (Hill et al., 1998), and that SitC is highly expressed *in vivo*, the substrate of SitC in *S. epidermidis* remains unknown.

In contrast to *S. epidermidis,* the *sitABC* homologue in *S. aureus* has an identified function in the transport of Mn^{2+}, and was consequently named *mntABC* (Horsburgh et al., 2002) to

reflect this activity, following the nomenclature previously used in *B. subtilis* (Que and Helmann, 2000). While the presence of Mn^{2+} facilitates repression of *mntABC* through the DtxR-family regulator, MntR (SirR) (see regulation section, above), the role of iron in *mntABC* expression is still unclear (Horsburgh et al., 2002; Ando et al., 2003). Furthermore, the structure and characterization of the substrate binding properties of MntC (SitC) have yet to be elucidated. Regardless, with MntC (SitC) being identified as a predominant lipoprotein in *S. aureus* and *S. epidermidis*, and the observation that lipoprotein-deficient mutants are both inhibited in iron-restricted growth and in Toll-like receptor 2 (TLR2) activation in the host, it is not surprising that MntC (SitC) contributes an important role in staphylococcal pathogenesis and survival (Stoll et al., 2005; Bubeck Wardenburg et al., 2006; Schmaler et al., 2009, 2010; Muller et al., 2010).

ROLES FOR IRLPs IN IMMUNE RECOGNITION

The recognition of bacterial invaders by the innate immune system occurs through the identification of pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs). Of the >10 human TLRs currently identified, TLR2, together with the adapter molecule MyD88, represents the main responsive element to Gram-positive cell wall components and, consequently, staphylococcal infection (Takeuchi et al., 1999, 2000a,b). Until recently, the predominant staphylococcal PAMP for induction of TLR2 and a potent inducer of cytokine release was considered to be lipoteichoic acid (LTA) (Schwandner et al., 1999; Morath et al., 2001, 2005). However, it has been shown that the LTA fractions in these experiments were likely contaminated by lipoproteins, the implied true stimuli for TLR2-mediated inflammation (Hashimoto et al., 2006a,b, 2007). The generation of staphylococcal *lgt* mutants (see biogenesis section, above) has revealed that lipid modification of prelipoproteins is essential to maximal activation of and subsequent NF-κB-dependent cytokine release via TLR2-MyD88, both *in vitro* and *in vivo* (Stoll et al., 2005; Bubeck Wardenburg et al., 2006; Kurokawa et al., 2009; Schmaler et al., 2009). Conversely, an LTA-depleted mutant was still capable of inducing TLR2 (Kurokawa et al., 2009). Furthermore, highly purified SitC (MntC) co-localized with and induced TLR2 (Kurokawa et al., 2009; Muller et al., 2010), showing direct evidence of IRLP recognition by the host immune system.

In vivo studies have revealed a paradox in the expression of lipoproteins during infection. Of particular note, sublethal challenge of mice with an *lgt* mutant resulted in increased mortality relative to the wild-type infection, suggesting that lipoproteins are required to initiate the host innate immune response (Bubeck Wardenburg et al., 2006; Schmaler et al., 2009, 2010). Conversely, the expression of lipoproteins increases bacterial burden during persistent tissue infections, shown to be due to their role in enhancing staphylococcal survival through iron acquisition (Schmaler et al., 2009, 2010). While SitC (MntC) represents the predominant iron-regulated staphylococcal lipoprotein, as previously discussed, *S. aureus* lacking SitC (MntC) can still stimulate TLR2 (Kurokawa et al., 2009). Given that staphylococcal lipoproteins involved in the acquisition of iron are highly upregulated during iron starvation and expressed *in vivo* and *in vitro* (Morrissey et al., 2000; Sebulsky and Heinrichs, 2001; Dale et al., 2004a,b; Skaar et al., 2004a,b; Allard et al., 2006; Hempel et al., 2011), they together likely serve as important PAMPs for TLR2 recognition. Together these results suggest that the elaboration of multiple and sometimes redundant IRLPs may ensure staphylococcal survival in an otherwise inflamed host, providing an intriguing option for the development of potential therapeutics.

CLINICAL APPLICATIONS

Given their cell surface exposure, propensity to be expressed *in vivo* and corresponding immunogenicity, the IRLPs represent a tangible target for staphylococcal vaccine development. Indeed, both Sanofi-Pasteur/Syntiron and Novartis are including IRLPs in pre-clinical multivalent vaccine preparations. A discussion of *S. aureus* vaccine attempts is beyond the scope of this review, so we refer the reader to several excellent, recent reviews on the subject (Otto, 2010; Daum and Spellberg, 2012; DeDent et al., 2012; Patti, 2011; Proctor, 2011). The focus of published passive and active immunization efforts targeting iron acquisition systems thus far have centered on the Isd cell wall anchored proteins IsdA, IsdB, and IsdH, with promising results in animal models of *S. aureus* infection (Clarke et al., 2006; Kuklin et al., 2006; Ebert et al., 2010; Kim et al., 2010; Ster et al., 2010; Arlian and Tinker, 2011; Daum and Spellberg, 2012; DeDent et al., 2012; Harro et al., 2012). Of the Isd proteins, Merck and Intercell took their single-antigen IsdB vaccine, V710 (Kuklin et al., 2006), through phase I clinical trials, demonstrating that the vaccine was immunogenic within 2 weeks of administration and had a good safety profile in humans (Harro et al., 2010, 2012). Phase II/III clinical trials were terminated following a statistical review of the data suggesting that V710 was unlikely to demonstrate a significant benefit to patients (Patti, 2011). Many experts in the field are now suggesting that as opposed to a single-antigen vaccine, multivalent strategies, at least in humans, offer the greatest chance for success. In support of this recommendation, IsdA and IsdB, when combined with two additional *S. aureus* surface antigens, offered greater protective immunity, at least in rodent models, than when the animals were immunized with the individual antigens alone (Stranger-Jones et al., 2006).

In addition to the challenges in mounting an antibody response against the staphylococci (Foster, 2005), the functional redundancy (in terms of supplying a vital iron source to the bacteria) and strain variation in iron-regulated proteins suggests multiple systems should be targeted through a combinatorial vaccine. Indeed, the reduced fitness of *sir hts sst* mutants relative to single or double mutants recommends targeting multiple iron acquisition lipoproteins to inhibit growth (Beasley et al., 2011). Moreover, effective antibody-based approaches may require antibodies that inhibit protein function, in addition to, or in lieu of, being opsonic. Certainly, protective anti-IsdA and anti-IsdB antibodies appear to function by inhibiting heme acquisition (Kim et al., 2010). Thus, puzzling out the substrate specificities, expression patterns, presence/absence in different strains or species, and relevance, alone or in combination, to *in vivo* growth/infectivity is paramount toward effective use of the IRLPs as therapeutic targets. Aside of vaccine strategies, other avenues do exist for taking advantage of iron acquisition systems for therapeutic intervention. It is possible that knowledge on the substrate specificities,

along with detailed structural information, will lead to the use of the uptake systems as portals for toxic "trojan horse" compounds that kill or limit growth of the staphylococci.

CONCLUDING REMARKS

IRLPs play an essential role in the acquisition of iron, and consequently the *in vivo* survival of the staphylococci. Paradoxically, these lipoproteins are also strongly immunogenic, inducing an inflammatory response through recognition by TLR2-MyD88. A balance, therefore, exists between the surface display of multiple IRLPs and evading immune recognition by the host. Both of these characteristics make IRLPs a good choice of targets for vaccine development, and the formulation of a combinatorial vaccine targeting multiple iron uptake systems has been suggested to maximize efficacy. Further research is required to identify the source of the activity responsible for the N-acylation of staphylococcal lipoproteins. More work should also focus on obtaining high-resolution structural information on each of the proteins discussed here. As iron represents an essential element to survival, the IRLPs remain at the interface of pathogenesis and potential therapeutic control of the often pathogenic staphylococci.

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Staphylococcal response to oxidative stress

Rosmarie Gaupp†, Nagender Ledala† and Greg A. Somerville*

School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Michael L. Vasil, University of Colorado Medical School, USA
Anthony Richardson, University of North Carolina at Chapel Hill, USA

***Correspondence:**
Greg A. Somerville, School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, VBS, Fair St. and East Campus Loop, Lincoln, NE, 68583-0905, USA.
e-mail: gsomerville3@unl.edu

† These authors contributed equally to this work.

Staphylococci are a versatile genus of bacteria that are capable of causing acute and chronic infections in diverse host species. The success of staphylococci as pathogens is due in part to their ability to mitigate endogenous and exogenous oxidative and nitrosative stress. Endogenous oxidative stress is a consequence of life in an aerobic environment; whereas, exogenous oxidative and nitrosative stress are often due to the bacteria's interaction with host immune systems. To overcome the deleterious effects of oxidative and nitrosative stress, staphylococci have evolved protection, detoxification, and repair mechanisms that are controlled by a network of regulators. In this review, we summarize the cellular targets of oxidative stress, the mechanisms by which staphylococci sense oxidative stress and damage, oxidative stress protection and repair mechanisms, and regulation of the oxidative stress response. When possible, special attention is given to how the oxidative stress defense mechanisms help staphylococci control oxidative stress in the host.

Keywords: *Staphylococcus*, oxidative stress

INTRODUCTION

The genus staphylococcus represents a broad family of related species and subspecies that are widely distributed in nature and that colonize skin, skin glands, and mucous membranes of humans and other warm-blooded animals (Götz et al., 2006). Several staphylococcal species have commercial value and are routinely used as starter cultures in fermentation processes (Schleifer and Fischer, 1982; Geisen et al., 1992; Tanasupawat et al., 1992; Hammes et al., 1995; Montel et al., 1996; Probst et al., 1998; Place et al., 2003), whereas others have achieved importance as animal and human pathogens. On this latter point, some staphylococci, such as *Staphylococcus epidermidis*, are pathogens primarily due to their ability to colonize indwelling medical devices and to form biofilms (von Eiff et al., 2002; Vuong and Otto, 2002; McCann et al., 2008; Rogers et al., 2009). In contrast, *Staphylococcus aureus* is capable of causing a variety of diseases ranging from soft tissue infections to life-threatening septicemia. The ability of *S. aureus* to cause this wide array of infections is due to its diverse range of virulence factors and its resistance to numerous antibiotics. In addition, *S. aureus* is a prevalent cause of infections due to the fact that 20–30% of humans carry *S. aureus* in their anterior nares (Kluytmans et al., 1997; von Eiff et al., 2001; Wertheim et al., 2005). Lastly, the prominence of *S. aureus* as a pathogen is also due to its ability to evade or defend itself from the host immune system (Voyich et al., 2005; Palazzolo-Ballance et al., 2008; Foster, 2009). For these reasons, *S. aureus* is the most prominent staphylococcal pathogen of nosocomial and community-acquired infections and a leading cause of human infections worldwide (Lowy, 1998; Diekema et al., 2001; Stevens, 2003; Grundmann et al., 2006; Chambers and DeLeo, 2009; Rosenthal et al., 2010; Johnson, 2011). As oxidative and nitrosative killing mechanisms are important for the host immune response, this review will focus on the ability of staphylococci to resist oxidative stress with an emphasis on *S. aureus*, for which the greatest amount of information is available.

ENDOGENOUS AND EXOGENOUS OXIDATIVE AND NITROSATIVE STRESS

Endogenous oxidative stress can be caused by many things, including aerobic respiration, autooxidation reactions, intracellular redox reactions, and antibiotics (Pomposiello and Demple, 2002; Imlay, 2003; Kohanski et al., 2007; Yeom et al., 2010). During aerobic respiration, oxygen functions as a final electron acceptor in the electron transport chain where its complete reduction results in the formation of H_2O. Occasionally, oxygen undergoes incomplete reduction on interaction with flavoproteins (e.g., oxidases and monooxygenases) and can generate reactive oxygen species (ROS) (Messner and Imlay, 1999). Upon interaction with the reduced FAD cofactor of flavoenzymes, one or two electrons are transferred to molecular oxygen (Müller, 1987), leading to the generation of endogenous superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) (Massey et al., 1969; Korshunov and Imlay, 2010). In addition to flavoenzyme catalyzed reactions generating ROS, other reactions can also produce reactive oxygen intermediates (Imlay et al., 1988); specifically, Fenton chemistry can produce the highly-reactive hydroxyl radicals (HO·). This chemistry occurs when iron reacts with H_2O_2 and generates HO· (**Figures 1** and **2**). Fenton chemistry has been hypothesized to be possible with other metals such as Cu and Cd; however, questions remain about the physiological significance of non-ferrous metals catalyzing this chemistry (Macomber and Imlay, 2009).

In addition to endogenous oxidative stress, exogenous oxidative stress is a common challenge that bacteria must overcome in order to survive. Host innate immune cells such as macrophages, monocytes, and neutrophils have NADPH oxidase (NOX) that is responsible for the generation of the O_2^- during an oxidative

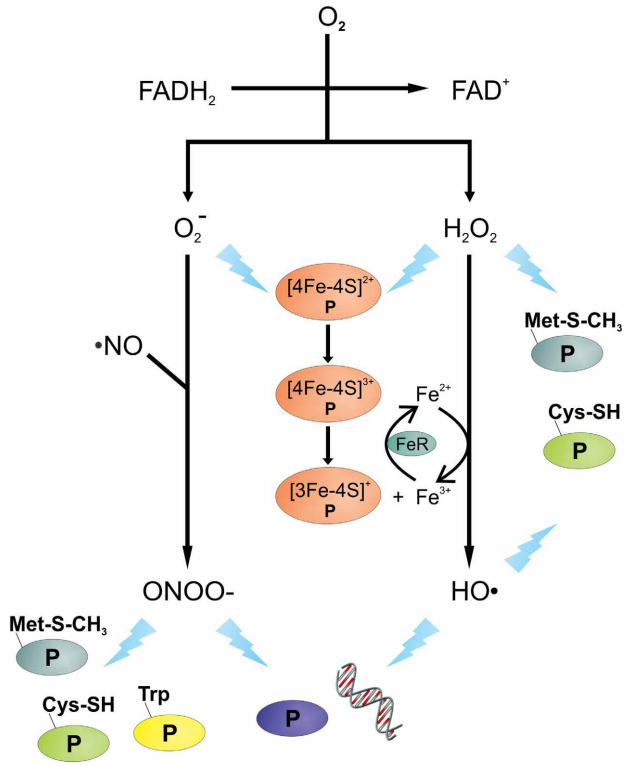

FIGURE 1 | Overview of oxidative and nitrosative stressors and their potential targets. The transfer of electron(s) from the reduced FAD of flavoenzymes to oxygen (O_2) can produce superoxide anions (O_2^-) and/or hydrogen peroxide (H_2O_2). Reaction of O_2^- with nitric oxide (·NO) can lead to the formation of peroxynitrite ($OONO^-$). Intracellular ferric (Fe^{3+}) reduction is catalyzed by ferric reductase (FeR), the Fe^{2+} can react with H_2O_2 to generate hydroxyl radicals (HO·). Damage to DNA and protein(s) is shown as a lightning bolt. Proteins are presented using letter "P".

burst. Superoxide is generated when the catalytic subunit of NOX transfers electrons from NADPH to oxygen; a process requiring FAD and heme (Nauseef, 2004). Once formed, O_2^- can undergo dismutation to H_2O_2. This H_2O_2 that is formed after dismutation of O_2^- can be used by the myeloperoxidase (MPO) complex to produce the bactericidal compound hypochlorite (OCl^-).

MPO, a 150 KDa heme-containing protein complex, is released into the phagosome when azurophilic granules fuse with the phagosome. In the phagosome, MPO binds to bacteria and catalyzes the H_2O_2-dependent conversion of Cl^- to OCl^- (Harrison and Schultz, 1976; Klebanoff, 1999). In addition to the generation of OCl^-, MPO is involved in the oxidation of L-tyrosine, such as that found in enkephalins, to the potent cytotoxic tyrosyl radical (o,o′-dityrosine) (Heinecke et al., 1993). That being said, the susceptibility of bacteria to MPO-mediated killing varies; hence, MPO is not considered essential for the innate immune response to bacteria (Lehrer et al., 1969; Lanza, 1998; Allen and Stephens, 2011).

Nitric oxide (·NO) is produced by all immune cells and it is important in the control of pathogens; however, like MPO, it is not equally effective against all pathogens (Bogdan et al., 2000). As an example, in mice, the function of ·NO in *S. aureus* control is limited, whereas for *Salmonella enterica* serovar Typhimurium it is critical (Nathan and Shiloh, 2000; Vazquez-Torres et al., 2000). Like ROS, NO is a reactive oxidant with potent cytotoxic properties against bacteria. In human macrophage, nitric oxide synthase (iNOS or NOS2) is induced on encountering a pathogen or by activation via cytokines. Once induced, iNOS or NOS2 catalyzes the conversion of L-arginine to L-citrulline and ·NO, a reaction that also reduces oxygen and oxidizes NADPH. While ·NO is toxic to bacteria by itself, ·NO has a synergistic effect with H_2O_2 to facilitate bacterial killing (Brunelli et al., 1995; Woodmansee and Imlay, 2003; Han et al., 2009). In addition, ·NO and O_2^- can form the bactericidal compound peroxynitrite ($OONO^-$) (**Figure 1**), a highly reactive nitrogen intermediate (Huie and Padmaja, 1993).

BACTERIAL TARGETS OF OXIDATIVE DAMAGE

The toxicity of ROS is due to its ability to damage any oxidizable moiety in a biological molecule. In *E. coli*, the importance of ROS damage has been demonstrated in mutants that lack components of the oxidative stress response system (i.e., superoxide dismutase, catalase, and peroxidase) (Carlioz and Touati, 1986; Park et al., 2005b; Jang and Imlay, 2007). In mutants lacking multiple genes of the oxidative stress response, the bacteria were highly sensitive to oxidants such as paraquat and H_2O_2. In this section we will attempt to address some of the consequences of oxidative stress in bacteria.

Superoxide and H_2O_2 can facilitate the release of iron from Fe-S cluster containing proteins, such as aconitase or serine dehydratase. Both O_2^- and H_2O_2 can oxidize the $[4Fe\text{-}4S]^{2+}$ to $[4Fe\text{-}4S]^{3+}$, which can be further oxidized, leading to the release of iron and the inactivation of the enzyme (Kuo et al., 1987; Flint et al., 1993; Jang and Imlay, 2007). In the presence of H_2O_2, the iron liberated from Fe-S clusters creates an intracellular environment permissive to Fenton chemistry, which generates the highly reactive HO·. Hydroxyl radicals will react with virtually the first molecule that it encounters; hence, to induce damage it must be in close proximity to its cellular target. DNA is a charged molecule that attracts positively charged molecules, like Fe^{2+}; hence, charge-charge interaction brings iron in close proximity to the DNA phosphodiester backbone. The close proximity of Fe^{2+} to DNA means that HO· generated by Fenton chemistry will likely react with DNA, inducing lethal or non-lethal mutations (Keyer and Imlay, 1996).

In addition to DNA being a target of ROS, amino acids, and proteins can be oxidized and/or modified by ROS. H_2O_2 can react with the Fe^{2+} of an iron-containing protein, presumably through Fenton chemistry, to cause irreversible protein carbonylation and the formation of protein aggregates (Dukan et al., 1999; Davies, 2005). Cysteine, methionine, and tryptophan can be oxidized by H_2O_2, HO·, and $ONOO^-$, which can lead to reversible or irreversible enzymatic inactivation. As an example, oxidation of cysteine residues can lead to reversible modifications (i.e., sulfenic acid or S-thiolation) or irreversible modifications (i.e., sulfinic acid, sulfonic acid) (Chouchani et al., 2011). Similarly, oxidation of methionine can lead to the formation of methionine sulfoxides, which are reversible through the action of the methionine sulfoxide reductase (discussed in Section "Methionine sulfoxide

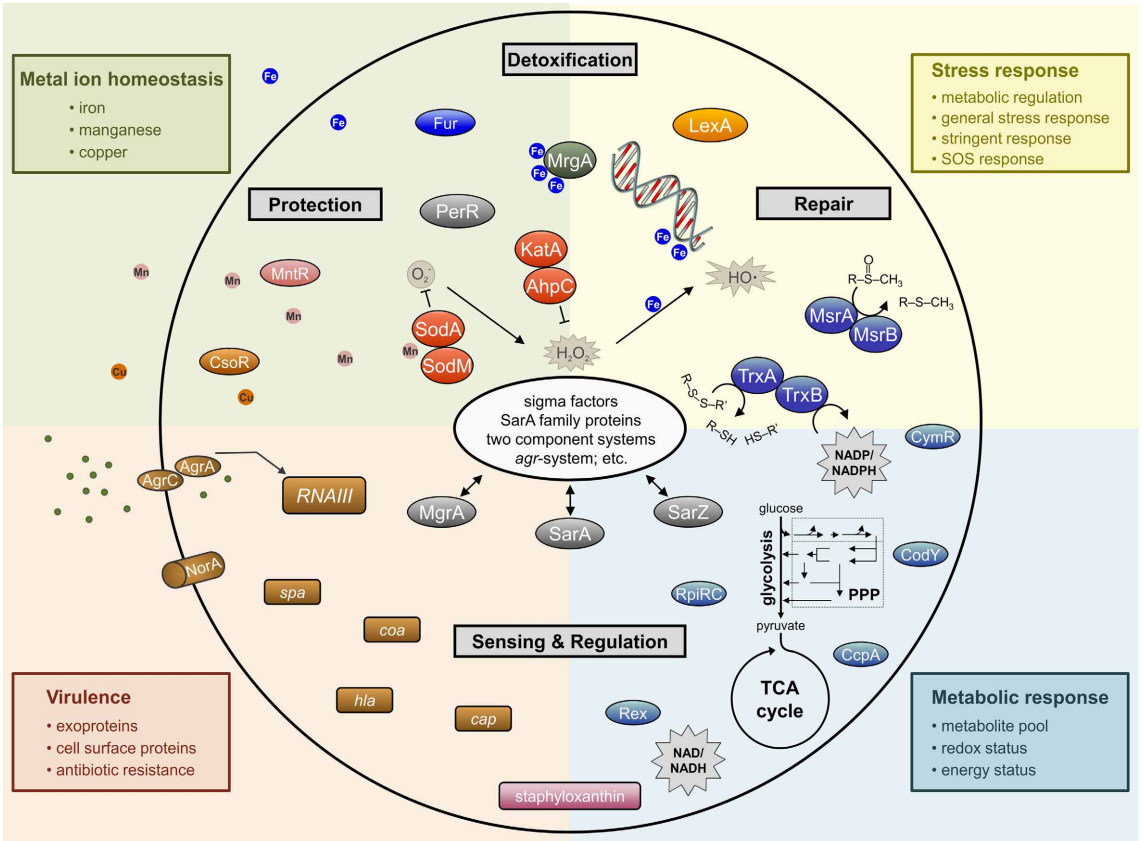

FIGURE 2 | Simplified schematic overview of important determinants involved in staphylococcal response to oxidative stress affecting whole cell physiology.

reductase"). The formation of some important ROS and the potential damage they cause are summarized in **Figure 1**.

OXIDATIVE STRESS RESISTANCE MECHANISMS

PIGMENTATION

As a general rule, most *S. aureus* strains isolated from human infections will form yellowish-orange or golden colonies due to the presence of carotenoid pigments. These pigments become more pronounced after 24 h of growth and when held at room temperature (Willis and Turner, 1962; Jacobs and Willis, 1964). An exception to this rule are the small colony variant (SCV) *S. aureus*, which are non-pigmented and may have auxotrophies for hemin, menadione, thiamine, or thymidine. SCVs are often associated with persistent and recurrent infections and are characterized by numerous phenotypic changes (Proctor et al., 2006). The main pigment of *S. aureus* is the membrane-bound orange-red C_{30} triterpenoid staphyloxanthin, which is synthesized from the enzymes coded within the *crtOPQMN* operon (Marshall and Wilmoth, 1981a,b; Pelz et al., 2005). The synthesis of staphyloxanthin involves the head-to-head condensation of two C_{15} isoprenoid molecules of farnesyl diphosphate to form dehydrosqualene, a reaction catalysed by dehydrosqualene synthase (CrtM). Dehydrosqualene is converted into 4,4′-diaponeurosporene by dehydrosqualene desaturase (CrtN), which is further oxidized, glycosylated, and esterified to yield staphyloxanthin (Wieland et al., 1994; Pelz et al., 2005). The *crtOPQMN* operon is under positive transcriptional control from the *rsbUVW*-σ^B system (Kullik et al., 1998; Giachino et al., 2001; Palma and Cheung, 2001; Bischoff et al., 2004) and under negative regulation by the small RNA, SsrA RNA (Liu et al., 2010).

Carotenoid pigments protect *S. aureus* against desiccation and photosensitization, and are known to quench toxic singlet oxygen. On this latter point, carotenoids are potent antioxidants due to their numerous conjugated double bonds, which make them an important survival factor for detoxifying ROS (Grinsted and Lacey, 1973; Mathews-Roth et al., 1974; Dahl et al., 1989; Krinsky, 1993; El-Agamey et al., 2004). The importance of staphyloxanthin in protecting *S. aureus* against ROS is seen in non-pigmented *crt* mutants, that grow normally, but have increased sensitivity toward ROS, $OONO^-$, and HOCl (Liu et al., 2005; Clauditz et al., 2006). One consequence of this increased sensitivity to oxidants is that *S. aureus* strains deficient in carotenoid biosynthesis are more readily cleared by the innate immune response (Salamah, 1992; Liu et al., 2005, 2008; Clauditz et al., 2006; Olivier et al., 2009). In a mouse subcutaneous abscess model as well as a systemic *S. aureus* infection model, non-pigmented *S. aureus* have reduced virulence and survival relative to the pigmented wild-type strain (Liu et al., 2005, 2008). The resistance to phagocytic

killing that is conferred by pigments is primarily attributed to the antioxidant properties of carotenoids, since the effect is not seen in NOX deficient mice or when the oxidative burst is inhibited (Liu et al., 2005). In addition to the antioxidant properties of pigments, staphyloxanthin can also alter membrane rigidity, which is important in protecting against non-oxidative host defences mediated by cationic peptides (Mishra et al., 2011). Because of the contribution of carotenoid pigments to staphylococcal fitness, inhibition of carotenoid biosynthesis is viewed as a potential therapeutic target in treating *S. aureus* infections (Daum, 2008; Haebich and von Nussbaum, 2008; Liu et al., 2008; Walsh and Fischbach, 2008; Song et al., 2009a,b; Oldfield, 2010).

DETOXIFYING ENZYMES

In addition to pigments, most staphylococci possess several enzymes that are used in the detoxification of reactive oxygen and nitrogen intermediates; particularly, superoxide dismutases, catalases, glutathione peroxidases, globins, and peroxiredoxins (**Figure 2**).

Superoxide dismutase (SOD)

Superoxide dismutases are metalloenzymes that catalyse the dismutation of O_2^- to oxygen and H_2O_2, which can be further reduced to water and oxygen by catalase or alkyl hydroperoxide reductase. By preventing the accumulation of O_2^-, SODs not only protect the bacterium from damage caused by O_2^-, but also against products that are derived from reactions requiring O_2^-, such as $OONO^-$ (**Figure 1**). SODs are classified into one of four types based on the metal ion cofactor; specifically, these are the copper-zinc type (Cu/Zn-SOD), the manganese type (Mn-SOD), the iron type (Fe-SOD), and the nickel type (Ni-SOD) (Fridovich, 1995; Kim et al., 1996, 1998). *S. aureus* possess two monocistronic superoxide dismutase genes, *sodA* and *sodM* (Poyart et al., 1995; Clements et al., 1999; Valderas and Hart, 2001); whereas, coagulase-negative staphylococci lack the *sodM* gene (Barrière et al., 2001a,b; Valderas et al., 2002).

The *S. aureus sodA* gene was identified during a screen for amino acid starvation survival mutants (Watson et al., 1998) and during characterization of that mutant, it was observed in a zymogram analysis that *S. aureus* had three zones of SOD activity (Clements et al., 1999). The three zones of activity were attributed to the presence of two homodimers and a heterodimer, which confirmed the presence of a previously identified second SOD (SodM) (Poyart et al., 1995; Valderas and Hart, 2001). During *in vitro* aerobic growth of *S. aureus*, the transcription and activity of both SODs increase in the post-exponential growth phase and remain high during the stationary phase, with SodA being responsible for the majority of SOD activity (Clements et al., 1999; Valderas and Hart, 2001; Karavolos et al., 2003). The transcription and activity of both SODs can also be increased by the addition of oxidants; specifically, *sodA* is induced by internal stressors and *sodM* by exogenous O_2^- stress. That being said, both SODs function in maintaining cell viability during exogenous O_2^- stress (Clements et al., 1999; Valderas and Hart, 2001; Karavolos et al., 2003). *In vivo*, the importance of superoxide dismutase in *S. aureus* infections is unclear, as some reports suggest that SOD is important (Kanafani and Martin, 1985; Karavolos et al., 2003; Das et al., 2008; Das and Bishayi, 2009), while others suggest that SOD has only a minimal effect on virulence (Mandell, 1975; Clements et al., 1999; Schneider et al., 2002). The difficulty in determining the function of SOD in virulence may be due to the observation that calprotectin sequesters Mn and Zn, decreasing SodA and SodM activity, and rendering the bacteria more susceptible to neutrophil-dependent killing (Kehl-Fie et al., 2011).

Both SODs in *S. aureus* are transcibed from σ^A-type promoters. The transcription of *sodA* initiates from one of two σ^A-type promoters; however, the first promoter is negatively influenced by σ^B. While the effect of σ^B on *sodA* transcription and activity remains unclear, transcription of *sodM* and activity are elevated in σ^B-deficient strains (Karavolos et al., 2003; Bischoff et al., 2004). Additionally, the staphylococcal accessory regulator (SarA) functions as a repressor of *sodM* transcription independent of σ^B. Other proteins of the SarA family have only slight effects (i.e., SarR) or no effect on *sodM* transcription. A less pronounced regulatory effect of SarA on *sodA* transcription has also been observed (Ballal and Manna, 2009).

Catalase and peroxiredoxin AhpC

As mentioned above, the detoxification of H_2O_2 is accomplished by catalases that catalyze the degradation of H_2O_2 to water and oxygen. The catalase family of proteins is divided into monofunctional or typical catalases, bifunctional catalase-peroxidases, and manganese-containing catalases (Chelikani et al., 2004). In the *Staphylococcus* genus, all species are catalase-positive with the exception of *S. saccharolyticus* and *S. aureus* subspecies *anaerobius* (Götz et al., 2006). *S. aureus* has a single monofunctional heme-containing tetrameric catalase encoded by the monocistronic *katA* gene (Sanz et al., 2000; Horsburgh et al., 2001a). In contrast, *S. xylosus*, *S. equorum*, and *S. saprophyticus* each have two catalase genes (Blaiotta et al., 2010). In addition to catalase, staphylococci have several peroxiredoxins that are induced upon treatment of *S. aureus* with H_2O_2 (i.e., Tpx, Ohr-like protein, and AhpC) (Wolf et al., 2008; Chen et al., 2009). Peroxiredoxins detoxify alkyl hydroperoxides by converting them to their corresponding alcohols using NADH or NADPH as the reducing equivalents. This reducing activity is dependent on redox-active cysteines in the active site. The alkyl hydroperoxide reductase (*ahpC*) gene forms an operon with *ahpF*, which codes for a homodimeric flavoenzyme that acts as a dedicated disulfide reductase to facilitate the reducing equivalent-dependent reduction and regeneration of AhpC (Poole, 2005). In *E. coli*, AhpC detoxifies low levels of H_2O_2, whereas KatA is the primary scavenger of H_2O_2 at high levels (Seaver and Imlay, 2001). Similar to *E. coli*, the *S. aureus* catalase is the major determinant in resistance toward H_2O_2 (Martin and Chaven, 1987; Horsburgh et al., 2001a), while AhpC confers resistance to a broader spectrum of ROS (Cosgrove et al., 2007). In *S. aureus* mutants lacking both catalase and AhpC, their ability to scavenge exogenous and endogenous H_2O_2 is inhibited, leading to the accumulation of H_2O_2 (Cosgrove et al., 2007).

In *S. aureus*, the *katA* gene and the *ahpCF* operon are negatively regulated by PerR (discussed in Section "PerR"), and putative PerR boxes are found in the promoter regions of both genes (Horsburgh et al., 2001a). In addition, the transcription of *katA* is positively affected by the ferric uptake regulator [Fur,

discussed in Section "Ferric uptake regulator (Fur)"] (Horsburgh et al., 2001b). As Fur normally functions as a repressor, it is likely that the positive regulation of *katA* is due to the repression of a positive regulator of *katA*, such as a small regulatory RNA. Together, PerR and Fur regulate transcription of the *katA* gene in response to peroxide and the availability of manganese and iron. As expected, maximal transcription of *katA* and catalase activity occur under aerobic conditions in the post-exponential growth phase, when tricarboxylic acid (TCA) cycle activity and electron transport are also maximal (Martin and Chaven, 1987; Horsburgh et al., 2001b; Cosgrove et al., 2007). The function of catalase in *S. aureus* virulence is not completely understood (Mandell, 1975; Kanafani and Martin, 1985; Horsburgh et al., 2001a; Messina et al., 2002; Cosgrove et al., 2007; Das et al., 2008; Das and Bishayi, 2009; Martínez-Pulgarín et al., 2009; Sen et al., 2009); however, both KatA and AhpC are important for nasal colonization (Cosgrove et al., 2007).

Flavohemoglobin (Hmp)

The discovery of a hemoglobin-like protein in *E. coli* (Vasudevan et al., 1991), and subsequent genome sequencing projects, led to realization that globins are widely distributed in nature. In *S. aureus*, the hemoglobin-like protein is a flavohemoglobin (Hmp), which has a N-terminal heme-containing globin domain and C-terminal NAD- and FAD-binding domains that together form a ferredoxin-$NADP^+$ oxidoreductase-like domain (Ermler et al., 1995). Hmp family members commonly demonstrate three enzymatic activities: NO-reductase, NO-dioxygenase, and alkylhydroperoxide reductase (Bonamore and Boffi, 2008). During aerobic growth the Hmp from *E. coli* utilizes NAD(P)H and O_2 to convert ·NO to nitrate; however, under anaerobic conditions it converts ·NO to N_2O, albeit less efficiently (Gardner et al., 1998; Kim et al., 1999). *In vitro* experiments using *E. coli* Hmp have demonstrated an NADH-dependent alkyhydroperoxide reductase activity; however, the *in vivo* significance of this activity remains to be elucidated (Bonamore et al., 2003). Like *E. coli*, *S. aureus* Hmp activity is greatest during microaerobic/anaerobic growth or during nitrosative stress conditions (Gonçalves et al., 2006; Richardson et al., 2006). Regulation of Hmp activity in response to reduced oxygen tension is mediated in part by the SrrAB two-component system; presumably, at the transcriptional level. That being said, the regulators of *hmp* transcription remain unknown in *S. aureus*, although, it has been postulated to involve the NO_2^--sensing transcription repressor, NsrR (Richardson et al., 2006).

METAL HOMEOSTASIS

Transition metal ions (i.e., Fe, Cu, Mn, and Zn) give structure to proteins, act as cofactors to enzymes, and are essential for electron transfer; hence, they are required by all forms of life (Lippard and Berg, 1994). The ability of transition metals to transfer electrons is beneficial under some circumstances; however, it is this same ability that facilitates the generation of ROS through Fenton chemistry (Gutteridge et al., 1982; Imlay et al., 1988). For this reason, the transport of metal ions is very tightly regulated to maintain an appropriate intracellular concentration and to avoid the accumulation of metals to toxic levels (**Figure 2**). To maintain metal ion homeostasis, bacteria have evolved active transporters, efflux systems, and metallochaperones (Finney and O'Halloran, 2003; Maier et al., 2007; Bagai et al., 2008).

Iron

Iron is an important cofactor for numerous enzymes; hence, it is essential for bacterial viability (Griffiths, 1999). Despite iron being one of the most abundant elements in nature, it primarily exists in the insoluble Fe^{3+} form, which is difficult for bacteria to acquire (Ratledge and Dover, 2000). In the host, the low solubility of iron and the presence of heme, ferritin, and lactoferrin, create an environment in which free iron is essentially non-existent (Brown and Holden, 2002). This iron-limited environment coupled with the bacterial need for iron, creates a major challenge for bacteria in the host (Weinberg, 1978). To counter this challenge, *S. aureus* have adapted to extract iron from heme, which is carried out in part using the proteins encoded by the iron-regulated surface determinant (Isd) genes, *isdA*, *isdB*, *isdCDEF*, *isdG*, *isdH*, and *isdI* (Skaar et al., 2004; Skaar and Schneewind, 2004; Torres et al., 2006). In addition, *S. aureus* can extract iron from transferrin using the siderophores staphyloferrin A and B (encoded by *sfaABCD* and *sbnABCDEFGHI*, respectively) (Dale et al., 2004; Park et al., 2005a; Cheung et al., 2009; Cotton et al., 2009). When complexed with iron, these siderophores are transported into the bacterial cytoplasm through the HtsABC and SirABC transport systems using the energy from FhuC catalyzed ATP hydrolysis to drive the importation (Speziali et al., 2006; Beasley et al., 2011). In addition, *S. aureus* can acquire iron from hydroxymate siderophores produced by other bacteria and from catecholamine (Morrissey et al., 2000; Sebulsky et al., 2003, 2004; Beasley et al., 2011). In *S. aureus*, these iron acquisition and transport systems are regulated in part by the Fur [(Xiong et al., 2000); discussed in Section "Ferric uptake regulator (Fur)"].

After iron has been acquired by *S. aureus*, it is utilized or it is bound by ferritin, bacterioferritin comigratory protein (Bcp), or the Dps homolog MrgA (Metallo regulated gene A), which function as iron chelator/storage proteins. Ferritin, a polypeptide with a ferroxidase center, is encoded by *ftnA* in *S. aureus* and *sefA* in *S. epidermidis* (Horsburgh et al., 2001a; Morrissey et al., 2004) and both function primarily as iron-storage proteins (Andrews, 1998). In contrast, MrgA has an iron-chelating function and protects DNA from oxidative damage (discussed in Section "MrgA"). Bcp is homologous to peroxiredoxin and as such is likely involved in the thiol-dependent reduction of peroxides. In *S. aureus*, transcription of *bcp*, *ftnA*, and *mrgA* is regulated by PerR, highlighting the importance of sequestering iron during periods of oxidative stress (Horsburgh et al., 2001a; Chang et al., 2006; Wolf et al., 2008).

Manganese

Like iron, manganese is an essential cofactor in bacteria that is involved in diverse cellular functions such as, sugar metabolism, signal transduction, the stringent response, and oxidative stress resistance (Kehres and Maguire, 2003; Papp-Wallace and Maguire, 2006). In contrast to iron, Mn^{2+} is soluble in a physiological pH range and it has a higher reduction potential than Fe^{2+}, meaning that it is less likely to facilitate deleterious redox reactions.

For these reasons, Mn^{2+} is important for the detoxification of ROS in bacteria. As examples, the activity of SodA (discussed in Section "Superoxide dismutase") requires Mn^{2+} and SodM is also believed to require Mn^{2+} (Clements et al., 1999; Valderas and Hart, 2001). To supply the *S. aureus* Mn^{2+} requirement, there are two transporters for manganese, encoded by *mntABC* (MntABC-type) and *mntH* (Nramp-type) and in *S. epidermidis*, *sitABC* codes for the MntABC-type transporter (Cockayne et al., 1998; Horsburgh et al., 2002).

The importance of Mn^{2+} to bacteria can be inferred from the observation that host phagocytic cells transport Mn^{2+} out of the phagosome upon engulfing a bacterium. To do this, phagocytic cells recruit the efflux protein Nramp1 to the phagosome; thus, reducing the availability of Mn^{2+} to the bacterium (Jabado et al., 2000). In addition, the heterodimeric host protein calprotectin decreases Mn^{2+} availability by chelating it (Corbin et al., 2008). Because bacteria need Mn^{2+} and host cells attempt to deny the bacteria Mn^{2+}, it is not surprising that *S. aureus* virulence is attenuated in mutant strains lacking both *mntA* and *mntH* relative to the isogenic wild-type bacteria. Similarly, mutation of *mntA*, *mntH*, or *mntR* reduces *S. aureus* survival in human endothelial cells (Horsburgh et al., 2001a, 2002; Ando et al., 2003).

Regulation of Mn^{2+} transport is critical for maintaining metal ion homeostasis. In *S. epidermidis*, the DtxR homolog SirR binds to a Sir box consensus sequence in the promoter region of *sitABC*, in a Mn/Fe-dependent manner, to repress transcription (Cockayne et al., 1998; Hill et al., 1998). Similarly, in *S. aureus*, the DtxR homolog MntR represses *mntABC*, but not *mntH*, in a Mn^{2+}-dependent manner by binding to a MntR box in the promoter region. In addition to MntR, PerR also regulates *mntABC* transcription and a putative PerR box is located in the promoter region of this operon. This communal regulation of *mntABC* by MntR and PerR is also seen with other members of the PerR regulon (Horsburgh et al., 2002).

Zinc

Zinc is an essential nutrient that is required as a cofactor for a few enzymes; however, it's more important function is in protein stability (Lippard and Berg, 1994). While Zn may be essential for bacterial viability, an over abundance of Zn is toxic due to the fact that it competes with other metals for binding to the active centers of enzymes (Beard et al., 1997; Xiong and Jayaswal, 1998). The essential nature of Zn and its potential to be toxic make obvious the importance of Zn ion homeostasis, and demonstrate why bacteria have evolved zinc transporters (Blencowe and Morby, 2003). In *S. aureus*, Zn homeostasis is maintained by the plasmid encoded CadCA (*cadCA*) transporter and/or the chromosomally encoded ZntRA (*zntRA* or *czrAB*) transporter (Endo and Silver, 1995; Xiong and Jayaswal, 1998; Kuroda et al., 1999). Both transport systems code for ArsR/SmtB family transcriptional regulators (CadC and ZntR) (Busenlehner et al., 2003) and metal-exporting membrane proteins CadA and ZntA (Guffanti et al., 2002). In the presence of excess zinc, CadC and ZntR bind Zn, decreasing their affinity for their cognate promoters and this de-represses transcription of *cadA* and *zntA* (Nucifora et al., 1989; Singh et al., 1999; Ye et al., 2005).

In *B. subtilis*, Zn transport is primarily mediated by the proteins encoded within the *znuABC* operon, and *zosA*. Transcription of *znuABC* is regulated by the Zn-responsive Fur homolog known as Zur, while the transcription of *zosA* is regulated by PerR [discussed in Section "PerR" (Lee and Helmann, 2007)]. In complex with Zn, the Zur homodimer functions as a transcriptional repressor by binding to a Zur-box (AAATCGTAATNATTACGATTT) present in the promoter/operator region of *znuABC* (Gaballa et al., 2002; Ma et al., 2011). When the availability of Zn is low, Zur releases from the DNA, de-repressing transcription of the ATP binding cassette Zn transporter coded by *znuABC*. The P-type metal-transporting ATPase coded by *zosA* is induced in response to H_2O_2, consistent with its proposed function in oxidative stress resistance (Gaballa and Helmann, 2002; Gaballa et al., 2002). Although *zosA* has not been identified in *S. aureus*, it does have genes homologous to *znuA* and *znuB*; designated as *mreA* and *mreB*, respectively. As stated, Zn has important physiological functions; however, the role of Zn transport in *S. aureus* pathogenesis remains to be elucidated (Lindsay and Foster, 2001).

Copper

Although limited in number, copper-requiring enzymes have critical roles in bacterial respiration, biosynthesis, and oxidative stress resistance; hence, Cu is considered an essential trace element (Halliwell and Gutteridge, 1984; Puig and Thiele, 2002). The enzymatic utility of Cu is due in part to its ability to act as an electron donor or acceptor by cycling between the Cu^{2+} to Cu^{1+} oxidation states. Like iron, the properties of Cu that make it a useful redox cofactor, also allow it to facilitate the generation of ROS (Baker et al., 2010). For this reason, bacteria have evolved mechanisms to protect themselves from the toxic effects of Cu, while maintaining the intracellular concentration of Cu at the minimum necessary for growth (Solioz and Stoyanov, 2003; Liu et al., 2007; Wolschendorf et al., 2011). In *S. aureus*, copper homeostasis is primarily maintained by the P_1-type ATPase CopA and the copper chaperone CopZ; CopZ sequesters intracellular Cu^{1+} and delivers it to the Cu exporter CopA (Sitthisak et al., 2007). While CopA and CopZ are highly conserved in *S. aureus*, some strains also have a second P_1-type ATPase copper transporter, CopB, and a Cu oxidizing enzyme, multicopper oxidase (*mco*) (Sitthisak et al., 2005). In contrast to the chromosomally encoded genes *copA* and *copZ*, both *copB* and *mco* are carried on a plasmid or on a plasmid integrated into the chromosome (Holden et al., 2004; Baker et al., 2011). Importantly, the plasmid carrying *copB* and *mco* can be transferred between *S. aureus* strains (Baker et al., 2011). Regulation of *copA*, *copZ*, *copB*, and *mco* is dependent upon the Cu^{1+} responsive copper-sensitive operon repressor (CsoR) (Baker et al., 2011; Grossoehme et al., 2011). CsoR binds to DNA in the absence of Cu^{1+} and represses transcription; however, as the intracellular concentration of copper increases, CsoR complexes with Cu^{1+}, releases from the DNA, and de-represses transcription of copper resistance genes. The *in vivo* importance of Cu resistance for *S. aureus* is likely due to the fact that macrophage increase the Cu concentration in phagosomes via the ATP7A Cu transporter, which enhances the bactericidal activity of the phagosome (Wagner et al., 2005; White et al., 2009).

DNA PROTECTION AND REPAIR

MrgA

The genomic DNA of bacteria is organized in a nucleoid that requires DNA supercoiling, molecular crowding, and several architectural proteins (e.g., Hu, H-NS, Fis, Dps) (Dame, 2005; Luijsterburg et al., 2006). Dps (DNA-binding protein from starved cells) is a member of the ferritin super family of proteins and a non-specific DNA binding protein that functions as a major determinant for protecting DNA by nucleoid condensation (Martinez and Kolter, 1997; Wolf et al., 1999; Nair and Finkel, 2004). During the stationary phase of growth in *E. coli*, the conformation of DNA changes from a relaxed state to a compacted state, a process requiring Dps (Kim et al., 2004; Ohniwa et al., 2006). This complex DNA condensation process is regulated during the exponential growth phase by Fis and H-NS binding to the *dps* promoter and repressing transcription. (Ohniwa et al., 2006; Grainger et al., 2008). Unlike *E. coli*, *S. aureus* appears to lack both Fis and H-NS homologs (Ohniwa et al., 2011); hence, transcription of the Dps homolog MrgA is likely linked to growth via other means. One possible linkage between growth and regulation of *mrgA* is PerR (discussed in Section "PerR").

Transcription of *mrgA* is induced by H_2O_2 and iron (Horsburgh et al., 2001a; Morrissey et al., 2004; Chang et al., 2006; Morikawa et al., 2006; Wolf et al., 2008), suggesting that PerR and Fur regulate *mrgA* transcription. This suggestion is partially true as PerR is a repressor of *mrgA* transcription; whereas, *mrgA* transcription is independent of Fur. Consistent with PerR regulation of *mrgA*, inactivation of *perR* results in a compacted nucleoid in the absence of oxidative stress, which is similar to that found in a strain overexpressing *mrgA* (Morikawa et al., 2006, 2007). The activating effect of iron on *mrgA* transcription, while not completely understood, is related to the fact that the MrgA/Dps protein is a ferritin-like Fe^{2+} binding and storage protein (Grant et al., 1998; Zhao et al., 2002; Su et al., 2005). The compact nature of the nucleoid and the susceptibility of DNA to oxidative damage (discussed in Section "Bacterial targets of oxidative damage") likely led to the evolution of a bi-functional protein involved in DNA condensation and protection from Fe^{2+} generated HO· (**Figure 2**).

Excision repair

Oxidative damage to DNA can occur at the bases or sugars, producing lesions such as strand breakage or base alterations (Demple and Harrison, 1994; Lu et al., 2001). Due to the obvious importance of DNA in species propagation, DNA repair mechanisms have evolved to maintain genetic integrity. These DNA repair mechanisms can be divided into two broad catagories; excision repair [i.e., base excision repair (BER), mismatch repair (MMR), and nucleotide excision repair (NER)] and recombinational repair. As with many aspects of bacterial physiology, much of our knowledge has been derived from the study of model bacteria; therefore, we will use this knowledge to draw inferences into staphylococcal DNA repair mechanisms. In time, these inferences will likely be proven correct as *S. aureus* has homologs of many of the DNA repair enzymes discussed below; specifically, for BER/MMR *S. aureus* has Nfo, MutM, MutY, MutT, MutS, MutL, RecJ, and Nth (O'Neill and Chopra, 2002; Prunier and Leclercq, 2005; Ambur et al., 2009).

Multistep BER pathways rely on damage-specific DNA glycosylases that scan DNA, recognize base lesions, and initiate removal by cleaving the base–deoxyribose glycosyl bond, forming apurinic/apyrimidimic sites (AP site). The next step in the repair process is restoration of the correct DNA sequence via short-patch (1-nucleotide patch size) or long-patch (multiple nucleotide patch size) pathways. Depending on the enzymatic properties of the initiating DNA glycoslyase, the activities of AP endonucleases and/or DNA deoxyribosephosphodiesterase (drPase) are required to break the DNA during this process. In *E. coli*, AP endonuclease activity is primarily due to exonuclease III (ExoIII or Xth) and endonuclease IV (EndoIV or Nfo), while drPase activity is due to RecJ and exonuclease I (ExoI) (Ljungquist, 1977; Lindahl, 1979; Rogers and Weiss, 1980; Franklin and Lindahl, 1988; Sandigursky and Franklin, 1992; Mol et al., 2000).

During periods of oxidative stress, a common lesion found in DNA is the oxidized base 7,8-dihydro-8-oxoguanine (8-oxoG or GO lesion), which can mispair with adenine (Shibutani et al., 1991). In *E. coli*, the formamidopyrimidine DNA glycosylase (Fpg or MutM) acts on oxidized and ring-opened purines, while the adenine DNA glycosylase MutY prevents mutagenic transversions by removing already misincorporated adenine (Michaels et al., 1992a,b; Michaels and Miller, 1992). In addition to repairing DNA, oxidized guanine is removed from the nucleotide pool by the pyrophosphohydrolase activity of MutT (Maki and Sekiguchi, 1992).

The 5,6-double bond of pyrimidines is susceptible to reaction with HO· creating a number of oxidatively damaged products, such as thymine glycol. In *E. coli*, pyrimidine lesions are recognized by endonuclease III (EndoIII or Nth) and endonuclease VIII (EndoVIII or Nei) (Radman, 1976; Demple and Linn, 1980; Katcher and Wallace, 1983; Breimer and Lindahl, 1984; Wallace, 1988). The removal of misincorporated bases occurs by the MMR system, which requires MutSL for recognition and initiation of excision repair (Modrich, 1991; Marti et al., 2002). Genetic defects in MMR and/or GO systems are associated with mutator phenotypes and as such, these damage repair systems are important in adaptive mutagenesis and the generation of genetic diversity, which was also demonstrated for *S. aureus* (O'Neill and Chopra, 2002; Chopra et al., 2003; Prunier and Leclercq, 2005; Vidales et al., 2009).

In contrast to BER, NER lesions are repaired in 12–13 nucleotide-long segments, followed by synthesis of a repair patch using the intact strand as a template and ligation of the repaired ends. Briefly, damaged DNA is detected by a complex of UvrA and UvrB. After the DNA damage is detected, UvrB binds the damaged DNA, displacing UvrA, and allowing UvrC to complex with UvrB. It is this UvrBC complex that cleaves the phosphodiester backbone to create a 12 base excision. UvrD facilitates dissociation of base pairing, causing the release of the nucleotide segment. At this point, the deletion can be filled by DNA polymerase I and then the newly synthesized DNA is ligated to the existing DNA. In addition to UvrAB, the transcription-repair coupling factor Mfd can recruit the DNA excision-repair machinery to damaged DNA (Sancar, 1996). This process is likely similar in *S. aureus* as it has the *uvrABC* genes and *mfd* (Ambur et al., 2009).

Recombinational repair

The predominant consequence of oxidative damage to the sugar in DNA is strand breakage, which can be repaired by mechanisms used in recombination. In *E. coli*, repair of DNA strand breaks initiates when RecBCD binds to the blunt end of a double stranded DNA break and the helicase activity of RecB and RecD unwinds the DNA. Due to severe consequences of strand breakage (i.e., death), there is redundancy in the initiation of recombinational repair systems (i.e. RecF and SbcCD pathways). While the repair of most double strand DNA breaks in *E. coli* are initiated by the RecBCD exonuclease/helicase complex, low-GC content Gram-positive bacteria, including staphylococci, lack this pathway. Gram-positive bacteria initiate double strand break repair using the AddAB nuclease/helicase complex, functional homologs of RecBCD, or homologs of the RecF and SbcCD pathways (Alonso et al., 1993; Eisen and Hanawalt, 1999; Ambur et al., 2009; Yeeles and Dillingham, 2010). After initiating recombinational repair and as the DNA is unwound, RecA binds to the single stranded DNA and pairs with the homologous DNA sequence and initiates strand invasion. Following strand invasion, RuvAB drive branch migration and then in concert with RuvC, cuts the DNA to resolve the Holliday junction. The process of branch migration and resolution can be catalyzed by RecG as well. These additional components of double strand break repair are conserved in *S. aureus* (i.e., RecA, RuvAB, and RecG) (Niga et al., 1997; Ambur et al., 2009).

PROTEIN DAMAGE REPAIR

Thioredoxin

In bacteria, the cytoplasm is in a reduced state; hence, protein thiols can be maintained in their reduced form (Pollitt and Zalkin, 1983; Derman and Beckwith, 1991). This reduced state of the bacterial cytoplasm depends heavily on the thioredoxin and glutaredoxin systems and the low-molecular-weight thiol reductants coenzyme A (CoASH) and bacillithiol (BSH) (Brown, 1959; Derman et al., 1993; Prinz et al., 1997; Di Simplicio et al., 2003). While many bacteria have both the thioredoxin and glutaredoxin systems, most Gram-positive bacteria, including *S. aureus*, lack the glutaredoxin system (Vido et al., 2005; Diep et al., 2006); thus, these bacteria rely heavily on the thioredoxin system to maintain a reduced cytoplasm (Scharf et al., 1998; Uziel et al., 2004). The importance of thioredoxin cannot be overstated as it is essential for a large variety of cellular processes, including acting as a hydrogen donor to ribonucleotide reductase and methionine sulfoxide reductases (Russel and Model, 1986; Aberg et al., 1989). As important, thioredoxins are major contributors to oxidative stress resistance by facilitating the reduction of H_2O_2, scavenging HO·, and donating reducing equivalents to peroxiredoxins and peroxidase (Arnér and Holmgren, 2000) (**Figure 2**).

The thioredoxin system is comprised of thioredoxin (*trxA*) and the thioredoxin reductase (*trxB*). Thioredoxins are small disulfide reductase proteins, while thioredoxin reductase uses the electrons from NADPH to maintain thioredoxin in a reduced state (Holmgren, 1985). In *B. subtilis*, *trxA* and *trxB* transcription is maintained at a basal level during growth but it is increased in response to diamide, H_2O_2, heat, salt, or ethanol stress (Scharf et al., 1998; Leichert et al., 2003; Mostertz et al., 2004). These increases in *trxA* and *trxB* transcription are primarily mediated by σ^A, σ^B, and Spx (Scharf et al., 1998), an RNA polymerase-dependent transcriptional activator that responds to diamide stress (Nakano et al., 2003). Similar to *B. subtilis*, transcription of *trxA* and *trxB* in *S. aureus* is maintained at a basal level under aerobic and anaerobic growth conditions, with Spx being required for transcription under all growth conditions (Horsburgh et al., 2001a; Pamp et al., 2006; Ballal and Manna, 2010). Also similar to *B. subtilis*, stressors such as copper, diamide, menadione, and tert-butyl hydroperoxide induce transcription of *trxA* and *trxB* (Uziel et al., 2004; Wolf et al., 2008; Baker et al., 2010).

As stated above, bacteria rely on the cysteine-containing small proteins thioredoxin and glutaredoxin to carry out the thiol-disulfide redox cycling reactions and maintain a reduced cytoplasm (Holmgren, 1989); therefore, cysteine biosynthesis is critical for sustaining the reducing environment of the cytoplasm. This can be seen by the fact that under diamide or H_2O_2-induced oxidative stress, *S. aureus* increases cysteine biosynthesis and uptake (Chang et al., 2006; Wolf et al., 2008). In *B. subtilis* and *S. aureus*, cysteine biosynthesis and transport are negatively regulated by CymR in complex with CysK [o-acetyl serine (OAS) thiol-lyase] (Even et al., 2006; Soutourina et al., 2009). Although cysteine is needed to maintain the reducing environment of the cytoplasm via thioredoxin, the intracellular concentration of cysteine is kept low due the ability of free cysteine to reduce Fe^{3+} to Fe^{2+}, which can facilitate Fenton chemistry (Park and Imlay, 2003). Thus, the intracellular concentration of cysteine must be finely balanced, or the reducing environment of the cytoplasm will be compromised. This can be seen in *cymR* mutants where cysteine accumulates in the cytoplasm and susceptibility to H_2O_2 also increases (Soutourina et al., 2009, 2010).

CoA reductase

Coenzyme A functions as a substrate for biosynthesis and the oxidation of pyruvate and fatty acids. These functions rely on the ability of CoA to form high-energy thioester bonds, such as that found in acetyl-CoA (Magnuson et al., 1993; del Cardayre et al., 1998). In addition to the metabolic importance of CoA, staphylococci use CoA and, possibly, bacillithiol as low molecular weight free thiols to help maintain the reducing environment of the cytoplasm (Newton et al., 1993; del Cardayre et al., 1998; Fahey, 2001; Pöther et al., 2009). To fill the need for reduced CoA (CoASH), coenzyme A disulfide reductase catalyzes the NADPH-dependent reduction of CoASSCoA to CoASH and protein-SH (Coulter et al., 1998). The importance of coenzyme A disulfide reductase is reflected in the observation that inactivation of the *S. aureus* coenzyme A disulfide reductase results in the attenuation of virulence for mice relative to the isogenic strain (Coulter et al., 1998; Schneider et al., 2002). Because of the metabolic demand for CoA and the use of CoA as a free thiol, it is difficult to predict which function is more responsible for this attenuation.

Methionine sulfoxide reductase

Methionine is highly susceptible to oxidation (Dean et al., 1997; Grimaud et al., 2001), which can lead to structural changes that alter or inhibit enzymatic functions. When oxidized, methionine forms diastereomeric S and R forms of methionine sulfoxide

(Brot et al., 1981; Moskovitz et al., 1996; Sharov et al., 1999). To repair this change, most life forms rely on methionine sulfoxide reductases (Moskovitz et al., 1996). The oxidized S form of methionine is reduced by the enzyme MsrA, while the R form is reduced by MsrB. The reduction of methionine sulfoxide to methionine is dependent on the thioredoxin thiol-disulfide redox system or other reducing agents that can donate electrons (Russel and Model, 1986). The importance of methionine sulfoxide reductase can be seen in *E. coli* where GroEL, a chaperone involved in the folding of polypeptides, is highly susceptible to inactivation by host immune cell generated $ONOO^-$ and HOCl (Khor et al., 2004; Sasindran et al., 2007).

In most bacteria genes encoding for the enzymes, MsrA and MsrB, are commonly present as a single copy for each one (Ezraty et al., 2005). In *S. aureus*, there are three paralogs of *msrA* (i.e., *msrA1*, *msrA2*, *msrA3*), and a single *msrB* gene. *msrA1* and *msrB* are present in an operon with a PTS permease and a fourth gene of unknown function; whereas, *msrA2* and *msrA3* are unlinked with *msrA1* and *msrB* (Singh et al., 2001; Singh and Moskovitz, 2003). As expected, *msrA1* deletion makes *S. aureus* more sensitive to H_2O_2; however, the other *msrA* paralogs do not complement for the *msrA1* deletion. In the case of *msrA2*, this may be due poor transcription rather than inability to function as a methionine sulfoxide reductase. Interestingly, *msrA1* transcription can be induced by oxacillin and other cell wall antibiotics, but not by peroxide (Singh et al., 2001; Singh and Moskovitz, 2003). While some work has been performed on methionine sulfoxide reductase in *S. aureus*, the fitness benefit of having three *msrA* paralogs remains to be determined.

Fe-S cluster repair

Iron-sulfur clusters are ubiquitous prosthetic groups that are involved in diverse cellular processes such as electron transfer, enzyme activity, environmental sensing, and gene regulation. The biogenesis of these Fe-S clusters requires the presence of assembly systems (e.g., *isc*, *suf*, and *csd* operons/systems), containing cysteine desulfurases, Fe-S scaffold proteins, and other accessory proteins (Johnson et al., 2005; Fontecave and Ollagnier-de-Choudens, 2008). As mentioned in Section "Bacterial targets of oxidative damage", Fe-S clusters are very susceptible to oxidative inactivation; hence, to survive in an aerobic environment, bacteria have evolved Fe-S cluster repair mechanisms. In some bacteria, the *suf* system appears to maintain Fe-S cluster assembly under oxidative stress conditions, while IscS is thought to repair Fe-S clusters (Nachin et al., 2003; Djaman et al., 2004). In addition to IscS, the recovery of aconitase and fumarase activity after oxidative damage is facilitated by the Fe-S cluster repair proteins YtfE in *E. coli* and ScdA in *S. aureus* (Justino et al., 2006, 2007; Overton et al., 2008; Vine et al., 2010). ScdA is a di-iron protein that is widely distributed among bacteria and is believed to be a new protein family that repairs Fe-S clusters (Overton et al., 2008). In *S. aureus*, the monocistronic *scdA* gene was first identified as being involved in autolysis and cell division (Brunskill et al., 1997). Transcription of *scdA* is de-repressed in the post-exponential growth phase when TCA cycle activity and electron transport are maximal and the bacterial demand for iron is at its greatest (Brunskill et al., 1997; Somerville et al., 2003), a process mediated by MgrA [(Ingavale et al., 2003); discussed in Section "MgrA"]. Consistent with the MgrA-dependent repression of *scdA*, transcription of *scdA* can be induced by exposure to H_2O_2 (Chang et al., 2006) and *scdA* inactivation leads to increased sensitivity to H_2O_2 (Overton et al., 2008). The importance of *scdA* to staphylococcal pathogenesis remains to be determined.

SENSING AND REGULATION

As oxidative stress affects all aspect of bacterial physiology, it is understandable that regulation of cellular processes that respond to oxidative stress is complex (**Figure 2**). In fact, the complexity is significantly increased when one considers that the staphylococcal response to oxidative stress depends on the chemical nature of the oxidant. This was demonstrated when *S. aureus* was treated with three different oxidative stress inducing compounds (i.e., paraquat, H_2O_2, diamide) and the protein profiles for each stress had only limited overlap (Wolf et al., 2008). Because many of the regulators that affect the oxidative stress response are discussed in other areas of this Frontiers Research Topic, we have limited our discussion to only a few of the sensing and regulatory systems in staphylococci.

ENVIRONMENTAL SENSING—THE METABOLIC RESPONSE

Despite the large percentage of the staphylococcal genomes dedicated to metabolism and physiology, staphylococci only require 13 biosynthetic intermediates to synthesize all macromolecules in the cell. These 13 biosynthetic intermediates are derived from the three metabolic pathways of central metabolism: glycolysis, the pentose phosphate pathway (PPP), and the TCA cycle. Because of the importance of these 13 intermediates, staphylococci have evolved metabolite responsive regulators (e.g., CcpA, CodY, RpiR) to "sense" the availability of these intermediates or compounds derived from them (Somerville and Proctor, 2009). As stated above, oxidative stress leads to the rapid inactivation of Fe-S cluster containing enzymes and the reversible and irreversible oxidation of some cysteine and methionine-containing proteins. Hence, oxidative stress alters enzymatic activity, resulting in changes in metabolite concentrations as well as the redox poise. These changes in the bacterial metabolic status create signals that alter the activity of redox-responsive and metabolite-responsive regulators (e.g., Rex, CcpA, CodY, RpiR) (Egeter and Brückner, 1996; Seidl et al., 2008a,b; Pagels et al., 2010). It is for this reason that metabolite-responsive regulators, such as CodY (Majerczyk et al., 2010) and RpiRC (Zhu et al., 2011) are involved in regulating components of the oxidative stress response (**Figure 2**).

FERRIC UPTAKE REGULATOR (Fur)

The ferric uptake regulator (Fur) is a transcriptional regulator that is partially responsible for maintenance of iron homeostasis in many bacteria, including *S. aureus* and *S. epidermidis* (Ernst et al., 1978; Hantke, 1981; Heidrich et al., 1996; Xiong et al., 2000). Fur is a homodimeric metalloprotein with an N terminal DNA binding domain and C terminal dimerization domain that may be occupied by structural zinc (Jacquamet et al., 1998; Gonzalez de Peredo et al., 1999; Sheikh and Taylor, 2009). Fur when complexed with iron can regulate the transcription of

genes by binding to a 19 bp inverted repeat sequence known as the Fur box (GATAATTGATAATCATTATC) in the promoter region (Ochsner et al., 1995; Escolar et al., 1999; Xiong et al., 2000; Baichoo and Helmann, 2002). Fur primarily functions as a repressor; therefore, changes in gene expression during growth in iron-limited medium are similar to those changes observed in a *fur* mutant (Johnson et al., 2011). Interestingly, *in vitro* studies of *fur* mutants have demonstrated that the number of genes regulated by iron far outweighs the number of genes identified as being directly regulated by Fur. It is hypothesized that Fur-independent transcription of genes that are regulated by the availability of iron may be regulated by *agr*, *rot*, and *sae*, as the transcription of these regulators is influenced by Fur during iron-limited growth. As stated, Fur is primarily a transcriptional repressor; however, Fur is a positive effector of *katA* transcription and consistent with this positive effect, *fur* mutants have reduced catalase activity and increased sensitivity to peroxide stress (Horsburgh et al., 2001b). The more likely explanation for the positive effect of Fur on *katA* transcription is that Fur represses transcription of a positive regulator of *katA* transcription; specifically, small RNAs have been implicated in regulating *S. aureus* transcription and translation (Allard et al., 2006; Felden et al., 2011). In *S. aureus*, Fur is speculated to regulate the accumulation of immunomodulatory proteins, cytolytic proteins, and to protect against neutrophil-mediated killing (Torres et al., 2010). Despite this speculation, *fur* inactivation has a slight effect on *S. aureus* virulence in one type of experimental model (Horsburgh et al., 2001b).

PerR

PerR is a second member of the Fur family of regulators in staphylococci, and it has been identified as a peroxide sensing protein (Horsburgh et al., 2001a; Lee and Helmann, 2006a, 2007). The PerR regulon includes many genes involved in the oxidative stress response and iron storage, including *katA*, *ahpCF*, *mrgA*, *bcp*, and *trxA* genes. The fact that PerR regulates part of the oxidative stress response would suggest it is important for surviving the host immune response during an infection; however, in a mouse model of infection, *perR* mutants are only slightly attenuated in virulence relative to the parental strain (Horsburgh et al., 2001a). As a member of the Fur family of regulators, the activity of PerR is dependent upon metal ions. In *B. subtilis*, PerR carries structural zinc and its DNA binding activity is enhanced when complexed with either Fe or Mn (Lee and Helmann, 2006a). PerR containing Fe or Mn will function as a transcriptional repressor by binding to a consensus DNA sequence, termed the PerR box (AAGTATTATTTATTATTATTA) (Chen et al., 1995; Horsburgh et al., 2001a). In the presence of H_2O_2, the iron in PerR leads to formation of HO·, which oxidizes the iron-coordinating histidines, causing the loss of iron and DNA binding activity (Lee and Helmann, 2006b). When PerR is complexed with Mn, it is less likely to be inactivated by H_2O_2 because Mn is a poor mediator of Fenton chemistry; thus, in the absence of HO· there is little oxidation of the metal coordinating histidines and PerR retains its DNA binding properties. Based on this mechanism of activity regulation, it is understandable that in the presence of high Mn^{2+} and low Fe^{2+} PerR regulon members remain repressed in the presence of H_2O_2 (Horsburgh et al., 2001a, 2002; Fuangthong et al., 2002).

MgrA

MgrA (multiple gene regulator) is a member of MarR family of regulators that positively affects capsule biosynthesis and nuclease accumulation, represses α-toxin, coagulase, and protein A synthesis, and represses autolysis (Ingavale et al., 2003; Luong et al., 2003, 2006). In addition, MgrA regulates transcription of several multidrug efflux pumps (i.e., NorA, NorB, NorC, and Tet38) (**Figure 2**); thus, MgrA functions in staphylococcal resistance to different antibiotics including fluoroquinolones, tetracycline, vancomycin or penicillin (Truong-Bolduc et al., 2003, 2006; Cui et al., 2005; Kaatz et al., 2005; Truong-Bolduc et al., 2005; Chen et al., 2006; Truong-Bolduc et al., 2006). In total, transcriptional profiling has revealed that MgrA affects the transcription of as many as 350 genes (Luong et al., 2006). This global reach of MgrA is achieved by binding to target genes as well as by indirect regulation through its affects on other regulators (i.e., SarS, SarV, SigB, LytRS, and ArlRS) (Ingavale et al., 2003, 2005; Truong-Bolduc et al., 2003; Manna et al., 2004; Luong et al., 2006). The global nature of MgrA is also reflected in the fact that it is required for the establishment and progression of *S. aureus* infections in murine abscess, septic arthritis, and sepsis models (Chen et al., 2006; Jonsson et al., 2008; Sun et al., 2011). *S. aureus* MgrA is structurally similar to the MarR of *E. coli* in that it contains a DNA binding, helix-turn-helix domain and a dimerization domain (Chen et al., 2006). In addition, MgrA contains a single cysteine (Cys12) in the dimerization domain that is accessible to oxidizing agents. This dimer interface domain is similar to that of the *B. subtilis* peroxide-sensing regulator OhrR (Fuangthong et al., 2001; Fuangthong and Helmann, 2002; Lee et al., 2007; Soonsanga et al., 2007), which when the cysteines of the two monomers are oxidized leads to dissociation of MgrA from the DNA (Chen et al., 2006). In addition, to regulating activity via Cys oxidation, the activity of MgrA is modulated by the eukaryotic-like serine/threonine kinase (Stk1 or PknB) (Truong-Bolduc et al., 2008).

SarZ

In addition to MgrA, a second MarR family regulator, SarZ, is involved in sensing oxidative stress. Like MgrA, SarZ has a DNA-binding helix–turn–helix motif, a single cysteine (Cys13), and a dimerization domain. The oxidation of Cys13 to sulfenic acid by peroxides is insufficient to disrupt SarZ DNA-binding properties; however, generation of a mixed disulfide or further oxidation to sulfinic acid or sulfonic acid leads to a de-repression of transcription. Thus, in *S. aureus*, SarZ and MgrA function as thiol switches, similar to the *B. subtilis* OhrR (Chen et al., 2006, 2011; Poor et al., 2009). Among the SarZ affected genes, many code for proteins involved in intermediary, amino acid, fatty acid, nucleotide, and sugar metabolism, including regulators of pyrimidine synthesis (*pyrR*) and gluconate catabolism (*gntR*, GntR-like protein). In addition, SarZ regulates transcription of the H_2O_2-inducible Ohr-like peroxiredoxin (Chen et al., 2009). Interestingly, there is little regulatory overlap between SarZ and MgrA affected genes (Luong et al., 2006; Chen et al., 2009). Since the Cys oxidation

mechanisms for regulating the activity of MgrA and SarZ are similar, this absence of overlap may only partially explain why different oxidants create different proteome profiles (Wolf et al., 2008).

SarA

The SarA (Cheung et al., 1992) is a promiscuous DNA binding protein containing a single cysteine (Cys9) at the dimerization interface that may be involved in oxidative stress sensing; however, SarA is more sensitive to alkylation than it is to oxidation (Chen et al., 2011). The DNA binding activity of SarA may be influenced by the redox poise or the oxidative status of the cytoplasm (Chan and Foster, 1998; Lindsay and Foster, 1999; Fujimoto et al., 2009). This may explain why SarA is a negative effector of superoxide dismutase and thioredoxin reductase transcription (Ballal and Manna, 2009, 2010). In addition to regulating some aspects of the oxidative stress response, SarA affects transcription of genes involved in many cellular processes, including virulence related genes and amino acid, nucleotide, and cell wall metabolism genes (Dunman et al., 2001).

SOS RESPONSE

If the ROS burden is high and the general stress response systems (e.g., σ^B-system) are overwhelmed, then the SOS response can become activated. As mentioned above, oxidative stress frequently induces DNA damage. For this reason, it is not surprising that exposure of *S. aureus* to H_2O_2 can induce the LexA regulated SOS response (Chang et al., 2006; Wolf et al., 2008). The SOS response is a highly conserved global DNA damage repair system that can be triggered by numerous DNA damaging agents, including fluoroquinolone or β-lactam antibiotics (Anderson et al., 2006; Cirz et al., 2007; Erill et al., 2007). During an SOS response, the sensor protein RecA becomes activated by non-specific binding to single-stranded DNA that is derived from recombinational repair or stalled replication. Activated RecA stimulates the autocatalytic cleavage of the SOS transcriptional repressor LexA in the C-terminal dimerization domain and in the N-terminal DNA binding domain, leading to the de-repression of SOS genes. When RecA no longer encounters ssDNA, the concentration of non-cleaved LexA increases and the SOS repair system is deactivated. While the SOS system is important for staphylococcal survival, it has also been linked to virulence, antibiotic resistance, and the dissemination of mobile genetic elements (Úbeda et al., 2005, 2007; Goerke et al., 2006; Kelley, 2006; Maiques et al., 2006). As an example, the gene encoding fibronectin binding protein B (*fnbpB*) is part of the LexA regulon in *S. aureus* (Bisognano et al., 2004).

CONCLUSION

Staphylococci face the near constant challenge of surviving in the presence of exogenous and endogenous oxidants. To meet this challenge, staphylococci have evolved a multitude of oxidative defense strategies that require a coordinated regulatory response (**Figure 2**). This regulatory response relies on molecular sentinels to detect oxidative stress or the damage caused by oxidative stress and to transduce these signals to regulators that enhance or repress transcription of the defence genes in proportion to the challenge. Once activated, the defence machinery must repair or degrade and replace damaged DNA and proteins. Disruptions in the ability of staphylococci to sense, respond, or repair oxidative stress, and the damage caused by oxidative stress, results in a fitness cost that makes the bacterium more sensitive to oxidative damage.

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Identification of a lactate-quinone oxidoreductase in *Staphylococcus aureus* that is essential for virulence

James R. Fuller†, Nicholas P. Vitko†, Ellen F. Perkowski, Eric Scott, Dal Khatri, Jeffrey S. Spontak, Lance R. Thurlow and Anthony R. Richardson*

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

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***Correspondence:**
Anthony R. Richardson, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, 116 Manning Dr, CB7290, Chapel Hill, NC 27599, USA.
e-mail: anthony_richardson@med.unc.edu

†*James R. Fuller and Nicholas P. Vitko have contributed equally to this work and are designated as co-authors.*

Staphylococcus aureus is an important human pathogen commonly infecting nearly every host tissue. The ability of *S. aureus* to resist innate immunity is critical to its success as a pathogen, including its propensity to grow in the presence of host nitric oxide (NO·). Upon exogenous NO· exposure, *S. aureus* immediately excretes copious amounts of L-lactate to maintain redox balance. However, after prolonged NO·-exposure, *S. aureus* reassimilates L-lactate specifically and in this work, we identify the enzyme responsible for this L-lactate-consumption as a L-lactate-quinone oxidoreductase (Lqo, SACOL2623). Originally annotated as Mqo2 and thought to oxidize malate, we show that this enzyme exhibits no affinity for malate but reacts specifically with L-lactate ($K_M = \sim 330\,\mu M$). In addition to its requirement for reassimilation of L-lactate during NO·-stress, Lqo is also critical to respiratory growth on L-lactate as a sole carbon source. Moreover, Δ*lqo* mutants exhibit attenuation in a murine model of sepsis, particularly in their ability to cause myocarditis. Interestingly, this cardiac-specific attenuation is completely abrogated in mice unable to synthesize inflammatory NO· ($iNOS^{-/-}$). We demonstrate that *S. aureus* NO·-resistance is highly dependent on the availability of a glycolytic carbon sources. However, *S. aureus* can utilize the combination of peptides and L-lactate as carbon sources during NO·-stress in an Lqo-dependent fashion. Murine cardiac tissue has markedly high levels of L-lactate in comparison to renal or hepatic tissue consistent with the NO·-dependent requirement for Lqo in *S. aureus* myocarditis. Thus, Lqo provides *S. aureus* with yet another means of replicating in the presence of host NO·.

Keywords: *Staphylococcus aureus*, lactate-quinone oxidoreductase, virulence, myocarditis, pericarditis, metabolism

INTRODUCTION

Staphylococcus aureus is a pathogen of tremendous importance to human health (Diekema et al., 2001). The Gram-positive bacterium can be commonly isolated from nasal passages, axillae, and perinea of healthy carriers (Mermel et al., 2011). While the most common disease presentations of *S. aureus* are skin/soft tissue infections, it is also a frequent cause of sepsis, endocarditis, and osteomyelitis (Klevens et al., 2007). The latter conditions can be complicated by bacterial spread to cardiac muscle resulting in purulent abscesses or myocarditis (Wasi and Shuter, 2003). *S. aureus* can also seed the pericardial space resulting in pericarditis necessitating surgical drainage followed by extensive intravenous antibiotic administration (Klacsmann et al., 1977; Mookadam et al., 2009). While pericarditis and myocarditis are not the most frequent disease presentations associated with *S. aureus* infections, this organism is the one of the most common causes of both life-threatening conditions (Wasi and Shuter, 2003; Mookadam et al., 2009). It is thought that myocarditis and pericarditis are rare complications of sepsis because bacteria in general do not efficiently colonize muscle tissue. Indeed, bacterial infections in skeletal muscles (pyomyositis/myositis) are even more rare than infections of cardiac muscle tissue. However, as with pericarditis and myocarditis, the most common cause of bacterial myositis and pyomyositis is *S. aureus* (Pannaraj et al., 2006). Thus, this pathogen has a propensity to colonize and thrive in nearly every sterile site in the body, even environments not conducive to supporting most other bacterial pathogens.

In order for *S. aureus* to inhabit sterile sites within the human body, it must be able to resist numerous host innate immune effectors (Foster, 2005). Nitric oxide (NO·) is a key broad-spectrum antimicrobial host effector that is essential for the efficient clearance of a variety of microbial pathogens (DeGroote and Fang, 1999). As with most other immune effectors, *S. aureus* is uncommonly resistant to the effects of exogenous NO· (Richardson et al., 2006, 2008; Hochgrafe et al., 2008). This NO·-resistance results from the induction of a metabolic state that circumvents the effects of this cytotoxic radical (Richardson et al., 2008). NO· is known to interfere with various bacterial metabolic pathways including the tricarboxylic acid cycle, aerobic respiration, fatty acid metabolism, pyruvate metabolism, and nucleic acid synthesis (Richardson et al., 2008, 2009, 2011). While we do not fully understand how *S. aureus* accommodates the loss of multiple NO·-sensitive targets, progress has been made with regard to *S. aureus* adaptation to the redox imbalance associated with NO·-stress. Upon exposure to exogenous NO·, *S. aureus* ceases to respire and shifts into a fermentative metabolic state concomitant with increased excretion of L-lactate

(Richardson et al., 2008). The reduction of pyruvate to L-lactate provides redox balance by regenerating NAD^+ in a cell unable to utilize the respiration-dependent NADH-dehydrogenase (Complex I of the electron transport chain). In *S. aureus* most of the L-lactate production is catalyzed by lactate dehydrogenase 1 (Ldh1), an allele that is unique to *S. aureus* and not found in other staphylococci (Richardson et al., 2008). Coincidentally, other staphylococcal species are incapable of robust growth during NO·-stress (Richardson et al., 2008). *S. aureus* additionally possesses another L-lactate dehydrogenase (Ldh2) that is shared among most other staphylococcal species. However, Ldh2 is expressed even in the absence of NO· and, given the strong induction of *ldh*1 following NO·-exposure, Ldh2 offers only a minor contribution to redox balance during NO·-stress (Richardson et al., 2008). Finally, *S. aureus* encodes a D-lactate dehydrogenase (Ddh) that also produces D-lactate during NO·-exposure, albeit to a much lower level than the L-enantiomer. Thus, *S. aureus* possesses three lactate-dehydrogenases all of which regenerate NAD^+ with Ldh1 playing the major redox-balancing role during growth in the presence of exogenous NO·.

However, a series of elegant studies conducted nearly a half-century ago characterized NAD-independent L-lactate dehydrogenase (*i*LDH) activity in *S. aureus* (Stockland and San Clemente, 1969). *i*LDHs oxidize L-lactate to pyruvate with a concomitant reduction of the respiratory quinone pool (Stevenson and Holdsworth, 1973). Electrons are eventually donated to terminal acceptors, and in *S. aureus* L-lactate oxidation was shown to be coupled to the reduction of ferric iron, oxygen, or nitrate (Theodore and Weinbach, 1974; Lascelles and Burke, 1978; Tynecka and Malm, 1995). While many *i*LDH enzymes exist in the microbial world, no such gene is annotated in any of the current *S. aureus* genome sequences (more than 14 to date; Stevenson and Holdsworth, 1973). Moreover, homology searches using known *i*LDH sequences were unsuccessful at identifying a gene encoding such an enzyme in *S. aureus*. Thus, the identification of a putative fourth lactate utilization gene responsible for observed enzymatic activity has proved elusive.

Here we identify the gene encoding the *S. aureus* *i*LDH as SACOL2623 originally annotated as Mqo2, a malate-quinone oxidoreductase. Given that malate is chemically similar to L-lactate and that malate- and L-lactate-dependent enzymes are phylogenetically related, the coding genes are occasionally misannotated (Madern, 2002). We show that purified Mqo2 has no affinity for malate and thus we suggest the protein be renamed Lqo. We characterize the contribution of Lqo to *S. aureus* catabolism of L-lactate both in the presence and absence of exogenous NO·. Lqo orthologs are present, within similar genetic environments, in the sequenced genomes of all *Staphylococcus* species. We also define a role for Lqo in *S. aureus* virulence in a murine model of sepsis. Specifically Δ*lqo* mutants are defective for growth within murine cardiac tissue in the presence of host NO·. We show that while *S. aureus* NO·-resistance is predicated on the presence of a glycolytic carbon source, specifically combining L-lactate with peptides also provides *S. aureus* with the means to replicate in the presence of NO· in an Lqo-dependent manner. The high levels of both L-lactate and protein in murine cardiac tissue are consistent with the specific NO·-dependent requirement for Lqo in murine septic myocarditis/pericarditis. Thus, Lqo represents a key metabolic enzyme allowing *S. aureus* to utilize elevated host L-lactate associated with active muscle tissue and, at the same time, resist the cytostatic effects of host NO·.

RESULTS

STAPHYLOCOCCUS AUREUS HARBORS AN UNIDENTIFIED L-LACTATE CATABOLIC ENZYME

Upon exposure to exogenous NO·, *S. aureus* evokes fermentative metabolism to contend with the propensity of NO· to block respiration. Accordingly, L-lactate, and to a lesser extent, D-lactate are both produced from pyruvate providing the cell with redox balance (**Figures 1A,B**). However, after prolonged exposure to NO·, L-lactate is specifically reassimilated but D-lactate is not (**Figure 1B**). It should be noted that this L-lactate-consumption occurs despite the presence of excess glucose (data not shown). We reasoned that reversal of the NAD-dependent Ldh1, Ldh2, or Ddh reactions was not responsible for the observed L-lactate-consumption, because: 1. the reverse reaction would be detrimental to the redox state of the cell and 2. both L-lactate and D-lactate would have been consumed since both reactions are reversible, enzymes for both L-lactate and D-lactate are highly expressed during NO·-stress (Richardson et al., 2006) and both enantiomers can support growth of WT *S. aureus*. Indeed, Ddh allows for D-lactate production anaerobically as well as its aerobic utilization (**Figure 1C**). Moreover, the Δ*ddh* mutant can neither produce nor consume D-lactate (**Figure 1C**). In contrast, while mutants lacking Ldh1 and Ldh2 do not produce L-lactate anaerobically, the Δ*ldh*1Δ*ldh*2 mutant still grows aerobically on L-lactate as a sole carbon source (**Figure 1C**). This is not due to racemase activity converting L-lactate to D-lactate as the triple Δ*ldh*1Δ*ldh*2Δ*ddh* mutant still grows on L-lactate aerobically (data not shown). Thus, *S. aureus* must encode another L-lactate catabolic enzyme that is irreversible (no L-lactate production in the Δ*ldh*1Δ*ldh*2 mutant) and likely does not use NAD^+ as a cofactor as this would represent a futile cycle with the high Ldh1 levels in NO·-exposed cells. The enzyme responsible for the previously described *i*LDH activity could explain these observations.

SACOL2623 (*mqo*2) ENCODES THE *S. AUREUS* *i*LDH ACTIVITY

No gene in any sequenced *S. aureus* genome is predicted to encode a putative *i*LDH, nor are there any identifiable homologs of well-characterized *i*LDH genes in *S. aureus*. However, *S. aureus* does encode two malate-quinone oxidoreductases (Mqo1 and Mqo2) that are thought to participate in the TCA cycle since this organism lacks an NAD-dependent malate dehydrogenase (**Figure 2A**). These enzymes are predicted to use an FAD-cofactor to assist in transferring electrons directly to the respiratory quinone pool, and therefore would oxidize their substrates independently of NAD^+. Since malate and lactate are structurally similar in that malate is essentially a carboxylated lactate (**Figure 2A**), we reasoned that one or both of the annotated Mqo enzymes may react with L-lactate and encode the *S. aureus* *i*LDH. Indeed, combining a deletion of *mqo*2, but not *mqo*1, with the Δ*ldh*1Δ*ldh*2 mutation abolished the ability to grow aerobically on L-lactate as a sole carbon source (**Figure 2B**). Thus, despite the juxtaposition of *mqo*1 to a gene encoding a predicted L-lactate permease (**Figure 2A**), it

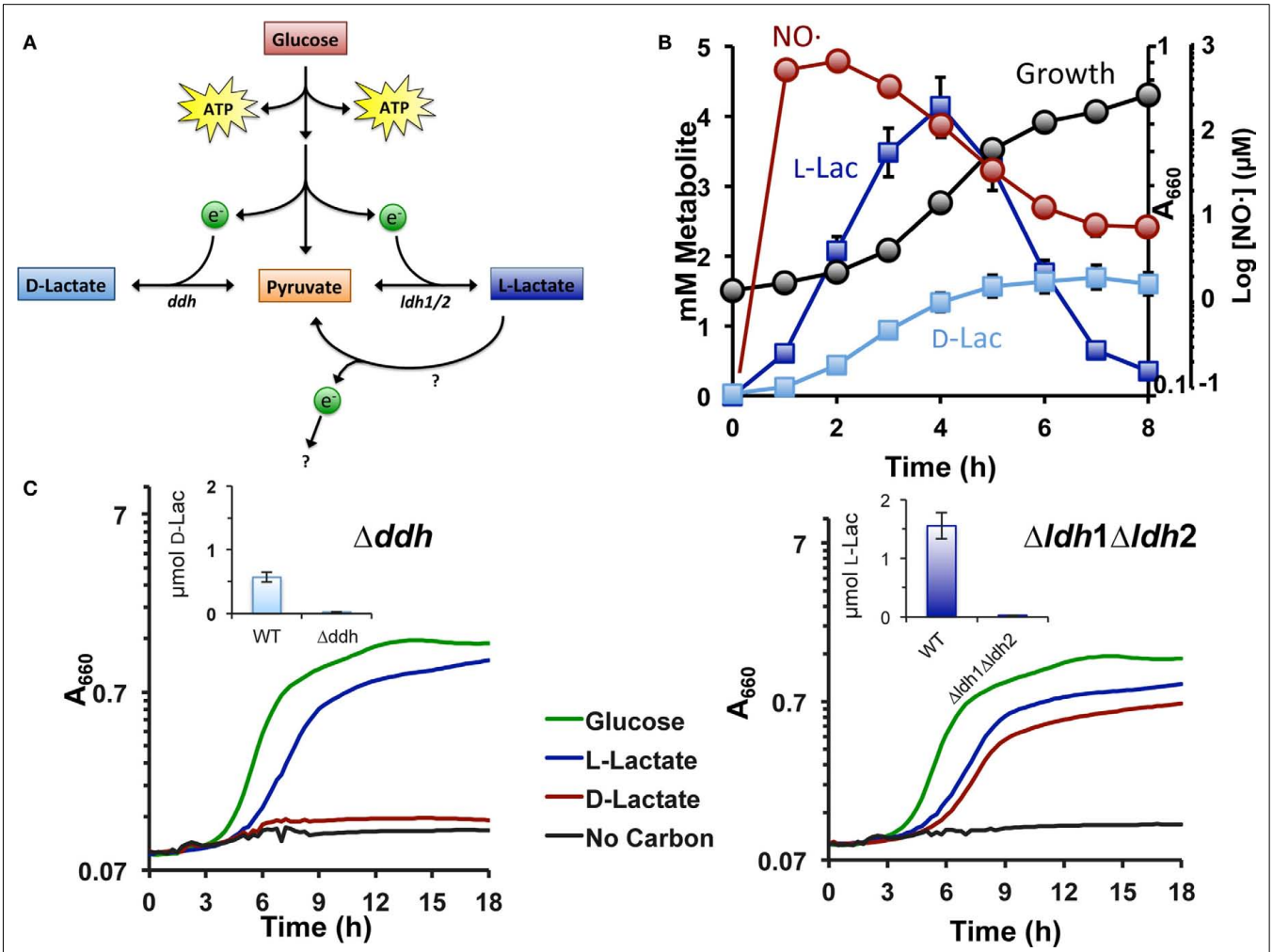


FIGURE 1 | *Staphylococcus aureus* encodes a fourth biologically relevant L-lactate utilization enzyme. (A) Known metabolic end products of *S. aureus* glucose catabolism. **(B)** Levels of extracellular D-lactate (light blue squares) and L-lactate (dark blue squares) following exposure to exogenous NO· (red circles). Cells were grown in chemically defined medium to an $OD_{660} = 0.15$ (black circles) at such time a combination of NO·-donors (10 mM NOC-12 and 1 mM DEA/NO) was added. The resulting NO· burst peaked at 1.5 h at ∼1 mM. **(C)** Δ*ddh* mutants (LEFT) no longer excrete D-lactate anaerobically (inset) and specifically lack the ability to grow on D-lactate as a primary carbon source. In contrast, while Δ*ldh*1Δ*ldh*2 mutants (RIGHT) do not produce measurable L-lactate anaerobically (inset), the double mutant is still able to utilize L-lactate as a primary carbon source. Residual growth on medium components was subtracted from depicted growth curves (see Materials and Methods).

appears that Mqo2 reacts with L-lactate (**Figure 2B**). Additionally, a Δ*mqo*2 mutant, but not Δ*mqo*1, was unable to reassimilate L-lactate during prolonged NO·-exposure (**Figure 2C**). However, the lack of L-lactate-reassimilation in the Δ*mqo*2 mutant had little reproducible effect on growth in the presence of NO· when glucose was used as the primary carbon source, and there were no effects on the rate of glucose consumption nor glucose yield (mg biomass/mg glucose consumed, data not shown). These data suggest that Mqo2 can oxidize L-lactate to pyruvate and therefore comprises the *S. aureus i*LDH activity. However, while *S. aureus i*LDH is active in the presence of NO· (**Figure 1B**), its role during growth on glucose under NO·-stress is still unclear.

In order to distinguish between Mqo2 having dual specificities and the possibility that it only has affinity for L-lactate, we performed enzymatic assays on membrane fractions from single mutant strains to determine substrate specificity. The Δ*mqo*1 mutant (only expressing Mqo2) exhibited L-lactate-specific activity but no real activity with malate (**Figure 3**). Conversely, a Δ*mqo*2 mutant membrane preparation reacted readily with malate, but not L-lactate (**Figure 3**). Membrane preparations from neither the Δ*mqo*1 nor the Δ*mqo*2 mutant reacted with D-lactate or pyruvate suggesting that Mqo2 was specific for L-lactate and the enzyme was non-reversible (data not shown). Finally, His_6-tagged versions of both enzymes were purified and affinities for malate and L-lactate were determined in an *in vitro* enzyme assay. While Mqo2 had significant affinity for L-lactate ($K_M = \sim 300\,\mu M$, **Figure 3**), it did not react readily with malate. Again, Mqo1 had high affinity for malate but no detectible reaction with L-lactate (**Figure 3**). Since Mqo2 does not exhibit any substrate specificity for malate and efficiently oxidizes

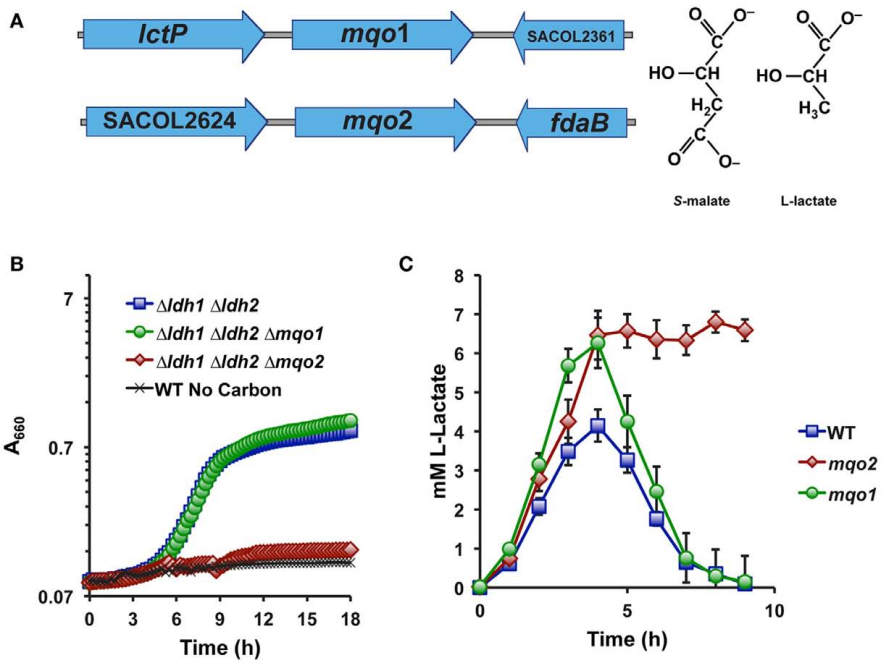
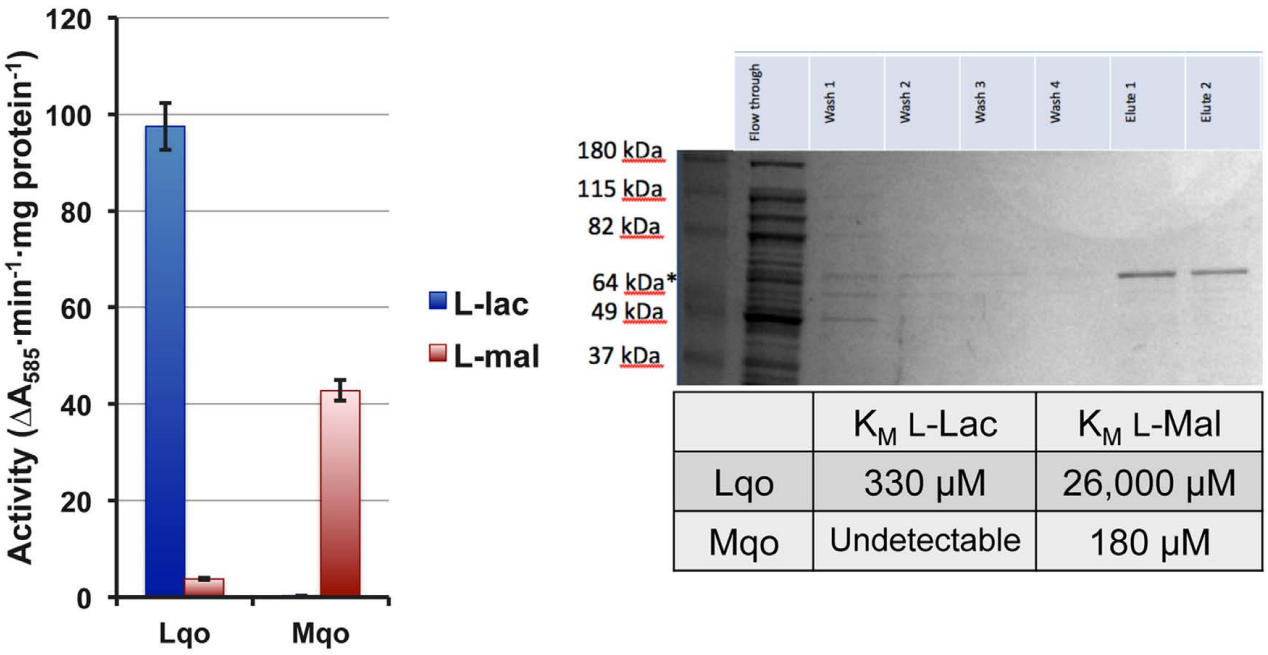


FIGURE 2 | Mqo2 is responsible for the elusive *S. aureus* iLDH activity. **(A)** Genetic organization of the two annotated malate-quinone oxidoreductase genes. *mqo*1 is between an L-lactate permease and a hypothetical gene, while *mqo*2 is found between a putative acetyl-CoA synthase (*acs*, SACOL2624) and a class I fructose 1,6-bisphosphate aldolase, *fdaB*. **(B)** Specifically combining a Δ*mqo*2 mutation to the Δ*ldh*1Δ*ldh*2 background abolishes the ability to utilize L-lactate. Cells were grown aerobically in chemically defined medium with 0.2% L-lactate as the primary carbon source. Residual growth on medium components was subtracted out of depicted curves (see Materials and Methods). **(C)** The L-lactate excreted following NO·-exposure cannot be reassimilated in a Δ*mqo*2 mutant. Cells were grown in chemically defined medium as described in **Figure 1B**.



	K_M L-Lac	K_M L-Mal
Lqo	330 μM	26,000 μM
Mqo	Undetectable	180 μM

FIGURE 3 | The *S. aureus* Lqo enzyme oxidizes L-lactate and has no affinity for malate. Left: Membrane preparations from single mutant *S. aureus* were assayed for Lqo and Mqo activity. Δ*mqo*1 (Lqo-containing) membranes react readily with L-lactate but not malate. In contrast, Δ*mqo*2 mutants (Mqo-containing) have activity against malate but not L-lactate. Right: Purified His6-tagged versions of Lqo and Mqo were used to determine affinities of each enzyme to its cognate substrate.

L-lactate, we suggest that it be renamed Lqo (L-lactate-quinone oxidoreductase).

We assessed the physiological roles for Lqo in growth on various carbon sources in the presence/absence of different electron acceptors. As expected, mutants lacking Lqo showed measurable defects when grown on L-lactate and these defects were additive when Δ*lqo* was combined with Δ*ldh*2 (**Figure A1** in Appendix). The loss of Ldh1 had little effect on L-lactate utilization consistent with its role in producing L-lactate for redox balance (**Figure A1** in Appendix). When *S. aureus* was grown aerobically in media with amino acid carbon sources, Lqo was dispensable (**Figure A2** in Appendix). In contrast a Δ*mqo* mutant was measurably impaired in this medium underscoring its role in the TCA cycle, a pathway essential to the utilization of amino acids (**Figure A2** in Appendix). Regardless of the medium used to cultivate *S. aureus*, *lqo* transcript was highly abundant, though expression was significantly lower in the presence of glucose (**Figure A3** in Appendix). Interestingly, *lqo* was still highly expressed in the Δ*ldh*1Δ*ldh*2 mutant grown on glucose, an environment completely devoid of L-lactate (data not shown). Anaerobically, *S. aureus* was unable to grow on L-lactate as a carbon source unless an electron acceptor (e.g., nitrate) was also present (**Figure A4A** in Appendix). This result highlights the respiratory nature of Lqo in that it reduces the quinone pool therefore necessitating the presence of a terminal electron acceptor such as oxygen or nitrate. Interestingly, Δ*lqo S. aureus* could still grow anaerobically on L-lactate in the presence of nitrate implying that Ldh1 and/or Ldh2 can also serve as catabolic enzymes provided the resulting NADH is recycled through a respiratory mechanism (**Figure A4A** in Appendix). Moreover, combining peptides (1% Tryptone) with L-lactate greatly improved anaerobic growth both in the presence and absence of nitrate (**Figure A4B** in Appendix). However, in the absence of nitrate, the added benefit of peptides combined with L-lactate was independent of Lqo and required Ldh1/2 in line with the absence of a defined electron acceptor (**Figure A4B** in Appendix). In contrast, as with L-lactate alone (**Figure A4A** in Appendix), addition of nitrate facilitated Lqo-dependent growth on peptide/L-lactate in that the Δ*ldh*1Δ*ldh*2 mutant grew nearly as well as WT (**Figure A4B** in Appendix). Collectively, these data show that Lqo plays a pivotal role in *S. aureus* metabolism when utilizing L-lactate specifically under respiratory conditions.

STAPHYLOCOCCUS AUREUS Lqo IS REQUIRED FOR FULL VIRULENCE IN A MURINE SEPSIS MODEL

Infecting mice i.v. with 5×10^6 cfu of *S. aureus* strain Newman results in reproducible weight loss over the first week of infection (**Figure 4**). Roughly half of the mice lost ≥30% original body weight within the first week post-inoculation and were sacrificed as per approved IACUC protocols. In contrast, mice infected with an isogenic Δ*lqo* mutant exhibited significantly ($p < 0.01$) slower weight loss kinetics with all of the mice maintaining ≥70% of their original body weight (**Figure 4**). In competitive i.v. infections with WT strain Newman, the Δ*lqo* mutant showed a marked defect in the heart, but only a modest ∼3-fold attenuation in the kidney (**Figure 4**). In mice unable to produce inflammatory NO· (*iNOS*$^{-/-}$), the heart-specific competitive defect was fully reversed while the modest defect in the kidneys was still observable (**Figure 4**). Thus, *S. aureus* Lqo contributes to virulence in both an *iNOS*-dependent and -independent fashion and mutants exhibit tissue-specific defects in competition with WT *S. aureus*.

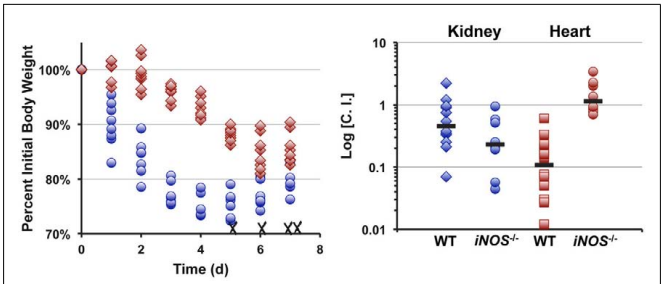


FIGURE 4 | Lqo is required for full *S. aureus* virulence in a murine sepsis model, particularly in establishing myocarditis. Left: Eight 4-to 6-week female C57BL/6 mice (two independent experiments each with four mice per group) were inoculated i.v. with 5×10^6 cfu of *S. aureus* strain Newman (blue circles) or isogenic Δ*lqo* (red diamonds). Weight loss was monitored following inoculation and presented as percent initial body weight. Once mice lose ≥30% of their original body weight, they are sacrificed as per IACUC protocol. WT infected mice exhibited significantly greater weight loss ($p < 0.01$, using longitudinal linear regression to model weight loss over time, SAS®, Cary, NC, USA) and four (50%) were sacrificed (indicated with X). Right: Competitive indices (C.I.) were established by infecting mice i.v. with 1×10^7 cfu of a 1:1 mixture of WT: Δ*lqo S. aureus* strain Newman. Organs were harvested 5 days post-inoculation and mutant:WT C.I. ratios were calculated as outlined in experimental procedures. In parallel, isogenic *iNOS*$^{-/-}$ mice unable to produce inflammatory NO· were also infected in a similar manner.

The fact that the Δ*lqo* virulence defect was partially reversed in *iNOS*$^{-/-}$ mice was surprising given the lack of an *in vitro* NO··phenotype when the mutant was grown on glucose (data not shown). Glucose is generally used in defined medium for *in vitro* experiments because glycolytic carbon sources support *S. aureus* NO··resistance while gluconeogenic sources do not (i.e., pyruvate, lactate, peptides, **Figure 5A**). However, combining L-lactate with peptides will support *S. aureus* growth under NO··stress, and this phenomenon is specific for L-lactate versus D-lactate and dependent on Lqo (**Figure 5A**). Furthermore, providing the product of Lqo (0.1% pyruvate) in combination with peptides also supported growth during NO··stress and as expected this was independent of Lqo (data not shown). We therefore hypothesized that Lqo was necessary for growth under NO··stress in the murine heart because of its requirement for replication using the combination of peptides and L-lactate during NO··exposure, and not because of its role in reassimilation of excreted L-lactate during growth on glucose.

Bacteria enumerated from murine heart tissue are found within abscesses associated with the cardiac muscle (myocarditis) or in some cases with the pericardial space (pericarditis; **Figure 5B**). Hematogenous myocarditis/pericarditis occurs regularly in the murine sepsis model in that bacteria are consistently isolated from cardiac tissue, though visible abscesses are not always present. Histological examination of infected tissue revealed infiltration of many granulocytic cells as well as other distinct cell types

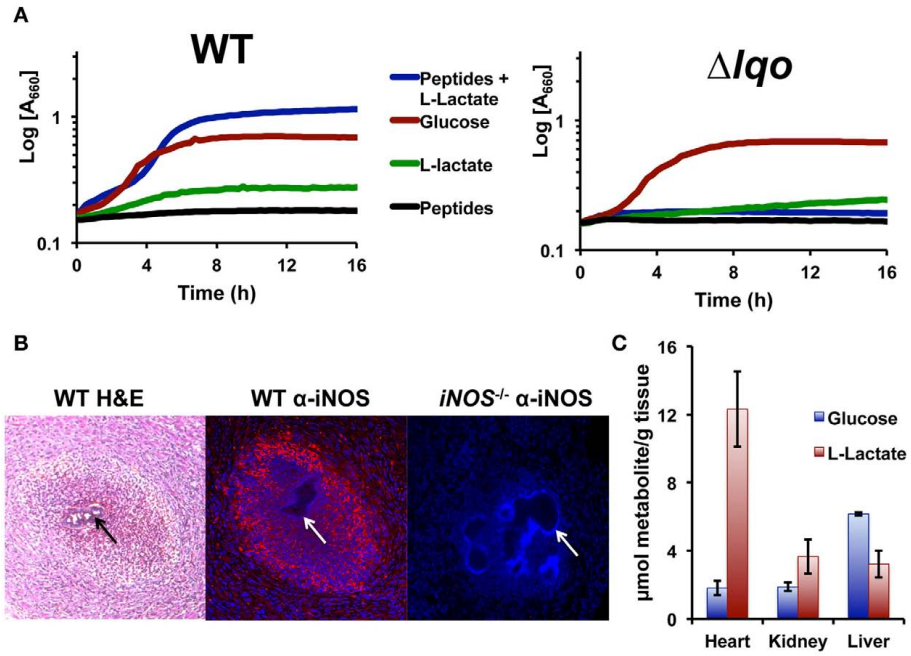


FIGURE 5 | *Staphylococcus aureus* growth in L-lactate-rich cardiac tissue in the presence of host NO· requires Lqo. (A) Individual gluconeogenic carbon sources (e.g., 1.0% L-lactate or 1.0% tryptone) do not support NO· resistance in chemically defined medium as does 0.5% glucose. However, combining 0.1% L-lactate with 1.0% tryptone allows *S. aureus* to grow in the presence of NO·. NO· in the form of 10 mM DETA/NO was added once cells reach an $OD_{660} = 0.15$ depicted here as t_0. **(B)** Myocardial abscesses in mice infected with WT *S. aureus* Newman 5 days post-inoculation. Left, hematoxylin and eosin staining of abscess reveal a central bacterial microcolony (black arrow) surrounded by infiltrating granulocytic cells. Center, immuno-histofluorescent staining of the same abscess with anti-iNOS monoclonal Ab reveals robust iNOS expression throughout the abscess. Tissue was counter-stained with DAPI to reveal host cell nuclei. Non-staining *S. aureus* microcolony is visible (white arrow). Right, identical immuno-histofluorescent staining of a similar abscess in an *iNOS*$^{-/-}$ mouse as a negative control for anti-iNOS Ab. Tissue was also counter-stained with DAPI. **(C)** Metabolite data from murine tissues were determined using enzymatic methods previously described and normalized to total organ weight.

surrounding micro-colonies of bacteria (**Figure 5B**). Immunohistological examination using anti-iNOS antibodies confirmed high-level iNOS expression in myocardial abscesses around *S. aureus* micro-colonies (**Figure 5B**). In comparison to other tissue commonly colonized hematogenously during *S. aureus* sepsis (e.g., hepatic and renal), cardiac tissue had by far the highest levels of L-lactate in addition to protein (**Figure 5C**). This is likely true for all active muscle tissue in mammals and is consistent with the specific requirement of Lqo for myocarditis/pericarditis in NO·-proficient hosts.

DISCUSSION

The existence of NAD-independent lactate dehydrogenase (*i*LDH) activity in *S. aureus* was first described over 50 years ago, but received little attention over the NAD-dependent enzymes (Stockland and San Clemente, 1969). The *S. aureus* *i*LDH activity was again reported 4 years later and shown to be specific for L-lactate (Stevenson and Holdsworth, 1973). Subsequently, this *i*LDH activity was shown to be membrane associated and was linked to the reduction of ferric iron, nitrate, or oxygen as terminal electron acceptors (Theodore and Weinbach, 1974; Lascelles and Burke, 1978; Tynecka and Malm, 1995). However, pure fractions containing only the *S. aureus* *i*LDH were never used to identify the protein. Thus, prior to this work, the gene(s) encoding the *S. aureus* *i*LDH was never identified, nor was this activity shown to be biologically relevant. We that the Δ*ldh*1Δ*ldh*2 mutant, which does not produce any L-lactate, was still able to grow on L-lactate as a sole carbon source (**Figure 1**). Since lactate-racemic activity has been reported in a singular strain of *S. aureus* (Stockland and San Clemente, 1969), the triple Δ*ldh*1Δ*ldh2*Δ*ddh* mutant was tested and shown to still utilize L-lactate. Thus, the Δ*ldh*1Δ*ldh*2 mutant was not racemically converting L-lactate to D-lactate and catabolizing the latter via Ddh. These data are consistent with *S. aureus* encoding a true *i*LDH that will promote growth on L-lactate as a carbon/energy source.

The ability of the Δ*ldh*1Δ*ldh*2 mutant to utilize L-lactate was used to screen two annotated malate-quinone oxidoreductases for their role in promoting growth on L-lactate given its chemical similarity with malate. Since malate and lactate utilizing enzymes are closely related and relatively few amino acid changes can distinguish the two activities (Yin and Kirsch, 2007), it seemed reasonable that either *mqo*1 or *mqo*2 encoded the *S. aureus* *i*LDH. Mqo2 alone was shown to promote growth of the Δ*ldh*1Δ*ldh*2 mutant on L-lactate thereby comprising the *S. aureus* *i*LDH activity (**Figure 2**). The enzyme catalyzes the oxidation of L-lactate (but not malate) with the concomitant reduction of menaquinone, thus we suggest renaming the enzyme Lqo (**Figure 3**). On the other hand, Mqo1 was shown to exhibit activity toward malate

alone and is the only enzyme in *S. aureus* capable of oxidizing malate to oxaloacetate, the penultimate reaction of the TCA cycle (**Figure 3**). Accordingly, Mqo is essential for maximal growth on amino acid carbon sources since their assimilation through the TCA cycle to oxaloacetate is necessary for gluconeogenesis (**Figure A2** in Appendix). Furthermore, Δ*mqo*1 mutants excrete excess lactate and acetate consistent with the lack of a functional TCA cycle resulting in "spill-over" metabolism at the pyruvate node (**Figure 2C**). In contrast, Δ*lqo* mutants do not excrete excess acetate since the TCA cycle is fully intact (data not shown). Thus, Mqo and Lqo are enzymes with distinct, non-overlapping substrates and functions. Notably, both enzymes are essential for full virulence and Δ*mqo* mutants are severely attenuated underscoring the importance of the TCA cycle for *S. aureus* pathogenesis (Somerville et al., 2002; **Figure A5** in Appendix).

At first glance, it seems illogical to express both Ldh1 and Lqo under NO·-stress because they would represent futile cycles. However, given that the electron carriers differ between the Ldh enzymes and *i*LDH, their net reaction resembles that of an alternative (non-proton pumping) NADH-dehydrogenase:

Ldh1/Ldh2	pyruvate + NADH	↔	L-lactate + NAD^+	(1)
Lqo	L-lactate + MQ = O	→	pyruvate + MQ-OH	(2)
	NADH + MQ = O	→	NAD^+ + MQ-OH	Net

Perhaps their dual expression provides *S. aureus* with an "uncoupled complex I" that can regenerate NAD^+ without affecting the proton-motive force. Interestingly, Lqo is still highly expressed in the Δ*ldh*1Δ*ldh*2 mutant grown on glucose, a scenario where there is no L-lactate in the environment at all (none provided exogenously and none produced endogenously from glucose). The constitutive expression of Lqo regardless of the presence/absence of L-lactate implies that in the host environment, *S. aureus* always requires Lqo activity eliminating the need for a complex regulatory system to control transcription.

When *S. aureus* is grown on L-lactate as a carbon source, the role for Lqo is clearly evident. Mutants lacking Lqo exhibit slower growth and lower overall yields on L-lactate (**Figure A1** in Appendix). This phenomenon is exacerbated in the Δ*ldh*2 background and not affected by deletion of *ldh*1 implying that the major enzymes for L-lactate catabolism in *S. aureus* are Lqo and Ldh2 (**Figure A1** in Appendix). Anaerobically, *S. aureus* cannot ferment L-lactate as a sole carbon source consistent with the requirement of a terminal electron acceptor for Lqo function and the fact that the reversal of Eq. 1 by Ldh1 or Ldh2 without a functioning respiratory chain would lead to redox imbalance (i.e., high NADH, **Figures 6** and **Figure A4A** in Appendix). Simply providing nitrate as an electron acceptor allows *S. aureus* to grow anaerobically on L-lactate using either Lqo or Ldh1/Ldh2 (**Figures 6** and **Figure A4A** in Appendix). The NAD-dependent LDHs (Ldh1 and Ldh2) catabolize L-lactate by the reversal of Eq. 1 presumably coupled to a respiratory NADH-dehydrogenase. Interestingly, adding peptides to L-lactate-containing media allowed for growth anaerobically in the absence of nitrate and this was dependent on Ldh1/Ldh2 (**Figure A4B** in Appendix). Presumably, excess carbon from peptides frees up L-lactate-derived carbon to establish redox balance. For instance, some pyruvate generated from L-lactate-oxidation could be used for energy production while the rest could be converted to ethanol providing redox balance. Whatever the mechanism behind peptide stimulated growth under anaerobiosis, Lqo is not involved because it directly requires active respiration to generate oxidized quinone for enzymatic activity (i.e., the Δ*lqo* mutant grows as well as wild type without nitrate, **Figure A4B** in Appendix). Thus, *S. aureus* has evolved multiple means of assimilating L-lactate under various conditions implying its central role in this pathogen's metabolic environment. Moreover, Lqo appears to provide *S. aureus* with an efficient means of L-lactate catabolism in the presence of aerobic/anaerobic respiration.

The ability of *S. aureus* to resist host NO· is key to its pathogenesis as demonstrated by the iNOS-dependent attenuation of the Δ*lqo* mutant, particularly in L-lactate-rich cardiac tissue (**Figure 5**). How *S. aureus* continues to catabolize host tissue in the presence of NO· given the numerous metabolic targets of this immune radical is still unclear. However, here we demonstrate that Lqo provides another NO·-resistant pathway to *S. aureus* when growing in a peptide/L-lactate-rich environment. Unlike glucose, individual gluconeogenic substrates do not allow *S. aureus* to circumvent the metabolic constraints imposed by exogenous NO· (**Figure 5A**). The reasons behind this phenomenon are unclear, but perhaps the multiple TCA cycle targets of NO· limit growth on amino acids. However, combining L-lactate with peptides does restore NO·-resistance to *S. aureus* in an Lqo-dependent fashion, though the physiological reasons are still unclear (**Figure 5A**). D-lactate was unable to support growth under NO·-stress in combination with peptides whereas pyruvate could independently of Lqo (data not shown). These data are consistent with the requirement for pyruvate combined with peptides to allow NO·-resistant growth in the absence of glucose. It should be noted that the difference in NO·-resistance between WT and Δ*lqo* in peptide/L-lactate medium was more robust with a slow releasing NO·-donor such as DETA/NO. There were reproducible differences between WT and Δ*lqo* growth in peptide/L-lactate medium under high NO· levels (∼1 mM as in **Figure 1**), but they manifested as extended lag phases rather than altered growth rates (data not shown). This is consistent with the requirement of Lqo for some level of respiratory activity involving a terminal electron acceptor. At millimolar NO·-levels, nearly all measurable respiration, both aerobic and anaerobic, is inhibited. However as NO· dissipates to sub-millimolar levels (**Figure 1B**), *S. aureus* may be able to respire using molecular oxygen mediated by a NO·-resistant aerobic respiratory system. Indeed, Lqo-dependent utilization of L-lactate/peptides in the presence of NO· was eliminated in the Δ*qoxBACD* mutant implying that the cytochrome-aa_3 quinol oxidase is essential for Lqo activity (**Figure A6** in Appendix). While the genes encoding the cytochrome *bd* quinol oxidase are induced by NO· (Richardson et al., 2006; Hochgrafe et al., 2008), this oxidase does not seem to support growth on L-lactate/peptides during NO·-stress (**Figure A6** in Appendix). The requirement for Lqo during infection of murine cardiac tissue implies that NO·/O_2 levels are likely amenable to active cytochrome-aa_3 supporting Lqo-dependent L-lactate utilization. Alternatively, during infection *S. aureus* may utilize flavohemoglobin (Hmp)-derived nitrate from NO·-detoxification as a

terminal electron acceptor supporting Lqo-mediated L-lactate utilization. More work is required to elucidate the nature of the relevant *S. aureus* electron acceptor under NO·-stress during infection.

The fact that either Lqo or Ldh1/2 can support growth on L-lactate anaerobically with nitrate represents a significant difference from the exclusive requirement for Lqo during L-lactate/peptide utilization under NO· stress (**Figure 6**). For Ldh1/2 to catabolize L-lactate via the reversal of Eq. 1, a respiratory system must be present to recycle NADH. Since respiration is also required for Lqo function during NO·-stress, that same respiratory activity should be able to recycle the NADH produced by Ldh1/2. This is not the case however, thus NO· must inhibit some other aspect of oxidative phosphorylation unique to the direct recycling of NADH. One explanation is that NO· may inhibit the NADH-dehydrogenase expressed during NO·-exposure (**Figure 6**). This would not affect Lqo activity but would limit the utility of Ldh1/2 even in the presence of a usable electron acceptor. Future experimentation will be directed at defining the effects of NO· that necessitate Lqo activity specifically for the utilization of L-lactate during NO·-stress.

While the role of Lqo in NO·-resistant L-lactate/peptide catabolism raises important metabolic questions, its disproportionate contribution to virulence in tissues with excess L-lactate (e.g., cardiac muscle tissue) has intriguing implications regarding *S. aureus* pathogenesis. While muscle tissue can have excessive amounts of L-lactate given the oxygen demand associated with muscle activity, other mammalian tissues relevant to *S. aureus* biology are also replete with high levels of L-lactate. For instance, human sweat can have as high as ∼50 mM L-lactate, possibly resulting in a role for Lqo in skin colonization (Sakharov et al., 2010). Moreover, nasal secretions have also been shown to contain high concentrations of L-lactate making the most common site of *S. aureus* colonization (the nasal cavity) a prime environment for Lqo activity (Stierna et al., 1991; Westrin et al., 1992). Thus, while Lqo may contribute to *S. aureus* disease, particularly in various forms of myocarditis and myositis, this newly characterized enzyme may also have more overarching roles in promoting *S. aureus* colonization. Thus, the identification of Lqo as comprising the elusive *S. aureus i*LDH activity will provide significant insight into the physiology, pathogenesis, and general biology of this very significant human pathogen.

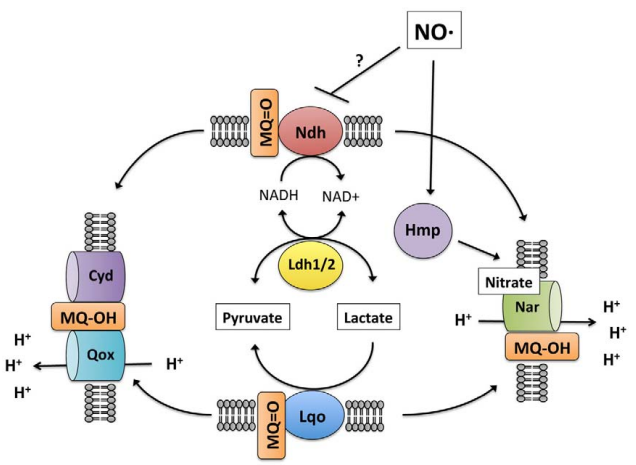


FIGURE 6 | Model of *S. aureus* lactate metabolism. During respiratory lactate catabolism (either aerobically or anaerobically on nitrate), *S. aureus* can use either Lqo or reversal of the NADH-dependent Ldh1/2 provided NADH can be recycled. In the absence of an external electron acceptor, *S. aureus* is unable to catabolize lactate because Lqo activity requires a functioning quinol oxidase as does Ldh1/2 for NAD^+ regeneration. Initially during NO· stress, respiration is inhibited and lactate is produced for redox balance (NAD^+ generation). Once respiration resumes due to waning NO· levels, lactate can be assimilated but only in an Lqo-dependent fashion. Despite the presence of active quinol oxidases (Qox, Cyd, or Nar) NADH-dependent Ldh1/2 do not promote lactate assimilation in the presence of NO·. This would be consistent with NO·-dependent inhibition of Ndh, limiting NAD^+ regeneration even in the presence of active quinol oxidases, though this remains to be demonstrated.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Staphylococcus aureus was cultivated in Brain Heart Infusion medium or in chemically defined medium (PN medium) in which primary carbon sources could be modified (Pattee, 1976). Briefly, PN medium is a phosphate-buffered medium composed of a primary carbon source (variable depending on experiment), nucleobases (Adenine, 5 mg/L; Guanine, 5 mg/L; Cytosine, 5 mg/L; Uracil, 5 mg/L; and Thymine, 20 mg/L), free amino acids (Ala, 60 mg/L; Arg, 70 mg/L; Asp, 90 mg/L; Cystine, 20 mg/L; Glu 100 mg/L; Gly, 50 mg/L; His, 30 mg/L; Iso, 30 mg/L; Leu, 90 mg/L; Lys, 70 mg/L; Met, 10 mg/L; Phe, 40 mg/L; Pro, 10 mg/L; Ser, 30 mg/L; Thr, 30 mg/L; Trp, 10 mg/L; Tyr, 50 mg/L; and Val, 80 mg/L), vitamins (thiamine, 1 mg/L; niacin, 1.2 mg/L; biotin, 5 μg/L; and pantothenate, 250 μg/L), $FeCl_3$ at 8 mg/L, $MgSO_4$ at 2.5 mg/L, and trace elements (ZnCl, 70 μg/L; MnCl, 63 μg/L; Boric Acid, 6 μg/L; $CoCl_2$, 190 μg/L; $CuCl_2$, 2 μg/L; $NiCl_2$, 13 μg/L; and Na_2MoO_4, 31 μg/L). Aerobic growth was achieved by shaking (250 rmp) in flasks or slanted test tubes where as anaerobic cultivation was performed in a Coy anaerobe chamber. Antibiotic selection in *S. aureus* (*E. coli*) was performed using the following concentrations: ampicillin (100 μg/ml), chloramphenicol 20 μg/ml, kanamycin 50 μg/ml (50 μg/ml), spectinomycin 100 μg/ml (500 μg/ml), erythromycin 5 μg/ml (300 μg/ml) unless otherwise indicated. Growth was monitored as change in absorbance (660 nm) assessed using a Tecan infinite M200 plate reader in 200 μl cultures within a 96 well plate. To assess the inability to grow in defined media with L- or D-lactate as a primary carbon source, residual growth on medium components was subtracted from growth in media replete with lactate carbon sources. Residual growth in media lacking primary carbon sources halted at $OD_{660} = 0.2–0.3$. Mutations were constructed using a modified allelic exchange method involving the cloning of flanking DNA sequence on either side of a selectable marker in the *S. aureus*/*E. coli* shuttle vector, pBT2ts. This construct was electroporated into *S. aureus* as previously described and grown with selection at 30°C. Cointegration was achieved by growing cultures overnight with selection at 43°C followed by plating on antibiotic containing media (Bruckner, 1997). Single colonies were isolated from 43°C and grown overnight for three consecutive days

without antibiotic selection at 30˚C. Finally, cultures were diluted 1:100 and grown to mid-exponential phase at 37˚C at such time chloramphenicol was added (10 μg/ml) to inhibit the growth of resolved cointegrates. After 30 min, cycloserine was administered (100 μg/ml) to selectively kill Cm^R-cointegrates. After ≥4 h incubation at 37˚C, surviving cells are plated on selective media and screened for successful allelic replacement.

VIRULENCE ASSESSMENT

Four to 6-week-old female C57BL/6 mice were inoculated i.v. via tail vein with 5×10^6 cfu in 100 μl of *S. aureus* strain Newman or isogenic mutants. Weight loss was monitored daily for 1 week as a metric for disease progression, mice exhibiting ≥30% weight loss were sacrificed as per IACUC approved protocol. Alternatively, WT mice or isogenic $iNOS^{-/-}$ mice (Jackson Laboratories) were similarly infected with 1×10^7 cfu of WT *S. aureus* strain Newman at a 1:1 mixture with isogenic Δ*lqo* and tissue harvested 5 days post-inoculation. Kidneys and hearts were extracted, and ratios of Δ*lqo*:WT were determined by plating on selective and non-selective media. Competitive indices (C.I.s) were calculated as d5 organ burden ratio (Δ*lqo*/WT) divided by inoculation ratio (Δ*lqo*/WT).

ENZYMATIC DETERMINATION OF METABOLITE LEVELS

Metabolites such as L- and D-lactate, glucose and nitrate were quantified as previously described as per manufacturer instruction (Richardson et al., 2008). Excreted metabolite levels from *S. aureus* cultures were determined using supernatants from 1 ml of pelleted, heat-inactivated (70˚C for 5 min) cells. Tissue metabolites were extracted from organs (liver, heart, kidneys) harvested immediately from individual mice (in triplicate) following euthanization via cervical dislocation as per approved IACUC protocols. The organs were quickly homogenized in PBS at 4˚C, quenched with ice cold methanol (final 80% v/v) and frozen at −80˚C. Subsequently, tissues were thawed and metabolites were extracted by adding chloroform and water to achieve a final ratio methanol:chloroform:water (4:4:3). The aqueous phase was removed, lyophilized, and resuspended in 100 μl ddH_2O for analyses. Lactate and glucose levels were normalized to organ weight (mg tissue).

FLUORESCENT IMMUNOHISTOCHEMISTRY

Immunohistochemistry on heart tissues was performed as previously described with minor modifications (Thurlow et al., 2011). Heart tissue was collected 5 days post-inoculation and fixed in 10% formalin then paraffin embedded and sectioned (10 μm) by the Histopathology Core Facility at UNC. Sections were deparaffinized using a graded series of xylene and ethanol washes followed by microwaving for 20 min in 10 mM NaCl buffer (pH 6) for antigen retrieval. Tissue sections were stained with primary rabbit-derived antibodies against iNOS (Abcam, Cambridge, MA, USA) and secondary donkey anti-rabbit biotin conjugated antibodies (Jackson ImmuoResearch Laboratories, West Grove, PA, USA) followed by the addition of DyLight™594-conjugated streptavidin (Jackson ImmunoResearch). Images were acquired using a Leica SP2 confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA).

MEMBRANE PREPARATIONS AND Lqo/Mqo ENZYMATIC ASSAYS

Overnight *S. aureus* cultures (Δ*lqo* to measure Mqo activity, Δ*mqo* to determine Lqo activity, and Δ*mqo*Δ*lqo* for background activity) were diluted 1:100 in 100 ml of LB medium (low glucose to prevent Mqo repression) and shaken at 37˚C until $OD_{660} = \sim 0.8$. Cells were pelleted, washed in PBS then incubated at 37˚C in the presence of Lysostaphin (12.5 μg/ml), Lysozyme (12.5 μg/ml), DNase (10 μg/ml), and RNase (10 μg/ml) for 1 h. Debris was pelleted and supernatants ultracentrifuged at 100,000 g for 1 h at 4˚C. Membrane fraction pellets were resuspended then sonicated and protein was quantified using BCA procedure (Pierce). Reaction mixtures contain 1 mg total membrane protein, 40 μg nitro blue tetrazolium (NBT), 5 mM KCN, and were initiated by addition of 20 mM substrate (malate/L-lactate) in a final volume of 200 μl. Reaction progress is monitored by measuring absorbance at 585 nm every 30 s for 20 min using a Tecan infinite M200 plate reader.

Lqo AND Mqo PURIFICATION AND ANALYSES

C-terminal His_6-tagged versions of Lqo and Mqo were constructed by amplifying alleles from *S. aureus* strain COL chromosomal DNA using primers Mqo2_His.1A/1B and Mqo1_His.1A/1B, respectively, and cloning into the *Nco*I/*Bam*HI sites of pQE-60 (Qiagen). Resulting constructs (pJF132 and pJF133) were transformed into *E. coli* M15 (pREP4; Qiagen) maintained at 37˚C with kanamycin (25 μg/ml) and ampicillin (200 μg/ml). For purification, 1 L cultures were seeded with 20 mL overnight cultures, and grown at 25˚C for 4 h with selection and the addition of IPTG (0.1 mM). Subsequently, cells were pelleted, washed in PBS and lysed via sonification. Enzymes were purified using HisPur Cobalt Purification System (Pierce) as per manufacturer instruction. Elutions were dialyzed twice for 2 h at 4˚C followed by an overnight dialysis at 4˚C against high-salt (300 mM NaCl) PBS. Yields were quantified using BCA Protein Quantification Kits (Pierce).

Enzymatic activity for Mqo and Lqo was monitored by addition of 100 ng purified His6-Tagged protein (1 μg if using non-specific substrate) in a 200 μl reaction mixture consisting of 1.4 mM menaquinone, 5 μM flavin adenine dinucleotide (FAD), 643 μg phosphatidylethanolamine (PE), 40 μg NBT and 20 mM substrate (malate/L-lactate). Prior to addition of enzyme, reaction mix was sonicated to uniformly disperse menaquinone within the PE vesicles. Reactions were initiated by addition of substrate and followed via monitoring absorbance at 585 nm using a Tecan infinite M200 plate reader.

RNA EXTRACTION AND QUANTITATIVE REVERSE-TRANSCRIPTASE PCR

Cells were grown in PN chemically defined medium supplemented with either 0.5% glucose, 1.0% L-lactate, or 1.0% tryptone as primary carbon sources. Once cells reached $OD_{660} = 0.5$, 25 ml of culture was added to 25 ml of ice cold ethanol:acetone (1:1) and incubated at −80˚C until further use. Frozen cell suspensions were thawed at room temperature, pelleted by centrifugation and resuspended in 500 μl of TE for mechanical disruption using Lysing Matrix B (MP Biomedicals, Solon, OH, USA) in a standard cell disruptor. Hundred microliter of lysates was used for RNA isolation using an RNAEasy® Mini Kit (Qiagen, Valencia, CA, USA) as per manufacturer instructions.

RNA was spectrophotometrically quantified and 50 ng of total RNA was analyzed per reaction using the OneStep™SYBR® RT-PCR kit (Quantace, Valencia, CA, USA) on an iCycler Real Time Machine (BioRad). Primers used for analysis are listed in **Table A1** in Appendix and both *mqo* and *lqo* transcript levels were normalized to those of *rpoD*.

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APPENDIX

Table A1 | Strains used in this work.

COL	WT *S. aureus*	W. Shafer
Newman	WT *S. aureus*	W. Shafer
AR0169	*S. aureus* strain COL Δ*ldh*1::Er^{R}Δ*ldh*2::Km^R	Richardson et al. (2008)
AR0173	*S. aureus* strain Newman Δ*ldh*1::Er^{R}Δ*ldh*2::Km^R	Richardson et al. (2008)
AR0194	*S. aureus* strain COL Δ*cydAB*::Km^R	This study
AR0309	*S. aureus* strain Newman Δ*ddh*::Sp^R	This study
AR0310	*S. aureus* strain Newman Δ*ldh*1::Er^{R}Δ*ldh*2::Km^{R}Δ*mqo*::Sp^R	This study
AR0312	*S. aureus* strain Newman Δ*ldh*1::Er^{R}Δ*ldh*2::Km^{R}Δ*ddh*::Sp^R	This study
AR0314	*S. aureus* strain Newman Δ*mqo*::Sp^R	This study
AR0327	*S. aureus* strain Newman Δ*cydAB*::Km^R	This study
AR0369	*S. aureus* strain COL Δ*lqo*::Sp^R	This study
AR0370	*S. aureus* strain COL Δ*ldh*1::Er^{R}Δ*ldh*2::Km^{R}Δ*lqo*::Sp^R	This study
AR0386	*S. aureus* strain Newman Δ*lqo*::Sp^R	This study
AR0387	*S. aureus* strain COL Δ*ldh*1::Er^R Δ*lqo*::Sp^R	This study
AR0388	*S. aureus* strain COL Δ*ldh*2::Km^{R}Δ*lqo*::Sp^R	This study
AR0418	*S. aureus* strain COL Δ*ddh*::Sp^R	This study
AR0419	*S. aureus* strain COL Δ*mqo*::Sp^R	This study
AR0420	*S. aureus* strain Newman Δ*lqo*::Sp^{R}Δ*mqo*::Sp^R	This study
AR0421	*S. aureus* strain COL Δ*narGH*::Sp^R	This study
AR0424	*S. aureus* strain COL Δ*lqo*::Sp^{R}Δ*mqo*::Sp^R	This study
AR0425	*S. aureus* strain COL Δ*ldh*1::Er^{R}Δ*ldh*2::Km^{R}Δ*mqo*::Sp^R	This study
AR0455	*S. aureus* strain COL Δ*ldh*1::Er^{R}Δ*ldh*2::Km^{R}Δ*ddh*::Sp^R	This study
AR0495	*S. aureus* strain Newman Δ*narGH*::Sp^R	This study
AR0630	*S. aureus* strain COL Δ*qoxBACD*::Sp^R	This study

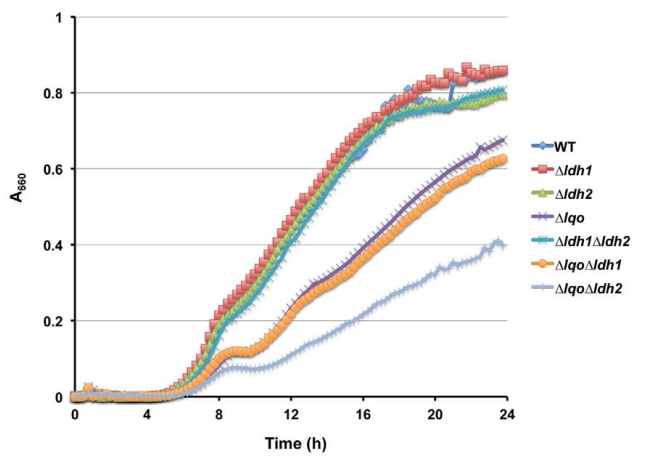

FIGURE A1 | Lqo, and to a lesser extent Ldh2, are required for maximal growth on L-lactate. Representative growth curve of WT *S. aureus* strain COL and isogenic mutants grown in chemically defined medium supplemented with 1.0% L-lactate as a primary carbon source. Growth as determined by absorbance at 660 nm was normalized to minimal growth in the absence of added carbon.

Table A2 | Primers/plasmids used in this study.

ddh-5′0.1A	5′-AAATGCGACCATCACTGATAAACC-3′
ddh-5′0.1B	5′-TAGGGCGATAGAAACAGAATACTC-3′
ddh-3′0.1A	5′-ACGTGGTGCAGTCATCAATACACC-3′
ddh-3′0.1B	5′-ACCAACAATGTGATTGATCTGACC-3′
mqo2-5′0.1A	5′-TACTTGCTGGGGATCCAACTCACATAATGCTGCATGACCC-3′
mqo2-5′0.1B	5′-CGACTCTAGAGGATCCAAGCATTCGTTATTTTACAGGACC-3′
mqo2-3′0.1A	5′-CAGTGCAGCGGAATTCGGTAACTTCACCTTCATTCTCACC-3′
mqo2-3′0.1B	5′-TACCGAGTCGAATTCAGAAGTATTAGAACGTAACTTCCC-3′
mqo1-5′0.1A	5′-GGGGAATTCCTGTATTTGGACTTTCATCTTGTC-3′
mqo1-5′0.1B	5′-GGGGAATTCACATTCTGCTTATATTGAATTCCC-3′
mqo1-3′0.1A	5′-GGGGATCCACCAAAGATTAAAGAAATGGTGCC-3′
mqo1-3′0.1B	5′-GGGGATCCCTATTACACTGCCAAATAATTCAC-3′
nar.1A	5′-AATTTAATGGGAATTGGTCGATCC-3′
nar.1B	5′-TCCTTTCACCTCTTATGCTTACAC-3′
cyd-5′0.1A	5′-TGGTAAGTTAAATATCATGTTCCC-3′
cyd-5′0.1B	5′-AACCGACTGATTTCAACTGTATCC-3′
cyd-3′0.1A	5′-ATGGAATTAGTAAATTGCCATACC-3′
cyd-3′0.1B	5′-AAGTTTTAATTAAACTCACCACCC-3′
qox-5′0.2A	5′-CGACTCTAGAGGATCCACTCAGAAATGACTTATCATTCG-3′
qox-5′0.2B	5′-TAAGGATCGGGGATCCATGGTGCATCTTACCAGATTCG-3′
qox-3′0.2A	5′-CTGATGAATTCACATATTGGGTTATGCAAGGC-3′
qox-3′0.3B	5′-CTGATGAATTCTTAGATCAAGCAGTTAAAGCG-3′
mqo2_RT.1A	5′-TGGAGCCGGTGTACTTAGCACAAC-3′
mqo2_RT.1B	5′-AACTCACATAATGCTGCATGACCC-3′
Mqo1_His.1A	5′-TTTACCATGGCTATGACAACACAACATAGCAAAACAG-3′
Mqo1_His.1B	5′-TTTAGGATCCTTTAACTTGTAAATACTTAGTTACTTCTTC-3′
Mqo2_His.1A	5′-TTTACCATGGCTAAGTCTAATAGTAAAGACATC-3′
Mqo2_His.1B	5′-TTTAGGATCCGTTTTCGTAGTAACCTAATTCTAAGTC-3′
pBT2ts	Temperature sensitive *E. coli*/*S. aureus* shuttle vector (Bruckner, 1997)
pBTE	1.2 kb *ermB* (Erythromycin^R) locus cloned into *Sma*I of pBT2ts
pBTK	1.4 kb *aph*-A3 (Kanamycin^R) locus cloned into *Sma*I of pBT2ts
pBTS	1.3 kb *aad*9 (Spectinomycin^R) locus cloned into *Sma*I of pBT2ts
pTR086	5′ and 3′ homology regions of *cydAB* cloned into pBTK to yield Δ*cydAB*::Sp^R
pTR108	5′ and 3′ homology regions of *mqo* cloned into pBTS to yield Δ*mqo*::Sp^R
pTR120	5′ and 3′ homology regions of *ddh* cloned into pBTS to yield Δ*ddh*::Sp^R
pDK004	5′ and 3′ homology regions of *lqo* cloned into pBTS to yield Δ*lqo*::Sp^R
pNV004	5′ and 3′ homology regions of *qoxBACD* cloned into pBTS to yield Δ*qoxBACD*::Sp^R
pJS006	1.4 kb Sp^R cassette replacing the 3.5 kb *Cla*I fragment within the 6.5 kb *narGHIJ* locus cloned into pBT2ts.
pJF132	C-term His_6-tagged Mqo in pQE-60
pJF133	C-term His_6-tagged Lqo in pQE-60

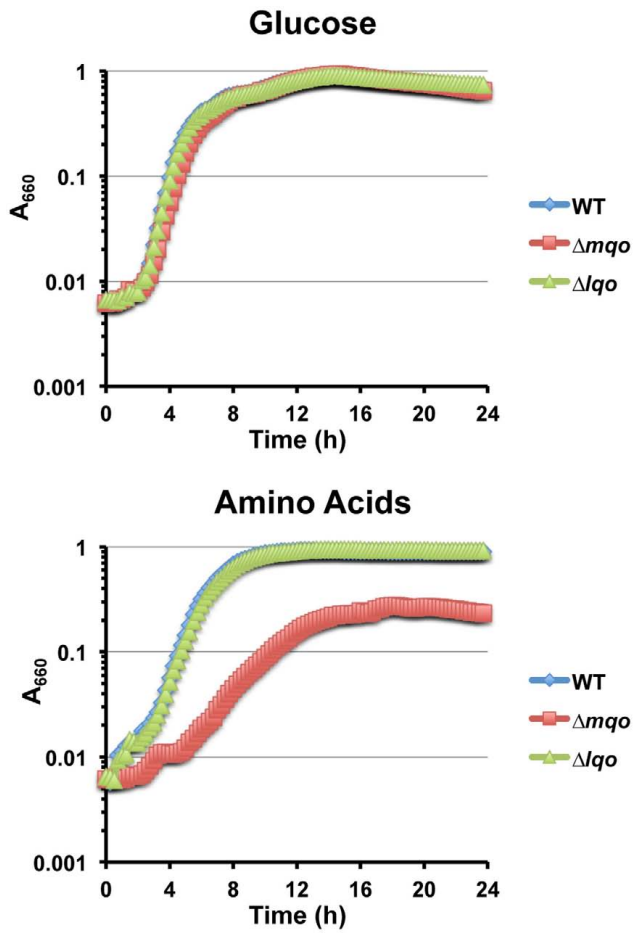

FIGURE A2 | Lqo and Mqo are differentially required for utilization of various carbon sources. TOP: Neither Mqo nor Lqo are essential for growth in chemically defined media using glucose (0.5%) as a primary carbon source. BOTTOM: While Mqo is dispensable for growth on L-lactate (Figure 2) it is essential for maximal growth in chemically defined media using amino acids (0.5% Cas Amino Acids) as a primary carbon source.

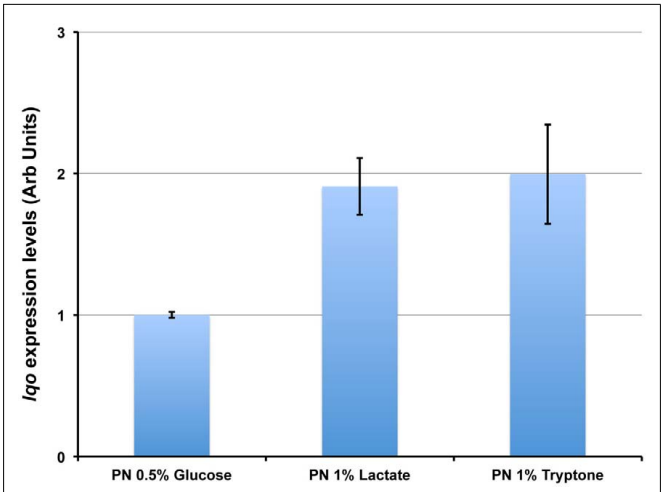

FIGURE A3 | *lqo* is highly expressed under a variety of culture conditions. Total RNA was extracted from *S. aureus* strain COL grown in chemically defined medium with either 0.5% Glucose, 1.0% L-lactate, or 1.0% tryptone as primary carbon sources. Quantitative RT-PCR was performed using primers specific for *lqo* and transcript levels were normalized to *rpoD*. Data are depicted relative to *lqo* transcript levels from cells grown on Glucose.

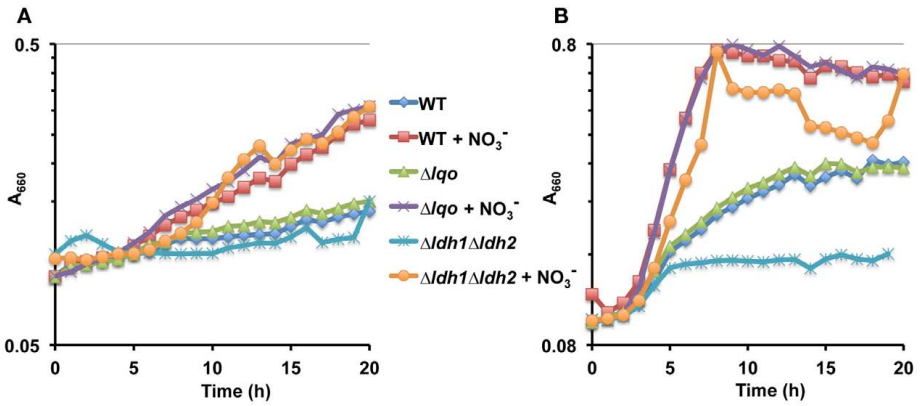

FIGURE A4 | Lqo-mediated growth on L-lactate under anaerobiosis requires an electron acceptor. (A) Representative growth curve of WT *S. aureus* strain Newman and isogenic mutants cultured anaerobically in chemically defined medium with 1.0% L-lactate as the primary carbon source. When indicated, nitrate was added (NO_3^- at 40 mM) as an anaerobic respiratory electron acceptor. **(B)** Representative growth curve of WT *S. aureus* strain Newman and isogenic mutants cultured anaerobically in chemically defined medium with a combination of 1.0% tryptone and L-lactate as the primary carbon source. When indicated, nitrate was added (NO_3^- at 40 mM) as an anaerobic respiratory electron acceptor.

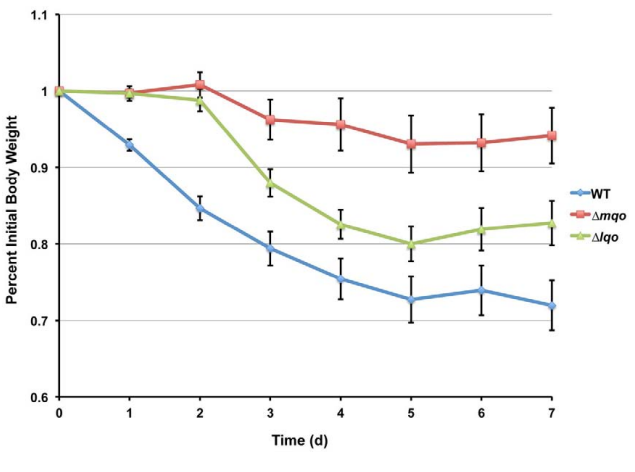

FIGURE A5 | Both Mqo and Lqo are required for full virulence in *S. aureus*. Eight female C57BL/6 mice (two independent experiments each involving four mice per group) were inoculated i.v. with 5×10^6 cfu of *S. aureus* strain Newman or isogenic mutants. Weight loss was documented and mice losing ≥30% of their body weight at the time of inoculation were sacrificed as per approved IACUC protocols.

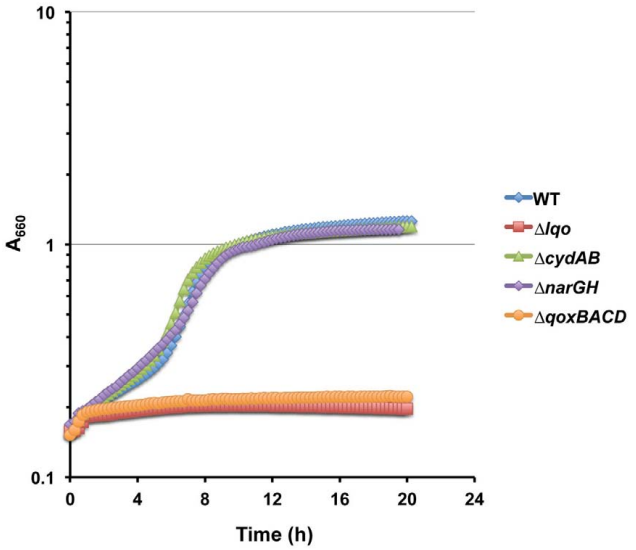

FIGURE A6 | Cytochrome-aa_3 (QoxBACD) is required for Lqo activity during NO·-stress. Representative growth curve on WT *S. aureus* strain COL and isogenic mutants grown in chemically defined medium using a combination of 0.1% L-lactate/1.0% tryptone as primary carbon sources in the presence of low level NO· (administered as 10 mM DETA/NO when cultures reach $A_{660} = 0.15$).

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Comparison of *Staphylococcus aureus* strains for ability to cause infective endocarditis and lethal sepsis in rabbits

Adam R. Spaulding[1], Erin A. Satterwhite[2], Ying-Chi Lin[3], Olivia N. Chuang-Smith[4], Kristi L. Frank[4], Joseph A. Merriman[1], Matthew M. Schaefers[3], Jeremy M. Yarwood[5], Marnie L. Peterson[3] and Patrick M. Schlievert[1]*

[1] Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA
[2] 3M Infection Prevention Division, 3M Company, St. Paul, Minneapolis, MN, USA
[3] Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, Minneapolis, MN, USA
[4] Department of Microbiology, Medical School, University of Minnesota, Minneapolis, MN, USA
[5] 3M Corporate Research Laboratory, 3M Company, St. Paul, Minneapolis, MN, USA

Edited by:
Martin J. McGavin, University of Western Ontario, Canada

Reviewed by:
Francois Vandenesch, Immunity Infection Vaccination, France
Vance Fowler, Duke University Medical Center, USA

***Correspondence:**
Patrick M. Schlievert, Department of Microbiology, Carver College of Medicine, University of Iowa, 51 Newton Road, 3–403 BSB, Iowa City, IA 52242, USA.
e-mail: patrick-schlievert@uiowa.edu

Staphylococcus aureus is a major cause of infective endocarditis (IE) and sepsis. Both methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) strains cause these illnesses. Common *S. aureus* strains include pulsed-field gel electrophoresis (PFGE) types USA200, 300, and 400 types where we hypothesize that secreted virulence factors contribute to both IE and sepsis. Rabbit cardiac physiology is considered similar to humans, and rabbits exhibit susceptibility to *S. aureus* superantigens (SAgs) and cytolysins. As such, rabbits are an excellent model for studying IE and sepsis, which over the course of four days develop IE vegetations and/or fatal septicemia. We examined the ability of MRSA and MSSA strains (4 USA200, 2 USA300, 2 USA400, and three additional common strains, FRI1169, Newman, and COL) to cause vegetations and lethal sepsis in rabbits. USA200, TSST-1$^+$ strains that produce only low amounts of α-toxin, exhibited modest LD_{50} in sepsis ($1 \times 10^8 - 5 \times 10^8$) colony-forming units (CFUs), and 3/4 caused significant IE. USA200 strain MNPE, which produces high-levels of α-toxin, was both highly lethal (LD_{50} 5×10^6 CFUs) and effective in causing IE. In contrast, USA300 strains were highly effective in causing lethal sepsis (LD_{50}s 1×10^6 and 5×10^7 CFUs) but were minimally capable of causing IE. Strain Newman, which is phylogenetically related to USA300 strains, was not highly lethal (LD_{50} of 2×10^9 CFUs) and was effective in causing IE. USA400 strains were both highly lethal (LD_{50}s of 1×10^7 and 5×10^7 CFUs) and highly effective causes of IE. The menstrual TSS isolate FRI1169, that is TSST-1$^+$, produces high-levels of α-toxin, but is not USA200, was both highly lethal and effective in causing IE. Additional studies showed that phenol soluble modulins (PSMs) produced by FRI1169 were important for sepsis but did not contribute to IE. Our studies show that these clonal groups of *S. aureus* differ in abilities to cause IE and lethal sepsis and suggest that secreted virulence factors, including SAgs and cytolysins, account for some of these differences.

Keywords: ***Staphylococcus aureus*, exotoxins, infective endocarditis, sepsis**

INTRODUCTION

Recently, the Centers for Disease Control and Prevention and collaborators published that *Staphylococcus aureus* is the most common cause of serious infectious diseases in the United States (Klevens et al., 2007). *S. aureus* is a common organism found in humans, with estimates of up to 40% of the population being colonized asymptomatically on mucosal and skin surfaces (Lowy, 1998; Schlievert et al., 2010). From these sites the organism causes many illnesses, ranging from benign soft tissue infections to life-threatening illnesses such as infective endocarditis (IE), sepsis, pneumonia, extreme pyrexia, and toxic shock syndrome (TSS) (Lowy, 1998; McCormick et al., 2001; Kravitz et al., 2005; Assimacopoulos et al., 2009). *S. aureus* is a serious pathogen both in hospital (Lowy, 1998) and community settings (Herold et al., 1998) with large numbers of severe infections emerging in the last decade.

In order to cause serious illnesses, *S. aureus* has many virulence factors that enable the microbe to interact with host tissues, defend itself from the immune system, and persist to cause organ dysfunction. Among the secreted virulence factors are multiple cytolysins and superantigens (SAgs) (Dinges et al., 2000; McCormick et al., 2001). The cytolysin α-toxin has been known for many years to be required for *S. aureus* strains to cause dermonecrotic and inflammatory skin infections. Recently, α-toxin has been shown to be important for causation of necrotizing pneumonia in mice (Bubeck Wardenburg et al., 2007; Bubeck Wardenburg and Schneewind, 2008). Other cytolysins include the hot:cold cytolysin β-toxin, which is a sphingomyelinase, biofilm ligase (Huseby et al., 2007, 2010), and participant in IE; δ-toxin and other phenol soluble modulins (PSMs) (Otto, 2010), which lyse cells as either surfactants or by forming small pores; and the hetero-heptamer pore-forming toxins, including γ-toxin and

Panton-Valentine leukocidin (Labandeira-Rey et al., 2007), may also contribute to serious illnesses.

Among the most studied secreted virulence factors are the SAgs, so named because of their unusual mechanism of dysregulating immune function (Marrack and Kappler, 1990; McCormick et al., 2001). SAgs include toxic shock syndrome toxin-1 (TSST-1), the emetic staphylococcal enterotoxins (SEs) serotypes A–E and I, and the non-emetic (or not tested) staphylococcal enterotoxin-like SAgs serotypes G, H, and J–X (McCormick et al., 2001). The biological activities of SAgs have been well described (Barsumian et al., 1978; Schlievert et al., 1981; Marrack and Kappler, 1990), and at least three (TSST-1, SEB, and SEC) cause the majority of cases of staphylococcal TSS (Schlievert et al., 2004). SAgs cause high fever (Schlievert et al., 1981; Schlievert, 1982), enhance host susceptibility to gram-negative lipopolysaccharide (Schlievert et al., 1981; Schlievert, 1982), and induce massive T-cell proliferation (Schlievert et al., 1981; Marrack and Kappler, 1990). SAgs stimulate T-cell proliferation by forming cross-bridges between the variable portions of the β-chains of the T-cell receptors (Vβ-TCRs) and invariant regions of the α- or β-chain of MHC II molecules on antigen presenting cells (Kotzin et al., 1993; Li et al., 1999; McCormick et al., 2001). SAg stimulation results in production of many cytokines, consequently leading to TSS. Recently, SAgs have been shown to induce proinflammatory responses in epithelial and endothelial cells, stimulating production primarily of chemokine responses, such as IL-8 and MIP-3α, which may play important roles in the early stages of infection through outside-in signaling from mucosal surfaces (Brosnahan et al., 2009; Brosnahan and Schlievert, 2011).

IE is a life-threatening infection of the heart endothelium most often caused by gram-positive bacteria (Bashore et al., 2006), with *S. aureus* being one of the most common (Fowler et al., 2005). IE is characterized by the formation of "cauliflower-like" vegetations, comprised of host factors and microorganisms, on the damaged endothelium of heart valves. There are two major animal models for the study of IE, rats, and rabbits. Both models require that the aortic valves of animals be damaged, usually with hard plastic catheters threaded through the left carotid arteries and against the aortic valves for two or more hours. Often in the rat model, investigators leave the catheters in place for the duration of experimentation. It is suggested that in using this method, the ability of *S. aureus* to form biofilms on the catheters as well as aortic valves is being studied, complicating assessments of endocarditis; it is well recognized that foreign bodies greatly increase the ability of *S. aureus* to cause illness and make it difficult to determine the contribution of individual virulence factors. This model also suffers from the inability to assess the role of SAgs in IE since rodents are highly resistant to SAgs (Schlievert, 2009). We and others have extensively used rabbits where catheters are removed in the animals after aortic valves are damaged (Schlievert et al., 1998), and rabbits are highly susceptible to secreted virulence factors produced by *S. aureus* that have been tested thus far (Schlievert, 2009). In rabbits, vegetations can be seen as early as one day after intravenous microbial challenge, and vegetations can become large enough in four days to obstruct the aortas completely. Since *S. aureus* is administered to animals intravenously, we also gain important information on ability to cause lethal septicemia.

This study was undertaken to compare abilities of various *S. aureus* clonal lineages to cause IE and lethal sepsis in rabbits. We also examined the possible roles of selected cytolysins and SAgs in these infections.

MATERIALS AND METHODS

BACTERIA

Well-characterized *S. aureus* isolates were tested for capacity to induce IE and lethal sepsis. Pulsed-field gel electrophoresis (PFGE) clonal group USA200 strains included menstrual TSS strains MN8 and CDC587 (Schlievert and Kelly, 1984), menstrual TSS community-associated methicillin-resistant (MRSA) *S. aureus* (CA-MRSA) MNWH, and post-influenza pneumonia TSS isolate MNPE (MacDonald et al., 1987). These strains produce TSST-1, and all except MNPE have a mutation in the α-toxin gene that reduces the amount of the cytolysin produced by approximately 50-fold (see **Table 1** for strains). We also studied menstrual TSS isolate FRI1169 and its naturally derived non-cytolytic variant termed JY3000. The variant emerged in biofilms from strain FRI1169, which is a TSST-1^{+}, α-toxin^{high} isolate (Yarwood et al., 2007); although from a patient with menstrual TSS, this strain does not belong to the USA200 clonal group based on dissimilarities in PFGE profiles. When cultured in the presence of serum and glucose, strain JY3000 became the dominant member of the FRI1169 population. Sequencing the *agr* locus in the organism yielded a single point mutation in *agrA*; however, this mutation did not explain the phenotype and gene expression patterns observed in the non-hemolytic variant. Microarray data confirmed that multiple virulence determinants were down-regulated, including *agrACDB* (9-fold), β-toxin (18-fold), RNAIII/δ-toxin (33-fold), γ-toxin (11-fold), and TSST-1 (5-fold). The following PFGE USA300 strains were used in our studies: CA-MRSA strain LAC, generously provided by Dr. F. R. DeLeo, NIH Rocky Mountain Laboratories, Hamilton, MT, and methicillin-sensitive (MSSA) strain MNLevy from a Minnesota case of extreme pyrexia complicating necrotizing pneumonia. USA400 strains included CA-MRSA MW2 and c99–529, both from the original description of necrotizing pneumonia in the upper Midwest (CDC, 1999). Finally, strain Newman, phylogenetically related to USA300 strains (Baba et al., 2008), and hospital-associated (HA)-MRSA strain COL were also evaluated. All strains used in these studies are maintained in the Schlievert laboratory as lyophilized stocks. For use in experimentation, the organisms were cultured on blood agar plates to ensure purity and then in Todd Hewitt broths (Difco Laboratories, Detroit, MI) at 37°C with 200 revolutions/min shaking.

RABBIT MODEL OF IE AND LETHAL SEPSIS

IE and lethal sepsis were evaluated using New Zealand white rabbits with approval from IACUC (protocol 0908A71722) (Pragman and Schlievert, 2004; Pragman et al., 2004a). Rabbits were anesthetized with ketamine and xylazine. Once anesthetized, the aortic valves were mechanically damaged with hard plastic catheters inserted into the left carotid arteries. Two hours

Table 1 | Lethality and infective endocarditis production by *Staphylococcus aureus*.

S. aureus strain	PFGE designation (α-Toxin and Superantigen Profile)	LD_{50} after intravenous injection after four days	Vegetation size
Pulmonary TSS MSSA MNPE	USA200 (α-Toxin^{high}, TSST-1$^+$, SEC$^+$)	5×10^6	Up to 100 mg
Menstrual TSS MSSA CDC587	USA200 (α-Toxin^{low}, TSST-1$^+$, SEC$^+$)	1×10^8	20–50 mg
Menstrual TSS MSSA MN8	USA200 (α-Toxin^{low}, TSST-1$^+$)	5×10^8	Up to 100 mg
Menstrual TSS CA-MRSA MNWH	USA200 (α-Toxin^{low}, TSST-1$^+$)	2×10^8	0
Menstrual TSS MSSA FRI1169	(α-Toxin^{high}, TSST-1$^+$)	5×10^6	Up to 100 mg
CA-MRSA LAC	USA300 (α-Toxin^{high}, SEI-X$^+$)	1×10^6	0
Extreme Pyrexia and Necrotizing Pneumonia MSSA Levy	USA300 (α-Toxin^{high}, SEI-X$^+$)	5×10^7	Up to 20 mg
MSSA Newman	(α-Toxin^{low}, SEA$^+$)	2×10^9	Up to 100 mg
Necrotizing Pneumonia CA-MRSA MW2	USA400 (α-Toxin$^+$, SEC4$^+$)	5×10^7	Up to 100 mg
Necrotizing Pneumonia CA-MRSA c99529	USA400 (α-Toxin$^+$, SEB$^+$)	1×10^7	Up to 100 mg
HA-MRSA COL	(α-Toxin^{low}, SEB$^+$)	2×10^9	Up to 200 mg

Note: We investigated the ability of a number of representative clonal strains to cause IE and lethal sepsis in a rabbit model. Strains were grown overnight in Todd Hewitt broths at 37°C shaking at 200 rpm. Dilutions were made (from 10^5/ml to 4×10^9/ml), and upon completion of surgery each rabbit was given a dose of 2 ml of the appropriate strain. Numbers for the LD_{50}s are given as the full 2 ml dose and were calculated to be the dose at which half of the rabbits died before the end of the 4-day trial.

later, catheters were removed, the rabbits were divided into groups, and the groups received varying doses of *S. aureus* strains washed one time and suspended in phosphate-buffered saline (PBS) intravenously in the marginal ear veins. The rabbits were allowed to awaken and were monitored daily for survival; rabbits were prematurely euthanized if they displayed symptoms 100% predictive of lethality (incapacity to right themselves and simultaneously failure to exhibit escape behavior) or euthanized (Beuthanasia D, Schering-Plough Animal Health Corp., Union, NJ) at the termination of experimentation after four days. Hearts were immediately removed to examine the aortas and aortic valves for the presence of vegetations, which were weighed.

IMMUNIZATION STUDIES

New Zealand white rabbits were immunized against a cocktail of PSMs α1, PSM α4, and δ-toxin (PSMγ) and then challenged in the IE/sepsis model with strain FRI1169 as above. Peptides were synthesized and purified at the University of Minnesota Biomedical Genomics Center (>90% purity by rHPLC). The lyophilized peptides were reconstituted in sterile distilled water. Rabbits received a series of three injections (days 0, 14, and 28) with the cocktail (120 μg of each per injection) diluted in PBS and then emulsified with incomplete Freund adjuvant (Difco Laboratories, Detroit, MI). Hyperimmunization (antibody titers > 2000) was verified by measuring the serum antibody titers to each antigen in all rabbits by ELISA. The immunized and non-immunized rabbits received $\sim 10^7$ CFUs of wildtype FRI1169 in the marginal ear veins.

SUPERNATE PREPARATION

Sterile supernates from 7 and 14 h cultures of strain FRI1169 and its non-cytolytic variant JY3000 grown in Todd Hewitt broths were collected by centrifugation followed by filtration (0.22 μm; Millipore, Carrigtwohill, Co. Cork, Ireland). Protein was measured by Bio-Rad Protein Assay (Hercules, CA). To collect ethanol-insoluble exoproteins, supernates were treated with 80% final concentration 4°C ethanol and centrifuged (1000 × g, 15 min). The ethanol-soluble fraction was lyophilized to collect exoproteins that did not precipitate. Ethanol (80%) insoluble exoproteins were collected and dried. Both ethanol-soluble and insoluble fractions were reconstituted in ultrapure water to their original volumes.

EXOPROTEIN CHARACTERIZATION

Supernate proteins from FRI1169 and JY3000 were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–20% gradient gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, Inc.) and then either stained with Coomassie brilliant blue or silver (SilverXpress, Invitrogen, Carlsbad, CA). Unique protein bands were cut from the Coomassie-stained gels, analyzed using MALDI-MS, and compared against the database staphylococcus_NCBI_952306_CTM, to determine protein identity (University of Minnesota Center for Mass Spectrometry and Proteomics).

TISSUE CULTURE EXPERIMENTS

A549 human lung epithelial cells (ATCC, Manassas, VA) were grown in 96-well plates (Nalco, Naperville, IL) to 80% confluence in RPMI 1640 medium (Gibco, Invitrogen), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and an antibiotic cocktail (25 μg/ml of penicillin, streptomycin, and fungizone; MP Biomedicals, Solon, OH), and then changed to antibiotic-free medium overnight. The next day, strain FRI1169 or JY3000 supernates were diluted to 20 μg/ml protein in RPMI medium and used to replace the medium on the A549 cells. After 4 h, interleukin-8 (IL-8) production was measured by ELISA (R&D Systems, Minneapolis, MN), and cell viability was measured with the MTT-based reagent (Cell Titer 96 AQeous One, Promega, Madison WI).

α-TOXIN WESTERN IMMUNOBLOTTING

Ten microliters of filtered overnight culture supernates from strain FRI1169 or JY3000 were analyzed by 12% SDS-PAGE, proteins transferred onto polyvinylidene fluoride (PVDF) membranes, and membranes immunoblotted with antiserum to α-toxin (Sigma-Aldrich). Secondary, anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Danvers, MA), were employed for detection via chemoluminescence using SuperSignal West Dura Extended Duration substrate (Thermo Scientific).

LD_{50} AND STATISTICAL ANALYSES

The LD_{50} of *S. aureus* strains following intravenous administration of washed bacteria, suspended in PBS, was determined by the method of Reed and Muench (Reed and Muench, 1938). Briefly, varying doses of *S. aureus* strains were administered to rabbits (3 per dose; 2 ml per rabbit), with doses ranging from 10^5/ml to 4×10^9/ml. Deaths were recorded over a 4-day time period. Tests for significance between means were carried out using Student's *t*-test or One-Way ANOVA with Dunnett post-test in Graph Prism (GraphPad Software, San Diego, CA). Significance in survival experiments was determined using Log-Rank Test (GraphPad Software).

RESULTS

COMPARATIVE ABILITIES OF *S. aureus* STRAINS TO CAUSE IE AND LETHAL SEPSIS

We evaluated the ability of multiple *S. aureus* isolates to cause IE and lethal sepsis (**Table 1**). CDC clonal groups USA200, USA300, and USA400 strains, as well as other commonly used strains, were evaluated. All studies were performed in accordance with IACUC approval.

USA200 isolates are common strains found in IE patients (Xiong et al., 2006). USA200 strains, nearly all of which in our large collection produce 3–20 μg/ml of TSST-1 *in vitro* in Todd Hewitt broths, were variably effective in causing vegetations of up to 100 mg, and there were differences in ability to cause lethal sepsis. The LD_{50} of the four USA200 strains, as determined by the method of Reed and Muench, ranged between 5×10^6 colony-forming units (CFU), for strain MNPE that has the wildtype α-toxin gene, and 1–5×10^8 CFUs, for the strains that have mutations in the α-toxin gene, thereby reducing α-toxin production 50-fold (Lin et al., 2011). Interestingly, one vaginal isolate, CA-MRSA MNWH, had an LD_{50} of 2×10^8 CFUs, comparable to the other strains with the α-toxin gene mutation, but did not cause IE.

The prototypical USA300 strains differed from the USA200 strains in ability to cause illnesses. CA-MRSA LAC especially had a low LD_{50} of 1.2×10^6 CFUs, like USA200 strain MNPE, but did not cause vegetations, unlike MNPE. The lethal sepsis activity of LAC and MNPE correlated with high-level production of α-toxin and production of SAgs, but the basis for lack of the ability of LAC to cause IE is unknown. MSSA USA300 strain MNLevy had a higher LD_{50} at 5×10^7 CFUs, but similarly caused only small vegetations. We have sequenced the genome of MNLevy and compared the sequence to the USA300 strain of Diep et al. (Diep et al., 2006); they are closely related, having a similar genome organization, except for the presence of the SCC*mec* DNA element in LAC. Strain Newman is not a USA300 strain but is phylogenetically related, and this organism appears to be unusually cardiotrophic in our studies, compared to other *S. aureus* tested. When injected intravenously, the organism caused extensive heart abscesses. Strain Newman caused IE, with large vegetations forming of up to 100 mg, but required more organisms to cause lethal sepsis (LD_{50} 2×10^9 CFUs) than the two USA300 strains.

CA-MRSA USA400 strains MW2 and c99–529 was highly capable of causing both IE and lethal sepsis. MW2 had an LD_{50} of 5×10^7 CFUs and the ability to cause vegetations of up to 100 mg. C99–529 was similar, with an LD_{50} of 1×10^7 CFUs and similar ability to cause IE. MW2 is known to produce α-toxin and the SAg SEC (Diep et al., 2008; Strandberg et al., 2010) and c99–529 produces α-toxin, as determined by lysis of rabbit erythrocytes (data not shown), and SEB in high amounts (Strandberg et al., 2010) (50–100 μg/ml in Todd Hewitt broths).

The HA-MRSA COL strain, which is not a USA400 strain but like many USA400 strains produces SEB in high amounts (Yarwood et al., 2002), was evaluated for its ability to cause IE and lethal sepsis; the strain was better at causing IE compared to other *S. aureus* strains, with vegetations reaching 200 mg in agreement with a prior publication (Huseby et al., 2010), but doing so with a high LD_{50} of 2×10^9 CFU.

Collectively, our data suggest that USA200 and USA400 strains are generally better able to cause IE than USA300 strains. The presence of high-levels of cytolysins and SAgs correlates with increased ability to cause lethal sepsis.

PSMS CONTRIBUTE TO LETHAL SEPSIS BUT NOT IE

Studies have shown that cytolysins and SAgs contribute to IE (Cheung et al., 1994; Pragman et al., 2004a; Huseby et al., 2010). However, studies have not evaluated the role of PSMs in IE. Through studies initiated with *S. aureus* FRI1169 and a non-cytolytic, natural variant JY3000, we evaluated the role. Our studies showed that wildtype FRI1169 is both highly lethal (LD_{50} 5×10^6 CFUs) and capable of causing IE (**Table 1**).

Based on our past experience with USA200 strain MNPE, in which lethal sepsis appeared to correlate with high α-toxin production, we hypothesized that FRI1169 lethality would be due to its wildtype α-toxin production. We thus initiated studies to compare the cytotoxicity of wildtype FRI1169 and the natural biofilm mutant JY3000 organism. A549 cells were exposed to early (7 h) and late stationary phase (14 h) culture supernates from both organisms for 4 h to compare cytotoxicity and pro-inflammatory responses. Supernates from two time points of growth were used to ensure that exoproteins expressed at different points were included. Epithelial cells were selected because they serve as a primary barrier to *S. aureus* infection on mucosal surfaces. Application of the 7 h supernates from wildtype FRI1169 resulted in approximately 50% reduction in cell viability, and the 14 h supernates resulted in over 90% reduction in cell viability (**Figure 1A**). In contrast, neither of the variant JY3000 supernates caused viability changes relative to media controls. The pro-inflammatory response, measured by IL-8 production to attract polymorphonuclear leukocytes,

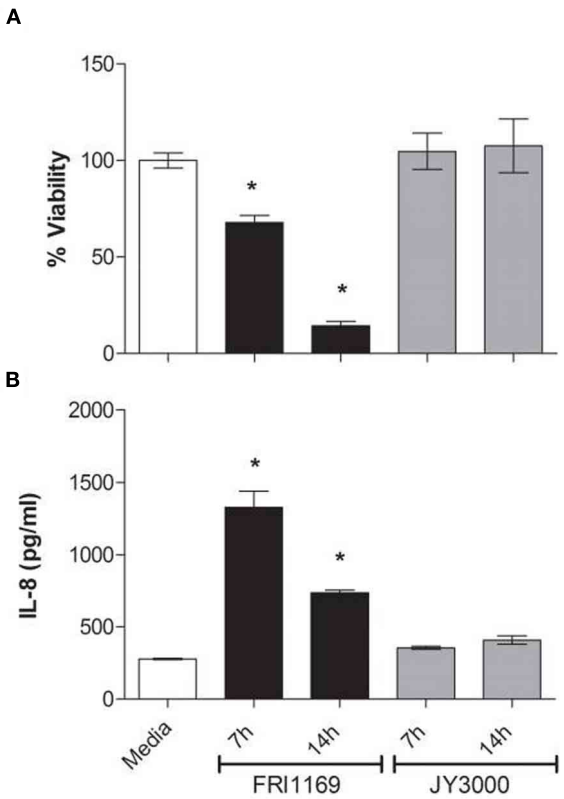

FIGURE 1 | *In vitro* comparison of wildtype FRI1169 and variant JY3000 cytotoxicity. A549 cell cytotoxicity **(A)** and IL-8 response **(B)** after 4 h exposure to *S. aureus* broth culture supernates of FRI1169 (wildtype cytolytic) and JY3000 (biofilm-derived variant, non-cytolytic) grown for 7 or 14 h. *Indicates $p < 0.05$ compared to medium control (RPMI medium alone) by One-Way ANOVA, data plotted as mean ± standard deviation.

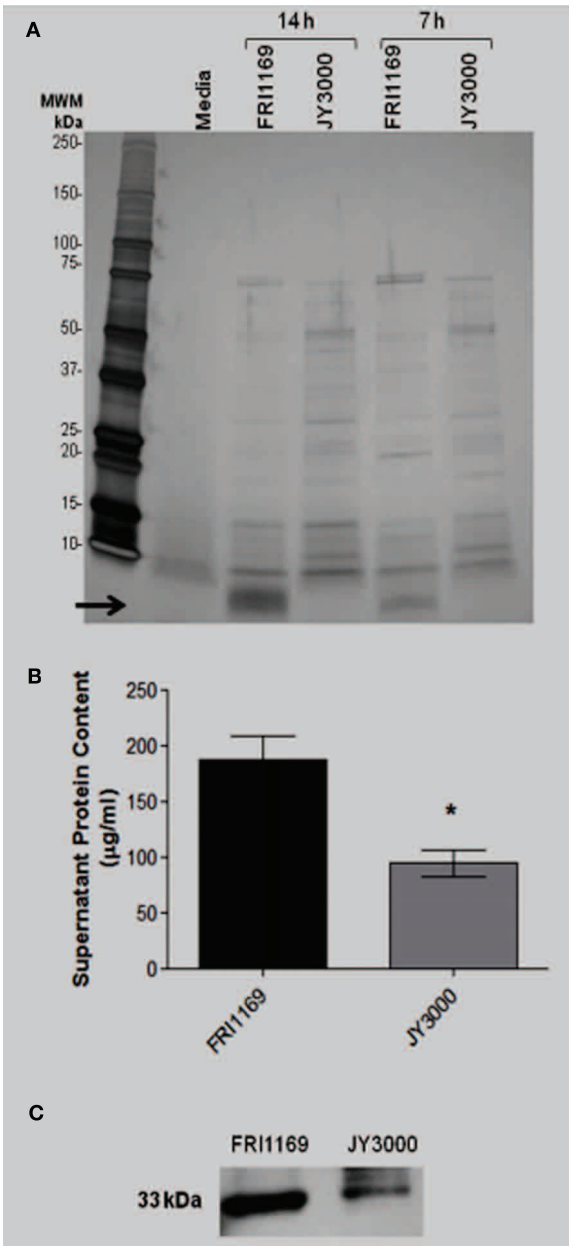

FIGURE 2 | Characterization of exoprotein production by wildtype FRI1169 and variant JY3000. (A) 14 and 7 h broth culture supernates (20 µg of protein loaded per lane) were separated by SDS-PAGE and silver-stained. Media denotes lane electrophoresed with Todd Hewitt media alone. Arrow denotes small molecular weight (<10 kDa) band only present in FRI1169 cultures. **(B)** Total protein content measured in three separate overnight broth culture supernates from JY3000 and FRI1169 via Bio Rad reagent. (*$P = 0.005$ by Student's *t*-test). **(C)** Western immunoblot results showing differences in α-toxin amounts produced by wildtype FRI1169 and JY3000.

also confirmed difference between wildtype FRI1169 and variant JY3000 (**Figure 1B**). Supernates from JY3000 at both time points did not alter IL-8 production relative to the medium-only control. However, supernates from wildtype FRI1169 caused significant increases in IL-8 production. We hypothesized that the major differences between FRI1169 and JY3000 cytolytic and pro-inflammatory activities depended on differential production of α-toxin, but as shown below the major differences were related to differential production of PSMs, including δ-toxin.

Supernates from 7 and 14 h broth cultures of FRI1169 and JY3000 were diluted to 20 µg/ml protein and subjected to SDS-PAGE. A unique band was observed for wildtype FRI1169 that was not present in the variant JY3000 fluids. This was a thick band containing low molecular weight species present in abundance in the 14 h FRI1169 supernates, and to a lesser extent in the 7 h FRI1169 supernates (**Figure 2A**). Aside from the difference in pattern, in three independent experiments we observed less total exoprotein in variant JY3000 supernates than in wildtype FRI1169 fluids ($p = 0.005$, **Figure 2B**).

Secreted virulence factors such as α-toxin and TSST-1 are known to be insoluble in 80% ethanol while smaller molecules are soluble in 80% ethanol, including cytolytic peptides known as PSMs. We demonstrated that wildtype FRI1169 and variant JY3000 produced detectable α-toxin by Western immunoblot, with FRI1169 producing more than JY3000. Late stationary phase supernates of wildtype FRI1169 were prepared and subjected to 80% ethanol treatment. Both the ethanol-insoluble

and ethanol-soluble fractions were reconstituted to their original volumes and compared using SDS-PAGE. The low molecular weight band observed in **Figure 2** was present exclusively in the ethanol-soluble fraction (**Figure 3A**). A549 cells were exposed to the reconstituted ethanol-soluble and ethanol-insoluble fractions, as well as the whole supernates. After 4 h, A549 cells exposed to the wildtype FRI1169 ethanol-soluble fractions and whole supernates showed reduced cell viability (**Figure 3B**). Surprisingly, no reduction in viability was detected for the re-solubilized wildtype FRI1169 ethanol precipitate, where α-toxin and TSST-1 were present. None of the JY3000 supernates demonstrated cytotoxicity (**Figure 3B**).

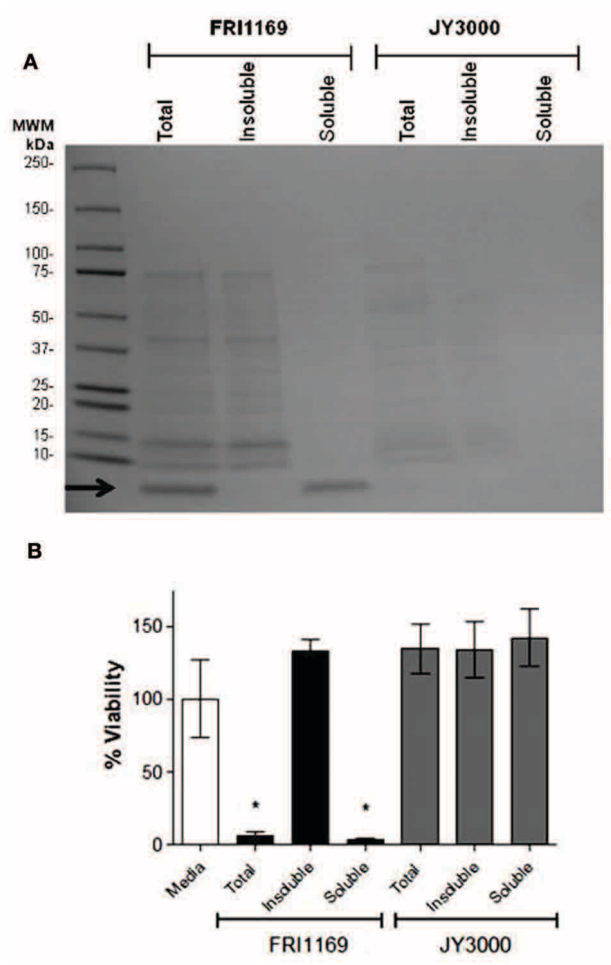

FIGURE 3 | Ethanol (80%) separation of exoprotein and determination of cytotoxicity. (A) 14 and 7 h broth culture supernatant fluids (20 μg of protein was loaded per lane) were treated with 80% (final concentration) ethanol and total, ethanol-insoluble, and ethanol-soluble fractions were separated by SDS-PAGE and Coomassie brilliant blue-stained. Arrow denotes small molecular weight (<10 kDa) band only present in total supernatant fluid or ethanol soluble fraction of FRI1169 culture **(B)** A549 cells were exposed to total, ethanol insoluble, and ethanol soluble fractions of FRI1169 and JY3000. Cytotoxicity was measured and plotted relative to media only (untreated) control. (*Indicates $P < 0.05$ relative to media control).

Because of the association between the presence of the low molecular weight species in wildtype FRI1169 and cytotoxicity for A549 cells, we analyzed the band via MALDI-MS analysis to identify the protein(s). Unique peptides were mapped to three, (potentially four) virulence elements: PSMα-1, PSMα-2 (redundant sequence with α-1), PSMα-4, and δ-toxin (PSMγ).

The rabbit IE/sepsis model was selected to understand preliminarily whether the *in vitro* cytotoxicity observations were predictive of *in vivo* virulence. Rabbits were injected with either wildtype FRI1169 or variant JY3000 at a concentration of $\sim 10^8$ CFUs/injection. After developing symptoms of serious infections, rabbits administered wildtype FRI1169 died within 24 h ($n = 5$) as a result of sepsis. Conversely, rabbits administered an equivalent dose of variant JY3000 survived with mild symptoms over the same time period. This experiment was terminated on day 2, as no FRI1169-inoculated rabbits survived long enough to develop significant aortic vegetations.

Since there are 3–4 PSMs that could account for cytotoxicity of strain FRI1169, and because one of these is δ-toxin in which its mRNA is RNA III, a global regulator of exotoxin production, it was straightforward to do immunization studies to assess their role in virulence, rather than attempt to make knockouts in the 3–4 PSMs. We thus immunized rabbits against PSM α-1, PSM α-4, and δ-hemolysin (PSMγ), verified that the animals were hyperimmunized by ELISA, and then challenged them and non-immune controls with $\sim 2.5 \times 10^7$ CFUs wildtype FRI1169. By 36 h post-inoculation, 5/5 of the non-immunized rabbits succumbed, while 4/4 immunized rabbits remained alive. Over the next 36 h, the immunized rabbits also died, indicating immunization delayed death, but ultimately did not block lethality (p value = 0.008, **Figure 4**). The rabbits presumably ultimately succumbed to TSS due to wildtype production of TSST-1. Vegetations on the aortic valves were recovered from all of the non-immunized rabbits (ranging from 1.0 to 9.0 mg per valve) and from 3/4 immunized rabbits (ranging from 0.5

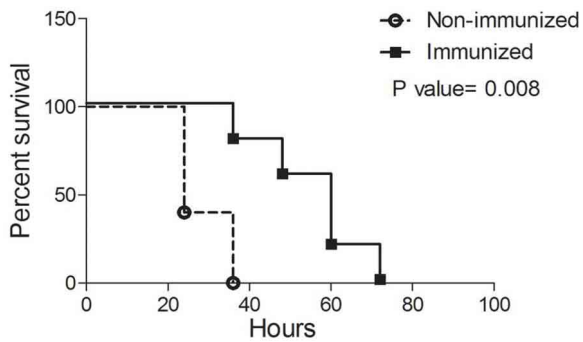

FIGURE 4 | Effect of immunization against PSMs on lethal sepsis/infectious endocarditis. Rabbits ($n = 4$ or 5 per group) were either immunized against a cocktail of PSMα-1, PSMα-4, and PSMγ/δ-toxin (squares) or left unimmunized (circles). Aortic valves were then mechanically damaged, and rabbits were challenged with $\sim 10^7$ CFUs of wildtype FRI1169 in the marginal ear veins. Rabbits were monitored for up to four days and were prematurely euthanized if displayed signs predictive of lethal illness. *P*-value determined by log rank test.

to 11.7 mg per valve). There was no significant difference in the number or weights of vegetations between the immunized and non-immunized rabbits.

DISCUSSION

The present studies have shown that there are differences among clonal groups of *S. aureus* with respect to causing IE and fatal sepsis. In general USA200 strains cause IE and have modest lethal activity in rabbits. In contrast, USA300 strains are only weakly able to cause IE, but the strains are highly lethal to rabbits. USA400 strains interestingly are both effective in causing IE and fatal sepsis. Our studies also show that PSMs, at least as produced by strain FRI1169, are important in causation of fatal sepsis in rabbits but do not contribute in major ways to IE.

We and others suggest that important cytolysins and SAgs contribute to the ability of strains to cause these and other illnesses. For example, USA200 isolates are the primary causes of TSS, accounting for nearly all menstrual TSS cases and 50% of non-menstrual TSS cases, including post-influenza cases (Bergdoll et al., 1981; Schlievert et al., 1981; MacDonald et al., 1987). These isolates and the additional menstrual TSS strain FRI1169 all produce TSST-1 as their dominant SAg. These strains lack the recently described SAg SEl-X which has been associated with necrotizing pneumonia caused by USA300 strains (Wilson et al., 2011). The majority of vaginal USA200 isolates, have a stop codon in the α-toxin gene (*hla*), preventing them from making wildtype amounts of α-toxin. Interestingly, these isolates have developed a mechanism by which they read through the stop codon and produce small amounts of α-toxin (Lin et al., 2011). MNPE and FRI1169 produce high-amounts of α-toxin as tested *in vitro* (up to 50 μg/ml). USA300 and USA400 strains, mainly CA-MRSA, are especially capable of causing skin and soft tissue abscesses and necrotizing pneumonia. Staphylococcal α-toxin is required for causation of skin infections (Kobayashi et al., 2011), and both α-toxin and SAgs, including a newly described SAg SEl-X, are required for fatal necrotizing pneumonia (CDC, 1999; Fey et al., 2003; Bubeck Wardenburg et al., 2007; Strandberg et al., 2010; Wilson et al., 2011).

The role of exotoxins in IE and lethal sepsis is only partially defined. In 1994, Cheung et al. showed that *sar*$^{-}$/*agr*$^{-}$ mutants were reduced in their abilities to colonize heart endothelium and cause IE, indicating that the regulation of production of exotoxins by these two component regulatory systems is critical for *S. aureus* to cause IE in a rabbit model (Cheung and Projan, 1994). Similarly, Xiong et al. showed that α-toxin regulation by *sae* is critical *in vivo* in the rabbit model for IE in that mutants, that had reduced *sae* activity and concurrent reduced *hla* production, were reduced in their abilities to cause IE compared to a wild-type isolate (Xiong et al., 2006). The SAg TSST-1 has been shown to be critical for IE in a rabbit model. Pragman et al. showed that TSST-1^{-} strains of *S. aureus* have much lower abilities to cause endocarditis than isogenic TSST-1^{+} strains (Pragman et al., 2004b). The TSST-1^{+} strains had much larger vegetations and on average 1×10^{6} CFUs more per vegetations than TSST-1^{-} strains (Pragman et al., 2004b). It is not known why the TSST-1^{+} CA-MRSA USA200 MNWH did not cause vegetations, but clearly the pro-IE role of TSST-1 can be modified by other factors in this strain.

In recent studies, Huseby et al. showed the pivotal role of β-toxin in IE (Huseby et al., 2010). Strain COL, known to produce β-toxin, was better able to cause vegetations than the COL strain knocked-out for β-toxin through bacteriophage integration into the β-toxin structural gene. While many *S. aureus* strains causing human illness do not produce β-toxin, many USA200 strains produce the toxin. USA200 strains are generally highly effective in causing endocarditis, and this may in part be due to the biofilm ligase activity of β-toxin (Huseby et al., 2010).

The data from our studies indicate that for *S. aureus* strain FRI1169, PSMs are important in determination of lethal sepsis, but are not critical for production of IE. These data are in agreement with prior studies of CA-MRSA USA300 strains that suggest PSMs contribute significantly to serious illnesses (Otto, 2010).

Finally, in agreement with the above studies, Seidl et al. recently showed that the ability of a *S. aureus* strain to induce endothelial damage *in vitro* was positively correlated to its ability to cause disease in a rabbit model of IE (Seidl et al., 2011). Taken together these data suggest that SAgs, cytolysins, and their regulatory mechanisms make for a highly virulent combination and are required for the progression of IE.

Recently, the Interscience Conference on Antimicrobial Agents and Chemotherapy published a historical account of their first 50 years. In that publication, it was noted that major symposia have been held each year to assess progress in management of IE. Additionally, large numbers of manuscript are published yearly studying IE. These symposia and papers indicate a clear need to continue research into understanding the fundamentals of IE caused by *S. aureus* to better treat patients and reduce the number of cases each year. We stress the importance of evaluating the role of the secreted virulence factors in these diseases, as many published studies have shown that both SAgs and cytolysins play definitive roles. It is only through a thorough understanding of their contributions in sensitive animal models that we will be able to find novel strategies to manage the illness. Our studies also demonstrate that different clonal groups, and even within clonal groups, variation in disease potential exists, making it difficult to make global statements about causative factors for groups of strains.

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Staphylococcus aureus hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors?

François Vandenesch[1,2,3]*, G. Lina[1,2,3] and Thomas Henry[1,2]*

[1] Bacterial Pathogenesis and Innate Immunity Laboratory, INSERM U851 "Immunity, Infection and Vaccination," Lyon, France
[2] Université de Lyon, Lyon, France
[3] Hospices Civils de Lyon, Lyon, France

Edited by:
Martin John McGavin, University of Western Ontario, Canada

Reviewed by:
Tammy Kielian, University of Nebraska Medical Center, USA
Juliane Bubeck Wardenburg, University of Chicago, USA

***Correspondence:**
François Vandenesch and Thomas Henry, Bacterial Pathogenesis and Innate Immunity Laboratory, INSERM U851, 21, Avenue Tony Garnier, 69365 Lyon Cedex, France.
e-mail: francois.vandenesch@univ-lyon1.fr; thomas.henry@inserm.fr

One key aspect of the virulence of *Staphylococcus aureus* lies in its ability to target the host cell membrane with a large number of membrane-damaging toxins and peptides. In this review, we describe the hemolysins, the bi-component leukocidins (which include the Panton Valentine leukocidin, LukAB/GH, and LukED), and the cytolytic peptides (phenol soluble modulins). While at first glance, all of these factors might appear redundant, it is now clear that some of these factors play specific roles in certain *S. aureus* life stages and diseases or target specific cell types or species. In this review, we present an update of the literature on toxin receptors and their cell type and species specificities. Furthermore, we review epidemiological studies and animal models illustrating the role of these membrane-damaging factors in various diseases. Finally, we emphasize the interplay of these factors with the host immune system and highlight all their non-lytic functions.

Keywords: *Staphylococcus aureus*, hemolysin, leukocidin, Panton Valentine leukocidin, PSM, inflammasome, pore-forming toxin, neutrophil

INTRODUCTION

Staphylococcus aureus is both a commensal bacterium found in about 30% of the healthy population and is one of the most virulent bacterial pathogens. The ability of this bacterium to cause diseases is associated with a large number of virulence factors allowing colonization and persistence, dissemination within the host, and evasion of the immune system. The set of virulence factors required to cause disease is likely to be highly dependent on the site of infection [e.g., skin and soft tissues infections (SSTI) versus infective endocarditis]. Particularly, *S. aureus* secretes numerous exotoxins, including a group of polypeptides capable of damaging the host cell plasma membrane. These polypeptides include pore-forming toxins [PFT: α-hemolysin and the bi-component leukocidins γ-hemolysin, the Panton Valentine leukocidin (PVL), LukED, and LukGH/AB], β-hemolysin (a neutral sphingomyelinase), and the phenol soluble modulins (PSMs, a family of small amphipathic peptides). While leukotoxic and hemolytic activities (the ability to lyse leukocytes and red blood cells, respectively) in *S. aureus* supernatant was described more than a century ago (Julianelle, 1922; Panton and Valentine, 1932), our understanding of the specific and physiological roles of the various toxins remains far from complete. The receptor for α-hemolysin, one of the most studied virulence factors since its discovery in the beginning of the past century, has only been identified recently, leading to novel important insights concerning the function of this toxin (Wilke and Bubeck Wardenburg, 2010; Inoshima et al., 2011). Furthermore, several highly expressed membrane-damaging (poly)peptides have been discovered in the past 5 years (Queck et al., 2009; Ventura et al., 2010; Dumont et al., 2011) highlighting both the gaps in knowledge and the extensive research efforts aimed at uncovering the function of these membrane-damaging toxins. The goal of this review is to give an update on the different membrane-damaging toxins and their role in *S. aureus*-mediated diseases. The molecular mechanisms of pore formation, which have been reviewed before (Kaneko and Kamio, 2004; Verdon et al., 2009), are not detailed here. Instead, we focus on understanding the interactions between these virulence factors and various host cells from different species and review their roles in *S. aureus*-mediated diseases. Furthermore, we describe the interplay between the innate immune system, which has evolved to sense membrane damage, and these virulence factors, several of which specifically target innate immune cells. Finally, we present the moonlighting activities of these toxins and discuss how these activities could be more relevant than the toxins' lytic activity in colonization and diseases.

OVERVIEW OF THE DIFFERENT CLASSES OF MEMBRANE-DAMAGING (POLY)PEPTIDES

α-HEMOLYSIN

α-Hemolysin is the most characterized virulence factor of *S. aureus*. Upon binding to the cell surface, α-hemolysin monomers assemble into a homoheptamer, forming a prepore. The prepore subsequently transitions to a mature β-barrel transmembrane pore (Bhakdi and Tranum-Jensen, 1991), thereby leading to the formation of a 14-Å diameter aqueous channel (Song et al., 1996). This pore allows the transport of molecules smaller than 2 kD (Menestrina, 1986), such as K^+ and Ca^{2+} ions, leading to necrotic death of the target cell.

γ-HEMOLYSIN AND BI-COMPONENT LEUKOCIDINS

Staphylococcus aureus possesses several other PFTs in addition to α-hemolysin. In contrast to α-hemolysin, the formation of a mature pore by these other PFTs involves two polypeptides that have been named S (slow) and F (fast) based on their electrophoretic mobility. The different *S. aureus* bi-component PFTs share significant homology (see Supplemental Figure S3 in Dumont et al., 2011). The PFTs include (i) γ-hemolysin corresponding to two combinations of a S component (HlgA or HlgC) with a F component (HlgB); (ii) the PVL (Panton and Valentine, 1932), made of LukS-PV and LukF-PV; (iii) LukED (Gravet et al., 1998; Morinaga et al., 2003); (iv) LukGH (Ventura et al., 2010), also known as LukAB (Dumont et al., 2011). The structure of these bi-component toxins (Olson et al., 1999; Pedelacq et al., 1999; Guillet et al., 2004) and their mechanism of pore formation have been mostly studied using γ-hemolysin and PVL, but are thought to be similar for all of the toxins. Each water-soluble component binds sequentially to the cell surface as a monomer before oligomerizing into a prepore formed by four S components alternatively arranged with four F components. The mature hetero-octamer forms a transmembrane β-barrel pore across the plasma membrane, leading to host cell lysis. The bi-component toxins are largely considered to be secreted proteins. LukGH/AB was identified both as a secreted protein in the extracellular medium and as one of the predominant surface protein of *S. aureus* at the late exponential phase of growth (Ventura et al., 2010). Surface association of this leukotoxin might be involved in targeting immune cells when they are in direct contact with the bacterium, such as during phagocytosis.

β-HEMOLYSIN

In contrast to the PFTs, the β-hemolysin does not form pores in the plasma cell membrane but instead is a neutral sphingomyelinase hydrolysing sphingomyelin, which is a plasma membrane lipid. β-hemolysin's enzymatic activity is required for its hemolytic activity (Huseby et al., 2007; Ira and Johnston, 2008). β-hemolysin lysis of red blood cells is only observed after the cells are switched to low temperature, suggesting that the lytic activity of β-hemolysin is not as efficient as that of other hemolysins, at least toward erythrocytes. β-hemolysin digests sphingomyelin into ceramide and phosphorylcholine (Doery et al., 1963). The mechanism leading to cytotoxicity is still poorly understood. Sphingomyelin is enriched in lipid-ordered membrane microdomains with high content in cholesterol. Sphingomyelinase treatment of synthetic lipid bilayers leads to aggregation of cholesterol-rich microdomains (Ira and Johnston, 2008), suggesting that cell death may result from the modification of host cell plasma membrane fluidity and destabilization of the bilayer structure. Alternatively, cell death might result from the formation of large ceramide-rich signaling platforms.

δ-HEMOLYSIN AND PSMs

This class of membrane-damaging peptides was identified over 60 years ago with the purification of δ-hemolysin (Wiseman, 1975). δ-hemolysin is a small amphipathic (one hydrophobic and one hydrophilic side) peptide (26 AA) with an α-helix structure. Three different mechanisms (reviewed in Verdon et al., 2009) have been proposed to explain its hemolytic activity. Briefly, δ-hemolysin could (i) bind to the cell surface and aggregate to form transmembrane pores; (ii) bind to the cell surface and affect the membrane curvature, thereby destabilizing the plasma membrane; or (iii) at high concentration, act as a detergent to solubilize the membrane. Recently, this family of small cytotoxic amphipathic peptides has grown with the identification of new peptides termed PSM, first in *S. epidermidis* (Mehlin et al., 1999) and subsequently in *S. aureus* (Wang et al., 2007; Queck et al., 2009). Two families of PSMs have been described based on their length (Wang et al., 2007). PSMα, which include δ-hemolysin, PSMα1–4, and PSM-mec (Queck et al., 2009), are 20–26 aminoacids long. In contrast, PSMβ1 and PSMβ2 are 44 aminoacids long.

RECEPTORS, CELL, AND SPECIES SPECIFICITIES

Comparison of toxin activity between different studies conducted using different cell types can be misleading, due to potential differences in specific activities or to the presence of contaminants linked to both the source of toxin (produced in *S. aureus* or in *E. coli*) and the nature of the purification process (see Craven et al., 2009). Keeping this possibility in mind, we review the data from the literature regarding toxin activity toward various cell types below and in **Table 1**.

α-HEMOLYSIN

α-hemolysin displays species- and cell type specificity. While rabbit erythrocytes are highly sensitive to α-hemolysin-mediated lysis, human erythrocytes are much less sensitive. Furthermore, while human lymphocytes and monocytes are α-hemolysin-susceptible cells (maximum lysis at 100 ng/ml), granulocytes are highly resistant to α-hemolysin lysis (no lysis at 10 μg/ml; Bhakdi et al., 1989; Valeva et al., 1997; Loffler et al., 2010). Similarly, lysis of human lung epithelial cells (A549) is only observed at high α-hemolysin concentrations (1 μM id 30 μg/ml; Rose et al., 2002; Wilke and Bubeck Wardenburg, 2010). The cell type and species specificities suggest the presence of a specific high affinity receptor (Bhakdi and Tranum-Jensen, 1991). Crystal structure of α-hemolysin bound to phosphocholine (Galdiero and Gouaux, 2004) validated early studies showing a requirement for phosphatidylcholine in generating α-hemolysin lytic activity against liposomes (Watanabe et al., 1987). However, the presence of phosphatidylcholine as a sole receptor cannot explain the low number of high affinity and saturable binding sites observed in rabbit erythrocytes. Valeva et al. (2006) showed that clustering of phosphocholine heads into lipid rafts (microdomains) could create high affinity binding sites. In agreement with this study, cholesterol depletion or sphingomyelinase treatment, which affect lipid rafts decreases the clustering of phosphatidylcholine lipids and abolishes the high affinity binding of α-hemolysin to rabbit erythrocytes. Recently, a biochemical approach led to the identification of A Disintegrin and Metalloproteinase Domain-containing protein 10 (ADAM10) as a α-hemolysin-interacting protein (Wilke and Bubeck Wardenburg, 2010). α-Hemolysin binding to different cell types correlates with ADAM10 expression. Furthermore, knock-down of ADAM10 gene expression in A549 cells decreases both α-hemolysin binding and lytic activity. ADAM10 is highly expressed in A549 cells and rabbit erythrocytes but not on human erythrocytes. To the best of our knowledge, the abundance of ADAM10 on human granulocytes has not been investigated. The resistance of

Table 1 | Species and cell type specificities of the various hemolysins, bi-component leukocidins, and PSMs.

Toxin/peptide	Target cell (% lysis)	Dose	Reference
α-Hemolysin	Human lymphocyte (80%)	100 ng/ml	Valeva et al. (1997)
	Jurkat cells and peripheral blood monocytes (0%: resistant)	100 ng/ml up to 20 μg/ml	Bantel et al. (2001), Bhakdi et al. (1989)
	Human granulocytes/neutrophils (0%: resistant)	10 μg/ml up to 20 μg/ml	Valeva et al. (1997), Loffler et al. (2010), Bhakdi et al. (1989)
	Human monocytes (90%)	80 ng/ml	Bhakdi et al. (1989), Bantel et al. (2001)
	A549 (40%)	375 nM (10 μg/ml)	Wilke and Bubeck Wardenburg (2010)
PVL	Murine alveolar macrophages[1]	(280 nM) 10 μg/ml	Ziebandt et al. (2010)
	Human macrophages (90%)	100 ng/ml	Perret et al. (in press)
	Human neutrophils (90%)	80 ng/ml	Loffler et al. (2010)
	Murine neutrophils (20%)	40 μg/ml	Loffler et al. (2010)
	Rabbit neutrophils (90%)	60 ng/ml	Loffler et al. (2010)
LukAB/GH	Human polymorphonuclear cells (60%)	2.5 μg/ml	Dumont et al. (2011)
LukED	Murine peritoneal exudate cells (60%)[2]	2.5 μg/ml	Alonzo et al. (2012)
β-Hemolysin	Sheep erythrocyte (50%)[3]	500 ng/ml	Tajima et al. (2009)
	HUVEC (resistant)[4]	1 μg/ml	Tajima et al. (2009)
	Human fibroblast (resistant)	1 μg/ml (5 μg/ml)	Walev et al. (1996)
	T lymphocyte (resistant)	1 μg/ml (5 μg/ml)	Walev et al. (1996)
	Granulocyte (resistant)	1 μg/ml (5 μg/ml)	Walev et al. (1996)
	Monocyte (50%)	0.001 μg/ml (5 ng/ml)	Walev et al. (1996)
	Lymphocyte (NA)[5]	10 μg/ml	Huseby et al. (2007)
δ-Hemolysin	HeLa	0.5 Hemolytic units/ml	Thelestam et al. (1973)
	Human neutrophils (5%)	10 μg/ml	Wang et al. (2007)
PSMα1	Human neutrophils (7.5%)	10 μg/ml	Wang et al. (2007)
PSMα2	Human neutrophils (10%)	10 μg/ml	Wang et al. (2007)
PSMα3	Human neutrophils (60%)	10 μg/ml	Wang et al. (2007)
PSM-mec	Human neutrophils (12%)	50 μg/ml	Queck et al. (2009)
PSMβ1	Human blood (weak)	1 μg/ml	Cheung et al. (2011)

[1] *Death monitored at 16 h post-intoxication.*

[2] *PEC were isolated from mice injected with S. aureus Newman strain 16 h before and intoxicated ex vivo with recombinant LukED.*

[3] *Hot–cold hemolysis.*

[4] *Human umbilical vein endothelial cells.*

[5] *Inhibition of proliferation.*

human granulocytes to α-hemolysin does not seem to be due to differences in α-hemolysin binding affinity but rather to the absence of insertion of the heptamer into the plasma membrane (Valeva et al., 1997). While this granulocyte resistance has been speculated to be associated with a specific response-inhibiting membrane insertion of pore-forming toxins (Valeva et al., 1997), the resistance might instead be linked to the absence of a co-receptor. Indeed, scanning α-hemolysin sequence for eukaryotic domains revealed a nine aminoacid motif resembling a caveolin-1-binding domain (Pany et al., 2004). Recombinant caveolin-1 added exogenously inhibits α-hemolysin-mediated red blood cell lysis, and α-hemolysin with a mutated caveolin-1 recognition site is inactive, due to impaired heptamerization on the cell surface (Pany et al., 2004). While ADAM10 could be involved in the first binding event, caveolin-1 might trigger a conformational change allowing α-hemolysin insertion into membrane. The relevance of caveolin-1-binding still needs to be thoroughly investigated, but caveolin-1 appears to be highly expressed in the lung and in epithelial cells (www.biogps.org). The expression patterns of caveolin-1 and ADAM10 might thus explain the cell and the species specificity observed for α-hemolysin. Furthermore, caveolae are caveolin-1, cholesterol and sphingomyelin-rich microdomains at the plasma membrane (Lajoie and Nabi, 2007). α-Hemolysin treatment relocalizes ADAM10 to caveolin-1 positive domains (Wilke and Bubeck Wardenburg, 2010). The connection between phosphatidylcholine clusters, lipid rafts, caveolin-1, and ADAM10 remains unclear. Sequential binding to those different receptors might be important to coordinate conformational changes and α-hemolysin oligomerization to trigger cell-specific lysis or localized ADAM10 activation in E-cadherin-containing caveolae (see below).

γ-HEMOLYSIN AND BI-COMPONENT TOXINS

γ-Hemolysin and, to a lesser extent, LukED are hemolytic to rabbit erythrocytes (Morinaga et al., 2003). PVL is non-hemolytic. To our knowledge, there is no report of hemolytic activity for LukAB/GH. While γ-hemolysin and PVL are highly cytotoxic toward human neutrophils (action at 10 ng/ml), LukAB/GH is only cytotoxic

toward the same cells at a 100-fold higher concentration (Dumont et al., 2011). No biological activity had been assigned to LukED for over 10 years (Gravet et al., 1998) although one variant, named $LukE_v$–$LukD_v$, was shown to display high leukotoxicity toward human neutrophils (Morinaga et al., 2003). Recent investigations of LukED indicate that the LukE and LukD proteins are highly conserved in *S. aureus* strains and have a sequence corresponding to the active $LukE_vD_v$ and not to the originally described sequence of LukED (Alonzo et al., 2012). In addition to neutrophils, the bi-component toxins are active against monocytes and macrophages. Different species specificities have been observed for the different bi-component toxins. Human and rabbit neutrophils are PVL-sensitive cells, while murine and Java monkey cells are, respectively, largely and fully resistant to PVL-mediated lysis (Loffler et al., 2010). It is therefore unlikely that the deleterious effects of PVL observed in the murine model of necrotizing pneumonia (see below) were caused by PVL-mediated neutrophils lysis (Labandeira-Rey et al., 2007; Zivkovic et al., 2011). In contrast, LukED is active against human, rabbit, and murine cells (Alonzo et al., 2012), while γ-hemolysin activity has been mostly studied against human and murine cells.

The receptors for bi-component toxins are still largely uncharacterized. The murine TLR2 ectodomain has been described to bind LukS-PV, leading to inflammation (Zivkovic et al., 2011). In addition, LukF-PV has been described to bind murine TLR4 (Inden et al., 2009). However, these receptors are unlikely to be the high affinity receptors triggering very fast lysis at very low doses in human and rabbit granulocytes and primary monocytes. Indeed, the murine TLR2/TLR4-dependent effects were observed at concentrations a 100-fold greater than the ones triggering 100% lysis of human neutrophils, monocytes, and macrophages (10 μg/ml vs. 100 ng/ml; Gauduchon et al., 2001; Perret et al., in press). As previously described for α-hemolysin, lipids have been described as receptors for LukS and LukF, although it is more likely that they act as co-receptors (Hakomori, 2003). LukS binds the ganglioside GM1, and LukF binds phosphatidylcholine (Noda et al., 1980). The latter interaction has been confirmed by crystallization of LukF-PV (Pedelacq et al., 1999), as well as with the homologous F component of γ-hemolysin, HlgB (Olson et al., 1999). Binding studies have shown that expression of the receptor for the S component of PVL and for HglC is limited to PVL-susceptible cells (Gauduchon et al., 2001). In contrast, the LukF-PV receptor is more broadly expressed (Meyer et al., 2009). Furthermore, HlgB seems to bind mainly to the corresponding S component on the host cell membrane (Meyer et al., 2009). These results indicate that the S components of PVL and γ-hemolysin drive the cell type susceptibilities. Interestingly, HlgC and LukS-PV share the same uncharacterized receptor on the surface of human granulocytes (Gauduchon et al., 2001). HlgC/HlgB and PVL thus probably target the same cell types. Furthermore, the two toxins are similarly potent at lysing granulocytes (Konig et al., 1997) and human macrophages (our unpublished results). Among recombinant bi-component toxins, PVL and γ-hemolysin are extremely potent, while LukAB/GH and lukED are active at concentrations 100-fold higher. The relative secretion of the different toxins in different conditions remains unknown, but this knowledge would help us to understand the physiological roles of the different leukotoxins.

β-HEMOLYSIN

The *P. aeruginosa* hemolytic phospholipase C (PlcHR), which is an extracellular sphingomyelinase, displays strong specificity for endothelial cells (Vasil et al., 2009). This targeting, which is mediated by a specific domain of the toxin and a saturable receptor, makes PlcHR cytotoxic to endothelial cells at concentrations 500-fold lower than the dosages toxic to epithelial cells or macrophages. Similarly, *S. aureus* β-hemolysin is selectively cytotoxic to monocytes and is inactive against lymphocytes, granulocytes, and fibroblasts (Walev et al., 1996). However, as monocytes are equally sensitive to sphingomyelinase C from *B. cereus* and from *Streptomyces* species, it is currently unknown if this result is due to a specific targeting of β-toxin to monocytes (possibly mediated by a specific receptor) or to a higher sensitivity of these cells to the toxin.

PHENOL SOLUBLE MODULINS

The current view concerning PSMs is that these peptides do not have a specific proteinaceous receptor required for cell lysis. Indeed, in contrast to other hemolysins, δ-hemolysin is hemolytic to erythrocytes from numerous species and can lyse organelles, as well as bacterial protoplasts and spheroplasts (Kreger et al., 1971; Verdon et al., 2009). Due to its amphipathic α-helix structure, δ-hemolysin has a natural affinity for lipids. Binding is preferential in liquid-disordered domains compared to liquid-ordered raft domains. Lipid composition of eukaryotic and prokaryotic membranes may explain the activity spectrum of PSMs. PSMs show moderate lytic activity toward granulocytes, and this activity is observed for only a subset of PSMs and at high concentrations (10 μg/ml for PSMα3 and δ-hemolysin, 50 μg/ml for PSM-mec). In contrast, detection of those peptides by the innate immune system occurs at concentrations as low at 50 nM (approximately 100 ng/ml; Kretschmer et al., 2010), thus it is unclear if the prime function of PSMs is to lyse neutrophils or to activate them. PSMs show activity toward human erythrocytes at lower concentrations (1 μg/ml or 0.1 μg/ml if co-incubated with β-hemolysin; Cheung et al., 2011).

PREVALENCE AND GENE ORGANIZATION

Both colonizing and clinical *S. aureus* isolates exhibit a large degree of heterogeneity. The observed heterogeneity can be due to (i) the presence or absence of a toxin gene, (ii) disruption of toxin-encoding loci, and (iii) variations in the expression level. Interestingly, some differences in the membrane-damaging toxin repertoire or in their level of expression have been linked to increased virulence or to antibiotic-resistance. The vast majority of *S. aureus* strains contain all the hemolysin genes, *hla*, *hlb*, *hld*, and *hlg*. The prevalence of the *lukED* genes is controversial. Indeed, one study found *lukED* genes in 87% of *S. aureus* strains (Morinaga et al., 2003; Alonzo et al., 2012) while another group described a prevalence of about 30% in human clinical and colonizing isolates (Gravet et al., 1998, 1999, 2001). To the best of our knowledge, the prevalence of LukAB/GH has not been investigated in a large number of clinical strains, but these toxins are well-conserved in all sequenced strains (Shukla et al., 2010; Ventura et al., 2010). In contrast, the *lukS-PV* and *lukF-PV* genes are present in less than 5% of MSSA strains (Vandenesch et al., 2003; Shukla et al., 2010).

LukS-PV and LukF-PV are encoded within lysogenic phages. A temperate phage ϕSLT can convert PVL^- strains into PVL^+ strains (Kaneko et al., 1997; Narita et al., 2001). Various lukS/lukF-PV-transducing phages have been described. This unique genetic organization makes PVL genes easily transmittable horizontally.

The β-hemolysin-encoding gene is a preferential locus for bacteriophage insertion. These insertions disrupt the β-hemolysin gene. Furthermore, most of the β-hemolysin-targeting bacteriophages simultaneously introduce genes encoding components of the immune evasion cluster (IEC), namely, the staphylokinase, the staphylococcal enterotoxins A and P, the staphylococcal complement inhibitor (SCIN), and the chemotaxis inhibitory protein of *S. aureus* (CHIPS; van Wamel et al., 2006). The vast majority (89%) of human clinical isolates carry two to four components of the IEC and thus are β-hemolysin-negative (van Wamel et al., 2006). In contrast, most *S. aureus* isolated from bovine do not have the IEC and express β-hemolysin (Monecke et al., 2007).

Interestingly, two PSMs are encoded in specific virulence loci. Indeed, δ-hemolysin is encoded within RNAIII, a highly structured 517 nt RNA (Benito et al., 2000) that is part of the agr regulon (a master virulence regulator system). The PSM-mec-encoding gene is found within the class A *mec* gene complex encoded on type II, III, and VIII SCC*mec* (Chatterjee et al., 2011), three methicillin-resistant cassettes integrated into the chromosome of certain MRSA strains. Importantly, this last finding provided a molecular link between the modulation of virulence and methicillin-resistance (Queck et al., 2009). However, the link between PSM-mec and increased virulence is controversial (Chatterjee et al., 2011; Kaito et al., 2011) and requires further investigation (see below). In addition, PSMα and α-hemolysin are produced at higher level in CA-MRSA strains than in hospital-acquired (HA)-MRSA strains (Wang et al., 2007; Li et al., 2009), and PVL is present in most of the community-acquired (CA)-MRSA lineages that have emerged worldwide (Vandenesch et al., 2003; Tristan et al., 2007). Although other factors, such as the arginine catabolic mobile element (ACME), have also been associated with CA-MRSA strains (Diep et al., 2006), the change in the repertoire or the quantity of membrane-damaging factors is also likely to participate in the high virulence of successful CA-MRSA clones.

EPIDEMIOLOGY AND ROLE IN *S. AUREUS*-DISEASES

The roles of *S. aureus* membrane-damaging factors have been investigated both by epidemiological studies in humans and by experimental infections with various mutants in diverse animal models.

EPIDEMIOLOGY

Robust epidemiological studies can only be performed on virulence factors that have a low prevalence and without association with a specific lineage. This requirement is true for PVL in CA-MSSA strains and for β-hemolysin regardless of methicillin-resistance status. To the best of our knowledge (and based on Vandenesch and Lina, unpublished data), the presence of β-hemolysin has not been associated with any specific human diseases or with severity criteria. The low prevalence of this factor in human isolates suggests that this factor is less important in human infection than the presence of the IEC components. The high prevalence of α-, γ-, and δ-hemolysin and α and β-PSMs prevents epidemiological studies from assessing their roles in specific human diseases or association with severity criteria. Nevertheless, their high prevalence suggests that these factors play a key role in colonization and/or diseases. Although LukAB/GH is highly prevalent (Ventura et al., 2010), it has been reported to be absent in certain colonizing and clinical isolates (see Figure S5 in Dumont et al., 2011). It is thus unclear if epidemiological studies on LukAB/GH would be informative. An increased prevalence of lukED genes has been observed in clinical strains isolated from cases of impetigo (Gravet et al., 2001) and from patients presenting antibiotic-associated diarrhea (Gravet et al., 1999). Due to the limited and controversial knowledge regarding LukED prevalence, the biological significance of these interesting associations remains unclear. PSM-mec has been only recently identified, and epidemiological studies have yet to be performed (Chatterjee et al., 2011).

There is compelling evidence that PVL is associated with a severe form of CA-MSSA pneumonia occurring in otherwise healthy children and young adults (Lina et al., 1999; Gillet et al., 2002). PVL is also associated with increased severity, treatment duration, and extra osseous complications in bone and joint infections, especially in children (Bocchini et al., 2006; Dohin et al., 2007). The role of PVL in SSTI is more debated (Bae et al., 2009), but several studies demonstrate a significant association between PVL and CA-SSTI (Shallcross et al., 2010; Del Giudice et al., 2011). This association is particularly strong in primary skin lesions, such as furunculosis (Gravet et al., 2001; Del Giudice et al., 2009, 2011). In contrast, no clear role of PVL has been identified in CA-MRSA infections. However, epidemiological studies regarding PVL and CA-MRSA-diseases have proven difficult, due to the high prevalence of PVL in CA-MRSA strains in North America and to the clonality of these CA-MRSA strains (Bae et al., 2009). In a similar vein, PVL is statistically associated with major MRSA abscesses but does not seem to affect SSTI patients' outcome (Bae et al., 2009). Furthermore, PVL is not associated with hospital-acquired/ventilation associated MRSA pneumonia (Peyrani et al., 2011). Of note, in these two studies, PVL^+ strains are statistically associated with younger patients than PVL^- strains. This observation suggests that PVL is a virulence factor in immunocompetent and not immunosenescent patients. Alternatively, an increase in the likelihood of being immunized against PVL with age might explain why PVL^+ strains are more often associated with infections in children and young adults than with infections in older patients. Indeed, serum antibody titers specific for *S. aureus* toxins increase with age (Hermos et al., 2010; Verkaik et al., 2010). However, Hermos and collaborators did not observe any protection against PVL^+ SSTI in patients with high anti-PVL antibody levels. In contrast, Rasigade et al. (2011) found that patients presenting a history of PVL-associated infections were protected from death in PVL-associated necrotizing pneumonia. Overall, these studies suggest that PVL-directed immunity does not protect the host from being re-infected and from developing severe infections, such as necrotizing pneumonia, but it may favor a positive outcome (survival) in severe diseases.

ANIMAL MODELS

Numerous animal models have been used to study the impact of membrane-damaging factors on *S. aureus* infection (**Table 2**). Strikingly, while iron is known to be a limiting nutrient during infections, it is still unknown if hemolysis occurs *in vivo* and which hemolysin is important in acquiring iron. α-Hemolysin is the key virulence factor emerging from *in vivo* studies. α-Hemolysin is a critical virulence factor in brain abscesses (Kielian et al., 2001), SSTI (Kobayashi et al., 2011), and pneumonia (Bubeck Wardenburg et al., 2007). The role of α-hemolysin in bacteremia is less obvious (Bayer et al., 1997), which is consistent with a major role of α-hemolysin at epithelial surfaces (see below). In contrast, the role of the other hemolysins is still poorly defined. β-hemolysin promotes inflammation and lung injury in a mouse model of pneumonia (Hayashida et al., 2009), and γ-hemolysin promotes bacterial growth and triggers inflammation in a rabbit model of endophthalmitis (Supersac et al., 1998). LukAB/GH is important for survival in human blood and increases the bacterial burden in the kidney during a murine model of bloodstream infection (Dumont et al., 2011). Similarly, deletion of the *lukE* and *lukD* genes attenuates *S. aureus* virulence and decreases lethality in a murine model of bloodstream infection (Alonzo et al., 2012).

As described above and in contrast to several other *S. aureus* virulence factors, PVL is poorly cytotoxic to murine cells (Loffler

Table 2 | Membrane-damaging factors and their role in various diseases in various animal models.

Disease	Animal	Inoculum (strain type)	Virulence factor	Phenotype of the animal infected with the mutant strain as compared to the WT strain	Reference
Skin infection	Rabbit	5×10^8 MRSA (USA 300 Lac)	hla	Decreased cfu, decreased abscess size	Kobayashi et al. (2011)
			PSMα	Decreased cfu, decreased abscess size	
			PVL	Decrease cfu, increased abscess size at resolution stage	
Skin infection	Rabbit	10^8 MRSA (USA 300 Lac)	PVL	Decreased lesion size, decreased necrotic lesion size	Lipinska et al. (2011)
Osteomyelitis	Rabbit	4×10^8 MRSA (USA 300 Lac)	PVL	No bone deformation at day 7, decreased cfu at day 28	Cremieux et al. (2009)
Necrotizing soft tissue infection	Mice	10^9 cfu	PVL	No difference in cfu, No difference in skin lesion size, decreased muscle lesions in BalB/c mice not in C57BL6J mice	Tseng et al. (2009)
Necrotizing pneumonia	Rabbit	10^{10} cfu (USA 300 SF8300)	PVL	Decreased mortality, decreased inflammation	Diep et al. (2010)
Necrotizing pneumonia	Mice	Purified PVL (3 μg LukS + 3 μg LukF)	PVL	Death, neutrophil recruitment	Labandeira-Rey et al. (2007)
Necrotizing pneumonia	Mice	2.10^7 cfu (LUG855)	PVL	Decreased mortality, decreased inflammation	
Bacteremia	Rabbit		PVL	Decreased cfu in the kidney at 24–48 h PI not at 72 h PI	Diep et al. (2008)
Bacteremia	Mice	1.10^6 cfu (USA 300 Lac)	LukAB/GH	Decreased cfu in the kidney at day 4 PI	Dumont et al. (2011)
Bacteremia	Mice	10^8 cfu (Mw2)	PSMα	Decreased mortality, less TNFα	Wang et al. (2007)
			PSMβ	No role	
			hld	Decreased mortality, less TNFα	
Skin infection	Mice	10^7 cfu (Mw2)	PSMα	Decreased lesion size	Wang et al. (2007)
			PSMβ	Increased lesion size	
			hld	No role	
Bacteremia	Mice	10^8 cfu (MSA890)[1]	PSM-mec	Decreased mortality	Queck et al. (2009)
Skin infection	Mice	10^7 cfu (MSA890)	PSM-mec	No dermonecrosis, no weight loss	
Pneumonia	Mice	$2–4 \times 10^8$ cfu (Lac)	hla	Decreased mortality	Bubeck Wardenburg et al. (2007)
Pneumonia	Mice	$2–4 \times 10^8$ cfu (MW2 or Lac)	PVL	No effect	Bubeck Wardenburg et al. (2007)
Pneumonia	Mice	1.2×10^8 cfu (8325.4)	hlb	Decreased inflammation, decreased lung injury	Hayashida et al. (2009)
Pneumonia	Mice	15 μg hlb	hlb	Increased inflammation, increased lung injury	Hayashida et al. (2009)
Endophthalmitis	Rabbit	10^2 cfu	hlg	Decreased cfu at day 4 PI, decreased inflammation at day 3 PI	Supersac et al. (1998)
Brain abscess	Mice	10^5 cfu (RN6390)	hla	Decreased cfu, decreased inflammation	Kielian et al. (2001)

Due to controversial results, we present a larger coverage on studies testing the role of PVL. [1]A strain with low production of core genome-encoded PSM.

et al., 2010). The low activity of PVL against murine cells has cast doubt on the results showing an effect of PVL in murine models of pneumonia and SSTI (Bubeck Wardenburg et al., 2007, 2008; Labandeira-Rey et al., 2007; Brown et al., 2009; Tseng et al., 2009; Otto, 2010; Vandenesch et al., 2010). These phenotypes might be linked with the high inocula used in these studies, which might bypass the low sensitivity of murine cells. In some cases, rabbit infection models have also led to controversial results concerning the role of PVL. Indeed, depending on the studies, PVL has been shown to play either a major role in skin inflammation and lesions (Lipinska et al., 2011) or only a minor role in promoting bacterial replication and survival early on and even accelerating infection resolution and healing at later stages (Kobayashi et al., 2011). Similarly, PVL plays a minor role in *S. aureus* rabbit bacteremia (Diep et al., 2008), while it contributes to persistence and extra osseous involvement in rabbit osteomyelitis (Cremieux et al., 2009). Finally, experimental infections in rabbit have proven a good model for studying necrotizing pneumonia. In this model, PVL triggers massive lung inflammation, neutrophil recruitment, and promotes lung damage and death in infected animals. Importantly, this activity is dependent on the presence of vinblastine-susceptible cells, which include neutrophils and all other circulating leukocytes, supporting a role for PVL targeting of neutrophils in this immune pathology (Diep et al., 2010).

Phenol soluble modulins α have also been involved in promoting death in bacteremia models and increasing the extent of the skin lesions in experimental SSTI (Wang et al., 2007; Queck et al., 2009). Importantly, the role of PSM-mec has been studied in a strain expressing low levels of core genome-encoded PSMs, suggesting that the presence of PSM-mec might not provide a novel virulence attribute in the presence of highly expressed core genome-encoded PSMα (Queck et al., 2009; Chatterjee et al., 2011). Conversely, in another study, PSM-mec was shown to reduce the severity of *S. aureus* sepsis in a mouse systemic infection model, owing to its regulatory effect on core genome-encoded virulence factors (Kaito et al., 2011).

INFLAMMATION AND DETECTION BY THE INNATE IMMUNE SYSTEM

While it is clear that certain membrane-damaging toxins have evolved to specifically target innate immune cells, the converse is also true. Indeed, the innate immune system has evolved several pathways to sense membrane damage (**Figure 1**; Henry and Lemichez, 2012).

PERTURBATION OF Ca^{2+} HOMEOSTASIS: NF-κB AND PLA2 ACTIVATION

There is a large difference in Ca^{2+} concentration between the extracellular environment ($[Ca^{2+}] > 1$ mM) and the host cytoplasm ($[Ca^{2+}]_i < 100$ nM). Pore formation leads to a rapid rise in intracellular calcium concentration associated with either direct influx through the pore or with a release from intracellular compartments, such as the endoplasmic reticulum. This calcium increase has been well exemplified in rabbit PMNs treated with sublytic α-hemolysin doses (Suttorp and Habben, 1988). Depending on their frequency, oscillations in $[Ca^{2+}]_i$ trigger activation of various transcription factors (Dolmetsch et al., 1998) including NF-κB. Calmodulin, a calcium binding protein, is the primary intracytoplasmic calcium sensor. Upon calcium binding-mediated conformation changes, calmodulin binds to and activates several proteins, including calcineurin, a protein phosphatase. Activated calcineurin activates the IKKb complex (I kappa B Kinase). Activated IKKb phosphorylates IκB (Frantz et al., 1994), a protein sequestering NF-κB into the cytosol, leading to IκB degradation, and the release of NF-κB (Henkel et al., 1993). One of the main NF-κB-inducible genes is IL-8 (CXCL8), a potent neutrophil chemoattractant. IL-8 is produced by PMNs treated with sublytic doses of α-hemolysin or PVL (Konig et al., 1994). This response is not limited to professional immune cells, as α-hemolysin-treated alveolar epithelial cells also release IL-8 (Rose et al., 2002).

In addition to regulating NF-κB activation, increases in $[Ca^{2+}]_i$ activate the cytoplasmic phospholipase A2 (PLA2). Cytoplasmic PLA2 hydrolyzes arachidonic acid-containing phospholipids, leading to the generation of arachidonic acid-derived lipid mediators, such as PGE2 and LTB4. An increase in $[Ca^{2+}]_i$ leads to relocation of cPLA2 to the phospholipid-containing plasma membrane and triggers its phosphorylation by a calmodulin-dependent kinase, leading to greater catalytic activity (reviewed in Hirabayashi et al., 2004). Treating human neutrophils or alveolar epithelial cells with sublytic doses of α-hemolysin triggers LTB4 generation (Suttorp et al., 1987; Rose et al., 2002). In PMNs, LTB4 generation is abolished by chelation of extracellular calcium (Suttorp et al., 1987). Similarly, PVL triggers LTB4 secretion, although the influence of extracellular calcium has not been investigated (Hensler et al., 1994).

PERTURBATION OF K^+ HOMEOSTASIS: MAPK AND INFLAMMASOME ACTIVATION

While calcium concentration is higher in the extracellular environment than in the cytosol, the potassium (K^+) gradient is inversely orientated. The intracellular potassium concentration ($[K^+]_i$) is 143 mM, while the extracellular potassium concentration does not exceed 5 mM.

α-Hemolysin triggers K^+ efflux upon insertion in the membrane of human keratinocytes. While the pore formed by α-hemolysin at low concentrations is specific for monovalent cations (Walev et al., 1993), at higher doses, Ca^{2+} can also flow through the pore. Cells are capable of detecting a drop in $[K^+]_i$, which leads to the activation of several immune pathways. Indeed, MAPK p38 phosphorylation is observed following α-hemolysin stimulation of a human keratinocyte cell line, and this phosphorylation is blocked by the presence of high extracellular $[K^+]$ (Kloft et al., 2009). Conversely, adding a K^+ ionophore, nigericin, to cells can recapitulate p38 activation in the absence of bacterial toxin, indicating that K^+ efflux is sufficient to activate p38 MAPK. In addition, osmotic stress triggered by sublethal α-hemolysin doses activates p38 MAPK in epithelial cells (Ratner et al., 2006). p38 MAPK activation results in NF-κB activation and IL-8 production.

In addition to activating the MAPK pathway, decreased $[K^+]_i$ is required to trigger another innate immune signaling pathway, namely, the inflammasome. The inflammasome is a cytosolic signaling platform assembled after sensing PAMPs or danger signals that leads to caspase-1 activation (reviewed in Brodsky and Monack, 2009). Caspase-1 is an inflammatory caspase that cleaves proIL-1β and proIL-18, leading to secretion of the corresponding

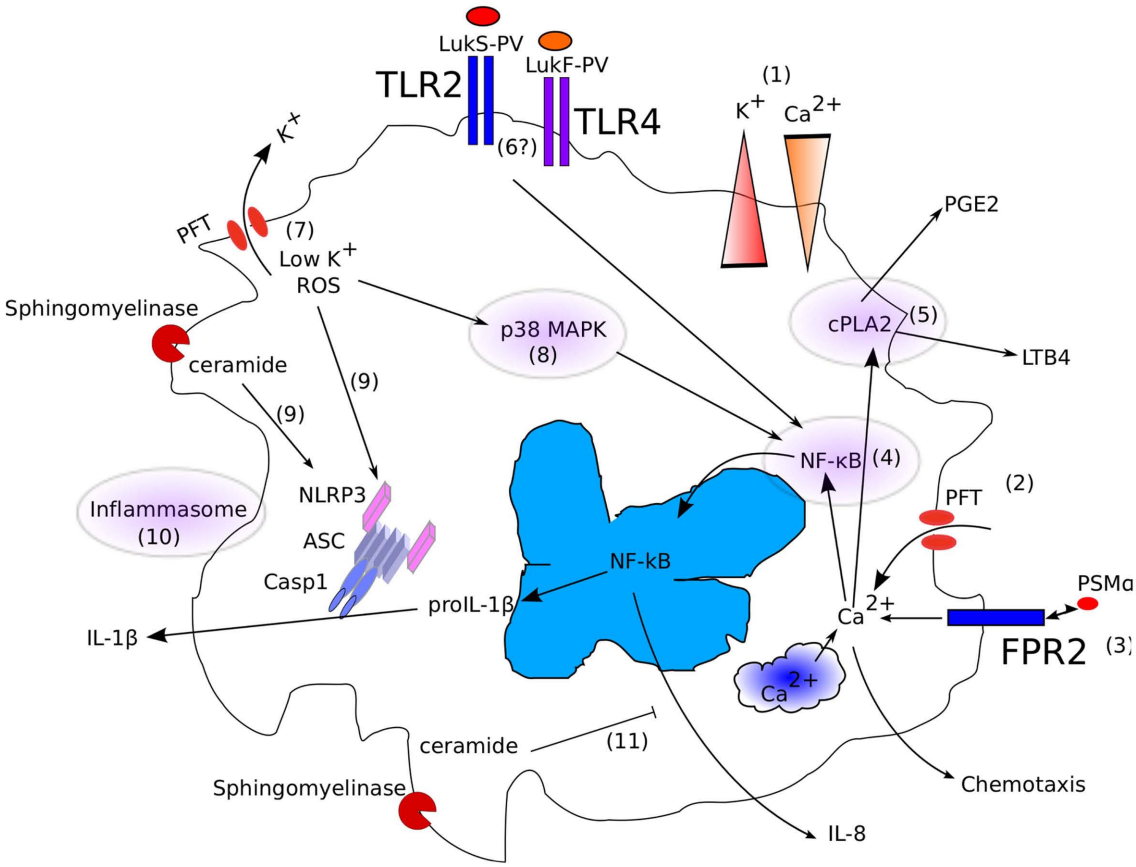

FIGURE 1 | Interplay between membrane-damaging (poly)peptides and the innate immune system. (1) In physiological conditions, differences in $[Ca^{2+}]$ and $[K^+]$ are actively maintained between the extracellular space and the cytosol. (2) Insertion of a PFT or membrane damage leads to a direct or indirect rise in $[Ca^{2+}]_i$. (3) Similarly, engagement of FPR2 by PSMα triggers an increase in $[Ca^{2+}]_i$. (4) $[Ca^{2+}]_i$ controls NF-κB and (5) cPLA2 activation. (6) TLR2 and 4 might also trigger NF-κB translocation in the nucleus by sensing LukS-PV and LukF-PV. (7) Insertion of a PFT or membrane damage leads to a rapid fall in $[K^+]_i$, leading to (8) MAP kinase and (9) NLRP3 inflammasome activation. (10) Ceramide generated by β-hemolysin might also directly activate the NLRP3 inflammasome. (11) In addition, ceramide has been reported to inhibit IL-8 secretion.

mature cytokines. Furthermore, caspase-1 triggers a form of programmed necrosis known as pyroptosis (Cookson and Brennan, 2001). Inflammasome-dependent IL-1β release is critical for neutrophil recruitment and bacterial clearance in a mouse model of *S. aureus* cutaneous infection (Miller et al., 2007). Numerous PFTs, including α-hemolysin and β-hemolysin on human monocytes (Bhakdi et al., 1989; Walev et al., 1996; Craven et al., 2009), PVL on human macrophages (Perret et al., in press), and α- and γ-hemolysins on murine microglial cells (Hanamsagar et al., 2011) activate IL-1β release. PFT activates the inflammasome via the inflammasome receptor NLRP3, which is also known as cryopyrin, and the adaptor protein ASC. It is still unclear how pore formation is translated into a NLRP3 signal (reviewed in Martinon, 2010). Intracellular K^+ depletion seems to be a key event controlling this activation. Indeed, α-hemolysin-mediated activation of the NLRP3 inflammasome can be inhibited in presence of high extracellular K^+ concentrations (Fernandes-alnmeri et al., 2007). Furthermore, as described before for MAP kinase activation, treatment of macrophages with nigericin, a bacterial K^+ ionophore, triggers NLRP3-mediated inflammasome activation (Mariathasan et al., 2006). Other possible mechanisms include toxin-mediated destabilization of lysosomes with the concomitant release of cathepsins into the host cytosol (Hornung et al., 2008) or sensing of ceramide by NLRP3, which might be relevant following β-hemolysin intoxication (Vandanmagsar et al., 2011).

FPR2 AND THE DETECTION OF PSMs

While the two immune responses described above are linked to membrane-damaging activity, PSMs can trigger intracellular Ca^{2+} flux, chemotaxis, and IL-8 secretion independently of membrane-damaging. Indeed, a host receptor, the formyl peptide receptor 2 (FPR2/ALX), can detect PSMs at nanomolar concentrations (Kretschmer et al., 2010). Despite its name, FPR2 detects non-formylated PSMs as efficiently as formylated PSMs. To date, it is not yet understood whether the innate immune system has evolved FPR2-mediated sensing of PSMs to detect *Staphylococcus* species or whether highly virulent *S. aureus* strains have evolved PSMs targeting of FPR2 to trigger a massive recruitment of neutrophils associated with massive leukotoxic activity (Kretschmer et al., 2010; Rautenberg et al., 2011).

TLR2, TLR4, AND PVL DETECTION

In necrotizing pneumonia, PVL is associated with massive inflammation leading to necrotic lesions in human lung necropsies (Gillet et al., 2002) and in animal models (Labandeira-Rey et al., 2007; Diep et al., 2010). While consensus view is that this inflammation is due to the inflammatory necrotic death of neutrophils and the release of associated DAMPs (Krysko et al., 2011), two studies have demonstrated the binding of LukS-PV and LukF-PV by TLR2 (Zivkovic et al., 2011) and TLR4 (Inden et al., 2009), respectively. While contamination with LPS, lipopeptides, or cell-wall component is always difficult to exclude, these studies suggest that PFT might be a novel PAMP recognized by TLRs.

NON-LYTIC FUNCTIONS OF THE MEMBRANE-DAMAGING POLYPEPTIDES

To date, most of these factors have been characterized for their hemolytic properties and their ability to lyse host cells. However, membrane-damaging activity might only be the tip of the iceberg, hiding more physiological functions. In this section, we review alternative functions recently uncovered for these virulent factors (**Figure 2**).

RECEPTOR ACTIVATION

As described above, the host receptors targeted by these different toxins remain unknown in most cases. However, ADAM10 has been recently identified as a α-hemolysin receptor (Wilke and Bubeck Wardenburg, 2010). Importantly, α-hemolysin binding to ADAM10 increases its metalloprotease activity and targets it to caveolae. Localized ADAM10 activation results in E-cadherin cleavage and disruption of intercellular tight junctions leading to epithelial barrier disruption (Tunggal et al., 2005). Surprisingly, the ability of α-hemolysin to form a pore is critical for ADAM10 activation, but it can be achieved at sublytic concentrations of α-hemolysin, suggesting that ion flux through α-hemolysin pores might be important for activating ADAM10 (Inoshima et al., 2011).

ESCAPE FROM THE PHAGOSOME

Staphylococcus aureus is classically considered to be an extracellular pathogen. However, clear evidence indicates that in the host, *S. aureus* can survive within an intracellular compartment (Bayles et al., 1998; Gresham et al., 2000; Giese et al., 2011), which may be relevant during persistent infections (Clement et al., 2005; Plouin-Gaudon et al., 2006). Importantly, *S. aureus* is found intracellularly in non-professional phagocytes, either in a membrane-bound compartment or in the host cytosol. Escape into the host cytosol involves a synergistic activity of δ-toxin or PSMβ and β-toxin (Giese et al., 2011). Furthermore, α-hemolysin is involved in phagosomal membrane lysis in CFTR-deficient cells, possibly explaining the persistence of *S. aureus* in cystic fibrosis patients (Jarry and Cheung, 2006; Jarry et al., 2008; Giese et al., 2009).

ADHESION

Panton Valentine leukocidin positive strains display stronger attachment to the cellular basement membrane than PVL^- strains (De Bentzmann et al., 2004). Surprisingly, this function was ascribed to the ability of LukS-PV signal peptides (SPs) to interact with heparan sulfates in the extracellular matrix (Tristan et al., 2009). While the interaction between LukS-PV SPs and heparan sulfate is of relatively low affinity, the large number of LukS-PV SPs associated with the cell-wall together with the large number of heparan sulfate ligands present could compensate for the low affinity by a high-avidity binding. The relevance of this binding to PVL-mediated pathogenesis remains to be addressed.

INTERFERENCE WITH INFLAMMATORY PATHWAYS

While β-hemolysin has mainly been characterized for its hemolytic activity, it differs from the other membrane-damaging (poly)peptides described above in its unique neutral sphingomyelinase activity. Sphingomyelin hydrolysis in eukaryotic membranes leads to the release of a very potent second messenger, ceramide, as well as to the release of the chemotactic molecule sphingosine-1-phosphate. β-hemolysin inhibits IL-8 production by endothelial cells in a manner similar to that of exogenous ceramide. This inhibition of IL-8 production correlates with a decrease in neutrophil transmigration across the endothelial barrier (Tajima et al., 2009), suggesting that β-hemolysin could cooperate with other *S. aureus* chemotaxis inhibitors, such as CHIPS (Foster, 2005).

BIOFILM REGULATION

Phenol soluble modulins-β in *S. epidermidis* have been convincingly demonstrated to participate in the maturation of the biofilm structure, specifically in formation of the intra-biofilm channels, and in mediating bacterial detachment from the biofilm at high concentrations. In a mouse model of catheter-related infection, PSMβ were essential in disseminating from the catheter biofilm to systemic organs (Wang et al., 2011). PSMβ in *S. aureus* share the α-helical amphipathic structure of *S. epidermidis* PSMβ. PSMβ are thus likely to perform the same biofilm regulatory functions in *S. aureus*. Similarly, β-toxin, in addition to its neutral sphingomyelinase activity, has a structure reminiscent of the DNAse I protein. In presence of exogenous DNA, β-toxin displays the unique ability to oligomerize covalently and precipitate DNA. β-hemolysin mutants have a reduced adherence and are defective in biofilm formation, suggesting that this oligomerization activity in presence of DNA could participate in biofilm formation, possibly by forming a nucleoprotein network facilitating biofilm structuration (Huseby et al., 2010).

GLOBAL DYSREGULATION

As mentioned above, PSM-mec was the first virulence factor identified in the SCCmec element, which carries the methicillin-resistance genes (Queck et al., 2009). Beside its direct cytolytic and pro-inflammatory functions, the PSM-mec peptide and its messenger RNA were found to suppress colony spreading and to enhance biofilm formation, while the PSM-mec mRNA also repressed the synthesis of the core genome-encoded PSMα (Kaito et al., 2011). However, as this latter effect seems to be observed only upon ectopic expression of PSM-mec from a plasmid, its relevance is questionable (Chatterjee et al., 2011). The mechanism for this dysregulation is still unknown but is reminiscent of the regulatory effect observed with the toxic-shock syndrome toxin-1 (TSST-1), which represses the transcription of many exoprotein genes (Vojtov et al., 2002). Similarly, PVL promotes the

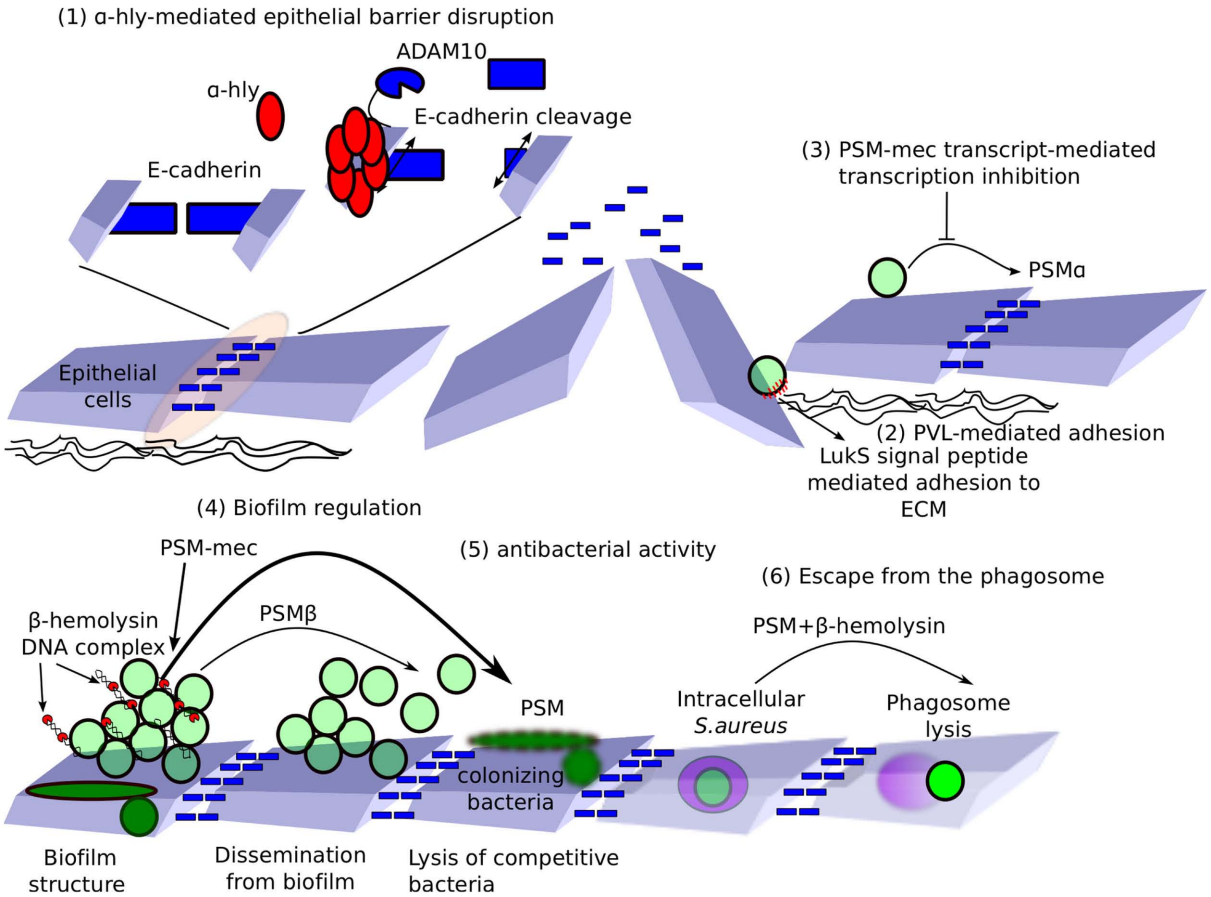

FIGURE 2 | Non-lytic functions of hemolysins, bi-component toxins, and PSMs. (1) E-cadherin is important for maintaining tight junctions and epithelial barrier function. α-Hemolysin binds ADAM10 and relocalizes it to E-cadherin-containing microdomains. ADAM10 cleaves E-cadherin, leading to loss in epithelial barrier function. (2) Exposure of the underlying glycosaminoglycans-rich extracellular matrix to LukS-PV signal peptide favors *S. aureus* adhesion. (3) PSM-mec RNA controls the transcription of virulence factors, including PSMα. (4) β-Hemolysin binds DNA, forming nucleoproteins nucleating *S. aureus* biofilm. PSMβ acts as surfactant, promoting the formation of intra-biofilm tunnels and bacterial dissemination from biofilms. PSM-mec enhances biofilm formation (5) In addition, PSMs have antibacterial activity, which may be relevant in lysing competing colonizing bacteria. (6) Finally, PSMs in synergy with β-hemolysin participate in phagosome lysis and escape into the host cytosol.

expression of staphylococcal protein A at the transcriptional level, which is an effect that could synergize with the pro-inflammatory activity of PVL and contribute to the pathogenesis of necrotizing pneumonia (Labandeira-Rey et al., 2007). Unexpectedly, this dysregulatory effect is restricted to the 8325-4 lineage (Vandenesch, unpublished), limiting the clinical relevance of this observation.

ANTIBACTERIAL ACTIVITY

δ-Toxin lyses bacterial protoplasts and spheroblasts. Similarly, the PSMβ-like peptides SLUSH-A, B, and C have been isolated from *S. lugdunensis* (Donvito et al., 1997; Rautenberg et al., 2011) and are described as having discrete anti-staphylococcal activity. PSMα and, to a lesser extent, PSMβ demonstrate bacteriostatic activity against *Legionella* at concentration 10-fold lower than the concentrations required to lyse 10% of a human erythrocytes suspension (Marchand et al., 2011). Finally, the antimicrobial activity against *S. pyogenes* and *M. luteus* present in the supernatant of CA-MRSA is abolished in mutants with the PSMα and β genes deleted. Furthermore, PSMα1 and PSMα2, when deleted from their two Nter aminoacids, display a high bacteriolytic activity against *S. pyogenes* (Joo et al., 2011). The processing mechanisms responsible for generating these potent antimicrobial peptides from inactive PSMs is still unknown but might be a key regulatory step, allowing a switch of target from host cells to competing bacteria. Overall, these studies suggest that PSMs, possibly in conjunction with host antimicrobial peptides (Cogen et al., 2010), help *S. aureus* to compete with other bacteria colonizing the same ecological niche. As CA-MRSA are robust PSM producers, this colonization advantage could explain its rapid spread worldwide (Joo et al., 2011). The relevance of this antibacterial activity in colonization and infection remains to be investigated.

CONCLUSION AND PERSPECTIVES

While the hemolytic and leukotoxic activities of *S. aureus* have been known for more than a century, the molecular nature of the factors involved in this bacterium's pathogenesis are still being uncovered. The identification of α-hemolysin receptors has highlighted the key role of this virulence factors in several *S.*

aureus-diseases involving epithelial barrier disruption. Similarly, identifying the host receptors for the other membrane-damaging toxins is likely to yield novel insights into the physiological and specific functions of the different factors and to help us progress beyond the apparent redundancy of all these factors. Furthermore, the putative role of hemolysins, leukotoxins, and PSMs in *S. aureus* colonization is still uncharacterized but should provide important knowledge on the balance between commensalism and infection.

While hemolysis and leukotoxicity have stood as the major role of these virulence factors for decades, it is increasingly clear that the membrane-damaging toxins and peptides might have other functions besides killing host cells. These other functions are now well-established for α-hemolysin, but secondary functions for other *S. aureus* secreted virulence factors are also emerging as a paradigm, as illustrated in this review. In most instances, the relevance of these functions as compared to their membrane-damaging functions remains to be established.

Another key challenge in the field will be to understand the temporal and spatial regulation of these different virulence factors. Indeed, different *in vitro* growth conditions are used to obtain secretion of the different membrane-damaging factors, suggesting that these virulence factors are secreted in response to different environmental cues or at different stages during colonization or infection. Furthermore, differential diffusion of the toxins and peptides within tissues might lead to different functions (e.g., sublytic vs. lytic concentrations) at various distances from the bacteria (e.g., local vs. systemic effects).

As noted in this review, these virulence factors interact intimately with the immune system by both actively targeting immune cells and being actively detected by the innate immune system. To date, most of the research has focused on neutrophils, but endothelial cells and epithelial cells are also emerging as central players in triggering and regulating the immune responses (Dolowschiak et al., 2010; Teijaro et al., 2011). Furthermore, dendritic cells and inflammatory monocytes are key in regulating inflammatory responses at the epithelial barrier. Study of the interaction between these virulence factors and host cells in more physiological models is thus likely to provide us novel important findings.

The spread of highly virulent CA-MRSA is a major concern. Increased prevalence of leukotoxins, such as PVL, and increased expression of membrane-damaging factors, such as α-hemolysin and PSMs, have been observed in these strains. A better understanding of the synergistic actions of these virulence factors in relevant animal models is required to understand their potential roles in the worldwide spread of these bacteria.

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Alpha-toxin promotes *Staphylococcus aureus* mucosal biofilm formation

Michele J. Anderson[1], Ying-Chi Lin[2], Aaron N. Gillman[1], Patrick J. Parks[1,3], Patrick M. Schlievert[4] and Marnie L. Peterson[1]*

[1] *Department of Experimental and Clinical Pharmacology, University of Minnesota, Minneapolis, MN, USA*
[2] *School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan*
[3] *3M Skin and Wound Care Division, 3M Company, St. Paul, MN, USA*
[4] *Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA*

Edited by:
Martin John McGavin, University of Western Ontario, Canada

Reviewed by:
Sang S. Yoon, Yonsei University College of Medicine, South Korea
Christiane Wolz, University Tübingen, Germany
Daniel O. Sordelli, University of Buenos Aires, Argentina

***Correspondence:**
Marnie L. Peterson, Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, 4-212 McGuire Translational Research Facility, 2001 6th Street SE, Minneapolis, MN 55455, USA.
e-mail: peter377@umn.edu

Staphylococcus aureus causes many diseases in humans, ranging from mild skin infections to serious, life-threatening, superantigen-mediated Toxic Shock Syndrome (TSS). *S. aureus* may be asymptomatically carried in the anterior nares or vagina or on the skin, serving as a reservoir for infection. Pulsed-field gel electrophoresis clonal type USA200 is the most widely disseminated colonizer and the leading cause of TSS. The cytolysin α-toxin (also known as α-hemolysin or Hla) is the major epithelial proinflammatory exotoxin produced by TSS *S. aureus* USA200 isolates. The current study aims to characterize the differences between TSS USA200 strains [high (*hla*$^+$) and low (*hla*$^-$) α-toxin producers] in their ability to disrupt vaginal mucosal tissue and to characterize the subsequent infection. Tissue viability post-infection and biofilm formation of TSS USA200 isolates CDC587 and MN8, which contain the α-toxin pseudogene (*hla*$^-$), MNPE (*hla*$^+$), and MNPE isogenic *hla* knockout (*hla*KO), were observed via LIVE/DEAD® staining and confocal microscopy. All TSS strains grew to similar bacterial densities (1–5 × 10^8 CFU) on the mucosa and were proinflammatory over 3 days. However, MNPE formed biofilms with significant reductions in the mucosal viability whereas neither CDC587 (*hla*$^-$), MN8 (*hla*$^-$), nor MNPE *hla*KO formed biofilms. The latter strains were also less cytotoxic than wild-type MNPE. The addition of exogenous, purified α-toxin to MNPE *hla*KO restored the biofilm phenotype. We speculate that α-toxin affects *S. aureus* phenotypic growth on vaginal mucosa by promoting tissue disruption and biofilm formation. Further, α-toxin mutants (*hla*$^-$) are not benign colonizers, but rather form a different type of infection, which we have termed high density pathogenic variants (HDPV).

Keywords: alpha-toxin, *Staphylococcus aureus*, biofilm, epithelium, vaginal mucosa, toxic shock syndrome

INTRODUCTION

Biofilms are a community of micro-organisms that are embedded in an extracellular matrix (ECM) composed of lipids, proteins, polysaccharides, and DNA. They are formed in a multi-stage process which includes initial attachment, accumulation, maturation, and dispersion (Gotz, 2002; Hall-Stoodley et al., 2004; Latasa et al., 2006; Heilmann, 2011). The members of a biofilm are protected from environmental factors such as ultraviolet light and dehydration, as well as from host immune factors such as neutrophils and macrophages (Hall-Stoodley et al., 2004). Biofilm-associated bacteria are also much more resistant to antibiotics and antimicrobial agents (Mah and O'Toole, 2001; Stewart and Costerton, 2001). This resistance is on the order of 100- to 1000-fold higher than the planktonic form and can be attributed to genetic factors, growth rate, metabolic factors, and the physical properties of the biofilm ECM (Gilbert et al., 2002).

Recently, there has been a great deal of interest in the role of biofilms in infectious diseases. The National Institutes of Health has estimated that ∼80% of human infections are caused by pathogenic biofilms (biofilms). Biofilm-mediated infections affect wounds, blood, mucosal surfaces (sinuses, respiratory, and genitourinary tracts), and medical devices (endotracheal tubes, intravascular and urinary catheters, orthopedic implants, and arterial stents; Towne et al., 1994; Donlan, 2001; Bakaletz, 2007; Wolcott et al., 2008; Rohrich et al., 2010). Device-related biofilm infections were the first to be described in the 1980s (Marrie et al., 1982). *Staphylococcus epidermidis* and *Staphylococcus aureus* are the most frequent causes of medically associated biofilms (Arciola et al., 2001; Lentino, 2003).

Staphylococcus aureus is an opportunistic pathogen which causes a wide variety of diseases in humans, ranging from relatively mild skin and soft tissue infections to severe, life-threatening necrotizing pneumonia, bacteremia, and Toxic Shock Syndrome (TSS; Lowy, 1998). This organism is the most common cause of ventilator-associated pneumonia, surgical site infections, and catheter-associated bloodstream infections in the healthcare setting (Kuehnert et al., 2005). *S. aureus* is also a common cause of skin and pulmonary infections in the community (Zetola et al., 2005). Infections caused by *S. aureus* are often preceded by colonization. In fact, carriers have a threefold higher risk for *S. aureus*

Table 1 | *Staphylococcus aureus* strains used in this study.

Strain ID	Clonal type	α-Toxin genotype	Characteristic	Reference
MNPE	USA200	*hla*$^+$	Wild-type α-toxin, post-influenza TSS lung isolate	MacDonald et al. (1987)
CDC587	USA200	*hla*$^-$	α-Toxin pseudogene, mTSS isolate	Schlievert et al. (1982)
MN8	USA200	*hla*$^-$	α-Toxin pseudogene, mTSS isolate	Schlievert et al. (1982)
MNPE *hla*KO	USA200	*hla deficient*	MNPE with α-toxin deletion (*hla::erm*), isogenic strain	Lin et al. (2011)

infections (Wertheim et al., 2005). *S. aureus* is carried asymptomatically in the anterior nares, skin, and vaginal mucosa by up to 30% of the population (Kuehnert et al., 2006; Gorwitz et al., 2008). USA200 methicillin-susceptible *S. aureus* (MSSA) are the most common pulsed-field gel electrophoresis (PFGE) type isolated from the nose and are a frequent cause of healthcare associated infections (Patel et al., 2008; Tenover et al., 2008). USA200 MSSA are also a major cause of TSS, as they are often associated with expression of the TSS toxin-1 gene (*tst*; Peacock et al., 2002; Diep et al., 2006; Tenover et al., 2008).

Staphylococcus aureus illnesses are usually initiated at mucosal surfaces or skin. Bacteremia is not typically associated with menstrual TSS; however, toxin penetration across the mucosa can be facilitated by the activity of other exotoxins, including the cytotoxin, α-toxin (Peterson et al., 2005; Brosnahan et al., 2009), which is an exotoxin known to play a role in the pathogenesis of *S. aureus* (Patel et al., 1987; Bramley et al., 1989). This cytotoxin is secreted as a water soluble monomer which forms a heptameric β-barrel pore in host cell membranes (Song et al., 1996). Targets of the toxin include lymphocytes, macrophages, epithelium, endothelium, and erythrocytes (Bhakdi and Tranum-Jensen, 1991).

Recently, the exoprotein profiles of two USA200 TSS *S. aureus* isolates, MNPE (α-toxin positive, *hla*$^+$) and CDC587 (α-toxin mutant, *hla*$^-$) were biochemically characterized (Lin et al., 2011). The MNPE strain is a pulmonary post-influenza TSS isolate, whereas CDC587 is a menstrual, vaginal TSS isolate which contains a pseudogene for α-toxin (Schlievert et al., 1982; MacDonald et al., 1987). The exoproteins from MNPE were significantly more proinflammatory and cytotoxic, *in vitro*, to immortalized human vaginal epithelial cells (HVECs) than those from the vaginal isolate CDC587 (Lin et al., 2011). This was due to the presence of large amounts of α-toxin in supernatants from MNPE.

An *ex vivo* porcine vaginal mucosa infection model was used to further characterize the differences between TSS USA200 *S. aureus* *hla*$^+$ and low α-toxin-producing *hla*$^-$ strains with regard to their abilities to infect and disrupt vaginal mucosal tissue. All TSS strains grow to similar bacterial densities on the vaginal mucosa. MNPE (*hla*$^+$) proved to be highly cytotoxic to the vaginal epithelium as early as 24 h post-infection. Cytotoxic effects of CDC587 (*hla*$^-$) and MN8 (*hla*$^-$) are much lower and do not appear until 48 h post-infection. Of particular interest, MNPE forms mature biofilms by 72 h post-infection, whereas CDC587, MN8, and MNPE *hla*KO isolates infect the tissue but do not form biofilms. Further, exogenous α-toxin restores the mature biofilm phenotype of the MNPE *hla*KO strain. These data suggest that α-toxin contributes to TSS *S. aureus* mucosal biofilm formation while causing significant disruption to the mucosa. Additionally, the absence of α-toxin did not inhibit TSS *S. aureus* isolates from infecting the vaginal mucosa, but growth occurred with a different, non-biofilm phenotype. As these non-biofilm-forming strains grow to a high density on the tissue and are proinflammatory, we have termed them high density pathogenic variants (HDPV).

MATERIALS AND METHODS

BACTERIA

USA200 isolates MNPE, CDC587, and MN8 were used extensively in this study (**Table 1**). All three strains are methicillin sensitive *S. aureus* (MSSA). MNPE was isolated from a post-influenza pulmonary TSS case in 1987 and most likely originated from a skin source (MacDonald et al., 1987). CDC587 and MN8 are typical menstrual vaginal TSS strains, isolated from patients in the 1980s (Schlievert et al., 1982). Previously described isogenic α-toxin deletion mutants, MNPE *hla*KO, CDC587 *hla*KO, were used in some studies (Lin et al., 2011). All bacteria are maintained in the laboratory as frozen glycerol stocks. Prior to experimentation, Tryptic Soy Agar containing 5% sheep's blood (TSB, Becton–Dickinson, Franklin Lakes, NJ, USA) is inoculated from frozen stocks. On the afternoon prior to initiation of experiment, Todd Hewitt broth (Becton–Dickinson) is inoculated with colonies from the fresh TSB plates. Stationary phase (overnight) cultures of each strain are washed in RMPI 1640 (Invitrogen, Carlsbad, CA, USA) and resuspended to a concentration of approximately 5×10^8 colony forming units (CFU/ml). Two microliters of this suspension are used to inoculate explants on the mucosal surface (1×10^6 CFU/explants). Explants are returned to 37°C and incubated for up to 4 days. All studies are performed in a biosafety level 2 (BSL2) laboratory.

EX VIVO PORCINE VAGINAL MUCOSA PREPARATION AND INFECTION

Porcine vaginal mucosa has been reported to be histologically and structurally similar to human vaginal mucosa (Squier et al., 2008). Therefore, we developed an *ex vivo* porcine vaginal mucosa infection model to characterize the role of α-toxin in *S. aureus* mucosal biofilm formation. Normal healthy porcine vaginal tissue is excised from animals at slaughter and transported to the laboratory in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin (50 IU/ml), streptomycin (50 mg/ml), and amphotericin B (2.5 μg/ml). Antibiotics are included to decolonize vaginal normal flora which may interfere with biofilm formation (Iwase et al., 2010). Tissue is utilized within 3 h of excision. Explants of uniform size are obtained from the porcine vagina using a 5-mm biopsy punch. Excess muscle tissue is trimmed away with a scalpel. Tissue explants are washed in antibiotic-free RPMI 1640 media three times. The explants are then placed mucosal side up on a PET track-etched 0.4 μm cell culture insert (BD Bioscience, Franklin Lakes, NJ, USA) in 6-well plates containing fresh serum- and antibiotic-free RPMI 1640 and incubated at 37°C. The

mucosal surface is continually exposed to air. The mucosal surface of each is inoculated with $\sim 1 \times 10^6$ CFU of bacteria suspended in antibiotic-free RPMI 1640. Infected explants are incubated at 37°C with 7% CO_2. At various times post-infection, samples are removed from the incubator and processed for bacterial recovery and IL-8 and α-toxin analysis. Some samples were stained and imaged by confocal laser scanning microscopy (CLSM) after removal from the incubator.

BACTERIAL BIOFILM AND MUCOSAL VIABILITY DETERMINATIONS

The physical properties of the LIVE/DEAD® staining kit from Invitrogen and CLSM are utilized to simultaneously determine microbial biofilm formation and corresponding toxicity to mucosal tissue. The FilmTracer™LIVE/DEAD® Biofilm Viability kit provides a two-color fluorescence assay of bacterial and mucosal viability based on membrane integrity. This kit utilizes mixtures of SYTO® 9 green fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy cells. When used alone, the SYTO® 9 stain generally labels all mucosal epithelial cells and/or bacteria in a population – those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only mucosal cells or bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. Thus, with the mixture of the SYTO® 9 and propidium iodide stains, mucosal cells or bacteria with intact cell membranes stain fluorescent green, while bacteria or mucosal cells with damaged membranes stain fluorescent red.

BACTERIAL RECOVERY FROM PORCINE VAGINAL EXPLANTS

Bacteria are enumerated from unwashed, infected explants by vortex mixing (highest setting for 2 min or medium high for 4 min) in 250 μl sterile phosphate buffered saline (Sigma, St. Louis, MO, USA). Supernatants are then serially diluted in PBS (or plated neat) and spread on Tryptic Soy Agar containing 5% sheep's blood (Becton Dickinson, Franklin Lakes, NJ, USA) using a spiral plater (Biotek, Microbiology International, MD, USA).

QUANTIFICATION OF VAGINAL MUCOSA IL-8

Interleukin-8 (IL-8) is a cytokine which is used as an indicator of inflammation or irritation. The amount of IL-8 produced in the vaginal mucosal explants is determined by homogenizing the explants in 250 μl sterile phosphate buffered saline. The homogenate is clarified by centrifugation and stored at −20°C until assayed. We use a porcine specific IL-8 ELISA (R&D Systems, Minneapolis, MN, USA) to quantify the amount of IL-8 present in each explant.

ALPHA-TOXIN DETECTION BY WESTERN IMMUNOBLOTTING

Clarified homogenates are concentrated by ethanol precipitation (five-fold) and resuspended in sterile water. Samples are mixed 1:1 with sample loading buffer and electrophoresed on a 4–20% gradient acrylamide gel (BioRad, Hercules, CA, USA) under reducing conditions, transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA, USA) and immunoblotted with rabbit α-toxin anti-sera (obtained as described in Lin et al., 2011). Proteins are detected by chemiluminescence using SuperSignal West Dura Extended Duration substrate (Pierce, Rockford, IL, USA).

STAINING AND EXAMINATION BY CLSM

Infected and control explants are stained using FilmTracer™LIVE/DEAD® Biofilm Viability kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. After staining, specimens are gently washed three times in Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA) and transferred to glass slides. A coverslip with 1 mm spacer (Electron Microscopy Sciences, Hatfield, PA, USA) is then applied and specimens are imaged on an Olympus FluoView 1000 BX2 or Zeiss Axio Scope II. Images are captured using either FluoView or LaserSharp software and processed using either FluoView or ImageJ software. All images are 60× original magnification.

PURIFICATION OF α-TOXIN

The cytolysin α-toxin is purified as previously described (Brosnahan et al., 2009). Briefly, α-toxin is isolated from *S. aureus* strain MNJA grown in beef heart medium. The culture is precipitated with ethanol at 4°C. The precipitate is resolubilized in water, and α-toxin is purified by isoelectric focusing (IEF). IEF is conducted in two phases: the first phase uses a pH gradient of 3.5–10, followed by a second phase using a pH gradient from 7 to 9. The isoelectric point of α-toxin is 8.5. Purity is confirmed by SDS-PAGE and quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

ALPHA-TOXIN TREATMENT

In some experiments, exogenous α-toxin is added to explants before or after infection with MNPE *hla*KO. As a pre-treatment, purified α-toxin (5 μg/explant) is added and explants are incubated for 6 h at 37°C. Explants are then infected with MNPE *hla*KO and further incubated for 3 day. Alternatively, explants are infected with MNPE *hla*KO and incubated at 37°C for 24 h, then 5 μg of purified α-toxin is added and explants are further incubated for two more days. Following incubation, explants are stained and imaged as described above.

BIOFILM FORMATION ON PLASTIC

The role of α-toxin in biofilm formation on plastic is evaluated using two different kinds of 96-well tissue culture plates (TDD 92096, MidSci, St. Louis, MO, USA and Corning 3595, Fisher Scientific, Pittsburgh, PA, USA) according to the procedure described previously (Christensen et al., 1985). Briefly, overnight cultures of *S. aureus* are diluted 1:20 in Tryptic Soy Broth (BD Bacto, Sparks, MD, USA) supplemented with 0.2% sterile glucose (Sigma Aldrich, St. Louis, MO, USA). Ninety-six well plates are inoculated with 200 μl of diluted bacteria and allowed to incubate for 24 or 72 h at 37°C; care was taken to prevent shaking during incubation. Following incubation, wells are aspirated and gently washed with sterile saline three times. Adherent bacteria are fixed overnight at room temperature with 200 μl of Bouin's fixative (Ricca Chemicals, Arlington, TX, USA). The fixative is then aspirated and bacteria are stained for 5 min at room temperature with Gentian Violet (Ricca Chemicals, Arlington, TX, USA). Plates are washed

under running water. Stain is resolubilized with 30% glacial acetic acid (Fisher Scientific, Pittsburgh, PA, USA) and optical density is read at 570 nm.

STATISTICAL ANALYSIS

The data in **Figures 2A,B** were analyzed by one-way ANOVA followed by Bonferroni's post-test using GraphPad Prism 5 Software (GraphPad Software Inc., La Jolla, CA, USA) and are representative of two experiments (pooled) with $n = 3$–4 per group, per experiment. The data in **Figure 5** were analyzed by one-way ANOVA followed by Bonferroni's post-test using GraphPad Prism 5 software. The results are representative of one experiment with $n = 5$–8 per strain.

RESULTS

hla$^+$ AND *hla*$^-$ CARRYING STRAINS PRODUCE DIFFERENT INFECTION PHENOTYPES ON PORCINE VAGINAL MUCOSA

Previous results indicated that secreted exotoxins from MNPE were highly proinflammatory and cytotoxic to immortalized HVECs when compared to secreted exotoxins from a mTSS strain, CDC587 (Lin et al., 2011); thus, it was hypothesized that an α-toxin^{high} strain of *S. aureus* (MNPE, *hla*$^+$) would be more cytotoxic to full thickness porcine vaginal mucosal epithelium compared to *hla*$^-$ carrying menstrual TSS isolates. To test this hypothesis, a LIVE/DEAD® staining kit from Invitrogen (green = live cells, red = dead cells) and CLSM were employed. Throughout the course of the experiment (up to 72 h), uninfected mucosal tissue remains alive and intact as demonstrated by a predominance of green color (**Figures 1A,E,I**). In contrast, at 24 h post-infection with MNPE, all of the epithelial cells are stained red and rounded, demonstrating disrupted membranes and loss of cell-to-cell adhesion (**Figure 1B**). The mucosal epithelium of explants infected with *hla*$^-$ strains (either CDC587 or MN8) remains highly viable (green staining) at 24 h post-infection (**Figures 1C,D**), although there is evidence of some cell rounding in CDC587-infected explants (**Figure 1C**), suggesting a loss of cell-to-cell adhesion. At the 48-h timepoint, there is still significant red staining in MNPE-infected explants (**Figure 1F**). Cytotoxicity and cell rounding are evident in CDC587-infected explants at 48 h post-infection (**Figure 1G**). The epithelium of explants infected with MN8 also shows signs of disruption at this intermediate time point, although there are still many viable cells and areas of intact cell junctions (**Figure 1H**). After 72 h, the only evidence of epithelium in MNPE-infected explants are faint red spots

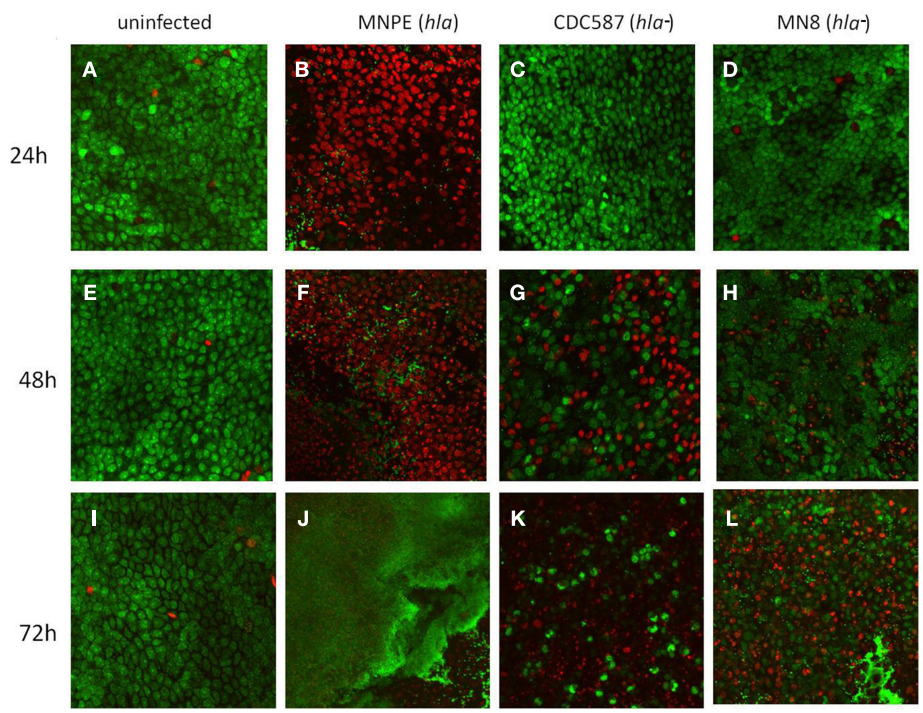

FIGURE 1 | *hla*$^+$ and *hla*$^-$carrying strains produce different infection phenotypes on porcine vaginal mucosa. Explants of normal, healthy porcine vaginal mucosa were infected with ∼1 × 10^6 CFU of live *S. aureus* strains MNPE, CDC587, or MN8. Uninfected or infected porcine vaginal mucosal epithelium explants were stained with FilmTracer™LIVE/DEAD stain, then gently washed to remove excess stain. CLSM was used to image explants at the indicated times post-infection. **(A,E,I)** Uninfected tissue remains live (green) and intact (asymmetrical) throughout the course of the experiment. **(B)** MNPE-infected tissue is dead (red) by 24 h. Some bacteria are attached to the tissue (small, green staining). **(C,D)** In contrast, the tissue infected with CDC587 or MN8 resembles uninfected tissue at 24 h. **(F)** At 48 h, more bacteria are evident in the MNPE-infected tissue. **(G,H)** Some of the tissue infected with CDC587 or MN8 has died (red) and sloughed away (black). Small, individual cocci (small, bright green punctuate staining) are beginning to appear. **(J)** At 72 h, MNPE biofilm (diffuse green) has overtaken the tissue. **(K)** At 72 h post-infection, most of the tissue has been killed by CDC587, very few cocci are observed. **(L)** At 72 h post-infection with MN8 results in ∼50% cell death (red/black areas). Some macro-colonies are beginning to form in the lower right quadrant. Original magnification was 60×.

in random patterns (**Figure 1J**, lower right quadrant). In contrast, bright green live epithelial cells remain (although they are sparse) in explants infected with CDC587 (**Figure 1K**). MN8 appears to be less cytotoxic to the mucosal tissue than either CDC587 or MNPE, as evidenced by larger areas of bright green stained epithelial cells (**Figure 1L**). These results indicate that strains-producing α-toxin are more cytotoxic to the mucosal epithelium than strains producing low amounts or no α-toxin.

The dyes used for CLSM also allowed imaging of the bacteria growing on the porcine vaginal mucosa over time. At 24 h, in MNPE-infected explants, some bacteria are observed (small green, **Figure 1B**). In contrast, the *hla*$^-$ bacteria are not visualized at this time point, based on a lack of small bright green punctate staining in explants (**Figures 1C,D**). At the intermediate time point (48 h post-infection), MNPE has entered the accumulation phase of biofilm development (**Figure 1F**). Microcolonies are observed to be interspersed between dead epithelial cells. Both *hla*$^-$ strains are beginning to be visible on the tissue (small, green, punctate staining, **Figures 1G,H**). Further, it appears that at least a portion of the MN8 bacteria are intracellular, as evidenced by the small, bright green punctate staining within the more diffuse green staining (**Figure 1H**). By 72 h post-infection, biofilm has overtaken most of the MNPE-infected explants (large green mass, **Figure 1J**), which is in stark contrast to explants infected with strains carrying the *hla* pseudogene (*hla*$^-$). Only sparse, individual colonies are observed in CDC587-infected explants (**Figure 1K**). *S. aureus* growth on MN8-infected explants was intermediate, with microcolonies beginning to form by 72 h (**Figure 1L**). These data suggest a role for α-toxin in the formation of mucosal biofilms.

S. AUREUS (*hla*$^+$ AND *hla*$^-$) GROW TO SIMILAR DENSITIES ON THE MUCOSAL SURFACE

The CFU recovered from infected, unwashed tissue were enumerated to determine if there were intrinsic differences in the ability of *hla*$^+$ or *hla*$^-$ strains to grow on porcine vaginal mucosa. No significant differences across strains were observed in the number of bacteria recovered from the tissue at any time point examined (**Figure 2A**, $n = 6$, $p > 0.05$). CFU values across strains were then pooled and evaluated for an effect of time. There is an increase in the amount of bacteria recovered from 24 to 48 h. Twenty-four hours after infection of the tissue with ∼6.0 Log10 CFU/explant, the bacterial densities are 7.83 ± 0.07 Log10 CFU/explant. By 48 h, the bacterial density is significantly increased to 8.41 ± 0.04 Log10 CFU/explant ($n = 18$, $p < 0.0001$). No further increase is observed between 48 and 72 h (8.41 ± 0.02 Log10 CFU/explants, $n = 18$, $p = 0.9543$). These data demonstrate that all of the strains grew to a density at which cytolysins would be expressed.

S. AUREUS STRAINS (*hla*$^+$ OR *hla*$^-$) ARE PROINFLAMMATORY TO THE VAGINAL MUCOSA

The cytokine interleukin-8 (IL-8) is chemotactic for neutrophils and is considered a marker of inflammation. A significant increase in the amount of IL-8 produced by vaginal explants at 24 and 48 h post-infection is observed with all strains of *S. aureus* tested (**Figure 2B**). At 24 h, explants infected with MNPE, CDC587, or MN8 produce 27.7 ± 2.16-fold, 26.7 ± 2.28-fold, and 32.3 ± 2.79-fold higher levels of IL-8 compared to uninfected explants, respectively. At 48 h post-infection, MNPE-infected explants contain the lowest amount of IL-8, with an 18.5 ± 2.70-fold increase, followed by CDC587-infected explants, which is increased 22.54 ± 5.16-fold. MN8-infected explants contain 34.3 ± 8.52-fold higher IL-8 than uninfected control tissue. By 3 days (72 h) post-infection, the differences in IL-8 production by MNPE and CDC587 compared to that of uninfected control are less than threefold and no longer significantly different. MN8-infected explants produce significantly higher IL-8, 8.09 ± 2.21-fold more than uninfected explants do. The decline in IL-8 detected in MNPE- and CDC587-infected mucosal tissue over time is likely due to a combination of

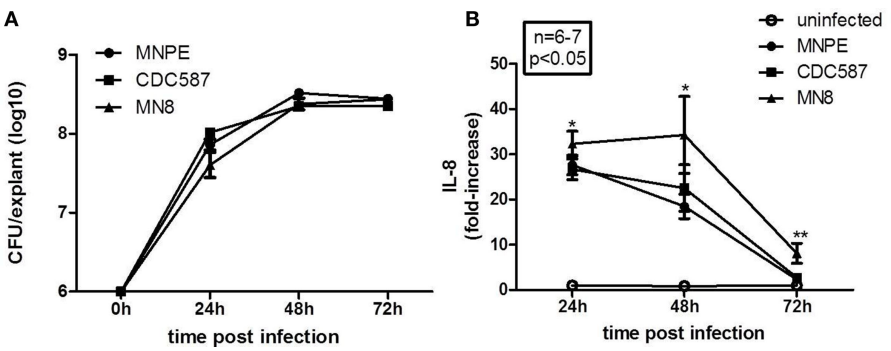

FIGURE 2 | *Staphylococcus aureus* (*hla*$^+$ *and hla*$^-$) grow to similar densities on the mucosal tissue and are proinflammatory.
(A) Quantitation of bacteria growth on tissue. Bacteria were recovered from unwashed explants infected with MNPE (circles), CDC587 (squares), or MN8 (triangles). The initial inoculum was $\sim 1 \times 10^6$ CFU/explant. After the indicated time, explants were transferred to tubes containing 250 μl PBS and vortex mixed for 4 min to recover bacteria. Supernatants were serially diluted in PBS and plated on TSB agar. Over the first 24–48 h, the bacteria grew logarithmically. There was no change in density from 48 to 72 h. Data are the combined results of two experiments, with $n = 3$ each. **(B)** Quantitation of IL-8 from the tissue during the course of infection. Explants of porcine vaginal mucosa were infected with $\sim 1 \times 10^6$ CFU of MNPE, CDC587, or MN8 and incubated at 37°C for the indicated times. Explants were then placed in tube containing 250 μl PBS and homogenized for 30 s. Homogenates were clarified by centrifugation and IL-8 was detected by porcine specific ELISA. Data were normalized to uninfected controls at each time point. $n = 3$–4 explants/experiment, results reflect pooled data from two experiments. IL-8 from infected explants was significantly higher than control (uninfected) at 24 and 48 h post-infection ($p < 0.05$). By 72 h, the amount of IL-8 detected in homogenates of explants from MNPE or CDC587 was reduced compared to earlier time points and is no longer statistically significant. IL-8 detected in MN8-infected explant homogenates was reduced compared to earlier time points, but remains significantly higher than controls.

increased cell death and proteolytic degradation. As time increases, the proportion of live cells decreases, as does the concentration of IL-8 detected. These data show that although CDC587 and MN8 are less cytotoxic than MNPE, they are equally proinflammatory to the mucosa.

α-TOXIN IS DETECTED IN THE MUCOSA AFTER INFECTION

The post-influenza TSS isolate (MNPE) and the mTSS isolates (CDC587 and MN8) are known to differ in their ability to produce α-toxin. MNPE, which contains the wild-type gene, expresses large amounts of α-toxin. CDC587 and MN8, which carry the pseudogene, is hypothesized to produce less α-toxin. To test this hypothesis, the accumulation of this toxin in the porcine vaginal mucosa at 72 h was examined by performing western blotting on tissue homogenates (**Figure 3A**). As expected, no toxin is detected in uninfected tissue, and α-toxin accumulates in the tissue infected with MNPE. Alpha-toxin accumulates in the tissue infected with CDC587 to a far lesser extent and appears to be slightly smaller in size (likely a truncated form of α-toxin). It was a bit surprising that no α-toxin bands are identified with *S. aureus* MN8 because in a previous exoprotein characterization (Lin et al., 2011), a small band was identified. Homogenates from tissue infected with wild-type MNPE, MNPE *hla*KO, wild-type CDC587 or CDC587 *hla*KO were analyzed for α-toxin expression to confirm α-toxin specificity of the immunoblotting antibody (**Figure 3B**). As expected, in the wt MNPE-infected tissue there is an abundance of α-toxin, but a lack of α-toxin in the homogenate from MNPE *hla*KO. Again, a small amount of slightly (1–2 kDa) truncated α-toxin in wt CDC587-infected homogenates is observed. This band is not detected in the CDC587 *hla*KO-infected homogenates, demonstrating a specific interaction of the anti-sera with α-toxin and indicates that CDC587 indeed expresses low amounts of α-toxin. These data demonstrate that α-toxin accumulates in the vaginal mucosa infected with strains carrying wild-type (hla^+) and in contrast is minimally detected in mutant (hla^-) strains.

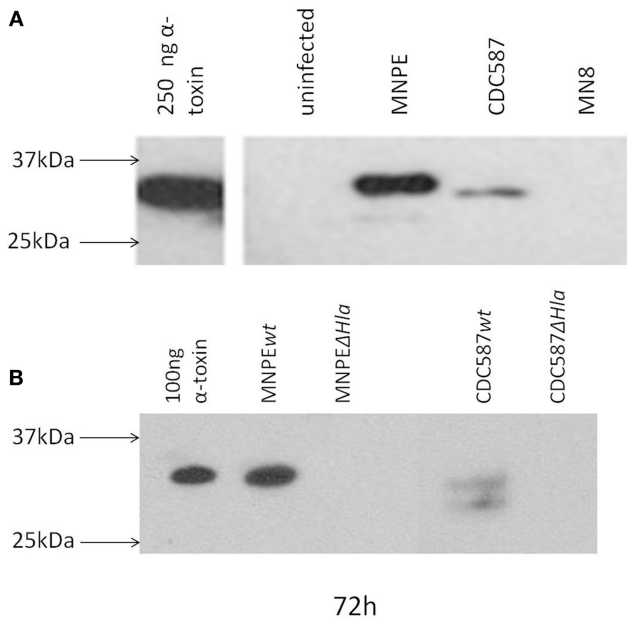

FIGURE 3 | α-Toxin is detected in the mucosa after infection. Detection of α-toxin in explants of porcine vaginal mucosa by western immunoblotting. At 72 h post-infection with different strains of *S. aureus*, explants were transferred to tubes containing 250 μl PBS and homogenized for 30 s. Homogenates were clarified by centrifugation and concentrated fivefold by ethanol precipitation. Proteins were separated by SDS-PAGE on a 4–20% gradient gel, transferred and probed with rabbit anti-α-toxin serum. **(A)** Based on the position of purified α-toxin, full-length α-toxin is only detected in tissue infected with MNPE. Truncated forms were detected in CDC587 and MNPE-infected tissue. No bands were observed in tissue infected with MN8 or in uninfected tissue. **(B)** No bands were detected in *hla*KO strains.

α-TOXIN PROMOTES *S. AUREUS* MUCOSAL BIOFILM FORMATION

Rescue experiments with exogenous α-toxin were performed to determine if α-toxin is both necessary and sufficient for biofilm formation. As expected with MNPE, large areas of biofilm (green) over ECM (black) are observed 72 h post-infection (**Figure 4B**). In contrast, the epithelium of the MNPE *hla*KO-infected explants contain many live cells (large green ovals) interspersed with dead cells (large red circles/ovals; **Figure 4C**). Live bacteria are also visible (small, green, punctate) as individual cocci or small aggregates. Treatment with 5 μg exogenous α-toxin for 72 h resulted in an increase in epithelial cytotoxicity as evidenced by more red staining (**Figure 4D**) compared to untreated, unifected control (**Figure 4A**). Treatment with 5 μg exogenous α-toxin for 6 h prior to infection (**Figure 4E**) or 24 h after infection with the MNPE *hla*KO restored the biofilm phenotype (**Figure 4F**). These data demonstrate that α-toxin is both necessary and sufficient for biofilm formation on a mucosal surface.

α-TOXIN IS NOT REQUIRED FOR *S. AUREUS* BIOFILM FORMATION ON PLASTIC

As the vast majority of biofilm experiments are done using a plastic substrate and optimal growth media, experiments were performed to determine if α-toxin plays a critical role in that model. Biofilms formed in all *S. aureus* strains tested by 24 h post-inoculation of 96-well tissue culture plates (**Figure 5A**). No significant differences were observed between strains. This was also the case at 72 h post-inoculation of the tissue culture plates (**Figure 5B**). These data suggest that under optimum conditions, α-toxin is not required to form biofilm on plastic.

DISCUSSION

Since the 1980s, biofilms have increasingly been recognized as an important component of human infectious diseases. Biofilm formation by *S. aureus* is a multi-stage process involving many gene products (Gotz, 2002; Heilmann, 2011). The majority of genes involved in biofilm formation have been identified using *in vitro* culture techniques. The classic *in vitro* microtiter plate assay for biofilm formation on abiotic surfaces has been a valuable tool; however, it does not represent the characteristics of biofilm-associated infections *in vivo* and may have led to an overestimation of the impact of some molecules in clinical biofilm formation (Otto, 2008). *In vivo* experiments are the gold standard in pathogenesis research, but they are highly labor intensive, expensive, and subject to regulation. Recognizing these limitations, we developed an *ex vivo* porcine mucosal infection model to study microbial colonization, infection, and biofilm formation on mucosal surfaces.

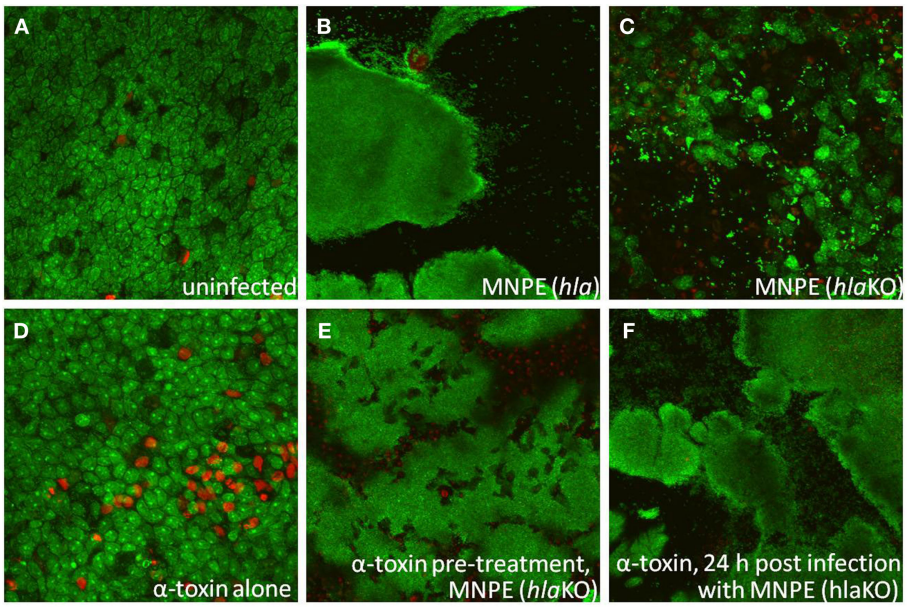

FIGURE 4 | α-Toxin promotes *S. aureus* biofilm formation. Explants of normal, healthy porcine vaginal mucosa were infected with ∼1 × 10^6 CFU of live *S. aureus* strain MNPE or an isogenic α-toxin deletion mutant (MNPE *hla*KO). FilmTracer™LIVE/DEAD stain was used to stain infected porcine vaginal mucosal epithelium explants. CLSM was used to image explants 3 days post-infection. **(A)** Mucosa is largely viable (green staining) and intact (asymmetry). **(B)** Large masses of biofilm are observed on mucosa infected with wt MNPE. **(C)** No biofilm is observed on mucosa infected with MNPE *hla*KO. Live individual cocci and small aggregates (small, bright punctuate, and irregular shaped green staining) are evident. Many cells of the mucosa remain alive (green ovals/circles). **(D)** Treatment of mucosa with 5 μg purified α-toxin alone for 72 h results in a slight increase in cytotoxicity (red staining). **(E)** Treatment of mucosa with 5 μg purified α-toxin for 6 h prior to infection with MNPE *hla*KO restores biofilm formation. **(F)** Treatment with 5 μg α-toxin 24 h post-infection with MNPE *hla*KO restores biofilm formation. Original magnification was 60×.

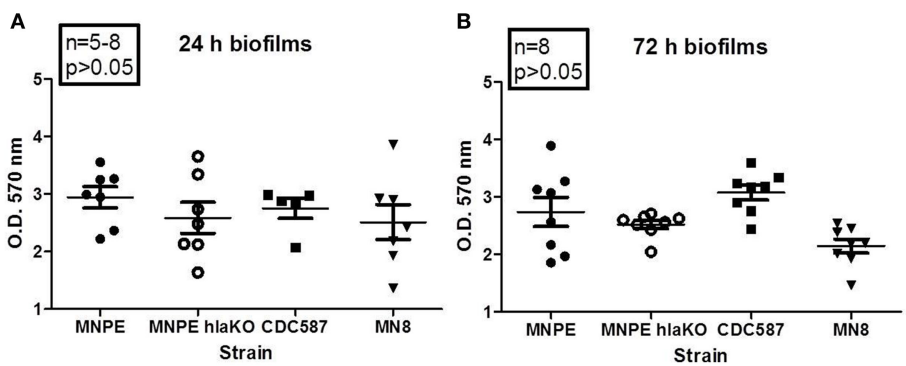

FIGURE 5 | α-Toxin is not required for *S. aureus* biofilm formation on plastic. Overnight cultures of various strains of *S. aureus* were diluted 1:20 in TSB + 0.2% glucose and inoculated into 96-well plates. Cultures were returned to 37°C incubator, without shaking, for the indicated times. After incubation, wells were washed, fixed and stained. O.D. at 570 nm was read after solubilizing dye with 30% glacial acetic acid. Robust biofilm forms at 24 h **(A)** and remains through 72 h **(B)**. No differences were observed between strains, regardless of α-toxin presence.

The histological and permeability characteristics of porcine vaginal mucosa are very similar to human vaginal mucosa (Squier et al., 2008).

Staphylococcus aureus initiates diseases at skin and mucosal surfaces, which are also frequent sites of *S. aureus* colonization. A previously published study identified differences in the secreted proteins between two clonally related USA200 TSS *S. aureus* isolates, MNPE (*hla*$^+$) and CDC587 (*hla*$^-$; Lin et al., 2011). Stationary phase supernatants from these two isolates differed in their epithelial cytotoxicity and proinflammatory potential, which was proportional to the amount of α-toxin produced by each strain. The current study investigates the ability of these strains to infect vaginal mucosa and form biofilms on the mucosal surface. The cytotoxicity and proinflammatory effects of the strains on vaginal mucosal tissue are also characterized. Using an *ex vivo* porcine vaginal mucosa model, a dramatic difference in infection

phenotypes and mucosal vaginal epithelial cytotoxicity in *hla*$^+$ strains compared to *hla*$^-$ strains was observed. Specifically, the *hla*$^+$ strain (MNPE) developed a mature biofilm over 3 days and was significantly more cytotoxic over the first 24 h. While the *hla*$^-$ strains CDC587 and MN8 did not form biofilm phenotypes, despite growing to similar bacterial cell densities and being proinflammatory, and were less cytotoxic. This observation is further supported by our findings that disruption of the *S. aureus* α-toxin gene (MNPE *hla*KO) abrogated biofilm formation on mucosal tissue, while the addition of exogenous α-toxin restored the biofilm phenotype. Our proposed mechanism is that *S. aureus* α-toxin contributes to the formation of biofilms on mucosal surfaces by disrupting the host epithelium, which exposes the ECM for attachment, provides nutrients for bacterial survival, and facilitates intercellular bacterial adhesion.

The western blot analysis revealed an abundance of α-toxin in porcine vaginal mucosa infected with the biofilm-forming MNPE but none in MNPE *hla*KO-infected tissue. This is the first study to examine α-toxin production by MNPE in a biofilm state on mucosal tissue and these data are consistent with previous findings of α-toxin in stationary phase supernatants (Lin et al., 2011). In this previous study by Lin et al. western blot analysis also revealed very low amounts of α-toxin secreted by *hla*$^-$ strains of *S. aureus* (CDC587 and MN8). Additionally, peptide reads on both sides of the stop codon at position 113 were observed via mass spectrometry. Furthermore, a rabbit red blood cell hemolytic assay demonstrated that CDC587 and MN8 had more biological activity (hemolysis) compared to their isogenic α-toxin knockouts. In the present study, tissue homogenates from CDC587 (*hla*$^-$) contained small amounts of α-toxin and the protein appears to be slightly truncated compared to MNPE and purified α-toxin (**Figure 3A**). Several truncated forms of α-toxin have been reported in the literature (Kwak et al., 2010; Vecsey-Semjen et al., 2010). These fragments demonstrate significantly lower biological activity than the full-length protein, requiring much higher concentrations and longer incubation times to achieve similar pathological effects (Kwak et al., 2010). Alpha-toxin is not detected from tissue homogenates infected with MN8 (*hla*$^-$) despite of this strain being very similar to CDC587. This may be a function of the concentration being below the limit of detection for this assay. Regardless, this finding is consistent with a role for α-toxin in biofilm formation in that MN8 and CDC587 strains failed to form biofilms over 3 days.

Classically, α-toxin is thought of as a cytolysin. The toxin binds to host cells, forms a pore and causes leakage of intracellular contents. A recent study reported that α-toxin not only causes irreversible pathological changes to the epithelial membrane by its insertion, but also activates a disintigrin and metalloproteinase 10 (ADAM10). This activation results in the shedding of E-cadherin and severing of homotypic, adherens junction-based linkages between adjacent cells, which causes disruption at the tissue level (Inoshima et al., 2011). Disruption of the tissue caused by α-toxin^{high} producing strains likely contributes to the penetration of TSST-1 and other superantigens (SAgs) across vaginal epithelial tissue as has been reported previously (Brosnahan et al., 2009). Low levels of α-toxin or other secreted exotoxins may also contribute to SAg accumulation within the tissue. TSST-1 accumulated within the tissue may then serve as a reservoir for toxin-mediated diseases.

Alpha-toxin has been proposed to play a role in the formation of biofilms on abiotic surfaces. A mutant defective in α-toxin failed to form biofilms *in vitro* under static conditions in microtiter plate assays and in flow cell chambers (Caiazza and O'Toole, 2003). The proposed mechanism for α-toxin promoting biofilm formation was to facilitate bacterial cell-to-cell interactions, which in turn facilitates macrocolony formation. In contrast to these findings, we observed no significant differences in the ability of the USA200 strains (*hla*$^+$ and *hla*$^-$) to form biofilms on plastic microtiter plates. This was not entirely surprising as other factors are known to be important for biofilm formation on abiotic surfaces when organisms are grown under optimal conditions, such as in nutrient-rich media on plastic substrates (Otto, 2008). Unlike plastic microtiter biofilm assays, the vaginal mucosa is a comparatively harsh environment, where nutrients are scarce and bacteria are subject to innate immune defenses, necessitating a role for α-toxin on the vaginal mucosa in biofilm formation. This is supported by our current observation of the *hla*$^+$ strain having a biofilm phenotype whereas *hla*$^-$ and *hla*KO do not.

In contrast to this study, *S. aureus* strain (UAMS-1), which carries the α-toxin pseudogene (*hla*$^-$), causes osteomyelitis, a disease considered to be biofilm-associated (Smeltzer et al., 1997). The reasons for this apparent discrepancy are unclear, but perhaps *hla*$^-$ carrying strains of *S. aureus* eventually form biofilms on mucosal surfaces beyond our tested 3 days. Due to limitations in *ex vivo* tissue viability, our experiments were conducted for only 3 or 4 days, whereas osteomyelitis takes weeks to develop. Alternatively, α-toxin may not play a role in colonization and subsequent infection of bone, especially since the biofilm substrate of bone is very different from the biofilm substrate of epithelial tissue. Together, *in vitro* and *in vivo* studies suggest that regulation of α-toxin by quorum sensing or by the presence of a pseudogene (*hla*$^-$) and the role of α-toxin in *S. aureus* biofilm formation on host tissues is complicated and not completely understood. This underscores the need for anatomical site specificity for accurate and clinically predictive models of staphylococcal diseases.

Although the *ex vivo* porcine mucosal tissue infection model is similar to human vaginal mucosa and was developed to more closely mimic *in vivo* conditions, the vaginal mucosa was not subjected to vaginal secretions or menstrual fluids, which may cause the outer mucosa to slough and/or impede the ability of the organisms to infect and/or produce exotoxins such as α-toxin. Further, due to the lack of a blood supply, there is no neutrophil influx, which is critical for controlling early bacterial growth. Taking these limitations into consideration, we still believe that our model is an improvement over *in vitro* abiotic biofilm models.

Our biofilm model is of much shorter experimental duration than some existing models due to tissue viability limitations. However, menstrual TSS commonly occurs within 2–3 days post initiation of menses, which is within the timeframe of our study. In addition, this study only describes one *hla*$^+$USA200 MSSA TSST-1$^+$ isolate compared to its isogenic *hla*KO and two *hla*$^-$ containing organisms. Therefore, whether all *hla*$^+$ TSST-1$^+$ *S. aureus* isolates would form a biofilm over 72 h on mucosal tissue is unknown. However, we have determined that a methicillin-resistant *S. aureus*

α-toxin^{high}, TSST-1^{+} isolate (I6) is also highly toxic to vaginal mucosal tissue and forms a biofilm by day 3 post-infection (unpublished data).

The role of α-toxin in *S. aureus* mucosal biofilm formation *in vivo* has not been characterized. However, this cytolysin is known to play a role in the pathogenesis of *S. aureus,* based on studies of mutants lacking α-toxin (Bramley et al., 1989). For example, in a murine model of peritonitis, α-toxin was shown to be of critical importance when only mice injected with wild-type *hla*$^{+}$ died (Patel et al., 1987). More recently, α-toxin has been reported to play a significant role in lung tissue damage during murine staphylococcal pneumonia (Bubeck Wardenburg and Schneewind, 2008). Researchers determined that virulence correlated with expression levels of α-toxin and further demonstrated that vaccination with a non-pore forming α-toxin (hla$_{H35L}$) protected mice from *S. aureus* pneumonia (Kennedy et al., 2010). Vaccines targeting α-toxin may prevent *S. aureus* vaginal mucosal biofilm formation, but not *S. aureus* infection or toxin (SAg)-mediated diseases. This is because α-toxin-deficient strains (MN8 and CDC587) were not avirulent, but rather caused a different type of infection.

We have termed these non-biofilm-forming isolates HDPV because they grow to a similar density on tissue, are proinflammatory to the tissue and can secrete other virulence factors, such as SAgs, which can lead to toxin-mediated diseases. These data also suggest that α-toxin may be a potent therapeutic target for some diseases. More specifically, neutralizing α-toxin may reduce the organism's ability to form biofilms, rendering it more sensitive to antimicrobial agents and host immune responses. Ultimately, *ex vivo* tissue models, like the one described, and follow-up *in vivo* studies are needed to determine the bacterial and host factors which contribute to *S. aureus* colonization, infection and biofilm formation in humans so optimal therapeutics to prevent and treat staphylococcal infections can be developed.

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Staphylococcal superantigens in colonization and disease

***Stacey X. Xu*[1] *and John K. McCormick*[1,2]*

[1] *Department of Microbiology and Immunology, Centre for Human Immunology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, ON, Canada*
[2] *Lawson Health Research Institute, London, ON, Canada*

Edited by:
Martin J. McGavin, University of Western Ontario, Canada

Reviewed by:
Thomas Proft, University of Auckland, New Zealand
Victor J. Torres, New York University School of Medicine, USA

***Correspondence:**
John K. McCormick, Department of Microbiology and Immunology, University of Western Ontario, 1151 Richmond St., London, ON N6A 5C1, Canada.
e-mail: john.mccormick@schulich.uwo.ca

Superantigens (SAgs) are a family of potent immunostimulatory exotoxins known to be produced by only a few bacterial pathogens, including *Staphylococcus aureus*. More than 20 distinct SAgs have been characterized from different *S. aureus* strains and at least 80% of clinical strains harbor at least one SAg gene, although most strains encode many. SAgs have been classically associated with food poisoning and toxic shock syndrome (TSS), for which these toxins are the causative agent. TSS is a potentially fatal disease whereby SAg-mediated activation of T cells results in overproduction of cytokines and results in systemic inflammation and shock. Numerous studies have also shown a possible role for SAgs in other diseases such as Kawasaki disease (KD), atopic dermatitis (AD), and chronic rhinosinusitis (CRS). There is also now a rich understanding of the mechanisms of action of SAgs, as well as their structures and function. However, we have yet to discover what purpose SAgs play in the life cycle of *S. aureus*, and why such a wide array of these toxins exists. This review will focus on recent developments within the SAg field in terms of the molecular biology of these toxins and their role in both colonization and disease.

Keywords: superantigen, staphylococcal enterotoxin, *Staphylococcus aureus*, colonization

INTRODUCTION

Bacterial superantigens (SAgs) represent a unique class of exotoxins which all function to activate enormous numbers of T lymphocytes (McCormick et al., 2001; Llewelyn and Cohen, 2002; Proft and Fraser, 2003). Although only a few SAgs have clear associations with specific human diseases, bacterial genome sequencing projects over the last decade have led to the characterization of a large and expanding family of exotoxins that includes many genetically and antigenically distinct proteins. These are found primarily in *Staphylococcus aureus* and *Streptococcus pyogenes*, but also found in a few other species of β-hemolytic streptococci, coagulase negative staphylococci, *Mycoplasma arthritidis*, and *Yersinia pseudotuberculosis*. In the following sections, we will update recent findings in the biochemistry of staphylococcal SAgs, and explore the role of SAgs in different lifestyles of *S. aureus* in the context of both infection and nasal colonization.

THE SUPERANTIGEN SUPERFAMILY

The staphylococcal SAgs include the staphylococcal enterotoxins (SEs), the staphylococcal enterotoxin-*like* (SE*l*s) proteins, and toxic shock syndrome toxin-1 (TSST-1) (Lina et al., 2004). The SEs were originally defined by their ability to cause staphylococcal food poisoning (SFP) including emesis, and currently include the SEs A, B, C, D, E, G, H, I, R, and T. The SE*l* toxins, although both homologous and structurally similar to the SEs, either do not induce emesis, or have not been formally demonstrated to induce emesis, and include the SE*l*s J, K, L, M, N, O, P, Q, S, U, V, and X. It is important to note that although designated as a "SE*l*" toxin, some of these may possess undemonstrated emetic activity and be reclassified in the future as bona fide enterotoxins.

An updated phylogenetic classification scheme of the SAg exotoxins (McCormick et al., 2001) is shown in **Figure 1** where SAgs from staphylococci and streptococci are placed into five evolutionary groups. TSST-1 sits as an evolutionarily distinct SAg that does not induce emesis (Schlievert et al., 2000) and is the only member of the Group I SAgs. TSST-1 is believed to be the major, if not sole cause of the menstrual form of toxic shock syndrome (TSS) (Bergdoll et al., 1981; Schlievert et al., 1981). The Group II SAgs contain both staphylococcal and streptococcal SAgs including SEB, SEC, and streptococcal pyrogenic exotoxin A (SpeA). After TSST-1, SEB has been historically most commonly linked with non-menstrual-associated cases of staphylococcal TSS (Schlievert, 1986), while SpeA has been historically most commonly linked with streptococcal TSS (Stevens et al., 1989). The Group III SAgs include only staphylococcal SAgs, and in general terms, this Group contains SAgs most commonly associated with SFP such as SEA, SED, and SEE, although the Group II SAgs SEB and SEC are often implicated as well (Argudin et al., 2010). Both Group II and III SAgs contain a unique "cysteine-loop structure" that is important for emetic activity (Hovde et al., 1994). The Group IV SAgs are only populated by streptococcal SAgs and will not be discussed here. The Group V SAgs, contain mostly staphylococcal SAgs (except SpeI and related orthologues), and other than SEI which has weak emetic activity, consists of only SE*l* toxins. In fact, SEI is the only SAg outside of the Group II and III SAgs demonstrated to have emetic activity, although this only occurred in one of four animals tested (Munson et al., 1998). Very recently, SE*l*-X was described as a novel SAg that does not align well within the currently classification system, but is encoded within the core chromosome of most *S. aureus* strains (Wilson et al., 2011). Also of note are the staphylococcal superantigen-like

proteins (SSLs) (Langley et al., 2010), and although these are also structurally similar to the staphylococcal superantigens (Baker et al., 2007; Chung et al., 2007; Ramsland et al., 2007) they do not possess SAg activity and will not be discussed within this review.

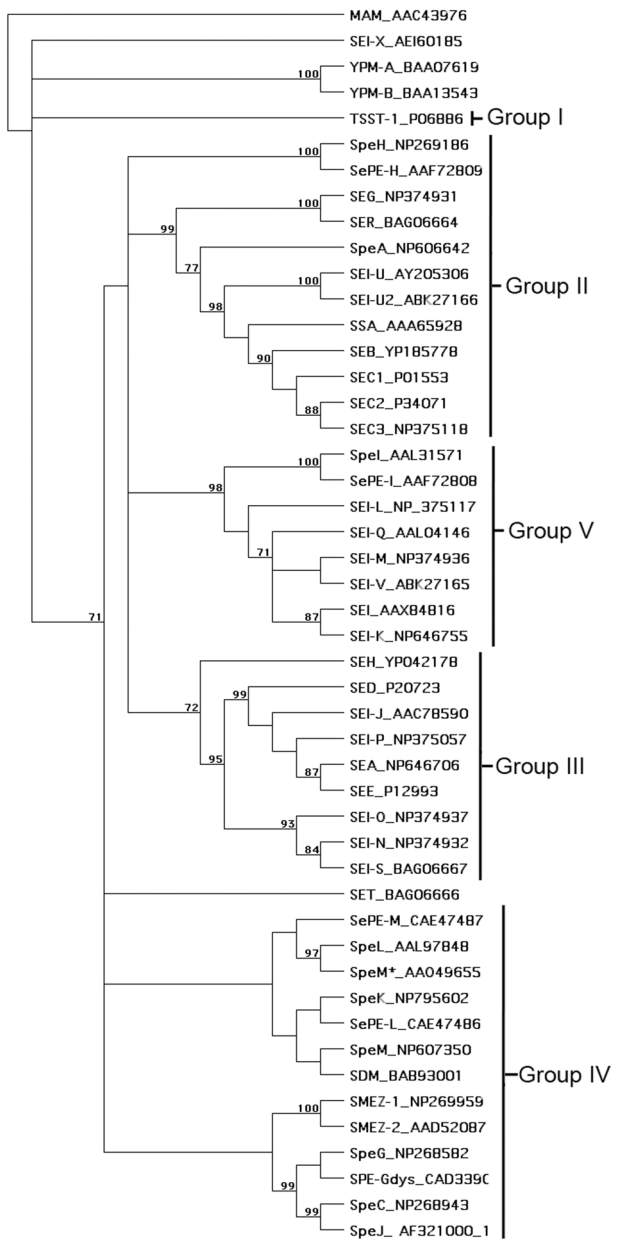

FIGURE 1 | Phylogenetic tree of known bacterial SAgs. The unrooted tree was based on the alignment of amino acid sequences constructed with the unweighted pair group method using arithmetic averages (UPGMA) in MacVector 7.2.3. The SAg abbreviations are indicated followed by the relevant accession number. As previously proposed (McCormick et al., 2001), the five main groups of SAgs belonging to the pyrogenic toxin class are indicated. MAM, YPM, and non-Group A streptococcal SAgs are also included in the analysis. The number of times each branch was supported from 1000 bootstraps is shown as a percentage.

CONVENTIONAL VERSUS SUPERANTIGEN-MEDIATED T CELL ACTIVATION

Normal T cell-mediated immunity is initiated through the interaction of an αβ T cell receptor (TCR) and a processed peptide antigen presented within self-major histocompatibility (pMHC) complexes **(Figure 2A)** (Garcia et al., 1999; Garcia and Adams, 2005). If the TCR specifically recognizes the antigen as foreign, these interactions will activate the tyrosine kinase Lck (associated with co-receptors CD4 and CD8), which in turn will activate downstream cell signaling resulting in activation of transcription factors to induce T cell proliferation and differentiation (Smith-Garvin et al., 2009). As TCRs are extraordinarily diverse molecules, only ∼0.01% of naïve T cells will recognize a given antigen (Givan et al., 1999).

SAg-mediated T cell activation is both quantitatively and qualitatively distinct from conventional T cell activation (Bueno et al., 2007). As the defining feature of the SAg toxin is the ability to activate T lymphocytes in a TCR β-chain variable domain (Vβ)-dependent manner (Marrack and Kappler, 1990), very large

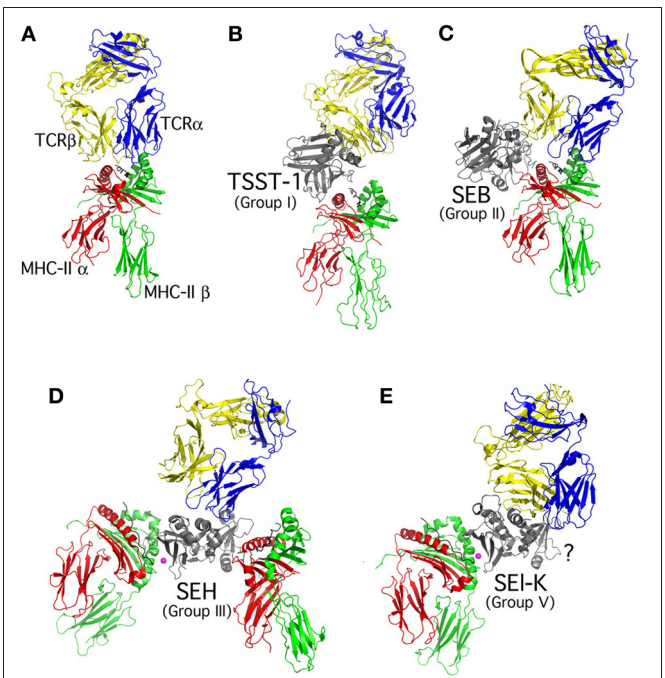

FIGURE 2 | Structural overview of the SAg-mediated T cell activation complexes. Ribbon diagram models show **(A)** conventional T cell activation (Hennecke et al., 2000), and SAg-mediated T cell activation complexes for **(B)** Group I (e.g., TSST-1) **(C)** Group II (e.g., SEB) **(D)** Group III (e.g., SEH) and **(E)** Group V (e.g., SE*I*-K). Colors for TCR and MHC class II chains are labeled in Panel **(A)**. The SAg activation complex models were generated by superposition of the TSST-Vβ (Moza et al., 2007) and TSST-MHC class II (Kim et al., 1994) structures, the SEC-Vβ (Fields et al., 1996) and SEB-MHC class II (Jardetzky et al., 1994) structures, the SEH-VαVβ (Saline et al., 2010), SEH-MHC class II β-chain (Petersson et al., 2001), and the SEA-MHC class II α-chain (Petersson et al., 2002) structures, and the SEK-Vβ (Gunther et al., 2007) and SEI-MHC II (Fernandez et al., 2006) structures. The TCR α-chain was modeled for clarity in each case from the conventional complex (Hennecke et al., 2000). The "?" in Panel **(E)** indicates that there is no current information regarding the presence, or absence, of the generic low-affinity MHC class II binding domain for Group V SAgs.

numbers of T cells can be activated upon SAg exposure. Although TCRs are extraordinarily diverse molecules, this diversity is concentrated within the CDR3 loops due to V(D)J (somatic) recombination during T cell development. However, there are a relatively limited number of possible TCR Vβ regions (∼50 are functionally expressed in humans), and thus SAgs can activate T cells in orders of magnitude above conventional processes. SAgs also do this in an extremely potent manner, and in general, most SAgs can induce measurable activation of T cells in the picogram (10^{-12} g) concentration range. It is often assumed that SAg-mediated T cell activation follows the normal signaling rules for conventional pMHC-mediated T cell activation and indeed this is the case with at least one major distinction. As predicted, TCR ligation by SAg will induce signals through Lck (Morgan et al., 2001), although Lck signaling is not actually required (Yamasaki et al., 1997; Bueno et al., 2006). However, signaling can proceed in the absence of Lck through a Gα11/PLCβ-dependent pathway that converges with the canonical Lck-dependent pathway at the level of ERK1/2 (Bueno et al., 2006). Since one function of the CD4/CD8 co-receptors is the recruitment of Lck, the ability of SAgs to bypass Lck is also likely related to the capability of SAgs to activate both $CD4^+$ and $CD8^+$ T cells, despite cross-linking with MHC class II molecules (Herrmann et al., 1992; Fuller and Braciale, 1998).

The human immune system has evolved to be able to recognize and eliminate pathogens and their antigens. However, SAgs represent the only known microbial virulence factor whose primary role is to deliberately force the activation of the adaptive immune system. This is counter-intuitive given the numerous staphylococcal virulence factors apparently designed for immune subversion and evasion (Nizet, 2007). This leads to the question as to why *S. aureus* produce SAgs. Given the wide variety and high prevalence of SAg genes, it is likely that these genes would be lost, especially since they are primarily encoded on mobile genetic elements, without a contribution to the fitness of *S. aureus*. The influence and architecture of SAg-encoding mobile genetic elements has been the subject of a comprehensive review in this issue (McGavin et al., 2012).

T cell anergy, a phenomenon where T cells become unresponsive to stimulation, has long been proposed to be an immune subversion tactic of *S. aureus*. Several studies have shown this *ex vivo* following *in vivo* stimulation (Kawabe and Ochi, 1990; Rellahan et al., 1990; Lee and Vitetta, 1992; Miller et al., 1999). However, SAg-induced anergy produced *ex vivo* does not necessarily translate into *in vivo* anergy (Heeg et al., 1995). In addition, high levels of purified toxin are often used in experimental mouse models that may not reflect physiological conditions. Also, there is no evidence to suggest that T cells are exhausted in nasal carriers of toxigenic *S. aureus* strains. Recently, a case study of TSS (likely induced by TSST-1) showed deletion followed by an expansion in the $V\beta2^+$ subset that normalized 70 days post-convalescence. In this important study, re-stimulation of peripheral blood mononuclear cells (PBMCs) taken during the acute phase of disease with exogenous SAg resulted in proliferation of $V\beta2^+$ cells suggesting that T cells were not rendered anergic (Rasigade et al., 2011). Recurrent cases of TSS occur, usually as a combined result of insufficient eradication of *S. aureus* and the inability to form anti-SAg antibodies. The proliferative response of T cells was assessed from a patient with recurrent TSS and there was no reduction in the ability of the patients T cells to respond *in vitro* (Arvand and Hahn, 1996). Thus, at least in some patients, true anergy of Vβ specific T cell subsets may not occur and suggests that this activity is not the purpose of SAg activity for *S. aureus*. Recurrent TSS also implies that T cells are not rendered anergic as they are able to react to SAg stimulation during multiple episodes.

Recently, Taylor and Llewelyn (2010) demonstrated that human PBMCs exposed to SAgs resulted in a dose-dependent, Vβ specific increase in CD25+ FoxP3+ cells, indicative of a regulatory T cell (Treg) phenotype. The immunosuppressive qualities of these SAg-induced Tregs have been attributed to the expression of IL-10 and may have a role in prolonging commensalism (Taylor and Llewelyn, 2010).

STAPHYLOCOCCAL SUPERANTIGENS AND THEIR HOST RECEPTORS

SAg pro-toxins include a secretion signal that is cleaved from the N-terminus upon export via the general Sec-dependent secretion pathway. SAgs are released as non-enzymatic, relatively small proteins, with the final toxin product ranging in size from ∼22 to 29 kDa. All SAgs are made of two structurally similar domains, linked through a central α-helix. The larger N-terminal domain contains a β-barrel motif similar to an OB-fold, while the smaller C-terminal domain contains the β-grasp motif, which is similar to immunoglobulin-binding domains (Mitchell et al., 2000).

Pioneering crystallographic studies with staphylococcal enterotoxins B in complex with human leukocyte antigen (HLA)-DR1 (Jardetzky et al., 1994), and SEC3 in complex with the mouse TCR Vβ8.2-chain (Fields et al., 1996), established a molecular framework by how SAgs can activate so many T cells (Li et al., 1999). These studies demonstrated that SAgs bind to lateral surfaces of both TCRs, and pMHC class II molecules, to "distort" the normal TCR-pMHC II interaction, such that the CDR3 loops of both TCR α- and β-chains (which are key for antigen recognition) are wedged away from the antigenic peptide (**Figures 2B–E**). Through this mechanism, activation of the T cell is no longer antigen specific, but now dependent upon which Vβs can be bound by that particular SAg explaining how SAgs are Vβ-specific (Li et al., 1999). Large numbers of SAg-activated T cells can then release a multitude of proinflammatory cytokines which in severe cases may lead to the "cytokine storm" phenomenon characteristic of TSS (McCormick et al., 2001). Activation of antigen presenting cells (APCs) by SAgs also contributes to cytokine release due to the involvement of MyD88, which upregulates NF-κB, leading to production of proinflammatory molecules (Kissner et al., 2011).

Recent years have seen a number of further advances in the structural characterization of the staphylococcal SAgs, and there is now a broader picture as to how SAgs from the different evolutionary groups (**Figure 1**) function to distort the normal process of T cell activation (**Figure 2**). For example, the Group I SAg TSST-1 (**Figure 2B**), which is extremely specific for the human $V\beta2^+$ T cells (Choi et al., 1989), forms a unique T cell activation

complex by binding the MHC II α-chain through a relatively low-affinity interface that is highly influenced by different antigenic peptides within MHC II (Kim et al., 1994; Wen et al., 1996). Also, TSST-1 recognizes unique amino acid insertions from Vβ2 within both CDR2 and framework region (FR) 3, explaining the extreme Vβ-specificity of this SAg (Moza et al., 2006, 2007; Rahman et al., 2011). There are no direct TCR-MHC II contacts in this T cell activation complex. Group II SAgs (**Figure 2C**) such as SEB, SEC3, and SpeA, are more "promiscuous" in their Vβ-targets, and engage TCR Vβ through "conformation-dependent" mechanisms that are thought to be less dependent on specific Vβ amino acid side-chains (Fields et al., 1996; Li et al., 1998; Sundberg et al., 2002). These SAgs bind the MHC II α-chain through an N-terminal, low-affinity binding domain, yet in contrast to TSST-1, this binding is antigenic peptide-independent (Jardetzky et al., 1994). Group III SAgs (**Figure 2D**) consist of only staphylococcal SAgs, and these toxins are thought to be able to cross-link MHC II molecules (Abrahmsen et al., 1995; Hudson et al., 1995) through both a low-affinity site similar to Group II, (Petersson et al., 2002) as well as a high-affinity, zinc-dependent MHC II β-chain interface located within the β-grasp domain of the SAg (Petersson et al., 2001). The only structural information for how Group III SAgs engage TCR is for SEH (Saline et al., 2010), which represents somewhat of an outlier within Group III, and is the only known Vα-specific SAg (Petersson et al., 2003; Pumphrey et al., 2007). Group IV SAgs are restricted to only streptococcal members, and these toxins bind Vβ similar to the Group II SAgs, although with a larger footprint (Sundberg et al., 2002), and contain a high-affinity MHC II β-chain binding domain similar to Group III (Li et al., 2001). Considerable evidence indicates the presence of a low-affinity MHC II α-chain interaction, likely similar to Group II (Swietnicki et al., 2003; Tripp et al., 2003; Kasper et al., 2008), although this interaction has not been characterized structurally. The Group V SAgs contain a high-affinity MHC II β-chain binding domain (Fernandez et al., 2006) similar to Group III, and bind the TCR Vβ with a more "lateral" position extending into FR4 (**Figure 2E**) (Gunther et al., 2007). There is currently no information relating to the presence, or absence, of the generic low-affinity MHC II interface with Group V SAgs.

Within the SAg family of toxins, each member is able to efficiently activate large numbers of T cells, regardless of subtle, or dramatic, differences within the different SAg-mediated T cell activation complexes. However, the one common structural feature of all characterized SAgs, with the exception of the Vα-specific SEH, is the engagement of the Vβ CDR2 loop, and this loop appears to be the critical determinant for Vβ-specificity (Rahman et al., 2011).

Recently, it was demonstrated that SEB can bind to the costimulatory molecule CD28, which is constitutively expressed on naïve T cells and binds B7 ligands on APCs. The CD28 binding site is divergent from both the TCR and MHC II binding domains of SEB, and is relatively conserved amongst the SAg family. Disruption of CD28 binding by peptide antagonists reduced mortality rates in mice administered with D-galactosamine and SEB by downregulating Th1, but not Th2 cytokines (Arad et al., 2011). These lines of evidence support the proposal that CD28 binding by SAgs is important to the function of SAgs. Further research elucidating downstream mechanisms will clarify the exact role of CD28 during T cell activation by SAgs.

STAPHYLOCOCCAL SUPERANTIGENS AND DISEASE

STAPHYLOCOCCAL FOOD POISONING

The first disease linked to the staphylococcal SAgs was SFP, and evidence that a staphylococcal toxin caused the illness dates back to 1930, where filterable supernatants from a "yellow staphylococcus" was able to induce SFP in human volunteers (Dack et al., 1930; Jordan, 1930). The role of the staphylococcal SAgs in foodborne disease has been reviewed recently in detail (Argudin et al., 2010; Hennekinne et al., 2011), and will not be discussed here.

TOXIC SHOCK SYNDROME

The other human disease clearly caused by the staphylococcal SAgs is TSS. This disease was described in 1927 by Franklin Stevens as staphylococcal scarlet fever (Stevens, 1927), and was named "toxic shock syndrome" by Todd and colleagues in 1978 to describe a systemic illness in seven children caused by non-invasive *S. aureus* (Todd et al., 1978). The pathogenesis of TSS is due to a SAg-induced cytokine storm owing to the massive activation of T cells in individuals lacking neutralizing antibodies to the particular SAg [reviewed in (McCormick et al., 2001)]. The disease is a capillary leak syndrome where patients develop fever, rash, hypotension, multiorgan involvement and convalescent desquamation (McCormick et al., 2001). *S. aureus* can cause the menstrual form of TSS, which historically occurred in young women in association with high absorbency tampons, and non-menstrual TSS, which can occur from virtually any *S. aureus* infection, although infrequently from bacteremia (McCormick et al., 2001). Although most staphylococcal SAgs are functionally capable of inducing TSS in experimental animals, only a few select SAgs have historically been associated with the disease. This is somewhat surprising given the large "collection" of these extremely potent toxins. The TSST-1 SAg was linked to the menstrual form of TSS in 1981 (Bergdoll et al., 1981; Schlievert et al., 1981), although it is also clear that other SAgs, primarily TSST-1, SEB and SEC, are capable of causing the non-menstrual form (Bohach et al., 1990; McCormick et al., 2001).

During the early 1980s, there were a high number of menstrual TSS cases in young women associated with the use of high absorbency tampons (Shands et al., 1980) and the estimated incidence of all forms of TSS at this time was 13.7/100,000 (Osterholm and Forfang, 1982). By the mid-1980s, following the removal of these products from the marker, and public awareness campaigns as well as product labeling, the overall incidence dropped to 0.53/100,000 with a case-fatality rate of ∼4% (Gaventa et al., 1989). A recent population based surveillance for TSS in Minnesota between 2000 and 2006 demonstrates that this rate has been relatively stable and that TSST-1 was still the major cause in most cases (DeVries et al., 2011). Of note, although the community acquired MRSA clonal strain USA300 has dramatically increased in prevalence in the U.S., this strain does not appear to cause many cases of TSS (DeVries et al., 2011). Although the overall incidence of TSS appears low, it has

been suggested that severe SAg-mediated disease remains underreported (DeVries et al., 2011), due to both the strict CDC case definition (Centers for Disease Control and Prevention, 2011) as well as prompt and appropriate medical attention that would prevent the most severe forms of SAg intoxication. Indeed, TSS is still a major problem, and cases of non-menstrual TSS pediatric burn patients can be extremely dangerous if not recognized early (White et al., 2005).

Apart from the more overt forms of SAg-mediated diseases, there is significant evidence that SAgs also can play a role in a number of other diseases and these will be discussed below.

KAWASAKI DISEASE

Kawasaki Disease (KD) was first described by Tomisaku Kawasaki in 1967 (Van Crombruggen et al., 2011) and is now the leading cause of acquired heart disease in children from developed nations. KD is an acute, self-limiting vasculitis, typically affecting the coronary arteries, and thought to be triggered by an infectious agent in genetically susceptible individuals (Yeung, 2010). Although the etiology of KD is not known, there is compelling evidence that bacterial SAgs are involved, and could be causal in association with host genetic factors (Matsubara and Fukaya, 2007). First, the clinical presentation of KD has features reminiscent of TSS, including fever, a desquamating rash and erythema of the mucous membranes. SAg producing *S. aureus* and *S. pyogenes* have been isolated from KD patients, and seroconversion with anti-SAg antibodies has also been demonstrated. Perhaps the strongest evidence of SAg involvement however, is the demonstration of Vβ skewing in KD patients (Abe et al., 1992). A number of studies have found primarily Vβ2 expansion (Leung et al., 1995b) providing a link to either TSST-1 or SpeC which are both Vβ2 specific (Rahman et al., 2011). Others, however, have found expansion of various Vβ families (Nomura et al., 1998; Yoshioka et al., 1999), potentially implicating other SAgs with different Vβ profiles. Treatment of KD involves the use of intravenous immunoglobulin (IVIG) (Newburger et al., 1986), and IVIG is well known to contain SAg neutralizing antibodies (Darenberg et al., 2004; Schrage et al., 2006). Although there is no direct evidence to suggest SAg involvement, there also exists the Kawasaki-like syndrome, which in contrast to KD occurs primarily in adults with severe immunosuppression including HIV/AIDS (Stankovic et al., 2007).

CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis (CRS) is a group of disorders characterized by inflammation of the nose and paranasal sinuses for at least 3 months duration (Van Crombruggen et al., 2011). CRS can occur with or without nasal polyps, and accumulated evidence is now convincing that *S. aureus* SAgs can contribute to, at least in some cases, CRS with nasal polyposis (Van Zele et al., 2004). In this disease, SAgs are thought to skew the cytokine response towards a T_H2 phenotype inducing both eosinophilia and the production of polyclonal IgE, which in turn could be further linked to asthma (Bachert et al., 2010). There is no single SAg associated specifically to this disease (Van Zele et al., 2008; Heymans et al., 2010), and as noted (Van Crombruggen et al., 2011), a causal relationship with *S. aureus* has not been established.

ATOPIC DERMATITIS

Atopic dermatitis (AD) represents a chronic and relapsing T cell-mediated inflammatory skin disorder with IgE-mediated sensitization to allergens. AD most often affects infants and young children, but may persist into adulthood, or may first develop in adults as late-onset AD. AD has both genetic and environmental contributions but nearly all AD patients are colonized by *S. aureus.* This is likely due to both the damaged skin barrier and impaired host immune responses. Significant evidence also indicates an important role for the staphylococcal SAgs in exacerbating the disease [reviewed in (Schlievert et al., 2010)]. SAgs have long been known to induce the skin homing receptor cutaneous lymphocyte-associated antigen (CLA) on T cells to recruit these cells to the skin (Leung et al., 1995a). Very recent evidence indicates that skin homing, phenotypically Treg (CD4+ FoxP3+) cells from AD patients may actually display a T_H2 phenotype in response to SEB stimulation (Lin et al., 2011). AD patients may also develop anti-SAg IgE antibodies that can further worsen the condition (Leung et al., 1993; Bunikowski et al., 1999; Lin et al., 2000). AD is often treated with glucocorticoids and SAgs have been shown to induce glucocorticoid resistance in PBMCs (Hauk et al., 2000). A recent study that examined essentially the entire staphylococcal SAg family found that isolates from steroid resistant AD patients contained significantly more SAgs genes than isolates from non-steroid resistant patients or menstrual isolates provoking the idea that steroid treatment may actually select for SAgs in these strains (Schlievert et al., 2008).

GUTTATE PSORIASIS

Guttate psoriasis is an acute form of psoriasis mediated by autoreactive T cells that typically develops in young adults and children. This inflammatory skin disease is typically preceded by streptococcal pharyngitis, and the streptococcal SAgs, in particular SpeC, and $V\beta2^+$ T cells have been implicated (Leung et al., 1995c). Some associations have also been made with *S. aureus* and chronic plaque psoriasis (Sayama et al., 1998; Balci et al., 2009).

NASAL COLONIZATION AND STAPHYLOCOCCAL SUPERANTIGENS

Staphylococcal colonization can be defined by the presence and multiplication of *S. aureus* in the absence of infection or disease. In humans, the anterior nares are the most common area colonized by *S. aureus* and the prevalence of nasal colonization is particularly high within the general population. Individuals have been typically classified into three separate groups based on their nasal carriage status: *persistent*, *intermediate,* or *non-carriers*. Approximately 20% of the general population are persistent carriers of *S. aureus*, ∼30% are intermittent carriers, and ∼50% are non-carriers (Wertheim et al., 2005). In the event of an infection, carriers have a better prognosis than non-carriers (von Eiff et al., 2001; Wertheim et al., 2004); however, nasal colonization increases the risk of infection by fourfold (Safdar and Bradley, 2008). Furthermore, it is believed that ∼80% of *S. aureus* bloodstream infections come from an endogenous source (von Eiff et al., 2001), and this can be particularly dangerous in a hospital setting if a nasal carrier is immunocompromised and the colonizing strain is resistant to antibiotics.

Although a myriad of bacterial factors play a role in determining nasal colonization, it has not yet been established whether or not SAgs are involved. Epidemiological studies evaluating *S. aureus* SAg gene distribution in nasal swabs compared with blood isolates concluded that there were no differences between blood and nasal isolates in the number of toxins, or a correlation to a particular toxin and that toxin gene distribution was widespread and highly varied (Holtfreter et al., 2007). *S. aureus* strains encoding the same SAg genes can produce different amounts of toxin (Varshney et al., 2009) and this may make correlations difficult in epidemiological studies, which often rely on genomic typing instead of protein quantification.

The particular molecular switch by how colonized bacteria become pathogenic is likely a mixture of host-pathogen and environmental factors that leads to a breach in the mucosa and subsequent infection. The role of the two-component regulatory system *agr* has been classically associated with dissemination and the release of secretory proteins and downregulation of surface associated proteins (Recsei et al., 1986). Many SAgs such as TSST-1 are regulated by *agr* (Recsei et al., 1986), which appears to be dampened during colonization, suggesting that *agr*-controlled SAgs may not be involved in colonization. This has been further supported where the constitutive expression of RNAIII, the effector molecule of the *agr* system, reduces nasal colonization in rats (Pynnonen et al., 2011). Thus, it is likely that *agr* is downregulated during colonization, which has been demonstrated in human studies (Burian et al., 2010). It has however, been suggested that certain SAgs such as staphylococcal enterotoxin A (SEA), which is not regulated by *agr*, may play a role early on in colonization (Bohach and Schlievert, 2007). Although many persistent carriers contain the bacteriophage that carries SEA, this genetic element does not appear to play a role early on during colonization (Verkaik et al., 2011). Furthermore, the *sea* gene has been correlated with sepsis (Ferry et al., 2005), although the presence of SEA has yet to be confirmed in blood during sepsis. This work also demonstrated a correlation between the *egc* operon of SAgs and colonization (Ferry et al., 2005). A follow-up study using recombinant SAgs found that both types (*egc* and non-*egc*) of SAgs induced similar proliferative activity on PBMCs (Grumann et al., 2008). However, the proliferative potential of supernatants taken from patients with strains containing *egc* genes demonstrated that strains encoding *egc* SAgs do not have as high proliferative activity as strains encoding non-*egc* SAgs, suggesting that *egc* SAgs are not made in quantities as high as non-*egc* SAgs. A lack of neutralizing antibodies against *egc*-encoded SAgs was also found in serum from healthy humans indicating either a lack of *egc* toxins being produced by *S. aureus* or an inability to produce neutralizing antibodies by the host (Holtfreter et al., 2004). It is interesting that only non-*egc* encoded SAgs have been implicated in toxin-mediated diseases. Thus, the role of *egc*-encoded SAgs in colonization requires further investigation.

It is difficult to directly ascertain whether or not SAgs are produced *in vivo* during colonization mainly due to the presence of *S. aureus* protein A, which binds the Fc portion of antibodies, thereby causing background levels of assays to be quite high. However, analysis of the immunological response can provide important information. In particular, both Vβ-specific T cell activation and SAg-neutralizing antibodies are indirect ways of determining if SAgs have encountered the immune system. While Vβ-skewing has been studied in the context of severe disease (Ferry et al., 2008b), it has long been known that the general population develops anti-SAg antibodies capable of neutralizing these toxins (Vergeront et al., 1983). Also, persistent nasal carriers of *S. aureus* have been found to have neutralizing antibodies against the SAgs produced by the colonizing strain (Holtfreter et al., 2006; Kolata et al., 2011). Levels of neutralizing antibodies against TSST-1 and SEA were significantly higher in persistent nasal carriers than non-carriers (Verkaik et al., 2009) suggesting that these SAgs are actively produced during nasal colonization.

To what extent, if any, do SAgs play during colonization has not yet been experimentally addressed. Intranasal vaccination in rodents with deactivated TSST-1 was able to decrease mortality rates from TSST-1 producing *S. aureus* septic challenge and significantly decreased the bacterial load in organs (Narita et al., 2008). This was a TSST-1 specific response, as challenge with non-TSST-1 producing *S. aureus* did not result in a significant reduction in bacterial load when compared to non-vaccinated mice. The same vaccination strategy protected against nasal challenge only during the initial colonization phase (days 1 and 3). Since the model only evaluated colonization up to day 7, it is difficult to assess whether or not this is able to have a lasting effect against *S. aureus* nasal persistence, since there were not significant effects at day 5 (Narita et al., 2008).

Staphylococcal peptidoglycan-embedded molecules have been found to downregulate the immune response stimulated by SAgs (Chau et al., 2009). This effect was most effective at high cell densities suggesting that it is important in a state of colonization or a biofilm as opposed to free-living planktonic cells. Thus, if a colonized population of *S. aureus* is producing SAgs, any invading "rogue" cells that are not a part of the main colony may be killed by an activated immune system, while the dense colony is able to downregulate this response in the local area to prevent clearance. This suggests a role for SAgs as checkpoints of dissemination. Evidence suggests that when SAgs are systemic in the case of TSS (Ferry et al., 2008b), *S. aureus* is able to prevent dissemination, which may be partly why bacteremia is rarely associated with staphylococcal TSS. This is also supported by the observation that sepsis patients lack Vβ-skewing unlike TSS patients (Ferry et al., 2008a), suggesting that bacterial dissemination prevents toxin production.

FUTURE DIRECTIONS FOR RESEARCH

The collective SAg research community has contributed enormously to an advanced understanding of SAg biology. Yet, there remain a number of important avenues for further study and consideration.

Although SAgs are defined by Vβ-specificity, different human MHC II molecules are also clearly important for the response to SAgs (Yeung et al., 1996; Medina et al., 2001; Kotb et al., 2002; Llewelyn et al., 2004; Goldmann et al., 2005; Nooh et al., 2007). Mouse models (such as C57BL/6 and BALBc) have been hampered by the fact that mouse MHC II do not respond in the same way, and are not as sensitive to SAgs, as human MHC (Yeung et al., 1996). Alternative models include rabbits that respond

more appropriately (Parsonnet et al., 1987; Dinges and Schlievert, 2001; Buonpane et al., 2007), as well as transgenic mouse strains that express human MHC class II molecules (Yeung et al., 1996; DaSilva et al., 2002). Models of TSS also often utilize a liver-damaging reagent such as D-galactosamine in conjunction with high levels of SAgs. Liver and gut pathology has recently been implicated in the course of TSS in a humanized transgenic HLA-DR3 mouse model without the use of D-galactosamine (Tilahun et al., 2011a,b) and thus, D-galactosamine may mask pathologies normally induced via TSS. Lastly, although many studies using purified recombinant SAgs have yielded many insights, SAg function is still rarely studied in the context of live infections using genetically defined knockout strains. More work using live infections with appropriate SAg-responsive models is needed to be able to coordinate SAg production with other virulence factors.

Although a number of studies have evaluated the presence of *S. aureus*, and correlations of particular SAg genes with particular clinical syndromes, the presence of the gene does not equate to expression and function of the actual toxin. Indeed, the original discovery of TSST-1 as the causal agent of menstrual TSS was made due to the high level production of this toxin from menstrual TSS strains (Bergdoll et al., 1981; Schlievert et al., 1981). For many human diseases where SAgs may contribute to, or drive the pathology, there is likely not being a single toxin responsible given that they can all activate numerous T cells. As we now know the Vβ skewing patterns of virtually all the known staphylococcal SAgs (Thomas et al., 2009; Seo et al., 2010; Wilson et al., 2011), further systematic evaluations focused on SAg expression coupled with function in relation to particular clinical syndromes (Ferry et al., 2008b), are warranted.

The large family of SAgs continue to grow, and the YPM and MAM SAgs seem to have developed their SAg-activity through convergent evolution as these toxins are not orthologous to the pyrogenic toxin SAgs, or to each other. Also, the animal model of KD utilizes an uncharacterized SAg from the cell wall preparation of *Lactobacillus casei* to induce the disease in mice (Yeung, 2007). *L. casei* is found commonly in the intestinal tract, is widely used by the dairy industry, and is clearly not a pathogen. It is easy to speculate that uncharacterized SAgs could be produced by other microorganisms associated with human immune mediated sequelae.

Arguably the most interesting question that remains in this field is why do *S. aureus* possess such a large, genetically and antigenically distinct, extremely potent, and seemingly redundant group of these toxins? SAgs skew responses toward T_H1 during severe disease, but toward T_H2 responses during atopic disease in genetically predisposed individuals. T_H1 skewing can result in delayed development of neutralizing antibody and perhaps this is an important *in vivo* survival strategy. Many patients following menstrual TSS fail to develop anti-TSST-1 antibodies (Stolz et al., 1985) so this can occur from TSS. Conversely, humans clearly develop anti-TSST-1 antibodies such that by age 1, ∼50% have antibody titers considered to be protective (Vergeront et al., 1983). An interesting hypothesis has been proposed where excessive T cell expansion may act as a sponge to titrate IL-2 necessary for further T cell expansion, essentially causing immunosuppression (Llewelyn and Cohen, 2002). Similarly, massive expansion of Vβ-specific T cells may induce a loss of overall receptor diversity filling up the "space," providing an alternative method of immune escape. Continued efforts into understanding the complex biology of SAgs will undoubtedly answer many of these questions. It is clear that these remarkable toxins represent a highly unique and well adapted virulence factor, although the evolutionary function of these toxin in the life cycle of *S. aureus* still remains unclear.

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Intracellular *Staphylococcus aureus*: live-in and let die

***Martin Fraunholz*[1]* and *Bhanu Sinha*[2]**

[1] *Department of Microbiology, Biocenter, University of Würzburg, Würzburg, Germany*

[2] *Department of Medical Microbiology, University Medical Center Groningen, Groningen, Netherlands*

Edited by:
Martin John McGavin, University of Western Ontario, Canada

Reviewed by:
Ross Fitzgerald, Roslin Institute, UK
Sung Ouk Kim, University of Western Ontario, Canada

***Correspondence:**
Martin Fraunholz, Department of Microbiology, Biocenter, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany. e-mail: martin.fraunholz@uni-wuerzburg.de

Staphylococcus aureus uses a plethora of virulence factors to accommodate a diversity of niches in its human host. Aside from the classical manifestations of *S. aureus*-induced diseases, the pathogen also invades and survives within mammalian host cells. The survival strategies of the pathogen are as diverse as strains or host cell types used. *S. aureus* is able to replicate in the phagosome or freely in the cytoplasm of its host cells. It escapes the phagosome of professional and non-professional phagocytes, subverts autophagy, induces cell death mechanisms such as apoptosis and pyronecrosis, and even can induce anti-apoptotic programs in phagocytes. The focus of this review is to present a guide to recent research outlining the variety of intracellular fates of *S. aureus*.

Keywords: *Staphylococcus aureus*, phagocytosis, phagosomal escape, autophagy, host cell death, bacterial persistence

INTRODUCTION

Most manifestations of *Staphylococcus aureus* disease involve extracellular bacteria (furuncles, carbuncles, impetigo, abscesses, septicemia, necrotizing pneumonia) or biofilm formation (catheter-induced infective endocarditis, atherosclerosis). Aside from this *S. aureus* infections have a second face: there is accumulating evidence that *S. aureus* is able to survive within its hosts cells and thus might be termed a facultative intracellular pathogen. Intracellularity of *S. aureus* has been implied as immune-evasive strategy thereby escaping detection by professional phagocytes.

INTERNALIZATION OF *S. aureus* BY HOST CELLS

Invasion of non-professional phagocytes by *S. aureus* is mediated by a zipper-type mechanism. To date many bacterial adhesins have been identified with Fibronectin (Fn)-binding proteins A and B (FnBPA, FnBPB) constituting the major staphylococcal adhesins for non-professional phagocytes such as epithelial, endothelial cells, fibroblasts, osteoblasts, and keratinocytes (Dziewanowska et al., 1999; Jevon et al., 1999; Lammers et al., 1999; Peacock et al., 1999; Sinha et al., 1999; Fowler et al., 2000; Ahmed et al., 2001; Kintarak et al., 2004; Sinha and Fraunholz, 2010; Edwards et al., 2011; **Figure 1**, Map Item 1). Fibronectin-bridging between FnBPs and $\alpha_5\beta_1$ integrins on the host cell surface is sufficient to induce zipper-type uptake of staphylococci (Sinha et al., 2000). However, FnBPs also have been shown to directly bind to human heat shock protein 60 (Hsp60) exposed on the cellular surface (Dziewanowska et al., 2000). FnBP-independent invasion was observed in *S. aureus* Newman, which produces C-terminally truncated FnBPs that are not covalently anchored to the cell wall of *S. aureus*. It has been shown that strain Newman gets internalized by epithelial cells and fibroblasts mediated by extracellular adherence protein (Eap; Harraghy et al., 2003) with its cellular receptor still not identified to date. Recently, the staphylococcal autolysin (Atl) was identified to function as adhesin/invasin with heat shock cognate protein Hsc70 being the direct cellular receptor (Hirschhausen et al., 2010). Further, wall teichoic acids (WTA) seem to be important for establishment of nasal colonization and there is evidence that a scavenger receptor is involved in WTA binding (Weidenmaier et al., 2004, 2005, 2008). Clumping factor B (ClfB) has been shown to bind to cytokeratins in the extracellular matrix (ECM) of host cells (O'Brien et al., 2002; Wertheim et al., 2008; Haim et al., 2010), and staphylococcal protein A can directly interact with tumor necrosis factor α receptor 1 (TNFR1; Claro et al., 2011). To what extent the internalization of the pathogen is mediated by WTA, ClfB, protein A, and a body of other molecules interacting with the ECM of host cells is not known thus far.

Since FnBPs contribute to the adherence of *S. aureus* to intact endothelium *in vivo* (Laschke et al., 2005; Kerdudou et al., 2006; Edwards et al., 2010), we can assume that staphylococcal invasion of epithelia or the endothelium is relevant in natural infections. The interaction of FnBP with ECM Fn is mediated by tandem β zipper structures via the binding of multiple fibronectin molecules by the repetitively arranged modules within a single FnBP (Schwarz-Linek et al., 2003; Rudino-Pinera et al., 2004; Bingham et al., 2008). As a result FnBP/Fn sequester $\alpha_5\beta_1$ integrins on the host cell surface. The resulting receptor clustering relays signals that result in cytoskeletal rearrangements (Agerer et al., 2005; Schröder et al., 2006b). The rearrangements initiated at focal adhesions, which are remodeled to fibrillar adhesions by loss of focal adhesion kinase (FAK), paxillin, and vinculin. The rearrangement is accompanied by a centripetal movement of *S. aureus* on the host cell surface that were observed by videomicroscopy (Schröder et al., 2006a). The repeated generation of actin comet tails beneath adherent staphylococci or FnbA-coated beads and the formation of actin cups without internalization of staphylococci is interpreted by the authors as a delay of phagocytosis (Schröder et al., 2006a; **Figure 1**, Map Item 2). Invasion signaling further involves src kinase (Agerer et al., 2003). Extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) p38 are required in osteoblasts (Ellington et al., 2001), whereas in HEp-2 cells p38 MAPK was found to be upregulated

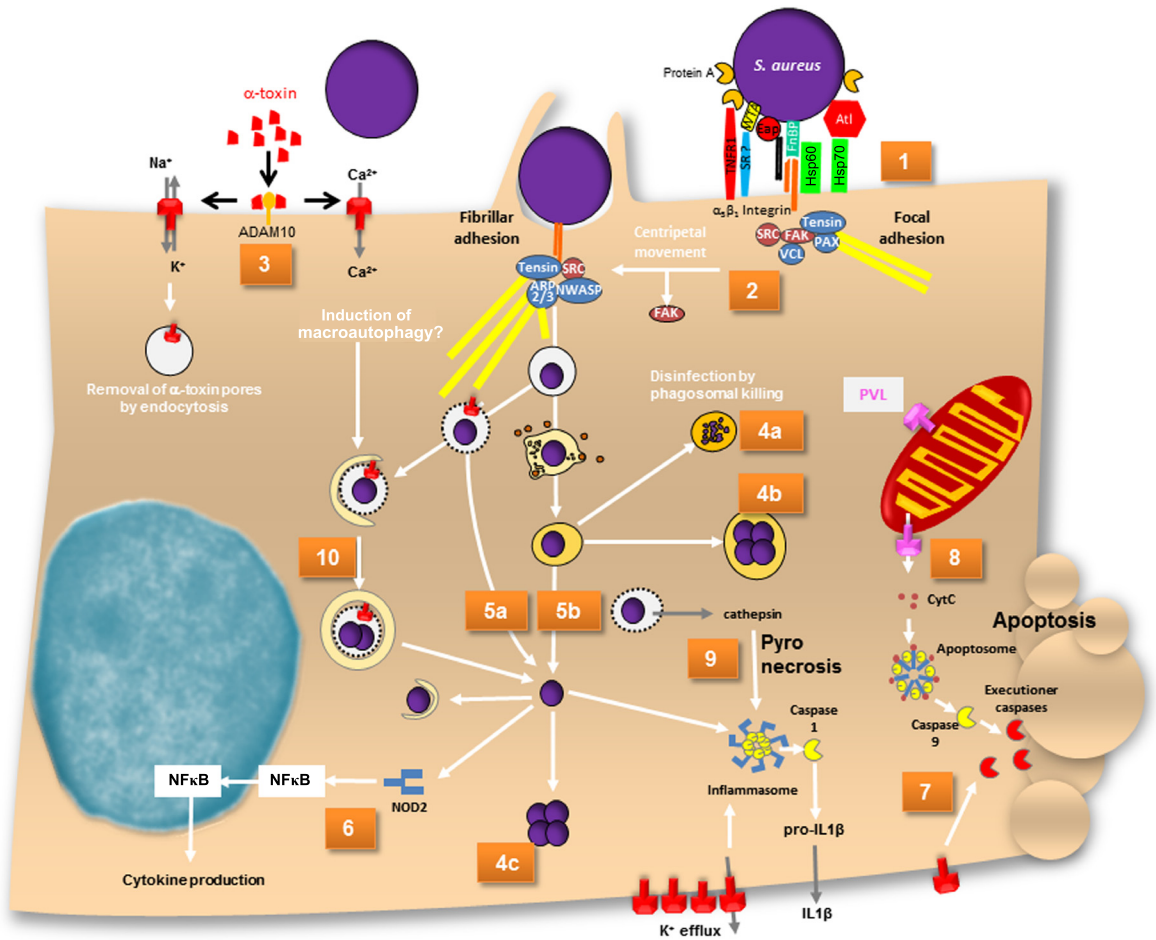

FIGURE 1 | A map of intracellular fates of *S.* aureus. (1) $\alpha_5\beta_1$ integrins are sequestered by FnBP-dependent fibronectin cross-linking at focal adhesions. (2) Centripetal movement and loss of FAK lead to development of fibrillar adhesions, at which phagocytic cups are formed and bacteria are eventually endocytosed. (3) Assembly of α-toxin pores on the plasma membrane of host cells leads is dependent on ADAM10. α-Toxin pores are permeable for cations. Ca^{2+} has been reported to induce macroautophagy. (4a) Bacteria are disinfected by phagolysosomes or (4b) survive and grow within endosomes or (4c) in the cytoplasm after phagosomal escape. (5a) Phagosomal escape can be mediated by α-toxin in cystic fibrosis cells and (5b) also by a combination of phenol-soluble modulins and phospholipases. (6) Cytoplasmic *S. aureus* peptidoglycan is recognized by NOD2, which activates NFκB and results in cytokine production. (7) The mode of cell death induced by *S. aureus* is not completely understood. While caspase-independent cell death exists, α-toxin is capable of inducing extrinsic apoptosis. Upon alpha-toxin induced potassium efflux caspase 2 has recently been shown to lead to mitochondrial outer membrane permeabilization. (8) PVL has been reported to permeabilize mitochondrial outer membrane thereby releasing cytochrome *c* and thus inducing the apoptosome in a Bax-independent pathway of intrinsic apoptosis. Caspase 9 subsequently activates executioner caspases. (9) Cathepsin release from permeabilized phagosomes activates the inflammasome. Activated caspase 1 leads to IL1β maturation and inflammatory pyronecrotic cell death. (10) Toxin-permeabilized endocytic vesicles are targeted by autophagy. During autophagy an isolation membrane engulfs leaky endosomes or cytoplasmically located bacteria. Within these autophagosomes bacterial replicate and eventually escape the organelle ultimately leading to host cell death. ADAM, a metalloprotease and disintegrin; ARP2/3, actin-related protein 2 and 3; Atl, autolysin; CytC, cytochrome *c*; Eap, extracellular adherence protein; FAK, focal adhesion kinase; FnBP, fibronectin-binding protein; HSP, heat shock protein; IL, interleukin; NFκB, nuclear factor κB; NWASP, neural Wiskott–Aldrich syndrome protein; PAX, paxillin; SR, scavenger receptor; VCL, vinculin; WTA, wall teichoic acid.

alongside ERK (Li et al., 2009). Further, phosphorylation of transcription factor c-Jun, but not of Elk-1 or ATF-2 has been demonstrated during invasion of osteoblasts (Ellington et al., 2001). The phosphoinositide-3-kinase (PI3K)-Akt pathway is active during *S. aureus* internalization by bovine endothelial cells (Oviedo-Boyso et al., 2011).

Eventually, *S. aureus* gets endocytosed by professional as well as non-professional cells where the pathogen faces a variety of intracellular fates.

Staphylococcus aureus INTRACELLULAR PERSISTENCE AND GROWTH

The fates of the pathogen and the infected host cell depend on staphylococcal isolate and genotype (Krut et al., 2003) as well as differential susceptibility of host cells to virulence factors, host cell gene expression, etc. For example, *S. aureus* produces different hemolysins. The majority of bovine mastitis strains were phenotypically positive for the sphingomyelinase β-toxin, whereas only a minority of human strains isolated from cases of septicemia

or nasal carriage was positive for β-toxin (Aarestrup et al., 1999). There seems to exist a selective pressure for *S. aureus* strains colonizing humans to acquire β-toxin converting phage (Goerke et al., 2009). This is most likely due to staphylococcal complement inhibitor SCIn and the chemotaxis inhibitor protein CHIPS (and additional factors) that are usually found to be encoded by the respective phage genomes. Purified β-toxin, selectively kills monocytes (Bhakdi et al., 1996) and destroys platelets, but barely affects other cell types (Wadstrom and Mollby, 1972). Similarly, most human cell types are fairly insensitive to the pore-former α-toxin, whereas human leukocytes and cells from other mammalian species are highly susceptible (Bhakdi and Tranum-Jensen, 1991). The metalloprotease ADAM10 has been recently identified as receptor of α-toxin monomers (Wilke and Bubeck Wardenburg, 2010; Inoshima et al., 2011; **Figure 1**, Map Item 3). The differential specificities of α-toxin possibly reflect ADAM10 expression differences in the respective cells or might result from differential capability of host cells to remove α-toxin pores by endocytosis (Husmann et al., 2009).

Staphylococcus aureus survival within host cells was highly dependent on multiplicity of infection (MOI; e.g., Mohammed et al., 2007; Schwartz et al., 2009; Pang et al., 2010) and also the growth phase of the bacteria used for infection (Schwartz et al., 2009). Green-fluorescent protein (GFP)-expressing *S. aureus* displayed bleaching of the fluorescent protein, which indicated degradation of the bacteria in polymorphonuclear neutrophils (PMN; **Figure 1**, Map Item 4a). The loss of fluorescence was not strain-specific and was seen in each of several different strains of *S. aureus*, including nosocomial and community-associated methicillin-resistant strains. When rapidly growing *S. aureus* was used for infection, the bacteria were found to be more susceptible to GFP bleaching (Schwartz et al., 2009). This indicated that these bacteria were cleared more efficiently. Bacterial disinfection was mainly dependent on hypochlorous acid (HOCl; Schwartz et al., 2009). Phagosomal acidification and digestion of *S. aureus* within professional phagocytes is required for MyD88-dependent toll-like receptor (TLR) responses to infection (Abdelzaher et al., 2010).

Not all bacterial cells are disinfected by the phagolysosomes. *S. aureus* has been reported to persist inside phagocytes or endothelial cells for prolonged periods (Hamill et al., 1986; Lowy et al., 1988; Vann and Proctor, 1988; Buisman et al., 1991; Hiemstra et al., 1992; Schröder et al., 2006a; Garzoni et al., 2007; Kubica et al., 2008; Tuchscherr et al., 2011; reviewed in Sendi and Proctor, 2009). Persistence is most often attributed to small colony variants (SCVs) of *S. aureus*. SCVs often present a metabolically quiescent, non-hemolytic, non-pigmented phenotype characterized by reversible auxotrophies in heme biosynthetic pathways or in oxidative phosphorylation (Proctor et al., 1994) as well as a defined transcriptome (Garzoni et al., 2007) and proteome (Kriegeskorte et al., 2011). Also, SCVs are generally found to be mutants in the accessory gene regulator locus (*agr*), thus failing to produce a variety of quorum sensing-controlled virulence factors. SCVs grow slowly and hence are more resistant to a variety of antibiotics (reviewed in Sendi and Proctor, 2009). Further, SCVs display a thick cell wall (Bulger and Bulger, 1967) and an up-regulation of alternative sigma-factor σB (Moisan et al., 2006), which enables *S. aureus* to cope with a variety of environmental stressors (Horsburgh et al., 2002). There is increasing evidence that *S. aureus* can persist *in vivo* in human infections presumably owing to its extreme durability and resistance against a variety of environmental conditions and thus can serve as a potential source for recurrent infection (Proctor et al., 1995; von Eiff et al., 2001; Kipp et al., 2003; Schröder et al., 2006a; reviewed in Garzoni and Kelley, 2009; Sendi and Proctor, 2009). SCVs of *S. aureus* even have been shown to survive and grow within host cell phagosomes (Schröder et al., 2006a; **Figure 1**, Map Item 4b). Also, the complementation of *rsb*U in laboratory strains restored activity of the alternative sigma-factor σB and led to intracellular growth of *S. aureus* within phagolysosomes of THP-1 phagocytes (Olivier et al., 2009). Contrasting these reports, staphylococcal growth has been described after pathogen translocation to the host cell cytoplasm (**Figure 1**, Map Item 4c): *S. aureus* strain Newman is able to escape the phagosome and persists within human monocyte-derived macrophages (hMDM) which resulted in host cell lysis on day 5 after infection (Kubica et al., 2008). The authors postulate that this survival within phagocytes might constitute a route for dissemination of staphylococcal infection. This is further corroborated by the identification of cytoprotective effects on macrophages after phagocytosis of *S. aureus*. Thus, the up-regulation of anti-apoptotic factors upon staphylococcal infection is responsible for extended phagocyte lifetime (Koziel et al., 2009). Both studies suggest that *S. aureus* might penetrate deeper into the tissue and even disseminate to different sites within "Trojan horse" phagocytes (Koziel et al., 2009). Survival within PMN is reported to depend on the accessory regulator Sar1, which was crucial to *S. aureus* survival inside spacious vacuoles, whereas *sar*$^-$ strains were localizing to so-called "tight vacuoles" (Gresham et al., 2000). Such different vacuoles can also be observed in non-professional phagocytes (Sinha and Fraunholz, 2010). The large vacuoles also are reminiscent of spacious *Listeria*-containing phagosomes, which were found to be non-acidified and non-degradative niches in macrophages (Birmingham et al., 2008). However, a more thorough characterization of the different vacuolar locations in intracellular *S. aureus* infections is lacking.

Staphylococcus aureus PHAGOSOMAL ESCAPE

Phagocytosed bacterial pathogens evade lysosomal killing, e.g., by disintegration of the organelle membrane in order to translocate into the host cell cytoplasm. *Listeria monocytogenes* co-opts the pore-forming toxin (PFT) listeriolysin O (LLO) and phospholipases (reviewed in Dramsi and Cossart, 2002; Schnupf and Portnoy, 2007), and Group A streptococci use the PFT streptolysin O (Hakansson et al., 2005). Phagosomal escape of *S. aureus* initially has been described by Bayles et al. (1998) and the *agr*-dependency of this process has been demonstrated (Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006; Kubica et al., 2008). The membrane destructive function of staphylococcal α-toxin thus suggested an involvement of the pore-former in phagosomal membrane disruption. The requirement for phagosomal escape and intracellular bacterial survival has been reported in CFT-1, a cystic fibrosis (CF) lung cell line (Jarry and Cheung, 2006) as well as in macrophages (Kubica et al., 2008; **Figure 1**, Map Item 5a). In CFT-1, *S. aureus* translocates into the cytoplasm in an α-toxin-dependent manner and the bacteria replicate within the

cytoplasm (Kahl et al., 2000; Jarry et al., 2008). However, in the LCSFN cell line complemented with the wild-type CF transmembrane conductance regulator, CFTR, α-toxin has no effect (Jarry and Cheung, 2006). Further, it has been demonstrated that neither α-toxin overexpressing strains (Lám et al., 2010) nor laboratory strains inducibly expressing α-toxin (Giese et al., 2009, 2011) are capable of releasing staphylococci into the host cell cytoplasm. By expression of the amphiphilic 26 amino-acid (AA) peptide δ-toxin in the non-cytotoxic laboratory strain *S. aureus* RN4220 *S. aureus* was capable of escape in rates similar to that of heterologously expressed LLO (Giese et al., 2011). δ-Toxin is encoded by the agr-effector RNAIII and is translated about 1 h after transcription of RNAIII (Balaban and Novick, 1995). It is capable of lysing bacterial protoplasts, lysosomes, lipid spherules, mitochondria, and erythrocytes *in vitro* in a temperature-independent manner. Its activity and mode of action is comparable to that of non-ionic detergents (Kreger and Bernheimer, 1971; Kreger et al., 1971; Rahal Jr., 1972; Kapral, 1976; reviewed in Verdon et al., 2009). δ-Toxin is encoded by RNAIII, the *agr* effector, and thus might constitute an immediate response to space limitation by phagosomal engulfment (**Figure 1**, Map Item 5b). However, membrane disruption by δ-toxin depended on the presence of the staphylococcal sphingomyelinase, β-toxin (Giese et al., 2011), which cleaves sphingomyelins (SM) to phosphorylcholine and ceramide moieties. δ-Toxin hardly binds to negatively charged phospholipids, binds strongly to liquid-disordered domains and poorly to cholesterol and sphingomyelin liquid-ordered raft domains (Pokorny et al., 2006). In one model, β-toxin thus may cleave SM to ceramides, which tend to accumulate in membrane microdomains. The hydrophobic nature of ceramide-rich domains thus might constitute regions of δ-toxin assembly, which eventually lead to target membrane permeabilization (**Figure 2**). *S. aureus* strain USA300 LAC, however, is escape proficient yet does not encode a functional β-toxin due to lysogeny of a β-converting phage (Diep et al., 2006). We thus have to hypothesize alternative factors that can act in phagosomal escape, such as a variety of lipases encoded by the staphylococcal genome or phenol-soluble modulins (PSMs; see below). Alternatively, prophages might be lost, e.g., during exposure to phagosomal reactive oxygen species, and then might contribute to phagosomal escape. A similar activation mechanism is used by *Streptococcus pneumoniae*, which produces hydrogen peroxide and thereby lyses *S. aureus* by a "remote control" prophage activation (Selva et al., 2009).

For complete hemolysis of erythrocytes in sheep blood agar plates either a shift to 4°C (hot–cold hemolysis), osmotic stress, or synergistic toxins are necessary. δ-Toxin belong to the class of PSMs, which initially had been shown to be present within a hot-phenol extraction of *S. epidermidis* extracts (Otto et al., 2004) and only later had been identified in *S. aureus* by sequence homology (Wang et al., 2007). The major groups of PSMs are divided into two operons referred to as PSMα and PSMβ. The PSMα operon is comprised of four open reading frames (ORFs) with approximately 20 AA. PSMβ encodes two ORFs, which are about 40 AA in length (Wang et al., 2007). The expression of PSMβ has

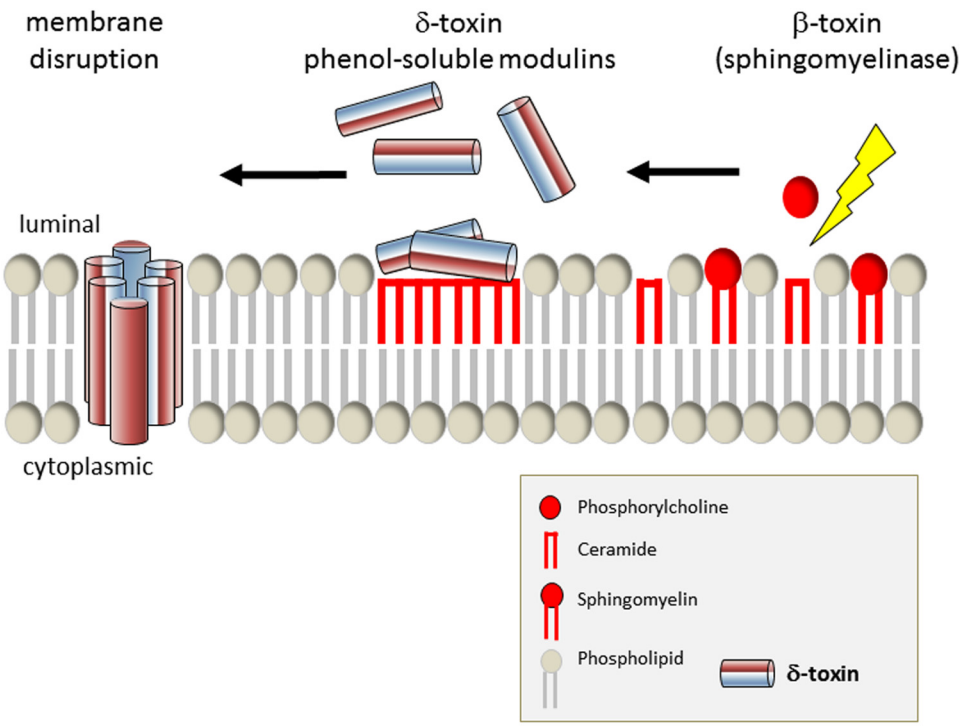

FIGURE 2 | Model of phagosomal escape by *S. aureus* by synergistic action of δ-toxin and β-toxin. After cleavage of sphingomyelin to ceramide and phosphocholine, δ-toxin is capable of interacting more efficiently with the outer leaflet of the eukaryotic plasma membrane. δ-Toxin accumulates in the resulting hydrophobic ceramide membrane domains and eventually permeabilizes the target membrane.

been demonstrated to result in phagosomal escape in a gain-of-function study (Giese et al., 2011). Just like δ-toxin, PSMα and PSMβ are *agr*-dependently expressed. It recently was shown that the staphylococcal *agr* system is confinement induced and thus comprises not only a quorum sensing system but also a diffusion sensing system active at the single cell level (Carnes et al., 2009). δ-Toxin and PSMs are hence produced upon phagosomal confinement and thus also low numbers of endocytosed staphylococci should be able to mount a toxin response in order to avoid lysosomal killing. Whereas gain of function assays demonstrated PSMβ activity in phagosomal escape (Giese et al., 2011), *in vivo* studies suggests a prominent role of PSMα, with the third ORF of the operon, PSMα3, being the most virulent principle (Wang et al., 2007). Despite these incongruities a common theme emerges: amphiphilic PSMs are involved in phagosomal escape. One possibility to explain the observed differences is the postulation of alternative pathways of phagosomal escape mechanisms for *S. aureus*.

Molecular patterns of pathogens that reside in the cytoplasm of their hosts are detected by nucleotide-binding and oligomerization domain proteins NOD1 and NOD2, which detect γ-D-glutamyl-diaminopimelic acid and muramyl dipeptide, peptidoglycan components of Gram-negative and Gram-positive bacteria. Peptidoglycan binding leads to a conformation change in NOD proteins, which initiates the recruitment of ubiquitin ligases and kinases and ultimately results in nuclear translocation of NFκB and activator protein 1 and expression of inflammatory genes (reviewed in Strober et al., 2006). NOD2 signaling upon *S. aureus* infection has been found to induce cytokine production (Kapetanovic et al., 2007) and thus might contribute to induction of inflammation, e.g., in the lung (Gomez and Prince, 2008; **Figure 1**; Map Item 6). NOD2-deficient mice exhibit a delayed inflammatory response and impaired bacterial clearance after infection with *S. aureus* (Hruz et al., 2009). α-Toxin facilitates NOD2-dependent recognition of *S. aureus* muramyl dipeptide (Hruz et al., 2009), possibly by interfering with phagosomal integrity.

The observation that *S. aureus* can translocate into the cytoplasm of host cells and grow without an immediately ensuing cell death (e.g., Kubica et al., 2008) illustrates that phagosomal escape is not identical with cytotoxicity. Thus, the link between phagosomal escape and host cell death still needs to be elucidated.

Staphylococcus aureus-INDUCED HOST CELL DEATH

The classical separation of host cell death into programed cell death (PCD; apoptosis) and accidental cell death or necrosis has long been superseded after the identifying a large variety of cell death mechanisms (for a reviews, see Taylor et al., 2008; Ting et al., 2008a; Bergsbaken et al., 2009). Many of which are defined by key factors that allow the assessment of death pathway activation following bacterial infection (e.g., reviewed in Rudel et al., 2010), although the synchronous activity of virulence factors from the staphylococcal arsenal renders unequivocal correlations between toxin and associated mode of cell death a daunting task. When virulent *S. aureus* strains are added to host cells in tissue culture, host cell death occurs via mechanisms that have been mainly identified as apoptotic (Bayles et al., 1998; Menzies and Kourteva, 1998, 2000; Wesson et al., 1998, 2000; Kahl et al., 2000; Nuzzo et al., 2000; Tucker et al., 2000; Haslinger et al., 2003; Genestier et al., 2005; **Figure 1**, Map Item 7). α-Toxin is both required and sufficient for induction of leukocyte cell death, either apoptotic or necrotic (Bantel et al., 2001; Essmann et al., 2003; Haslinger et al., 2003). Leukocytes are sensitive to staphylococcal α-toxin. Already low toxin doses induce apoptosis accompanied by a breakdown of the mitochondrial transmembrane potential (Bantel et al., 2001; Haslinger et al., 2003). A recent study suggests that caspase-2 acts as an initiator caspase during cell death of non-professional phagocytes. Caspase 2 was induced by potassium efflux due to pore-forming toxins such as staphylococcal alpha-toxin and aerolysin (Imre et al., 2012). By contrast, high doses induce necrotic cell death (Walev et al., 1993; Bantel et al., 2001; Essmann et al., 2003; Haslinger et al., 2003). Human endothelial cells are virtually insensitive to the action of *S. aureus* α-toxin, however, comparatively low numbers of *S. aureus* cells with a combined invasive and strongly hemolytic phenotype readily induce apoptotic cell death in HUVEC (Haslinger-Löffler et al., 2005). This suggests that cell death mechanisms are activated from within their intracellular location. The effect is highly specific, since fixed, non-hemolytic, rifampin-treated or weakly invasive staphylococci are not cytotoxic toward endothelial cells (Haslinger-Löffler et al., 2005). Multiple other studies indicate that *S. aureus* might kill its other types of host cells from within (Bayles et al., 1998; Menzies and Kourteva, 1998; Wesson et al., 1998; Nuzzo et al., 2000; Tucker et al., 2000; Krut et al., 2003; Haslinger-Löffler et al., 2005; Chatterjee et al., 2008; Jarry et al., 2008; Kubica et al., 2008; Lám et al., 2010). The virulence factors required for *S. aureus*-induced apoptosis in endothelial cells depend on *agr* and the alternative stress–response sigma-factor σB (Wesson et al., 1998; Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006; Kubica et al., 2008), but mainly seem to be independent of SarA (Haslinger-Löffler et al., 2005; Jarry et al., 2008) – although there are contradicting results on the involvement of SarA (Wesson et al., 1998).

Phage-encoded Panton-Valentine leukocidin (PVL) predominantly destroys leukocytes, although there also is some species specificity for human and rabbit PMN (Loffler et al., 2010). In PMN, PVL induced a rapid caspase-9/3-dependent cell death *in vitro* (Genestier et al., 2005). The authors further identified to a mitochondrial localization of the PVL toxin. Isolated mitochondria were permeabilized for pro-apoptotic factors such as cytochrome *c* (CytC) by PVL which suggested that PVL is able to create pores in the mitochondrial outer membrane and thus triggers a Bax-independent mitochondrial pathway of host cell apoptosis (Genestier et al., 2005; **Figure 1**, Map Item 8). During the intrinsic pathway of apoptosis release of CytC from injured mitochondria leads to activation of apoptosis-activating factor-1 (APAF-1). Oligomerizing CytC/APAF-1 recruits and subsequently activates pro-caspase 9. Caspase 9 then proteolytically activates effector caspases, which finally cleave their respective substrates resulting in membrane blebbing, and DNA fragmentation (reviewed in Rudel et al., 2010). Similarly, α-toxin has been described to activate caspases via the intrinsic death pathway (Bantel et al., 2001; Haslinger et al., 2003) independently of death receptor signaling (CD95/Fas/APO-1). Bcl-2 overexpressing Jurkat cells were protected from α-toxin mediated cell death (Bantel et al.,

2001; Essmann et al., 2003) although recent result suggest that the function of Bcl-2 in autophagy might be responsible for the observed phenomena (Schnaith et al., 2007; Mestre et al., 2010).

Interestingly, *S. aureus* also seems to be able to exert anti-apoptotic host cell responses. The pathogen was shown to suppress staurosporine-induced apoptosis in hMDM although early apoptotic features such as phosphatidylserine display at the outer plasma membrane leaflet, reduced mitochondrial membrane potential, CytC release, and caspase-3 activation are still observed (Koziel et al., 2009). *S. aureus* infection strongly upregulated the expression of mitochondrial membrane potential stabilizing Bcl-2 and Mcl-1 gene products. Since also heat-killed *S. aureus* was able to suppress apoptosis in the host cells staphylococcal products such as lipoteichoic acid or peptidoglycan might activate the macrophages through intracellular pattern recognition sensors such as nucleotide oligomerization domain receptors (NOD; Kapetanovic et al., 2007; Koziel et al., 2009).

Aside from apoptosis *S. aureus* is also able to induce pyronecrosis (**Figure 1**; Map Item 9). There, caspase-1 is activated as part of an inflammasome, which further consists of NOD-like receptor protein 3 (NLRP3) and the adaptor protein, apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (ASC). *S. aureus* can function as a stimulus for NLRP3 (Munoz-Planillo et al., 2009), however, the molecular identity of the stimulating signal is not known thus far (Mariathasan et al., 2006; Ting et al., 2008b; Wright and Nair, 2010). Lysosomal permeabilization is one NLRP3-activating principle, wherein release of the lysosomal protease cathepsin B into the cytoplasm contributes to NLRP3 activation (reviewed in Willingham and Ting, 2008; Bergsbaken et al., 2009). Thus, it seems likely, that lysosomal rupture or permeabilization by *S. aureus* toxins releases cathepsin which then activates the inflammasome. Indeed staphylococcal α, β, and γ-hemolysins have been shown to be important activation of the NLRP3 inflammasome (Craven et al., 2009; Munoz-Planillo et al., 2009; Kebaier et al., 2012). The pore-forming α- and γ-toxins of *S. aureus* permeabilize membranes and thus might be involved in cathepsin release and subsequent inflammasome activation. α-Toxin further is known to permeabilize the plasma membrane for potassium ions. In turn, potassium efflux activates the inflammasome (Petrilli et al., 2007). β-Toxin has been shown to be involved in phagosomal escape (Giese et al., 2011) and thus also might act in cathepsin release.

Staphylococcus aureus AND THE SUBVERSION OF AUTOPHAGY

Autophagy sequesters cytoplasmic contents via an isolation membrane. Engulfment of cargo by the so-called phagophore forms double membrane-bound autophagic vesicles that eventually fuse with lysosomes to yield autolysosomes. Autophagy serves for degradation of organelles or self-digestion during nutrient limiting conditions such as starvation and is generally thought to constitute a cellular survival mechanism. During bacterial infections autophagy disposes of leaky vesicles or intracellular bacteria, however, bacterial pathogens have found multiple ways to subvert autophagy (reviewed in Dorn et al., 2002; Kirkegaard et al., 2004; Levine, 2005; Campoy and Colombo, 2009; Orvedahl and Levine, 2009; Ogawa et al., 2011).

Staphylococcus aureus was shown to interact with autophagosomes in a rather unique way (Schnaith et al., 2007) when compared to autophagy-subvertive strategies of other bacterial pathogens (Campoy and Colombo, 2009; Ogawa et al., 2011). *S. aureus* inhibits fusion of phagosomes with lysosomes. It permeabilizes HeLa phagosomes by a mechanism dependent on *Staphylococcus*-secreted toxins. The leaky phagosomes are targeted by autophagy and within autophagosomes *S. aureus* replicates. Eventually the bacteria escape from their intracellular confinement into the host cell cytoplasm in an *agr*-dependent manner (Schnaith et al., 2007; **Figure 1**, Map Item 9). Finally, host cell death is induced, which is independent of a caspase activation cascade but was blocked by overexpression of anti-autophagic Bcl-2. Induction of autophagy by rapamycin resulted in an increased number of recovered colony-forming units, whereas inhibition with wortmann in reduced the colonies recovered from the intracellular environment. *S. aureus*-induced autophagy resulted in a vacuolization of the host cell cytoplasm ("Swiss cheese phenotype"). *agr*-deficient *S. aureus* fail to induce autophagy, which results in maturation of bacteria-containing phagosomes followed by lysosomal degradation of the pathogens. α-Toxin is able to permeabilize membranes for Ca^{2+}, an inducer of autophagy (Brady et al., 2007) and autophagy targets phagosomes perforated by α-toxin in Chinese hamster ovary cells (Mestre et al., 2010). Whereas latter observation is in line with the results obtained by Schnaith et al. (2007) it contrasts findings that α-toxin is not sufficient to permeabilize HeLa phagosomes (Jarry and Cheung, 2006; Giese et al., 2009; Lám et al., 2010).

CONCLUSION

With about 200–300 virulence factors, *S. aureus* is able to exert a multitude of effects upon its eukaryotic host cells. Although many details have emerged through ground-breaking and recent research, only a minority of pathogenicity factors of *S. aureus* has been functionally annotated to date. Particularly the assessment of intracellular staphylococcal virulence is often hampered by the difficulty to discriminate between toxin effects that result from extracellular or intracellular bacteria, although inducible toxin-expressing might provide valuable tools for molecular dissection of host–pathogen interactions.

With our advancing knowledge of cell death mechanisms ground-breaking experiments will have to be re-evaluated in order to understand the mechanisms of *S. aureus*-induced host cell killing. When comparing experimental research originating from different labs the influence of a body of factors should be taken into account that could lead to different infection outcomes:

Staphylococcus aureus strain used for infection, its growth phase at the time of infection (and hence the bacterial growth medium), as well as MOI are important, whereas on the host side the cell type and hence the protein profile will drastically influence infection outcome by altering host cell susceptibility to bacterial toxins, response to pathogen-associated molecular patterns, expression of receptors, caspases, and other host factors.

In order to identify activities of single toxins or virulence factors gain-of-function studies can be useful, e.g., using the a pathogenic *S. carnosus* as toxin delivery vehicle. However, such strategies

will not easily identify pathways during which an orchestrated interplay of multiple virulence factors is required. However, novel high-throughput sequencing technologies of transposon insertions (Gawronski et al., 2009; van Opijnen et al., 2009) open up new possibilities for analysis of genome-wide mutant libraries of clinically relevant strains. Using such novel tools we will be able to address a lot of open questions with regard to intracellular staphylococcal infections: do different adhesins result in employment of different uptake mechanisms into non-professional phagocytes as these would subsequently result in different infection outcomes? What is the nature of tight and spacious vacuoles (Gresham et al., 2000) that are occupied by, for example, *sarA*− and *sar*A+ *S. aureus*, respectively? Which alternative pathways for phagosomal escape do exist? Which modes of cell death are activated by a single strain in different host cell types or different strains in a single host cell line? There is still a lot to learn about the versatile facultative intracellular pathogen, *Staphylococcus aureus*.

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Deciphering mechanisms of staphylococcal biofilm evasion of host immunity

Mark L. Hanke and Tammy Kielian*

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Jeffery A. Hobden, LSU Health Sciences Center New Orleans, USA
Pietro Speziale, University degli Studi di Pavia, Italy

***Correspondence:**
Tammy Kielian, Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198-5900, USA.
e-mail: tkielian@unmc.edu

Biofilms are adherent communities of bacteria contained within a complex matrix. Although host immune responses to planktonic staphylococcal species have been relatively well-characterized, less is known regarding immunity to staphylococcal biofilms and how they modulate anti-bacterial effector mechanisms when organized in this protective milieu. Previously, staphylococcal biofilms were thought to escape immune recognition on the basis of their chronic and indolent nature. Instead, we have proposed that staphylococcal biofilms skew the host immune response away from a proinflammatory bactericidal phenotype toward an anti-inflammatory, pro-fibrotic response that favors bacterial persistence. This possibility is supported by recent studies from our laboratory using a mouse model of catheter-associated biofilm infection, where *S. aureus* biofilms led to the accumulation of alternatively activated M2 macrophages that exhibit anti-inflammatory and pro-fibrotic properties. In addition, relatively few neutrophils were recruited into *S. aureus* biofilms, representing another mechanism that deviates from planktonic infections. However, it is important to recognize the diversity of biofilm infections, in that studies by others have demonstrated the induction of distinct immune responses during staphylococcal biofilm growth in other models, suggesting influences from the local tissue microenvironment. This review will discuss the immune defenses that staphylococcal biofilms evade as well as conceptual issues that remain to be resolved. An improved understanding of why the host immune response is unable to clear biofilm infections could lead to targeted therapies to reverse these defects and expedite biofilm clearance.

Keywords: *S. aureus*, *S. epidermidis*, biofilm, macrophage, neutrophil, immune evasion, alternative macrophage activation, fibrosis

INTRODUCTION

Biofilms are adherent communities of bacteria contained within a complex matrix. From a clinical standpoint, biofilm infections of native tissues or medical devices represent a serious therapeutic challenge, since organisms are typically recalcitrant to conventional antibiotics (Stewart and Costerton, 2001; Donlan and Costerton, 2002). Medical device-related infections are typified by high morbidity, with their clinical management often requiring device removal (Garvin and Hanssen, 1995; Morscher et al., 1995; Replacement et al., 1995; Lew and Waldvogel, 1997). In addition, despite prolonged therapy, the failure rate associated with infected devices is high, primarily due to their failure to be cleared by conventional antibiotics (Schoifet and Morrey, 1990; Wilson et al., 1990; Burger et al., 1991; Hartman et al., 1991; Rasul et al., 1991; Tsukayama et al., 1991; Brandt et al., 1997). To date, staphylococcal species remain one of the major causes of both health care-associated (HA) as well as community-associated (CA) infections. *Staphylococcus epidermidis* (*S. epidermidis*) is a frequent etiological agent of biofilm infections on medical devices, including indwelling catheters and prostheses (Fitzpatrick et al., 2005a; Otto, 2008; Fey, 2010), whereas *Staphylococcus aureus* (*S. aureus*) is commonly associated with tissue infections, such as endocarditis (Fitzsimmons et al., 2010) and osteomyelitis (Zuluaga et al., 2006). With the emergence of drug-resistant strains in the 1960s, primarily methicillin-resistant *S. aureus* (MRSA), this ubiquitous pathogen is becoming an even greater therapeutic challenge. Consequently, based on their chronicity, debilitating nature, and economic impact, biofilm infections are of paramount significance in modern medicine. Therefore, it is imperative that we understand the mechanisms whereby staphylococcal biofilms alter immune recognition pathways to devise novel therapies for treating these devastating infections.

STAPHYLOCOCCAL BIOFILMS AND TOLL-LIKE RECEPTORS (TLRs)

Cells of the innate immune system recognize highly conserved pathogen-associated molecular patterns (PAMPs) that are expressed by large groups of microorganisms (Kawai and Akira, 2011). These conserved bacterial motifs are identified by a series of germ-line encoded receptors of the innate immune system termed pattern recognition receptors (PRRs). Toll-like receptors (TLRs) represent one PRR class expressed by cells of the innate immune system that mediate cellular activation in response to PAMPs (Kaisho and Akira, 2004; O'Neill, 2004). Thirteen TLRs have been described in the human and 10 in the mouse, each conferring responsiveness to various infectious agents as well as

some endogenous ligands (Kopp and Medzhitov, 2003; Kawai and Akira, 2011). Staphylococcal species harbor a complex cell wall containing PAMPs that represent TLR2 ligands, namely lipoteichoic acid (LTA) and peptidoglycan (PGN) (Morath et al, 2002; Dziarski, 2003; Weber et al., 2003). PGN is released during normal bacterial growth as well as from dying organisms within staphylococcal biofilms (Mercier et al., 2002; Cerca et al., 2006; Moscoso et al., 2006; Qin et al., 2007; Strunk et al., 2010). Likewise, polysaccharide intercellular adhesin (PIA) and phenol-soluble modulin (PSM) expression in *S. epidermidis* promotes biofilm formation and can be recognized by TLR2 (Hajjar et al., 2001; Stevens et al., 2009). Staphylococcal lipoproteins (Lpp), a large family of membrane-anchored proteins, have also been identified as potent TLR2 ligands (Hashimoto et al., 2006a,b; Kurokawa et al., 2009). Some reports indicate that Lpp contaminating LTA and PGN preparations is responsible for most of the observed TLR2 stimulatory action (Travassos et al., 2004; Hashimoto et al., 2006a,b; Kurokawa et al., 2009). However, a synthetic LTA analog devoid of lipoproteins has also been shown to possess immune activity (Morath et al, 2002; Deininger et al., 2003). Regarding the role of PGN as a TLR2 agonist, a subsequent report demonstrated that the solubility characteristics of purified PGN dictated whether it was capable of triggering TLR2 (Dziarski and Gupta, 2005). Importantly, the ability of PGN to activate TLR2 can be destroyed by certain purification methods, leading to discrepancies in potency for TLR2 activation. Therefore, the immunostimulatory role of LTA and the innate immune receptor specificity of staphylococcal PGN for TLR2 remains an issue of debate. TLR9 is an intracellular receptor that recognizes unmethylated CpG motifs characteristic of bacterial DNA (Hemmi et al., 2000; Bauer et al., 2001). Mammalian DNA is methylated on guanine residues, which serves as a critical self vs. non-self discriminator. Upon phagocytosis and digestion of bacteria in the phagosome, bacterial DNA is liberated and engages TLR9. However, it is well recognized that extracellular DNA (eDNA) can also trigger TLR9-dependent activation, which is relevant to biofilms due to the extensive amount of eDNA within the matrix (Whitchurch et al., 2002; Allesen-Holm et al., 2006; Rice et al., 2007). Innate immune cells, including macrophages, neutrophils (PMNs), and dendritic cells, express TLR2 and TLR9 and are competent to respond to both Lpp/PGN/LTA and eDNA, respectively, which culminates in the induction of a wide array of classical pro-inflammatory mediators and bactericidal activity (Takeuchi et al., 1999; Bauer et al., 2001; Hertz et al., 2001; Jones et al., 2001; Kirschning and Schumann, 2002; Hayashi et al., 2003).

The role for TLRs in mediating innate immune recognition of staphylococcal species during planktonic growth has been well-characterized (Yoshimura et al., 1999; Takeuchi et al., 2000; Mullaly and Kubes, 2006; Stevens et al., 2009; Strunk et al., 2010). However, recent reports have determined that one mechanism utilized by biofilms to evade host immunity is by circumventing TLR2 and TLR9 recognition (**Figure 1**) (Bernthal et al., 2011; Thurlow et al., 2011). This agrees with the finding that patients bearing mutations which inactivate TLR2 have no increased risk of developing post-arthoplasty *S. aureus* infections (El-Helou et al., 2011). In contrast, the ability of *S. aureus* biofilms to evade TLR9 recognition differs from *P. aeruginosa* biofilms, since eDNA has been demonstrated to be a major proinflammatory stimulus during *P. aeruginosa* biofilm growth (Fuxman Bass et al., 2010). This emphasizes the importance of bacterial species and growth state in dictating whether innate immune sensor mechanisms will be effective at clearing infection. It is also possible that *S. aureus* biofilms may be recognized by alternative PRRs besides TLR2 or TLR9. For example, eDNA could also be sensed by other intracellular PRRs such as AIM2 or DNA-dependent activator of IFN-regulatory factors (DAI) (Vilaysane and Muruve, 2009; Hornung and Latz, 2010). In addition, the degradation product of staphylococcal PGN, muramyl dipeptide, can be sensed by the cytoplasmic PRR nucleotide-binding oligomerization domain-containing protein 2 (NOD2) to elicit proinflammatory mediator release (Girardin et al., 2003; Volz et al., 2010). The mechanism(s) responsible for TLR2/TLR9 evasion by *S. aureus* biofilms are not known but could also be explained by ligand inaccessibility. Biofilms are encased within a complex three-dimensional structure with few free bacteria exposed at the outer surface, thus avoiding detection by PRRs expressed on the surface of phagocytes (Thurlow et al., 2011). Likewise, complex polysaccharide polymers that are known components of the biofilm matrix (Flemming and Wingender, 2010), may interfere with optimal engagement of potential ligands with TLRs. Understanding the receptor repertoire triggered by staphylococcal biofilms may enable the selective targeting of these molecules to facilitate pathogen elimination and/or render the biofilm more sensitive to conventional antibiotic therapies.

ROLE FOR INTERLEUKIN-1β (IL-1β) AND OTHER MyD88-DEPENDENT PATHWAYS IN IMMUNE RECOGNITION OF STAPHYLOCOCCAL BIOFILMS

In contrast to a lack of TLR2 and TLR9 involvement during staphylococcal biofilm infections, a recent report has revealed a role for IL-1β in controlling early bacterial burdens in a post-arthroplasty *S. aureus* biofilm infection model (Bernthal et al., 2011). Specifically, biofilm formation was enhanced in IL-1β KO mice concomitant with decreased PMN recruitment; however, PMN infiltrates were visualized by H&E staining in this study and quantitative assessment by flow cytometry was not performed. It is worth noting that PMN influx is minimal in a model of s.c. catheter-associated *S. aureus* biofilm infection (**Figure 2**), which may represent differences in the biofilm locale or degree of planktonic infection surrounding the biofilm. Studies from our laboratory have recently investigated the role of myeloid differentiation factor 88 (MyD88), the common downstream adaptor utilized by both the IL-1 receptor (IL-1R) and TLRs (Hanke and Kielian, in revision), in regulating *S. aureus* biofilm growth. MyD88 signaling culminates in NF-κB-mediated transcription and until our work, nothing was known regarding the role of MyD88- or NF-κB-dependent signaling during staphylococcal biofilm infections. Utilizing a well-characterized model of catheter-associated *S. aureus* biofilm infection (Rupp et al., 1999; Cassat et al., 2007; Thurlow et al., 2011), MyD88 KO mice displayed significant increases in bacterial burdens on catheters as well as surrounding tissues throughout the course of infection compared to WT animals. Additionally, *S. aureus* titers

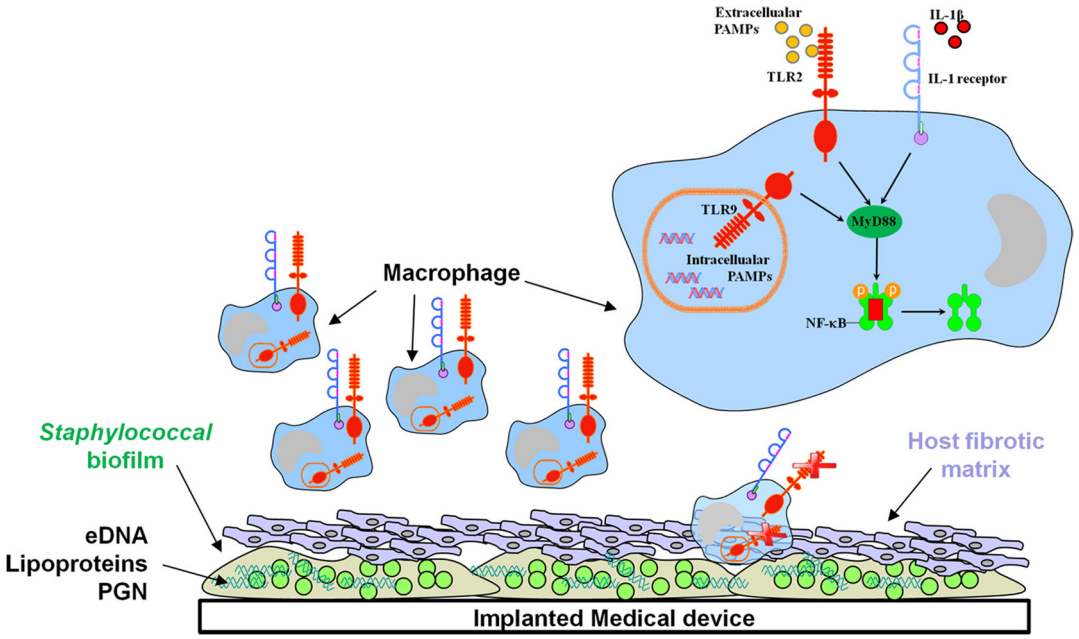

FIGURE 1 | The signaling adaptor MyD88 is pivotal for regulating biofilm development. Recent studies have revealed important roles for IL-1β and MyD88-dependent pathways in controlling bacterial burdens during *S. aureus* biofilm growth, whereas biofilms evade TLR2/TLR9 recognition. The macrophage is a major cellular infiltrate during device-associated biofilm infections; however, current evidence indicates that macrophage microbicidal properties are inhibited by the biofilm.

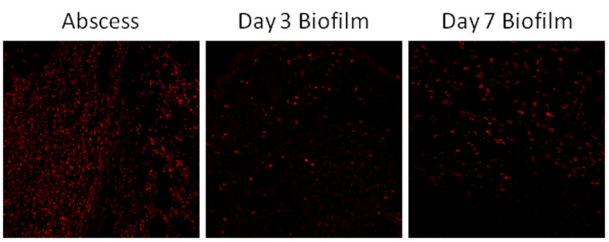

FIGURE 2 | Neutrophil recruitment into *S. aureus* biofilms *in vivo* is limited. C57BL/6 mice were infected with 5×10^5 CFU USA300 LAC either in the lumen of surgically implanted catheters or s.c. in the absence of any indwelling device to establish biofilm and abscess infections, respectively. Animals were sacrificed at days 3 or 7 following *S. aureus* exposure, whereupon tissues surrounding infected catheters or s.c. injection sites were collected and subjected to immunofluorescence staining for the neutrophil-specific marker Ly-6G (red) and visualized by confocal microscopy (original magnification ×20).

were significantly elevated in the heart and kidney of MyD88 KO mice, demonstrating a role for MyD88 in bacterial containment at the site of biofilm infection. Furthermore, immunofluorescence staining revealed an increased fibrotic response associated with biofilms in MyD88 KO animals, which coincided with increased recruitment of alternatively activated M2 macrophages (Hanke and Kielian, in revision). Collectively, these studies reveal a role for IL-1β, potentially mediated through MyD88 signaling, in *S. aureus* biofilm containment (**Figure 1**). Although it is evident that these pathways influence biofilm development, they are not sufficient to eradicate staphylococcal biofilms, since these infections persist in an immunocompetent host. Instead, tonic MyD88/IL-1β action could account for the finding that biofilm burdens remain relatively constant throughout the course of infection (Thurlow et al., 2011), which likely represents an equilibrium between bacterial dispersal from the biofilm, clearance of planktonic organisms (presumably via MyD88/IL-1β pathways), and continued biofilm growth.

Although it is clear that IL-1β and MyD88-dependent pathways can influence *S. aureus* biofilm development, the precise molecular pathways remain to be defined. In particular, numerous receptors utilize MyD88, including multiple TLRs, IL-1R, IL-18R, and IL-33R and it is uncertain which is most relevant or alternatively, if multiple pathways are triggered and act in an additive/synergistic manner. Based on the findings with IL-1β-deficient mice (Bernthal et al., 2011), it is likely that IL-1RI signaling plays a key role, but this remains to be determined. Importantly, IL-1β is produced as an inactive precursor that requires proteolytic cleavage for its release. This process requires the coordinate induction of a two-signal model mediated by TLRs (signal 1) and Nod-like receptors (NLR; signal 2), the latter of which forms the inflammasome that is responsible for processing the inactive forms of IL-1β and IL-18 into their mature states (Craven et al., 2009; Schroder and Tschopp, 2010). Signal 1 is elicited by TLR signaling though MyD88 that triggers the transcriptional induction of pro-IL-1β and pro-IL-18, which remain inactive until a second signal is initiated. Signal 2 has been shown to originate within the host cell cytoplasm and is mediated by the sensing of a danger signal by members of the NLR family of intracellular PRRs. With regard to staphylococcal infections, signal 2 can be triggered by intact bacteria or purified α- and γ-hemolysins, which lead to K^+ efflux from the cell (Craven

et al., 2009; Hanamsagar et al., 2011). Currently, we know nothing about what receptors upstream of MyD88 are required for staphylococcal biofilm recognition or signals that lead to IL-1β transcription or proteolytic processing. These issues will be important to evaluate in the quest to unveil promising therapeutic targets for staphylococcal biofilm eradication.

Signals emanating from TLRs, IL-1R, and numerous cytokine receptors trigger activation of the transcription factor NF-κB. The NF-κB family regulates the expression of numerous genes associated with proliferation, differentiation, and cell death, as well as innate and adaptive immune responses, which represents an attractive target for exploitation by staphylococcal species (Rahman and McFadden, 2011). Indeed, NF-κB signaling is the most frequent intracellular pathway targeted by numerous microbes to subvert the immune response (Finlay and McFadden, 2006). *S. epidermidis* PIA-, accumulation-associated protein (Aap)-, or extracellular matrix-binding protein (Embp)-dependent biofilms were protected from macrophage phagocytic uptake and induced minimal NF-κB activation and IL-1β production (Schommer et al., 2011). Interestingly, similar to our previous results where physical disruption of the biofilm structure allowed for macrophage phagocytosis, mechanical dispersal of *S. epidermidis* biofilms partially restored NF-κB activation (Schommer et al., 2011; Thurlow et al., 2011). Together, these results indicate that staphylococcal biofilms possess mechanisms to circumvent macrophage phagocytic uptake and limit proinflammatory activity by attenuating NF-κB activity.

Another study has shown that inflammatory cytokines were augmented during early *S. aureus* biofilm infection, whereas anti-inflammatory infiltrates predominated later. Based on these findings, the authors proposed that the acute inflammatory response induced tissue damage to facilitate biofilm growth (Prabhakara et al., 2011). However, the infectious inoculum utilized was rather high and the implanted device was coated with bacteria prior to surgical implantation, which might account for the initial proinflammatory response if a significant degree of planktonic organisms were present prior to mature biofilm formation. Since it takes time for biofilm maturation to occur, the anti-inflammatory response that was reported at later time points might be a better indication of the immune response to a mature biofilm, which is supported by other studies (Prabhakara et al., 2011; Thurlow et al., 2011). However, differences in the sites of biofilm infection, methods of inoculating foreign devices (i.e., infecting devices prior to insertion or inoculation directly into the implanted device *in vivo*), and infectious inoculums may influence the nature of the host immune response to staphylococcal biofilms. The relative impact of each of these factors remains to be determined.

ANTIMICROBIAL PEPTIDES AND STAPHYLOCOCCAL BIOFILMS

The innate immune response to numerous microbial infections, including staphylococcal species, is mediated, in part, by pre-existing soluble factors that recognize and destroy pathogens or target them for killing by macrophages and PMNs. Two examples are antimicrobial peptides (AMPs) and the complement pathway Medzhitov and Janeway, 2000; Medzhitov, 2007, both of which are induced immediately following infection and represent a first line of defense against invading microbes. The complement system consists of blood-derived proteins that are activated in an enzymatic cascade to eliminate pathogens from the host. PMN killing of planktonic *S. epidermidis* is complement-dependent and biofilm formation has been shown to trigger complement activation in a PIA-dependent manner (Clark and Easmon, 1986; Kristian et al., 2008; Fredheim et al., 2011). However, *S. epidermidis* biofilms impair IgG and complement deposition, resulting in increased resistance to opsonization and phagocyte-mediated killing (Kristian et al., 2008; Fredheim et al., 2011). AMPs are universal innate defense molecules in humans and other higher organisms, which despite co-evolution with bacteria have retained their efficacy, as bacteria have yet to develop wide-spread resistance. These peptides show narrow or broad-spectrum activity against bacteria, fungi, viruses, and/or parasites. The net-positive charge, amphipathicity, and small size of AMPs allow for the disruption of microbial membranes and also inhibit cell wall, nucleic acid, and protein biosynthesis (Yeaman and Yount, 2003; Brogden, 2005). Currently, only a few AMPs have shown efficacy against *S. aureus* biofilms by either inhibiting biofilm formation or down regulating the expression of genes involved in biofilm development (Lopez-Leban et al., 2010; Dean et al., 2011). Recent collaborative studies from our laboratory have identified a synthetic AMP, DASamP1, that selectively kills MRSA and prevents biofilm formation *in vivo* (Menousek et al., 2012). Although AMPs have not been extensively explored for their anti-biofilm activity, these initial studies suggest that this class of antimicrobials deserves attention and could conceivably exert additive/synergistic effects with other approaches for the treatment of staphylococcal biofilm infections.

INNATE CELLULAR IMMUNITY

To date, most studies investigating innate immune responses to biofilms have been performed with *P. aeruginosa* and *S. epidermidis*, where PMNs have been shown to phagocytose biofilm-associated bacteria and produce oxidative bursts, albeit at reduced levels compared to planktonic bacteria (Jensen et al., 1990; Jesaitis et al., 2003; Walker et al., 2005; Chandra et al., 2007; Kristian et al., 2008; Graves et al., 2010). Neutrophils represent a first line of cellular defense against bacterial infections and possess a potent arsenal of bactericidal compounds, including defensins, cathelicidins, and lysozyme (Nathan, 2006; Nauseef, 2007). In terms of their bactericidal activity, PMNs are most notable for their ability to produce large amounts of reactive oxygen intermediates (ROI) catalyzed by NADPH oxidase. In addition to ROI production, PMNs can also secrete several proinflammatory cytokines, including TNF-α and IL-1β as well as chemokines, such as CXCL2 (macrophage inflammatory protein-2; MIP-2) and CXCL1 (KC, keratinocyte-derived chemokine; both are functional mouse homologs of IL-8) and CCL3 (MIP-1β) (Cassatella, 1995; Witko-Sarsat et al., 2000; Nathan, 2006). Although PMNs exert *S. aureus* bactericidal activity under planktonic conditions (Fournier and Philpott, 2005; Graham et al., 2007), the direct role of these cells in modulating *S. aureus* biofilm growth has not yet been examined. Although a previous report had suggested that PMNs were competent to invade a *S. aureus* biofilm, this study

was performed with peripheral blood leukocytes that represented a mixed cell population of PMNs, monocytes, and T and B lymphocytes (Leid et al., 2002). Therefore, the dynamics of PMN interactions with biofilms could not be definitively determined. Interestingly, this study also detected mammalian cytokine production in biofilm-conditioned supernatants, which may be explained by interference of *S. aureus* protein A, a MSCRAMM that binds the Fc portion of immunoglobulins, non-specifically binding to capture/detection antibodies in the ELISA assay. More recent studies using 85–95% pure PMN populations suggested that both *S. aureus* and *S. epidermidis* biofilms are phagocytosed by PMNs albeit to different extents, with *S. aureus* being more susceptible to phagocytic uptake (Günther et al., 2009; Graves et al., 2010; Meyle et al., 2010). However, it is important to note that these studies evaluated immature *S. aureus* biofilms grown under what might be considered sub-optimal conditions, such as continuous shaking and on non-coated surfaces (Wagner et al., 2011). Therefore, the thickness and complexity (i.e., presence of secondary tower structures) of biofilms should be considered when assessing the degree of phagocytosis by various immune cell populations.

As previously mentioned, PMN infiltrates were reduced during post-arthroplasty *S. aureus* biofilm infection in IL-1β deficient mice; however, the functional importance of PMNs in controlling bacterial burdens was not explored in this report (Bernthal et al., 2011). Future studies with neutrophil-depleted mice will provide definitive evidence regarding the role of PMNs in biofilm clearance in models where significant infiltrates are observed. Likewise, potential bystander damage originating from the cytotoxic, proteolytic, and proinflammatory effector functions of PMNs in tissue degradation and osteolysis should also be examined. A recent study reported a prominent leukocyte infiltrate associated with post-traumatic osteomyelitis, which consisted predominantly of activated PMNs. The authors proposed that during the ineffective "frustrated" attempt to phagocytose bacteria, PMNs release cytotoxic and proteolytic molecules that, in turn, amplify tissue injury in conjunction with biofilm-derived toxins and proteolytic enzymes (Wagner et al., 2003, 2005). As mentioned earlier, PMN recruitment is minimal in a different model of *S. aureus* biofilm infection (**Figure 2**), and instead macrophage infiltrates predominate (Thurlow et al., 2011). The reasons responsible for differential PMN recruitment in various biofilm models may be influenced by the extent of tissue vascularization, maturity, and extent of biofilm development, and whether the inoculum used to establish biofilm formation may lead to an initial planktonic component that results in early PMN recruitment that is independent of the developing biofilm. The latter point is particularly relevant, since it is reasonable to assume that not all organisms will attach to an artificial device when introducing bacteria adjacent to the implant. In addition, larger inoculums may result in more planktonic bacteria neighboring the device, which are known to elicit a robust proinflammatory response, and conceivably augment PMN infiltrates. In contrast, in the context of lower inoculums, even if some organisms do not attach, the small number of planktonic bacteria would not be expected to elicit a robust inflammatory response. In addition, it could be argued that low inoculums more accurately mimic the events that would be encountered during native device seeding *in vivo*. Another factor to consider is the type of device studied. In the case of a hollow catheter, bacteria introduced directly into the lumen may be afforded additional protection from immune recognition by shielding provided by the catheter wall. In the case of a solid device, bacteria are immediately exposed to host tissues, in theory enabling an immediate proinflammatory response. Regardless, these issues should become clearer as additional *in vivo* studies examining staphylococcal biofilm pathogenesis are performed.

Several studies investigating innate immunity to staphylococcal species have focused on PMNs (Kobayashi et al., 2003; Anwar et al., 2009; Graves et al., 2010), whereas macrophage responses have received relatively less attention. Although PMNs are important antimicrobial effectors, their transcriptional capacity is limited, and their short lifespan requires constant recruitment into infection sites (Yamashiro et al., 2001; Borregaard, 2010; Mantovani et al., 2011). In contrast, resident macrophages are present in virtually all host tissues and represent a critical antimicrobial effector population and immediate line of defense against microbial invasion (Serbina et al., 2008; Gonzalez-Mejia and Doseff, 2009). Macrophages are more long-lived compared to PMNs and produce numerous proinflammatory mediators that are critical for immune cell recruitment and activation (Silva, 2010, 2011). In addition, macrophages possess potent phagocytic capacity and, like PMNs, can produce ROI and RNI species. However, macrophages are a major source of pro-inflammatory cytokines and chemokines, since activated cells survive significantly longer compared to PMNs (Furze and Rankin, 2008).

Examination of several proinflammatory signals responsible for macrophage and PMN recruitment (CCL2 and CXCL2, respectively) and activation (TNF-α and IL-1β) were significantly reduced in *S. aureus* biofilm infected tissues (Thurlow et al., 2011). Of note, the disconnect between reduced CCL2 levels and prominent macrophage infiltrates during biofilm formation indicate that existence of alternative macrophage chemoattractant signals, the identity of which remains unknown. Inducible nitric oxide synthase (iNOS) expression was also decreased, whereas arginase-1, a key enzyme involved in the collagen biosynthetic pathway was increased in macrophages surrounding the biofilm. Because both iNOS and arginase-1 compete for arginine to initiate their respective biosynthetic pathways, the preferential induction of arginase-1 expression in biofilm-associated macrophages likely results in skewing the immune response away from bacterial killing to favor fibrosis (Thurlow et al., 2011). Indeed, this possibility appears plausible based on three pieces of evidence. First, biofilm-associated device infections in animal models as well as humans are typified by a robust fibrotic response (Pickering et al., 1989; Buret et al., 1991; Duch and Yee, 2001), which might inadvertently serve to further sequester the biofilm from immune recognition. Second, *in vitro* and *in vivo* studies from our laboratory demonstrate that *S. aureus* biofilms skew macrophages toward an anti-inflammatory M2 phenotype (Thurlow et al., 2011), which contributes to fibrosis. Likewise, *S. epidermidis* biofilms with higher proportions of viable, but non-culturable dormant bacteria have recently been shown to induce less macrophage activation as revealed

by reduced pro-inflammatory cytokine production *in vitro* and expression of surface activation markers *in vivo* (Cerca et al., 2011). Third, various ECM molecules have been shown to attenuate immune cell proinflammatory activity, in accordance with a wound healing environment (Adair-Kirk and Senior, 2008; Korpos et al., 2009; Rodero and Khosrotehrani, 2010; Sorokin, 2010; Mahdavian Delavary et al., 2011; Wight and Potter-Perigo, 2011). Based on these observations, we propose that staphylococcal biofilms skew the host innate immune response from a classical pro-inflammatory bactericidal phenotype toward an anti-inflammatory, pro-fibrotic response to favor bacterial persistence (**Figure 3**). The typical response of macrophages to bacterial infections involves the induction of genes related to proinflammatory M1 polarization that is usually associated with protection during acute infections. However, a growing number of studies demonstrate that some organisms have evolved sophisticated strategies to interfere with M1 polarization by either inhibiting macrophage microbicidal activity (Vazquez-Torres et al., 2000), hindering M1 cytokine expression/secretion (Bost and Clements, 1997; Dornand et al., 2002), or by producing virulence factors that directly impede NF-κB activation (Pathak et al., 2007). In contrast, bacterial persistence is often linked to macrophage reprogramming toward an anti-inflammatory M2 state (reviewed in Benoit et al., 2008). Additional studies are warranted to tease apart this relationship from a mechanistic perspective.

Although we and others have previously demonstrated that PMNs are capable of phagocytosing *S. aureus* biofilms (Günther et al., 2009; Graves et al., 2010; Thurlow et al., 2011), macrophages are limited in their ability to ingest *S. aureus* when organized within a biofilm compared to planktonic bacteria (Thurlow et al., 2011). Importantly, macrophages were capable of phagocytosing bacteria from mechanically disrupted biofilms, suggesting either (1) the failure to physically engulf the complex biofilm structure that exceeds the size of a macrophage by several orders of magnitude, or (2) inability opsonize intact biofilms. The latter is supported by a previous study showing that IgG and C3b deposition is reduced on the surface of *S. epidermidis* biofilms compared with planktonic bacteria (Kristian et al., 2008). Interestingly, macrophages incubated with conditioned supernatants from *S. aureus* biofilms *in vitro* were unable to phagocytize latex beads or planktonic bacteria, which were readily internalized by untreated macrophages (**Figure 4**). Consequently, it appears that biofilms not only alter macrophage activation states, but also paralyze their phagocytic potential in response to particulate material that would be readily internalized under normal conditions. The implications of this phenomenon during biofilm infections are envisioned to be significant, in that macrophages would be impaired in their ability to scavenge dead cells/debris or contribute to tissue remodeling in the vicinity of the biofilm. Although these possibilities remain speculative, they could conceivably contribute to biofilm persistence *in vivo*.

The majority of macrophages that invaded *S. aureus* biofilms *in vitro* were dead compared to those that remained above the biofilm surface (Thurlow et al., 2011). There are a number of potential mechanisms that may account for the differential sensitivity of macrophages to cell death based on their physical distance from the biofilm, which can be framed in the context of metabolic "layers" that have been proposed for the biofilm (Spormann, 2008; Stewart and Franklin, 2008; Bester et al., 2010). These zones represent a complex relationship between anaerobic and aerobic microenvironments within the biofilm mass, bacterial-influenced fluctuations in pH, and bacterial metabolic profiles that may affect macrophage survival due to the release of toxic byproducts. Furthermore, the biofilm proper may contain a high concentration of lytic toxins, which in combination with the bulky biofilm matrix may lead to frustrated phagocytosis and cell death (Hoiby et al., 1995; Costerton et al., 1999). Additional studies are warranted to investigate the factor(s) that are responsible for innate immune cell death upon contact with staphylococcal biofilms.

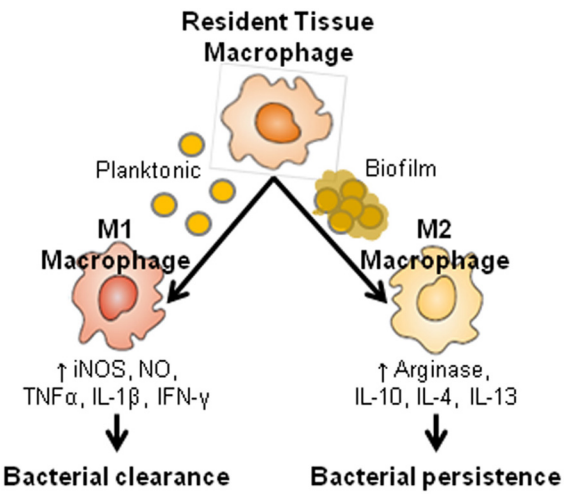

FIGURE 3 | Macrophage activation pathways. Depending on the local cytokine milieu, macrophages can be polarized toward a classical M1 or alternatively activated M2 phenotype, which possess potent antimicrobial and pro-fibrotic activities, respectively. Based on these attributes, and the finding that *S. aureus* biofilms are associated with a prominent M2 macrophage infiltrate, we propose that the ability of biofilms to elicit a M2 dominant response is responsible, in part, for biofilm persistence in an immunocompetent host.

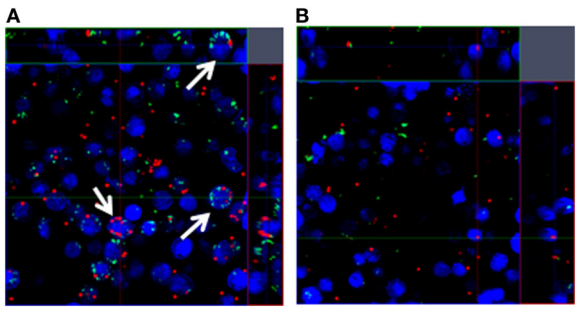

FIGURE 4 | Phagocytosis is impaired in response to *S. aureus* biofilms. Bone marrow-derived macrophages labeled with CellTracker Blue (blue) were incubated with **(A)** fresh medium or **(B)** biofilm-conditioned supernatants for 2 h, whereupon planktonic *S. aureus* expressing GFP (green) and fluorescent latex beads (red) were added for 1 h to determine macrophage phagocytic ability by confocal microscopy. Arrows indicate phagocytic macrophages.

FIBROSIS

Biofilm infections typically become surrounded by a fibrous capsule that is likely driven by host pro-fibrotic pathways (Pickering et al., 1989; Buret et al., 1991; Duch and Yee, 2001). However, little information is available regarding the host-derived factors that trigger biofilm encapsulation or its consequences on the evolving immune response. Although it is presumed that biofilm encapsulation by the host represents a protective response to contain the infection, this process may inadvertently provide survival advantages to the bacteria. For example, despite an early macrophage infiltrate, immunofluorescence staining demonstrated that the majority of cells remained distant from the infection site, with only a few macrophages recruited to the biofilm surface (Thurlow et al., 2011). Therefore, the fibrotic capsule may provide a protective barrier to physically sequester invading immune cells from the biofilm and/or limit antibiotic penetration into the infection site (Xu et al., 2000; Singh et al., 2010). Alternatively, fibrosis may enable the dissemination of *S. aureus* when organisms are released from the biofilm through their ability to produce proteases and adhesion molecules that demonstrate affinity for host fibrotic molecules (Gordon and Lowy, 2008). What remains unknown is whether encapsulation is triggered by virulence factor(s) released from the biofilm and the consequences of fibrosis formation.

The host immune response plays a critical role during both physiological and pathological fibrosis by releasing several pro-fibrotic cytokines and other molecules that participate in extracellular matrix (ECM) remodeling to induce fibrosis (Mauviel, 2005; Lupher and Gallatin, 2006; Wynn, 2008). Fibrosis has been linked to the transition of macrophages into an alternatively activated M2 phenotype, which is dictated, in part, by the pro-fibrotic Th2 cytokines IL-4, IL-5, and IL-13 (Wynn, 2004; Lupher and Gallatin, 2006). Recent studies suggest that certain pathogens favor the transition of the immune response from a classical pro-inflammatory to an anti-inflammatory state (Bouhlel et al., 2007; Gallardo-Soler et al., 2008). Since fibrosis typically ensues following the dampening of inflammation (Lupher and Gallatin, 2006), which coincides with the increased expression of anti-inflammatory mediators, this provides a link between the two processes. By extension, it appears plausible that biofilm growth actively directs the ensuing fibrotic response. Our recent studies in the mouse catheter-associated biofilm infection model demonstrated that infected catheters become rapidly surrounded by a fibrotic capsule composed primarily of type I collagen and fibronectin (Hanke and Kielian, in revision). The coating of artificial surfaces and implanted medical devices with platelets and host ECM proteins, such as fibronectin, facilitate *S. aureus* adherence *in vitro* (Foster, 1996). In terms of coagulase-negative staphylococci, others have reported enhanced adhesion with various plasma proteins, including fibronectin (Herrmann et al., 1988). These interactions are facilitated by the numerous MSCRAMMs that are expressed by *S. aureus*, which have known binding affinity for monomeric collagen and fibronectin (Rivera et al., 2007). However, others have demonstrated that fibronectin and its proteolytic fragments inhibited *S. epidermidis* adhesion to plastic surfaces (Dunne and Burd, 1993). This might be explained by either the growth phase when bacteria were harvested (since MSCRAMMs are maximally expressed during log phase growth in broth cultures) or the fact that *S. epidermidis* is more adept at binding to inert surfaces compared to *S. aureus*. In this case, coating with exogenous molecules is more dispensable and conceivably, could deter *S. epidermidis* binding due to charge repulsion, although this remains highly speculative.

As previously mentioned, numerous studies have identified macrophages as a major effector cell in fibrosis (Lupher and Gallatin, 2006). It is now clear that macrophages can be programmed toward distinct activation phenotypes categorized as classical M1 and alternatively activated M2 macrophages (Gordon, 2003; Mantovani et al., 2004). Th1 cytokines, such as IFN-γ, activate iNOS and proinflammatory cytokine expression in M1 macrophages, leading to their potent antimicrobial activity. Recently, M2 macrophages have been further subdivided into three types, based on the cytokine milieu encountered and the mediators they secrete. M2a represent what the field collectively considers "M2" macrophages, which are driven by IL-4 and IL-13 and are associated with TGF-β production and arginase-1 (ARG1) activity, both of which are pro-fibrotic (Munder et al., 1998; Hesse et al., 2001; Gordon, 2003). M2b macrophages are induced following exposure to PAMPs in combination with IL-1β and are typified by their production of IL-10 and select chemokines. M2c macrophages arise from exposure to TGF-β and IL-10; however, less is known about their secretory profile. It is important to note that these activation states likely represent a continuum that is influenced by the local inflammatory milieu and importantly, macrophages exhibit plasticity in their ability to adapt to changing microenvironments (Gordon and Taylor, 2005; Martinez et al., 2009; Biswas and Mantovani, 2010). With regard to antibacterial responses and fibrosis, iNOS and arginase-1 are involved in opposing pathways (**Figure 3**). Namely, iNOS is proinflammatory and part of a conserved "core host response" to infection, whereas ARG1 is considered anti-inflammatory and drives fibrotic reactions (Benoit et al., 2008). Therefore, the transition of macrophages from an M1 to an M2 phenotype facilitates fibrosis. Future studies are needed to investigate these macrophage populations throughout the course of staphylococcal biofilm infection to elucidate whether a particular type predominates, particularly with regard to the M2a, M2b, and M2c states. In addition, it will be interesting to determine whether differences in macrophage polarization are observed in *S. aureus* compared to *S. epidermidis* biofilms, since the former is significantly more invasive and capable of causing systemic disease (Gordon and Lowy, 2008). In this case, one could envision that the fibrotic response may be more extensively developed with *S. aureus* in an attempt to prevent infection dissemination.

ADAPTIVE IMMUNITY AND STAPHYLOCOCCAL BIOFILMS

Although little information is currently available regarding adaptive immune responses against staphylococcal biofilms, there is great interest in developing a vaccine to prevent biofilm infections. One challenge toward the development of such a vaccine is identifying appropriate immunodominant antigen(s) that are capable of eliciting robust antimicrobial activity. In fact, the failure to find one such an antigen has led many groups to design multivalent vaccines in hopes of inducing antibody-mediated protection (Brady et al., 2011). The efficacy of a quadrivalent

vaccine consisting of cell wall- and membrane-associated proteins whose expression is increased during *S. aureus* biofilm growth has been recently examined (Brady et al., 2011). Neither the quadrivalent nor monovalent vaccines were effective at clearing *S. aureus* biofilms *in vivo*, and only in combination with powerful antibiotic treatment was any effect achieved (Brady et al., 2011). It is reasonable to predict that antibodies may be capable of clearing planktonic bacteria released from the biofilm; however, it seems less likely that antibodies will be effective in neutralizing bacteria deep within the biofilm, since many organisms are embedded within the matrix and not accessible to antibody opsonization. Indeed, even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host unless the biofilm itself is physically removed. For this reason, biofilms are typically persistent infections even after repetitive cycles of antibiotic therapy, which is a testament to the powerful array of factors that staphylococci possess to thwart the host immune response. The reader is referred to several recent reviews discussing the challenges facing the development of an anti-biofilm vaccine based on the multi-factorial nature of the obstacles involved from both the biofilm and immune perspectives (Visai et al., 2007; Harro et al., 2010; Ohlsen and Lorenz, 2010; Montanaro et al., 2011).

POINTS FOR FUTURE CONSIDERATIONS

When considering the host immune response to staphylococcal biofilms, one must take into account several factors. Particularly, the method of biofilm propagation *in vitro* (i.e., static versus shear flow), *in vivo* model, type of immune cell population being examined, genetic background when utilizing mouse models, pathogen niche within the host, variability between various staphylococcal isolates, and the type of biofilm formed (protein vs. polysaccharide) can all conceivably influence experimental outcomes. In terms of *in vitro* biofilm propagation, the compatibility of medium formulations to achieve optimal survival of both the biofilm and immune cells must be considered. Although typical bacterial broth formulations induce robust biofilm growth, they are likely to induce immune cell apoptosis as we have observed (**Figure 5**). Although biofilm maturation was slightly delayed in mammalian cell culture medium compared to equivalent growth periods in TSB, biofilms still achieved a relatively uniform thickness and density, along with the presence of tower structures (Thurlow et al., 2011). In addition, it might be argued that this medium formulary may better model the host environment that the bacteria must colonize to establish a biofilm.

Intercellular adhesive mechanisms play a key role in biofilm development, particularly for *S. epidermidis* biofilms (Heilmann et al., 1996; Cramton et al., 1999; Rohde et al., 2007). PIA was among the first factors identified for mediating biofilm accumulation in *S. epidermidis* and was later described in *S. aureus* (Rohde et al., 2007). Recently the surfactant-like PSM peptides have been identified as contributors to *S. epidermidis* and *S. aureus* biofilm formation and maturation (Wang et al., 2011; Periasamy et al., 2012). Proteins have also been found to play an important role in staphylococcal biofilm formation and in *S. epidermidis*. Aap was identified as a PIA-independent intercellular adhesin (Rohde et al., 2005; Sun et al., 2005). Likewise, PIA-independent *S. aureus* biofilms can also form in humans (Fitzpatrick et al., 2005b; Toledo-Arana et al., 2005).

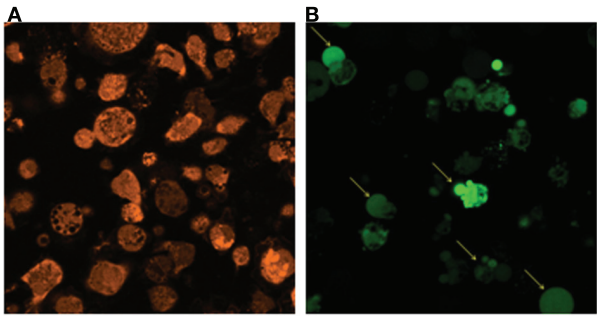

FIGURE 5 | TSB medium does not support macrophage survival. **(A)** Primary thioglycollate-elicited peritoneal macrophages isolated from C57BL/6 mice were stained with 5 μM CellTracker Orange (orange) and incubated in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. **(B)** Primary macrophages isolated from GFP transgenic mice were incubated in TSB supplemented with 0.5% glucose and 3% NaCl for 24 h at 37°C. Arrows indicate apoptotic macrophages.

While there is an abundance of information on staphylococcal biofilm mutants *in vitro* (Mann et al., 2009; Beenken et al., 2010; Kiedrowski et al., 2011) there is limited number of studies characterizing their impact on host immune mechanisms *in vivo*. Moreover, those studies that do investigate the ability of staphylococcal mutants to establish biofilms *in vivo* often use outbred or severely immune compromised mouse strains, which may have a significant impact on the results obtained. Indeed, the mouse strain used can greatly influence the experimental outcome and should be thoughtfully considered when designing *in vivo* experiments. Additional studies are needed to further our understanding of immunity during staphylococcal biofilm infections in multiple clinically relevant models to identify pathways that may be exploited for therapy.

CONCLUSIONS

Since staphylococcal biofilms represent a serious clinical situation based on the propensity of organisms to detach and colonize new sites of infection (Lowy, 1998; Fätkenheuer et al., 2002), understanding host immune-biofilm dynamics is an important issue that warrants further investigation. Although there is a now a good foundation documenting immune responses to staphylococcal biofilms, much work remains to be done. In particular, relatively little is known regarding mechanisms of neutrophil and macrophage recognition and activation of staphylococcal biofilms and pathways contributing to fibrotic encapsulation of these infections. These issues are of particular importance and may help explain the phenomenon as to why staphylococcal biofilms are recalcitrant to therapy and could conceivably unveil candidate molecules for targeted therapy to augment host immunity to biofilm infections.

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The staphylococcal accessory regulator, SarA, is an RNA-binding protein that modulates the mRNA turnover properties of late-exponential and stationary phase *Staphylococcus aureus* cells

John M. Morrison[1], Kelsi L. Anderson[1], Karen E. Beenken[2], Mark S. Smeltzer[2] and Paul M. Dunman[1,3]*

[1] *Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA*
[2] *Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
[3] *Department of Microbiology and Immunology, School of Medicine and Dentistry, University of Rochester, Rochester, NY, USA*

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Mark Estes, University of Georgia, USA
Sebastien P. Faucher, McGill University, Canada
Pierre Fechter, Centre National de la Recherche Scientifique, France
Tom Geissmann, Institue de Santé et Recherche Médicale, France

***Correspondence:**
Paul M. Dunman, Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Box 672, Rochester, NY 14642, USA
e-mail: paul_dunman@urmc.rochester.edu

The modulation of mRNA turnover is gaining recognition as a mechanism by which *Staphylococcus aureus* regulates gene expression, but the factors that orchestrate alterations in transcript degradation are poorly understood. In that regard, we previously found that 138 mRNA species, including transcripts coding for the virulence factors protein A (*spa*) and collagen-binding protein (*cna*), are stabilized in a *sarA*-dependent manner during exponential phase growth, suggesting that SarA directly or indirectly affects the RNA turnover properties of these transcripts. Herein, we expanded our characterization of the effects of *sarA* on mRNA turnover during late-exponential and stationary phases of growth. Results revealed that the locus affects the RNA degradation properties of cells during both growth phases. Further, using gel mobility shift assays and RIP-Chip, it was found that SarA protein is capable of binding mRNA species that it stabilizes both *in vitro* and within bacterial cells. Taken together, these results suggest that SarA post-transcriptionally regulates *S. aureus* gene expression in a manner that involves binding to and consequently altering the mRNA turnover properties of target transcripts.

Keywords: *Staphylococcus aureus*, SarA, RNA degradation

INTRODUCTION

Staphylococcus aureus is a human pathogen that causes nosocomial and community-associated infections that result in high rates of morbidity and mortality (Klevens et al., 2007; Deleo et al., 2010). The organism largely owes its ability to cause infection to the production of an array of virulence factors which, in the laboratory setting, are coordinately regulated in a cell density-dependent manner. Cell surface-associated factors are predominantly expressed during exponential phase growth whereas extracellular factors are predominantly produced during stationary phase growth (Novick, 2003; Bronner et al., 2004). The organism's virulence factors are also coordinately regulated in response to endogenous and exogenous cues, including cellular stresses and sub-inhibitory concentrations of antibiotics. A plethora of two component regulatory systems (TCRS) and nucleic acid-binding proteins have been hypothesized to modulate *S. aureus* virulence factor expression.

Of the 17 TCRS identified in *S. aureus* to date, the best-characterized is the accessory gene regulator (*agr*) locus. The *agr* locus encodes a quorum-sensing TCRS, AgrAC, whose regulatory effects are generally thought to be mediated by a regulatory RNA molecule, RNAIII. Within laboratory culture conditions, RNAIII expression peaks during the transition to stationary phase growth (Novick, 2003). RNAIII has been shown to modulate virulence factor expression by directly binding to target mRNA species, thereby affecting their stability and translation properties (Morfeldt et al., 1995; Huntzinger et al., 2005; Geisinger et al., 2006; Boisset et al., 2007). For instance, RNAIII binding to the cell surface factor protein A (*spa*) transcript creates a substrate for ribonuclease III digestion, which, in-turn, accelerates *spa* mRNA digestion and consequently limits Spa production (Huntzinger et al., 2005). Conversely, the binding of RNAIII to the extracellular virulence factor α-hemolysin (*hla*) transcript liberates the mRNA's Shine–Dalgarno sequence and increases Hla production (Morfeldt et al., 1995). Similar mechanisms of RNAIII regulation have been documented for the virulence factor *coa* (Chevalier et al., 2010) and the regulatory locus repressor of toxins (*rot*; Boisset et al., 2007).

In addition to TCRS, *S. aureus* produces a family of DNA-binding proteins that regulate virulence factor expression. The best-characterized to date is the staphylococcal accessory regulator nucleic acid-binding protein, SarA. The *sarA* locus consists of a 1.2 kb DNA region that produces three overlapping transcriptional units (*sarB*, *sarC*, and *sarA*), each of which terminates at the same site and encodes for the SarA protein (Chien and Cheung, 1998). Unlike RNAIII, SarA is constitutively produced throughout *S. aureus* growth phases, however the expression of the individual *sar*

transcripts occurs in a growth phase-dependent manner; *sarA* and *sarB* are primarily transcribed during exponential phase growth whereas *sarC* is predominantly expressed during stationary phase growth (Manna et al., 1998; Blevins et al., 1999). SarA has been characterized as a pleiotropic transcriptional regulator of virulence factors that can bind to the promoter regions of a subset of genes that it regulates, such as *hla* (α-hemolysin) and *spa* (protein A; Chien and Cheung, 1998; Chien et al., 1999). Nonetheless, several observations have suggested that SarA's regulatory effects might be more complex than initially appreciated. Arvidson and colleagues have reported that, in addition to affecting *spa* transcript synthesis, SarA may also indirectly regulate Spa production (Tegmark et al., 2000). Further, no clear SarA consensus binding site has been defined; Cheung and colleagues found that SarA binds a 26 base pair (bp) region termed the SarA box, whereas Sterba et al. (2003) have defined the SarA box to be a 7 bp sequence, which is present more than 1000 times within the *S. aureus* genome, indicating that the protein may have the capability of binding the chromosome more frequently than one might expect for a *bona fide* transcription factor (Chien et al., 1999). In that regard, others have suggested that SarA is a histone-like protein whose regulatory effects are a function of altering DNA topology and, consequently, promoter accessibility (Schumacher et al., 2001).

In *Escherichia coli*, histone-like proteins can post-transcriptionally regulate gene expression by binding directly to mRNA molecules and influencing the transcript's stability and translation (Balandina et al., 2001; Brescia et al., 2004). Accordingly, based on the possibility that SarA may behave as a histone-like protein, we hypothesized that the protein's regulatory effects may, in part, be due to its ability to bind and subsequently modulate the mRNA turnover properties of target transcripts. As a first step toward testing that prediction, we found that 138 mRNA species that are produced during *S. aureus* exponential phase growth, including the known SarA-regulated genes *spa* and *cna*, are also stabilized in a *sarA*-dependent manner (Roberts et al., 2006). More specifically, these mRNA transcripts are stabilized in a $sarA^+$ background as compared to isogenic $sarA^-$ cells, raising the possibility that SarA protein may bind these transcripts in a manner that affects their stability and, consequently, expression. In the current body of work we extended our evaluation of this phenomenon by investigating whether the mRNA turnover properties of late-exponential and/or stationary phase transcripts are also modulated in a *sarA*-dependent manner. Results revealed that this is indeed the case; the *sarA* locus affects the mRNA turnover properties of transcripts produced during both phases of growth. Further, using ribonucleoprotein immunoprecipitation (RIP-Chip) assays, we found that SarA binds these transcripts within *S. aureus* cells. Results were verified via gel-shift mobility assays. Taken together, these results indicate that SarA is capable of binding cellular mRNA species and that the protein's regulatory effects could be attributable to its ability to directly modulate the mRNA turnover properties of target mRNA species.

MATERIALS AND METHODS

GROWTH CONDITIONS

Bacterial strains and plasmids used in this study are listed in **Table 1**. Overnight *S. aureus* cultures were diluted 1:100 into fresh brain–heart infusion (BHI) broth with a flask to volume ratio of 5:1 and were incubated at 37°C with aeration on a rotary shaker at 225 rpm. When cultures reached mid-exponential ($OD_{600\ nm} = 0.25$–0.30), late-exponential ($OD_{600\ nm} = 1.1$–1.2), or stationary (24 h post-inoculation) phase, rifampicin (200 μg ml^{-1}; Sigma; St. Louis, MO) was added to arrest transcription. Aliquots of cells were removed at 0, 2.5, 5, 15, and 30 min post-transcriptional arrest, mixed with an equi-volume of ice-cold acetone:ethanol (1:1) and RNA was isolated, as previously described (Anderson et al., 2006; Roberts et al., 2006; Olson et al., 2011).

Table 1 | Bacterial strains and plasmids used in this study

Strains	Relevant genotype or phenotype	Reference
UAMS-1	Wild type *S. aureus* osteomyelitis isolate	Gillaspy et al. (1995); Cassat et al. (2005)
UAMS-929	UAMS-1 (Δ*sarA*)	Blevins et al. (1999)
KLA43	UAMS-929 (pKLA40)	This study
RN4220	Restriction-deficient *S. aureus*	de Azavedo et al. (1985)
INVαF	*E. coli* competent cells; *lacZ*ΔM15	Invitrogen
DH5α	*E. coli* competent cells	Invitrogen
Plasmids	**Relevant genotype**	
pCRII	TOPO-TA cloning vector	Invitrogen
pBK123	Cam^R derivative of pCN51	Charpentier et al. (2004)
pKLA40	pBK123::c-Myc-SarA	This study

GENECHIP® MICROARRAYS

All microarray studies were performed as previously described (Anderson et al., 2006, 2010; Roberts et al., 2006; Olson et al., 2011). Briefly, 10 μg of each bacterial RNA sample was labeled and hybridized to a *S. aureus* GeneChip®, following the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix; Santa Clara, CA). Average GeneChip®signal intensities for biological replicates ($n \geq 2$) for each strain and time point were obtained from values normalized to exogenous transcripts. The half-life of each RNA transcript was determined using a "twofold" algorithm (Selinger et al., 2003) and was measured as the time point at which a given RNA signal intensity decreased two-fold as compared to that transcript at 0 min post-transcriptional arrest using GeneSpring 7.2 software (Agilent Technologies; Redwood City, CA).

IN VITRO TRANSCRIPTION

DNA templates consisting of the full-length *spa* transcriptional unit or derivatives containing 3′ or 5′ deletions were created by PCR amplification using UAMS-1 chromosomal DNA as a template and oligonucleotides listed in **Table 2**. Resulting PCR products contained a 5′ T7 RNA polymerase promoter and transcription start site and were used as templates for generating corresponding RNA species using a T7 MegaScript *In vitro* Transcription Kit (Ambion; Austin, TX) according to the manufacturer's recommendations. Briefly, for transcription reactions, approximately 1 μg of PCR product was incubated for 3 h at 37°C with 7.5 mM of a NTP mix and T7 RNA polymerase to generate the

Table 2 | Oligonucleotides used in this study

Primer name	Sequence (5′ → 3′)
RT-PCR	
cna F	AACGAACAAGTATACACCAGGAGAG
cna R	TTTGCTTTTTCATCTAATCCTGTC
norA F	GCAGGTGCATTAGGCATTTTAGC
norA R	TGCCGATAAACCGAACGCTAAG
spa F	GCAGAAGCTAAAAAGCTAAATGATG
spa R	GCTCACTGAAGGATCGTCTTTAAGG
sarA F	TTTTAACCATGGCAATTACAAAAAT
sarA R	TTTCTCTTTGTTTTCGCTGATGTAT
PCR-MEDIATED *IN VITRO* TRANSCRIPTION*	
L 1–450 *spa* T7 F	**TAATACGACTCACTATAGGG**CATACAGGGGGTATTAATTTGAAAA
L 1–450 *spa* R	ATCCTAGAATTCTCTTCGTTCAAGTTAGGCATGTTCA
L −250 *spa* T7 F	**TAATACGACTCACTATAGGG**GTTCAACAAAGATCAACAAAGCGCC
L −250 *spa* R	AGTAGAAAGTGTTGAGGCGTTTCAG
CLONING†,‡	
c-Myc-SarA F	ATCCTAGTCGACGCTAACCCAGAAATACAATCACTGTGTC
c-Myc-SarA R	ATCCTAGG*ATCCTTACAGATCTTCTTCGCTGATCAGTTTCTGTTC*TA GTTCAATTTCGTTGTTTGCTTCAGTG

**Bolded nucleotides indicate T7 promoter sequence.*

† Underlined nucleotides indicate restriction sites.

‡ Italicized nucleotides indicate the c-Myc epitope.

indicated RNA species. Template DNA was degraded with four units of TurboDNase I for 15 min at 37°C, and *in vitro* transcribed RNA was recovered by lithium chloride precipitation and resuspended in nuclease-free water. RNA concentration of the synthesized product was determined spectrophotometrically ($OD_{260\,nm}$ $1.0 = 40\,\mu g\,ml^{-1}$) and the integrity of the transcript was evaluated on a 1.2% agarose–0.66 M formaldehyde denaturing gel.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

In vitro transcribed *spa* mRNA or a PCR product containing the endogenous *hla* promoter were 3′ end-labeled with digoxigenin-ddUTP (DIG; Roche Applied Science; Indianapolis IN) following the manufacturer's recommendations. One picomole DIG-mRNA or -DNA was mixed with 0, 7.3, 36.6, 73.3, or 366.5 pmol purified SarA protein then, incubated for 15 min at 37°C in gel-shift buffer (20 mM HEPES-KOH, pH 8.0, 8 mM $MgCl_2$, 100 mM NaCl), cooled on ice and the entire sample volume was electrophoresed in 1.2% agarose–0.66 M formaldehyde denaturing gels. Next, RNA-protein or DNA-protein complexes were transferred via capillary action to nylon membranes overnight in 20× SSC buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0). Nucleic acid was then cross-linked to the membrane by UV irradiation in a Stratalinker®UV crosslinker (Stratagene; La Jolla, CA) twice at 1200 (×100) microjoules. Membranes were probed for presence of the DIG-labeled RNA or DNA using anti-DIG Fab fragments conjugated to alkaline phosphatase (1:10,000; Roche Applied Science) and visualized chemiluminescently with CSPD reagent (Roche Applied Science). For all binding assays, bovine serum albumin (BSA) was used as a negative control for non-specific RNA-protein and DNA-protein interactions.

c-Myc-SarA CONSTRUCTION

A c-Myc epitope was translationally fused to the C-terminus of the SarA open reading frame. To do so, 615 bp of the *S. aureus* strain UAMS-1 *sarA* gene and its corresponding P1 promoter region were amplified by PCR using primers c-Myc-SarA F and c-Myc-SarA R (**Table 2**); the latter included a c-Myc epitope and 3′ flanking restriction enzyme site. The resultant PCR product was gel-purified and digested with restriction enzymes *Sal*I and *Bam*HI and subcloned into pBK123 (Charpentier et al., 2004) to generate the plasmid pKLA40. Plasmid pKLA40 was then electroporated into the restriction-negative strain of *S. aureus*, RN4220 (de Azavedo et al., 1985) and transfected into UAMS-929 (Δ*sarA*) via φ11-mediated phage to generate strain KLA43 and the plasmid was sequenced to confirm the integrity of the insert. c-Myc-SarA functionality was confirmed by examining the ability of the epitope-tagged protein to complement the exoprotein profile of UAMS-929(Δ*sarA*) cells. Briefly, supernates from stationary phase cultures of *S. aureus* UAMS-1, UAMS-929(Δ*sarA*), and KLA43 (Δ*sarA*; c-Myc-SarA) were collected, filtered through a 0.22 μm nylon membrane, and compared by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and silver staining (Bio-Rad Life Science, Hercules, CA).

WESTERN BLOTTING

Cultures were grown to the indicated growth phase and cells were mechanically disrupted in TE buffer in BIO101 lysing matrix B tubes using a FastPrep120 shaker (MP Biomedicals; Solon OH). Cell debris was removed by centrifugation at 4°C for 15 min and the protein concentration of supernatants were quantified by Bradford protein assays. For confirmation of c-Myc-SarA expression, 1 μg of protein was electrophoresed in a 15% sodium dodecyl

sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 10% milk in Tween-TBS (TTBS; TBS containing 0.1% Tween 20) and rabbit polyclonal anti-c-Myc antibody (1:1000; Sigma Aldrich; St. Louis, MO) was used to probe for presence of the c-Myc-SarA protein. Following incubation with the primary antibody, membranes were washed in TTBS and probed with peroxidase-conjugated anti-rabbit secondary antibody (1:1000; Promega; Madison, WI). Membranes were washed and c-Myc-SarA was detected chemiluminescently by ECL^{TM} (Amersham BioSciences; Piscataway NJ). Confirmation of successful RIP was confirmed by loading 10 μl of cell lysate, cell supernate, wash, or elution fractions and subjected to SDS-PAGE and western blotting, as described above.

RIBONUCLEOPROTEIN IMMUNOPRECIPITATION (RIP-Chip)

Ribonucleoprotein immunoprecipitation was performed using a c-Myc Tag/Co-IP Kit (Pierce Biotechnology; Rockford, IL). To do so, *S. aureus* strains UAMS-929 (Δ*sarA*; negative control) and KLA43 (Δ*sarA*/pKLA40::c-Myc-SarA) were grown to mid-exponential phase and RNA-binding proteins were cross-linked to RNA by incubating the cells for 30 min with 1% formaldehyde at room temperature. Cross-linking was quenched by adding 125 mM glycine for 5 min at room temperature. Cells were centrifuged at 3000 RPM for 10 min at 4°C and cell pellets were washed twice in 1 ml ice-cold TBS, resuspended in 350 μl of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA, 50 mM NaCl, and 1 mM PMSF) and were lysed by the addition 100 μg ml^{-1} lysostaphin (Ambi; Lawrence, NY) at 37°C for 30 min. Next, an equal volume of 2× immunoprecipitation (IP) buffer (100 mM Tris pH 8.0, 10 mM EDTA, 300 mM NaCl, 2% Triton X-100, 1 mM PMSF) was added and suspensions were incubated for an additional 10 min at 37°C. Nucleic acids were fragmented with a Sonic Dismembrator (Fisher) on ice twice for 15 s using an output setting of one with 15 s rests on ice between each pulse and cell debris was subsequently removed by centrifugation for 10 min at 4°C. Next, 800 μl of the supernatant was mixed with 10 μl of anti-c-Myc agarose (Pierce Biotechnology; Rockford IL) and then added to IP spin columns (Pierce Biotechnology) and incubated overnight at 4°C to collect c-Myc-SarA/RNA and DNA complexes. Columns were washed five times with 1× IP buffer and c-Myc-SarA complexes were eluted in 50 μl of elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) by first incubating at 65°C for 10 in followed by centrifugation at 4°C to collect eluate. Cross-linking was reversed by incubating eluate for 2 h at 42°C and then 6 h at 65°C in 0.5× elution buffer containing 0.8 mg ml^{-1} pronase. For chromatin immunoprecipitation (ChIP-chip) experiments used to detect known SarA-DNA interactions, liberated DNA was purified using a PCR Clean-Up Kit (Qiagen) per manufacturer's recommendations and hybridized to a GeneChip®and processed as described above. For RIP-chIP experiments, liberated nucleic acid was treated with 17 U of RNase-Free DNase I (Qiagen) and RNA was purified using a Clean and Concentrator Kit (Zymo Research; Orange, CA) according to the manufacturer's recommendations. All assays were performed in triplicate for each strain and purification of the c-Myc-SarA protein was confirmed via Western blotting. Following IP, bacterial RNA was amplified and reverse-transcribed using a MessageAmp II-Bacteria Prokaryotic RNA Amplification Kit (Ambion). 1.5 μg of the amplified cDNA was then hybridized to a GeneChip®and processed as described above. The fold change in average signal intensity of KLA43 replicates as compared to the average signal intensity of UAMS-929 (negative control) replicates was calculated for each GeneChip®qualifier. SarA was considered to bind DNA or transcripts that exhibited a signal intensity that was ≥ two-fold in KLA43 samples and greater than two standard deviations from the average signal intensity (background), as compared to UAMS-929 cells.

QUANTITATIVE REVERSE-TRANSCRIPTION PCR

For standard qRT-PCR reactions, 25 ng of total bacterial RNA were reverse-transcribed, amplified, and measured using a LightCycler®RNA Master SYBR Green I kit per the manufacturer's recommendations (Roche Applied Science). As an internal control by which to standardize RNA loading, 0.5 pg of RNA was used to quantitate the amount of 16S rRNA in each sample. Transcript concentrations were calculated using LightCycler®software and LightCycler®Control Cytokine RNA titration kit as a standard for determining the copies of each transcript present per reaction. Final concentration values are listed as normalized to 16S rRNA abundance. Transcript half-lives were calculated as the time point at which RNA titers exhibited a ≥ two-fold decrease in signal intensity.

RESULTS

It is well recognized that SarA is a pleiotropic regulator of *S. aureus* virulence factors, yet the mechanism(s) by which the protein affects gene expression is incompletely understood. Several studies have shown that the molecule affects the transcript synthesis of target genes, however the lack of a consensus SarA-binding sequence and evidence of indirect control of gene expression have implicated SarA in post-transcriptional control of gene expression. Accordingly, it has also been hypothesized that SarA regulates *S. aureus* gene expression via modulating the mRNA turnover properties of target transcripts. In support of this hypothesis, we have previously shown that 138 mRNA species, including the virulence factor transcripts protein A (*spa*) and collagen-binding protein (*cna*) are stabilized in a *sarA*-dependent manner (Roberts et al., 2006). Our current efforts are designed to expand upon these initial studies and further define the role for *sarA* in modulating mRNA turnover.

sarA STABILIZES SUBSETS OF mRNA SPECIES IN A GROWTH PHASE-DEPENDENT MANNER

Herein, we set out to establish whether the degradation properties of mRNA species that are expressed during late-exponential and/or stationary phase growth are affected by a product of the *sarA* locus. To do so, *S. aureus* UAMS-1 (wild type) and UAMS-929 (UAMS-1Δ*sarA*) were grown to late-exponential or stationary phase growth and transcription was halted by the addition of rifampicin. Total bacterial RNA was then purified from aliquots of cells at 0, 2.5, 5, 15, and 30 min post-transcriptional arrest and the mRNA titers of expressed transcripts were then measured using Affymetrix GeneChips®. A comparsion of each transcript's titer at 0 min post-transcriptional arrest to that of each subsequent time point allowed the half-lives of each mRNA to be measured, as

previously described (Anderson et al., 2006, 2010; Roberts et al., 2006; Olson et al., 2011).

As shown in **Figure 1**, in general terms, the global RNA turnover properties of UAMS-1 and UAMS-1Δ*sarA* cells exhibited a similar trend that is consistent with our previous observations; bulk mRNA turnover occurs more rapidly in exponential phase as compared to stationary phase cells (Olson et al., 2011). Despite these similarities, a more detailed analysis of RNA degradation properties of individual mRNA species within each strain background indicated that *sarA* influences the stability of many transcripts. More specifically, 93.2 and 48.4% of transcripts exhibited half-lives of <2.5 min during late-exponential and stationary phase growth, respectively, in the wild type strain (**Table 3**). Conversely, 76.9 and 32.5% of transcripts were degraded within 2.5 min in late-exponential and stationary phase Δ*sarA* cells, suggesting that *sarA* affects the stability of a subset of transcripts. Within this subset, 68 (3.2%; Supplemental Table S1 in Supplementary Material) and 325 (15.3%; Supplemental Table S2 in Supplementary Material) of late-log and stationary phase transcripts, respectively, were less stable in Δ*sarA* cells, indicating that they are stabilized in a *sarA*-dependent manner. Conversely, 385 (18.2%; Supplemental Table S3 in Supplementary Material) and 580 (27.2%; Supplemental Table S4 in Supplementary Material) of transcripts were more stable in Δ*sarA* cells, indicating that, like RNAIII, SarA may facilitate degradation of a particular subset of mRNA species.

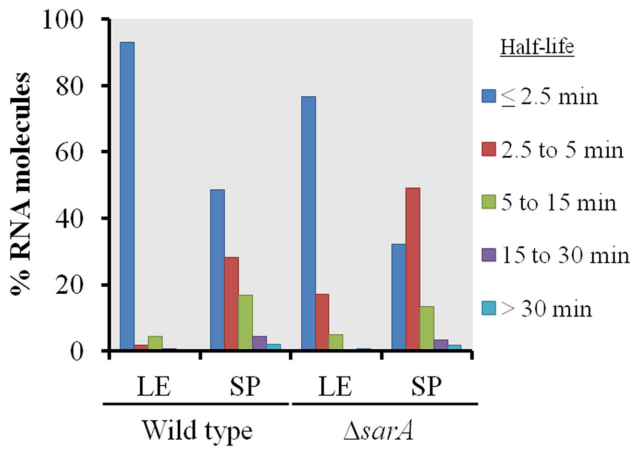

FIGURE 1 | Global transcript turnover properties of wild type and Δ*sarA* cells. The global RNA turnover properties of UAMS-1 (wild type) and isogenic Δ*sarA* (Δ*sarA*) cells during late-exponential (LE) and stationary phase (SP) growth are graphed. Percentages of total transcripts with half-lives of ≤2.5, 2.5–5, 5–15, 15–30, and ≥30 min are shown.

sarA AFFECTS VIRULENCE FACTOR mRNA STABILITY

Results also indicated that the mRNA turnover properties of many virulence factors are influenced by *sarA*, but that these effects predominantly occur during stationary phase growth. More specifically, during late-exponential phase growth the transcripts coding for three virulence-associated genes were stabilized in a *sarA*-dependent manner (**Table 4**). Among these were two metabolic enzymes, enolase (*eno*; Carneiro et al., 2004) and carbamate kinase (*arc*; Beenken et al., 2004; Diep et al., 2006; Diep and Otto, 2008), both of which exhibited half-lives of 30 min in wild type cells but only 15 min in Δ*sarA* cells. Likewise, the ATP-dependent protease *clpP* (Frees et al., 2003, 2005;Michel et al., 2006), exhibited a half-life of 15 min in wild type cells, but was reduced to five min in the *sarA*-mutant. During stationary phase growth at least 13 virulence factor transcripts were stabilized in a *sarA*-dependent manner (**Table 4**). Included among these were: *spa* (protein A), *hla* (α-hemolysin), *hlb* (β-hemolysin), and *hlgCB* (γ-hemolysin), as well as members of the capsule operon (*cap*) and the cell surface-associated factors *fnbA* (encoding fibronectin binding protein A) and *cna* (encoding a collagen-binding protein). Quantitative RT-PCR-based determination of select virulence factor half-lives within stationary phase wild type and Δ*sarA* cells verified the microarray-based RNA turnover measures (**Table 5**). In addition to virulence factors, three virulence factor regulatory molecules, *agrA*, *sarS*, and *saeS*, were stabilized in a *sarA*-dependent manner.

Table 3 | *S. aureus* growth phase mRNA degradation

	UAMS-1				Δ*sarA*			
	Late-log		Stationary		Late-log		Stationary	
	Transcripts	Percentage	Transcripts	Percentage	Transcripts	Percentage	Transcripts	Percentage
≤2.5 min	2044	93.2	1115	48.4	1620	76.9	691	32.5
2.5–5 min	36	1.6	648	28.1	359	17.0	1032	48.5
5–15 min	92	4.2	389	16.9	104	4.9	294	13.8
15–30 min	16	0.7	103	4.5	9	0.4	69	3.2
>30 min	4	0.2	47	2.0	15	0.7	40	1.9
Total	2192[a]	100	2302[b]	100	2107[c]	100	2126[d]	100

[a] *55 RNA species could not be measured.*
[b] *27 RNA species could not be measured.*
[c] *38 RNA species could not be measured.*
[d] *18 RNA species could not be measured.*

Table 4 | *sarA*-stabilized virulence-associated transcripts

Common name[†‡]	Half-life*		Description
	Wild type	Δ*sarA*	
LATE-EXPONENTIAL			
arcC	30 min	15 min	Carbamate kinase
clpP	15 min	5 min	ATP-dependent protease
eno	30 min*	15 min	Enolase
spa[‡]	Stable*	Stable*	Protein A
STATIONARY			
agrA	Stable*	30 min	AgrAC TCRS response regulator
arcB	15 min	5 min	Ornithine carbamoyltransferase
cap5A	5 min	2.5 min	Capsular polysaccharide biosynthesis
cap5C	15 min	5 min	Capsular polysaccharide biosynthesis
cap5D	15 min	5 min	Capsular polysaccharide biosynthesis
cna	15 min	5 min	Collagen-binding protein
fnbA	15 min	5 min	Fibronectin binding protein A
hla[‡]	Stable	30 min	α-hemolysin
hlb	30 min	15 min	Phospholipase C
hlgB	30 min	15 min	γ hemolysin component B
hlgC	15 min	5 min	γ hemolysin component C
norA	15 min	5 min	Multi drug transporter
rsbV	5 min	2.5 min	Anti–anti-sigma factor
SACOL0390	Stable	30 min	Lipase
saeR	15 min*	5 min	SaeRS TCRS response regulator
sarV	5 min	2.5 min	SarA homolog
spa[‡]	30 min*	15 min*	Protein A

**Estimated half-life due to detection limits of the system.*

[†] S. aureus strain N315 loci unless otherwise noted.

[‡] Transcript synthesis affected by sarA in UAMS-1 cells (Cassat et al., 2006).

Table 5 | Stationary phase mRNA half-lives of selected virulence factor transcripts as calculated by qRT-PCR*

	Wild type[†]	Δ*sarA*[†]
spa		
0 min	1.00	1.00
5 min	1.06	1.34
15 min	1.22	1.63
30 min	2.43	5.56
60 min	2.47	11.28
norA		
0 min	1.00	1.00
5 min	1.45	3.14
15 min	2.20	3.22
30 min	3.14	6.99
60 min	3.20	5.38
cna		
0 min	1.00	1.00
5 min	1.02	2.49
15 min	2.68	2.07
30 min	18.66	6.22
60 min	83.91	35.33

**Time point corresponding to ≥ two-fold decrease (half-life) is shaded gray.*

[†] Values represented as fold-change normalized to mRNA titers at 0 min.

SarA BINDS mRNA *IN VITRO*

The results presented here are consistent with previous observations suggesting that the *sarA* locus affects *S. aureus* mRNA turnover properties with exponential phase cells (Roberts et al., 2006). The two most likely scenarios that could account for these observations are that a product of the *sarA* locus may directly interact with and subsequently affect the mRNA degradation properties of target transcripts or that SarA indirectly modulates the organism's mRNA turnover properties by regulating other factors that affect *S. aureus* RNA decay. As a preliminary means of distinguishing between these two possibilities we set out to determine whether SarA is an RNA-binding protein. To do so, electrophoretic mobility shift assays (EMSAs) were performed to investigate whether purified SarA protein directly binds to *spa* transcripts, an mRNA species that is stabilized in a *sarA*-dependent manner (Roberts et al., 2006). Accordingly, various amounts (0, 7.3, 36.6, 73.3, or 366.5 pmol) of purified SarA protein were incubated with DIG-labeled, *in vitro* transcribed *spa* mRNA, during experimental conditions that have previously been used to establish that SarA binds to the promoter region of the α-hemolysin (*hla*) gene (Chien et al., 1999). As shown in **Figure 2A**, during these assay conditions SarA protein did indeed bind to the *hla* promoter region, confirming that the experimental conditions are appropriate to measure SarA-nucleic acid interactions and further validating the work of Chien et al. (1999). Similarly, the EMSA revealed that SarA elicited a dose-dependent shift in mobility of labeled *spa* mRNA, suggesting that SarA may be capable of binding RNA molecules (**Figure 2B**). Gel-shift assays were also performed with BSA and a *S. aureus* stable RNA, SSR42 (Olson et al., 2011), to determine if non-staphylococcal proteins would bind to the *in vitro* transcribed mRNA and whether SarA alters the mobility of any RNA species, respectively. Results of those studies indicated that BSA did not affect the mobility of *spa* transcript and, likewise, that SarA did not affect the migration of *in vitro* transcribed SSR42 (**Figure 2C**).

RNA-binding proteins have been shown to bind to specific regions of target mRNAs (Folichon et al., 2003; Wang et al., 2005); to test this possibility with SarA, we used EMSAs to measure the protein's ability to bind different regions of *in vitro* transcribed *spa* mRNA: a region lacking bases 1–320 of the 5′ end (L-250) and a fragment of *spa* containing only bases 1–450 (L 1–450; **Figure 2D**). As seen in **Figure 2E**, SarA affected the mobility of both RNA species. Further, a higher molecular weight product was observed when SarA was incubated with *spa* RNA fragments containing an intact 5′region (450 nt) in comparison to fragments lacking bases 1–250 of the transcript (1,100 nt), suggesting that the 5′ end of the *spa* transcript may harbor a greater number of SarA-binding sites than the 3′ terminus. Taken together, these results suggest that SarA may be capable of binding both DNA and RNA molecules.

SarA BINDS mRNA *IN VIVO*

Based on the observations that many exponential phase transcripts, including *spa*, are stabilized in a *sarA*-dependent manner and that SarA protein is capable of altering the mobility of *spa*

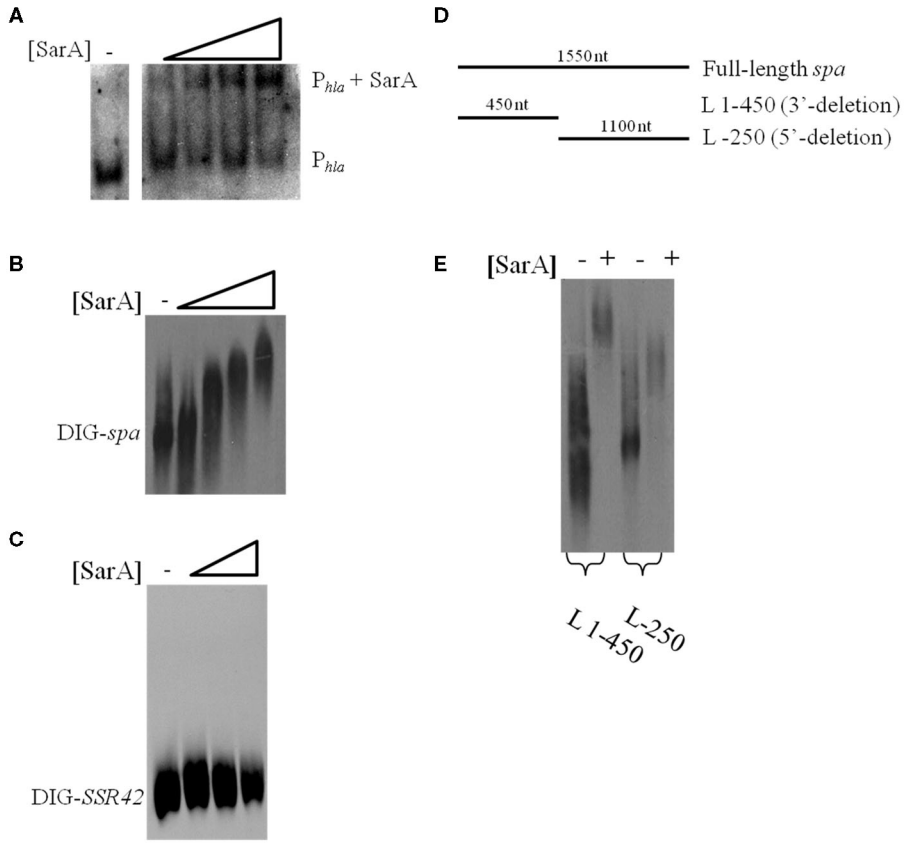

FIGURE 2 | SarA binds mRNA *in vitro*. (A) Gel mobility shift assays evaluating the mobility of DIG-labeled *hla* promoter DNA (P_{hla}) in the presence of increasing amounts of purified SarA protein (0, 7.3, 36.6, 73.3, or 366.5 pmol). **(B)** Gel mobility shift assays evaluating the mobility of DIG-labeled, *in vitro* transcribed *spa* in the presence of increasing concentrations of SarA protein (0, 7.3, 36.6, 73.3, or 366.5 pmol). **(C)** Gel mobility shift assays evaluating the mobility of DIG-labeled, *in vitro* transcribed *SSR42* in the presence of increasing concentrations of SarA protein (0, 36.6, 73.3, or 366.5 pmol). **(D)** Schematic of transcript variants containing deletions of 3′ or 5′ regions of *spa* that were DIG-labeled for use in subsequent gel-shift assays. **(E)** Gel mobility shift assays evaluating the mobility DIG-labeled *spa* deletion variants in the presence of 73.3 pmol SarA.

transcripts *in vitro*, we set out to determine whether the protein is capable of binding mRNA species within bacterial cells using ribonucleoprotein immunoprecipitation-microarray (RIP-Chip) assays. To do so, we needed to create a means of capturing cellular SarA protein. Accordingly, the SarA open reading frame was translationally fused to a C-terminal c-Myc epitope. This construct was placed under control of the *sarA* endogenous P1 promoter and was inserted into plasmid pCN51 to generate pKLA40. This plasmid was transfected into UAMS-929 (Δ*sarA*) cells to generate strain KLA43 and the expression properties of C-Myc-SarA protein were evaluated. It should be noted that the c-Myc epitope has been shown to exhibit little or no non-specific nucleic acid binding in previous assays (Pannone et al., 1998). As expected, RT-PCR and Western blotting using anti-c-Myc antibodies established that the chimeric protein was expressed within KLA43 cells (**Figures 3A,B**) during mid- and late-exponential as well as stationary phase growth. We also confirmed that c-Myc-SarA was capable of complementing SarA's regulatory effects within Δ*sarA* cells. Specifically, *sarA*-mutant cells have been reported as exhibiting altered exprotein profiles due to alterations in the control of gene expression (Tegmark et al., 2000). Thus, we evaluated the ability of the c-Myc-SarA construct to complement the exoprotein profile of supernates from stationary phase cultures of *S. aureus* strain UAMS-929 (Δ*sarA*). As shown in **Figure 3C**, the c-Myc-SarA construct at least partially complemented the exoprotein profile of Δ*sarA* cells, indicating that presence of the epitope did not significantly affect SarA's regulatory capacity and, consequently, the chimeric protein was appropriate for studying SarA's cellular role as an RNA-binding protein.

Having confirmed that the chimeric protein was expressed and functional within Δ*sarA* cells, RIP-chIP assays were performed using anti-c-Myc immunoglobulin immobilized on agarose beads to capture cellular c-Myc-SarA-bound transcripts. To do so, KLA43 (Δ*sarA* c-Myc-SarA) was grown to mid-exponential phase and the proteins were cross-linked to nucleic acids with 1% formaldehyde to stabilize any interactions between c-Myc-SarA and RNA molecules. Cells were lysed and c-Myc-SarA protein was then immunoprecipitated with the anti-c-Myc agarose beads, co-precipitating any bound RNA species. Western blotting of the cell lysates and RIP intermediates indicated the presence of the epitope-labeled SarA through sequential steps of the IP process (**Figure 3D**). After reversal of the RNA cross-linking, the RNA was

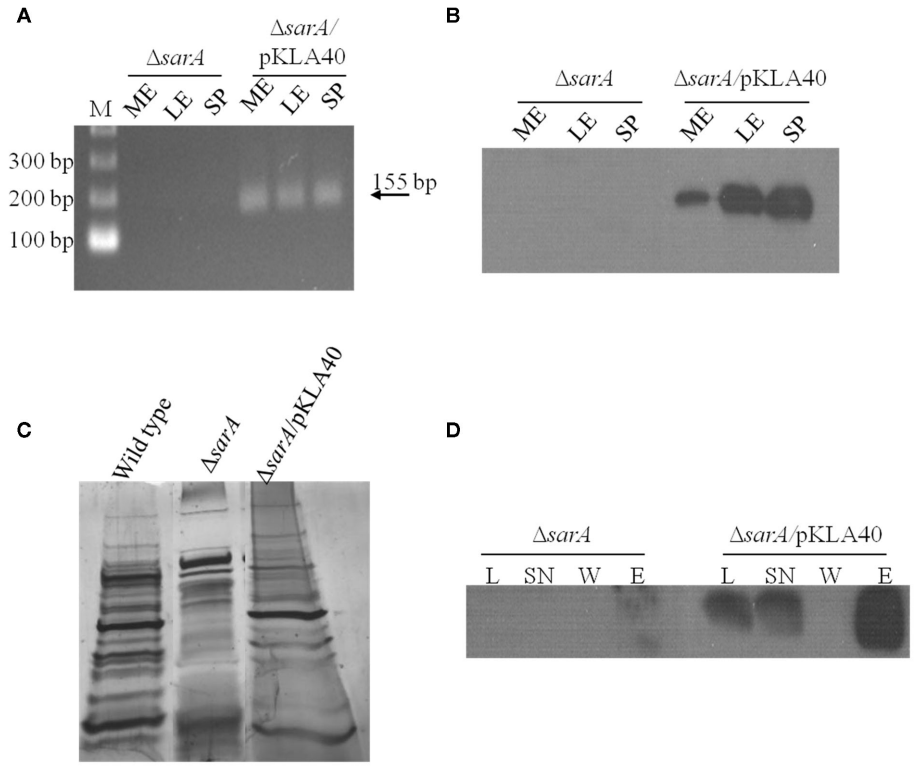

FIGURE 3 | SarA binds mRNA *in vivo*. **(A)** RT-PCR based detection of c-Myc-SarA transcript expression in Δ*sarA* and Δ*sarA* harboring plasmid pKLA40 harboring a c-Myc-SarA construct under control of the *sarA* P1 promoter during mid-exponential (ME), late-exponential (LE), and stationary (SP) phase growth. (**B**) Western blotting based detection of c-Myc-SarA chimeric protein from *sarA* and Δ*sarA* harboring plasmid pKLA40; lysates collected during mid-exponential (ME), late-exponential (LE), and stationary (SP) phase growth. **(C)** SDS-PAGE and silver staining of exoproteins purified from wild type, Δ*sarA* and Δ*sarA* harboring plasmid pKLA40 stationary phase culture supernates. **(D)** RNA immunoprecipitation (RIP) was performed by capturing c-Myc-SarA protein cross-linked to nucleic acids. Successful immunoprecipitation was confirmed by Western blotting of cell lysate (L), lysate supernate (SN), wash (W), and elution (E) fractions with anti-c-Myc antibody.

purified, treated with DNase, amplified, labeled, and applied to a *S. aureus* GeneChip®. All RIP-Chip assays were performed in triplicate for both KLA43 (Δ*sarA* c-Myc-SarA) and Δ*sarA* cells (negative control). A transcript was considered to be bound by c-Myc-SarA if its average signal intensity value was ≥ two-fold in KLA43 cells as compared to the negative control. It should be noted that as a prerequisite to performing RIP-Chip assays we exploited SarA's known ability to bind the *hla* promoter region and the fact that the microarray used in this study contains intergenic regions representing gene promoters to determine whether our experimental approach was appropriate for studying cellular SarA-nucleic acid interactions. Results from ChIP-chip experiments revealed that the signal intensity of the intergenic region representing the *hla* promoter region exhibited a signal increase beyond the established threshold (≥ two-fold) in samples from KLA43 cells as compared to UAMS-929 cells, indicating that the experimental approach was capable of detecting known *in vitro* protein-nucleic acid reactions *in vivo* (data not shown).

RIP-Chip assays results indicated that cellular c-Myc-SarA is capable of binding to a total of 115 RNA species, including eight virulence factor transcripts (Supplemental Table S5 in Supplementary Material). Of the virulence factor transcripts bound by SarA (**Table 6**), *spa* and *arcB* were also stabilized in a *sarA*-dependent manner in at least one of the growth phases studied here (late-log and stationary phase) or by our laboratory previously (mid-exponential phase; Roberts et al., 2006). Thus, our collective results indicate that *spa* mRNA is stabilized in a *sarA*-dependent manner

Table 6 | Virulence-associated transcripts bound by cellular c-Myc-SarA

Common name*	Fold change†	mRNA stability‡	Description
arcB	8.3	Stabilized	Ornithine carbamoyltransferase
pfoR	3.7	Destabilized	Perfringolysin O regulator protein
rsbW	7.6		Anti-sigma B factor
SA0097	8.3	Destabilized	Transcriptional regulator AraC/XylS family
sarZ	2.7		SarA homolog
sbi	3.3	Destabilized	IgG-binding protein
spa	4.2	Stabilized	IgG-binding protein A
srtA	7.4		Sortase

**S. aureus strain N315 locus.*

† Fold change of signal intensities present in c-Myc-SarA samples in comparison to Δ sarA samples ($p < 0.05$).

‡ Effect of sarA on transcript turnover.

(Roberts et al., 2006) and that SarA protein is capable of binding the transcript during *in vitro* conditions and within bacterial cells. When taken together, these results suggest that SarA may post-transcriptionally regulate gene expression by binding to and modulating the RNA degradation properties of target transcripts.

DISCUSSION

Staphylococci produce an array of TCRS and DNA-binding proteins that are thought to modulate virulence factor transcript synthesis, providing the pathogen with a means for sensing and responding to environmental stresses including exposure to sub-inhibitory concentrations of antibiotics (Novick, 2003). By definition, transcript titers are a function of both transcript synthesis and degradation, and mounting evidence strongly suggests that orchestrated changes in mRNA degradation mediate the organism's ability to regulate gene expression. Indeed, the global mRNA degradation properties of *S. aureus* are significantly altered in response to growth phase, stringent response-inducing conditions, and pH and temperature stress (Anderson et al., 2006, 2010;Olson et al., 2011). With respect to growth phase, RNA turnover is rapid in exponential phase cells whereas a global stabilization of RNA transcripts occurs during stationary phase growth; ∼90% of transcripts decay within 5 min during mid-exponential growth while only ∼50% of all transcripts decay within 5 min during stationary phase growth (Olson et al., 2011). Presently, the factors that mediate this transition between RNA degradation profiles are poorly characterized. Because all transcripts do not degrade at the same rate, it has been hypothesized that *trans*-acting factors, such as RNA-binding proteins or regulatory RNAs, may affect mRNA stability. Indeed, the mRNA turnover properties of nearly 150 exponential phase mRNA species, including the cell surface-associated virulence factors *spa* and *cna*, have been reported to be stabilized in a *sarA*-dependent manner (Roberts et al., 2006). However, those studies were limited in scope, in that the effects of *sarA* on RNA stability were only studied within mid-exponential phase cells. In the present work, we evaluated the effects of *sarA* on late-exponential and stationary phase RNA turnover.

A comparison of mRNA turnover within wild type and Δ*sarA* cells indicated that global RNA decay is similar between the two strains; mRNA is rapidly degraded in late-exponential phase cells but transcripts become more stable during stationary phase growth. Despite these similarities, transcript turnover within each growth phase was slightly decreased in Δ*sarA* cells indicating that a product of the *sarA* locus affects the stability of a subset of mRNA species. Indeed, *sarA* was found to both stabilize and destabilize transcripts in each growth phase; 3.4 and 16.5% of transcripts were stabilized, whereas 16.4 and 23.5% of transcripts were destabilized in a *sarA*-dependent manner within late-exponential and stationary phase cells, respectively. Among the stabilized transcripts were those encoding for translation machinery, suggesting that *sarA* may modulate protein production indirectly by post-transcriptionally altering expression of the translation apparatus. Perhaps more relevant to *S. aureus* pathogenesis, 19 virulence-associated factors were stabilized in a *sarA*-dependent manner, including cell surface components, secreted enzymes and toxins, transcriptional regulators, and antibiotic resistance determinants. Interestingly, a subset of these virulence factors, including *spa*, *hla*, *sak*, and *sspB* have been previously shown to be regulated by *sarA* at the transcriptional level (Cassat et al., 2006). These results suggest that the effects of *sarA* on gene regulation in *S. aureus* may be more complex than previously thought, and that a product of the locus may post-transcriptionally regulate gene expression.

The *sarA* locus is capable of producing three distinct transcripts, each of which encode for and result in the production of SarA protein. Thus, any potential post-transcriptional regulation could occur via the SarA protein or via one of the transcribed mRNAs as has been described with other regulatory RNAs in *S. aureus* (reviewed in Felden et al., 2011). The mRNA turnover properties of Δ*sarA* cells harboring plasmid-borne copies each of these three transcriptional units demonstrated that their RNA degradation properties mimicked one-another, suggesting that the SarA protein (as opposed to a particular *sarA* transcriptional unit) accounts for the *sarA*-dependent modulation of mRNA turnover (data not shown). Thus, we predicted that SarA directly or indirectly affects the RNA degradation properties of target transcripts. Accordingly, gel-shift assays were performed to determine whether SarA protein directly binds to *spa* mRNA *in vitro*, a transcript that is stabilized in a *sarA*-dependent manner. EMSA results revealed that SarA alters the mobility of *spa* mRNA in a concentration-dependent manner, suggesting that the protein is capable of binding the transcript in a cooperative manner. We hypothesize that this may reflect two potential dynamic properties of the interaction: (1) SarA oligomerizes when binding RNA or (2) the *spa* transcript contains multiple SarA-binding sites such that in the presence of excess SarA the number of sites bound by the protein are increased. Further, gel-shift assays indicated that SarA exhibits a greater propensity to cause a higher molecular shift of the 5′-end of *spa* mRNA, suggesting that either the 5′ end of the message harbors a higher incidence of SarA-binding sites or that the secondary structure of the transcript's 5′ end reduces the amount of steric hindrance. Regardless, we conclude from the results of these assays suggest that the SarA-mRNA interaction is not likely to be equal across the entire length of the *spa* transcript.

In order to characterize the physiological relevance of SarA's potential RNA-binding properties, we performed *in vivo* RIP-chIP assays to identify the transcripts with which the protein could interact within bacterial cells. To do so, c-Myc epitope-tagged SarA was expressed in Δ*sarA* cells and immunoprecipitated; c-Myc-SarA-bound RNA molecules were subsequently identified using GeneChips®. In total, SarA was found to interact with 115 mRNA species, including eight virulence factors. Specifically, two of the virulence factor transcripts that bound to SarA *in vivo* were also found to be stabilized in a *sarA*-dependent manner, including the *spa* transcript, an mRNA species that SarA appears to bind *in vitro*. Taken together, these data suggest that SarA may alter the stability of target transcripts by directly binding to these mRNA species, which could, in-turn, limit the RNA molecule's accessibility to degradation machinery. Additionally, SarA also interacted with three transcripts that are destabilized in a *sarA*-dependent manner, suggesting that the protein:mRNA interaction may catalyze a conformational change in the substrate RNA molecule that makes it more susceptible to ribonuclease attack. In addition to ORFs,

SarA also bound five transcripts that mapped to intergenic regions and may represent non-coding RNA molecules.

Collectively, the data presented here indicate that SarA interacts with RNA in a biologically relevant setting and that these interactions correlate with alterations in the degradation properties of a subset of those mRNA species. Thus, we hypothesize that SarA post-transcriptionally regulates gene expression via binding to and consequently modulating the mRNA turnover properties of target transcripts. There is precedence for this prediction in other organisms. For instance, in *Escherichia coli*, CsrA is an RNA-binding protein that modulates virulence factor expression, in part, by both stabilizing and destabilizing target transcripts. For instance, CsrA associates with the 5′ untranslated region of the *pgaA* transcript, which blocks translation of the molecule and subsequently destabilizes the transcript (Wang et al., 2005). CsrA has also been shown to bind the *flhDC* transcript, stabilizing the molecule and ultimately contributes to bacterial motility (Wei et al., 2001). Given the similarities of CsrA and other RNA-binding proteins to SarA, scenarios by which SarA can post-transcriptionally modulate gene expression emerge. In the case of *spa*, whose transcript is stabilized but protein production is repressed by SarA, binding of the RNA may simultaneously inhibit activity of the translational machinery and ribonucleases (RNases). This mechanism would presumably provide the cell with a mechanism to repress protein A production, in a manner that when inactivated would allow for efficient induction of Spa production without having to expend energy to synthesize new transcripts. Our finding that SarA seems to preferentially interact with the 5′ terminus of the *spa* transcripts supports this prediction, as the 5′end of mRNA not only serves as a potential entry point for the RNA degradation machinery, it is also the loading site for the translation apparatus. Similarly, the binding of SarA to transcripts which are normally stabilized and for which protein levels are increased, SarA could function by inhibiting degradation of the transcript by RNases, thus facilitating increased production of protein. As what occurs with CsrA, SarA also destabilizes transcripts that it binds *in vivo*, suggesting that this interaction could expose transcripts to cleavage by RNases. It was also observed that virulence factor transcripts that were stabilized in a *sarA*-dependent manner were not bound by SarA *in vivo* (Supplemental Table S5 in Supplementary Material). Thus, one can assume that the modulation of RNA stability of these transcripts is indirect; presumably, SarA regulates another factor, such as a regulatory RNA or other RNA-binding protein, which ultimately mediates the alteration in stability. While the biological significance of *sarA*-dependent control of mRNA stability is not yet fully understood, these results indicate that SarA is an RNA-binding protein with the potential to post-transcriptionally regulate the mRNA and consequently protein production of target transcripts. These results may have expanded significance in that SarA represents a prototypical regulatory molecule with a multitude of homologs within *S. aureus* and other bacterial pathogens.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2012.00026/abstract

Table S1 | Late-exponential phase transcripts stabilized in a *sarA*-dependent manner.

Table S2 | Stationary phase transcripts stabilized in a *sarA*-dependent manner.

Table S3 | Late-exponential phase transcripts destabilized in a *sarA*-dependent manner.

Table S4 | Stationary phase transcripts destabilized in a *sarA*-dependent manner.

Table S5 | Mid-exponential growth phase transcripts bound by SarA *in vivo*.

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Genetic regulation of the intercellular adhesion locus in staphylococci

David Cue, Mei G. Lei and Chia Y. Lee*

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Motoyuki Sugai, Hiroshima University, Japan
Ambrose Cheung, Dartmouth Medical School, USA

***Correspondence:**
Chia Y. Lee, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 Markam Street, Slot 511, Little Rock, AR 72205, USA.
e-mail: clee2@uams.edu

The formation of biofilms by *Staphylococcus aureus* and *Staphylococcus epidermidis* is an important aspect of many staphylococcal infections, most notably endocarditis, osteomyelitis and infections associated with indwelling medical devices. The major constituents of staphylococcal biofilms are polysaccharides, such as poly *N*-acetyl glucosamine (PIA/PNAG), cell surface and secreted bacterial proteins, and extracellular DNA. The exact composition of biofilms often varies considerably between different strains of staphylococci and between different sites of infection by the same strain. PIA/PNAG is synthesized by the products of four genes, *icaADBC*, that are encoded in a single operon. A fifth gene, *icaR*, is a negative regulator of *icaADBC*. Expression of *icaADBC* is tightly regulated, but can often be induced *in vitro* by growing staphylococci in the presence of high salt, high glucose, or ethanol. Regulation of *icaADBC* is complex and numerous regulatory factors have been implicated in control of *icaADBC*. Many of these are well known global transcriptional regulatory factors like SarA and sigmaB, whereas other regulators, such as IcaR, seem to affect expression of relatively few genes. Here, we will summarize how various regulatory factors affect the production of PIA/PNAG in staphylococci.

Keywords: *Staphylococcus aureus, Staphylococcus epidermidis,* biofilm, intercellular adhesion locus, *ica,* PIA, PNAG

Staphylococcus aureus is a major nosocomial and community acquired pathogen causing a diverse array of infections ranging from superficial infections of the skin and mucosa to highly invasive and potentially lethal infections. Perhaps not surprisingly, *S. aureus* encodes a large array of virulence factors that enable the organism to infect different tissues within its host. Despite the potential of *S. aureus* to cause disease, the organism asymptomatically colonizes approximately one third of the adult population with the nares being the most common niche (Iwase et al., 2010). A number of *S. aureus* infections are associated with the formation of biofilms, including endocarditis, septic arthritis and osteomyelitis, and infections associated with implanted medical devices such as prosthetic heart valves, skeletal prostheses, and catheters. The formation of biofilms not only facilitates bacterial colonization of a host, but also provides resistance to antibiotics and the host immune system. Biofilms can also serve as foci of infection for metastatic spread of bacteria and release of toxins into the bloodstream (Gotz, 2002; Fitzpatrick et al., 2005; O'Gara, 2007; Otto, 2008; Boles and Horswill, 2011).

S. epidermidis is a human commensal and an opportunistic pathogen capable of causing disease in immunocompromised individuals. In healthy individuals, *S. epidermidis* typically causes infections only if introduced into subcutaneous tissues by some form of trauma especially in the presence of foreign bodies. *S. epidermidis* is also a common cause of biofilm-associated infections. Because it is present on skin and mucosal surfaces, the organism has the potential to be introduced into deeper tissues during the implantation of medical devices. *S. epidermidis* is much less virulent than *S. aureus* and the capacity to form biofilms is considered the most important virulence trait of the organism (O'Gara, 2007; Otto, 2009; Fey and Olson, 2010).

FORMATION AND COMPOSITION OF BIOFILMS

Bacterial biofilms are complex communities of organisms containing layers of bacteria within a glycocalyx. A mature biofilm contains specific three dimensional structures referred to as towers or mushrooms separated by fluid filled channels (Costerton et al., 1999; Stoodley et al., 2002). The formation of biofilms occurs in multiple stages, initial attachment, microcolony and macrocolony formation, and detachment or disassembly (Otto, 2008; Fey and Olson, 2010; Boles and Horswill, 2011). The initial attachment of staphylococci is often mediated by cell surface proteins that bind to mammalian extracellular matrix/plasma proteins such as fibrinogen, fibronectin, collagen, vitronectin, or laminin. Collectively these bacterial proteins are frequently referred to as MSCRAMMs (*m*icrobial *s*urface *c*omponents *r*ecognizing *a*dhesive *m*atrix *m*olecules) (Patti et al., 1994). Staphylococci have dozens of MSCRAMMs which can be covalently or noncovalently bound to the cell surface. Many staphylococci are capable of binding directly to plastic surfaces and researchers have often measured attachment to plastic as an *in vitro* model of attachment *in vivo*. Implanted medical devices are usually coated by plasma proteins, however, possibly obviating a need to bind directly to abiotic surfaces (Tsang et al., 2008; Beenken et al., 2010).

Non-MSCRAMM, surface localized proteins can also mediate attachment. The major cell wall autolysins, AtlA, and AtlE, (Heilmann et al., 1997; Houston et al., 2011) promote binding to hydrophobic surfaces for initial attachment and possibly biofilm accumulation (Heilmann et al., 1997; Hirschhausen et al., 2010; Houston et al., 2011). Teichoic (TA) and lipoteichoic (LTA) acids can also aid in initial attachment (Qin et al., 2007). TAs and LTAs are common components of the cell envelopes of Gram-positive bacteria that often play a role in bacterial adherence to host cells. *S. aureus* strains with a mutation in the *dlt* operon or *tagO*, both involved in TA/LTA synthesis, exhibit reduced binding to polystyrene and glass or other abiotic surfaces (Gross et al., 2001; Vergara-Irigaray et al., 2008).

The formation of microcolonies and biofilm accumulation require mechanisms for intercellular aggregation of bacteria. The production of exopolysaccharides is a common and important factor in biofilm accumulation. In both *S. aureus* and *S. epidermidis*, the major exopolysaccharide produced is termed polysaccharide intercellular adhesion (PIA), also known as poly-*N*-acetyl-glucosamine (PNAG) (Mack et al., 1996). PIA/PNAG, which has a net positive charge, may promote intercellular interactions by binding to the negatively charged surfaces of bacterial cells. PIA/PNAG may or may not interact with TAs and LTAs to foster intercellular interactions (O'Gara, 2007; Vergara-Irigaray et al., 2008). PNAG has been found to be essential for biofilm formation by many strains of *S. aureus* and *S. epidermidis*. In addition to PIA/PNAG, biofilms contain bacterial proteins and DNA as essential components with the ratios of these components being variable.

A number of staphylococcal strains exhibit PIA/PNAG-independent biofilm formation. In the latter strains, secreted proteins and extracellular DNA appear to substitute for PIA/PNAG. The fibronectin-fibrinogen binding MSCRAMMs, FnbA, and FnbB (O'Neill et al., 2008) the IgG binding Spa protein (Merino et al., 2009), and the adhesin SasG (Geoghegan et al., 2010) all contribute to biofilm formation in *S. aureus*. The biofilm-associated protein (Bap) encoded by some *S. aureus* strains that cause bovine mastitis, appears absent in human isolates. Bap is important for both initial attachment and biofilm accumulation (Cucarella et al., 2004). The accumulation-associated protein (Aap) is commonly found in *S.epidermidis* isolates. Aap forms fibrillar structures on the cell surface and may facilitate intercellular interactions (Rohde et al., 2005). Bhp, a homolog of Bap, is another accumulation-associated protein produced by some *S. epidermidis* strains (Cucarella et al., 2001). At least some strains of staphylococci appear able to switch from PIA-dependent to PIA/PNAG-independent biofilm formation (Hennig et al., 2007).

A number of soluble extracellular proteins can also affect biofilm formation. Beta toxin is a *S. aureus* sphingomyelinase capable of lysing sheep erythrocytes under the appropriate assay conditions, and killing lymphocytes (Marshall et al., 2000; Huseby et al., 2007). Huseby et al. (2010) demonstrated that beta toxin promotes biofilm accumulation by forming crosslinks with itself in the presence of extracellular DNA, producing an insoluble nucleoprotein matrix. Alpha hemolysin, a small pore forming toxin, has also been shown to be required for biofilm production in the 8325-4 strain of *S. aureus*. Inactivation of the *hla* gene, encoding alpha hemolysin, resulted in a strain capable of initial attachment but incapable of the cell to cell interactions required for biofilm accumulation (Caiazza and O'Toole, 2003).

Detachment of biofilms is widely regarded as a mechanism for bacterial spread in an infected host, probably initiated by changes in pH, nutrient depletion, and waste accumulation within the biofilm. Detachment involves the degradation of the biofilm matrix by proteases and nucleases (Otto, 2008; Beenken et al., 2010; Boles and Horswill, 2011). Degradation of PNAG apparently does not occur in staphylococcal biofilms, as staphylococci do not seem to have a PNAG hydrolytic enzyme (Otto, 2009). A group of small amphiphilic α-helical peptides, known as phenol-soluble modulins seem to function as surfactants, disrupting cell-to-cell interactions within the biofilm. It has been proposed that phenol-soluble modulins may play a more important role in detachment of biofilms than do proteases (Otto, 2008; Boles and Horswill, 2011).

REGULATION OF PIA/PNAG PRODUCTION AND *icaADBC* EXPRESSION

Production of PIA/PNAG is tightly regulated and, at least *in vitro*, seems to occur primarily at the transcriptional level. Although the signals controlling PIA/PNAG production *in vivo* are not clearly defined, a number of environmental conditions affect production *in vitro*. High temperature, anaerobiosis, high osmolarity, glucose, and ethanol can all induce PIA/PNAG production although there is strain-to strain variation in regard to which conditions result in increased PIA/PNAG production. Subinhibitory concentrations of specific antibiotics, including tetracycline, gentamicin, and the streptogramins, quinopristin and dalfopristin, can also increase PNAG (Rachid et al., 2000b; Nuryastuti et al., 2011).

PIA/PNAG is synthesized by four proteins, IcaA, IcaD, IcaB, and IcaC, encoded by the *ica* operon (**Figure 1A**). The transmembrane proteins, IcaA, and IcaD, work in concert as an *N*-acetylglucosaminyltransferase to synthesize PNAG oligomers that are less than 20 residues in length. IcaC is a membrane protein believed to transport IcaAD-synthesized oligomers across the cell membrane. IcaC is also involved in the formation of long oligomers of PIA/PNAG. The IcaB protein, which can be found in association with the bacterial cell surface and culture supernatants, deacetylates PIA/PNAG resulting in a positively charged polymer. Deacetylation is believed to promote the interaction of PIA/PNAG with the negatively charged cell surface.

The *ica* locus was originally identified by screening a library of *S. epidermidis* transposon insertion mutants for isolates with defects in biofilm formation. A mutant with an insertion in the *ica* locus exhibited defects in biofilm formation, intercellular aggregation, and PIA synthesis (Heilmann et al., 1996a,b). The transposon insertion could be complemented by a plasmid carrying the *icaADBC* genes. Moreover, the *icaADBC* plasmid could confer a biofilm positive, aggregation, and PIA producing phenotype on the heterologous host species, *S. carnosus* (Heilmann et al., 1996b; McKenney et al., 1998). Not every isolate of *S. epidermidis* carries the *ica* locus, but *ica* genes seem to be more common in nosocomial and invasive isolates than in skin isolates (Rogers et al., 2008; Fey and Olson, 2010). It has been argued that carriage

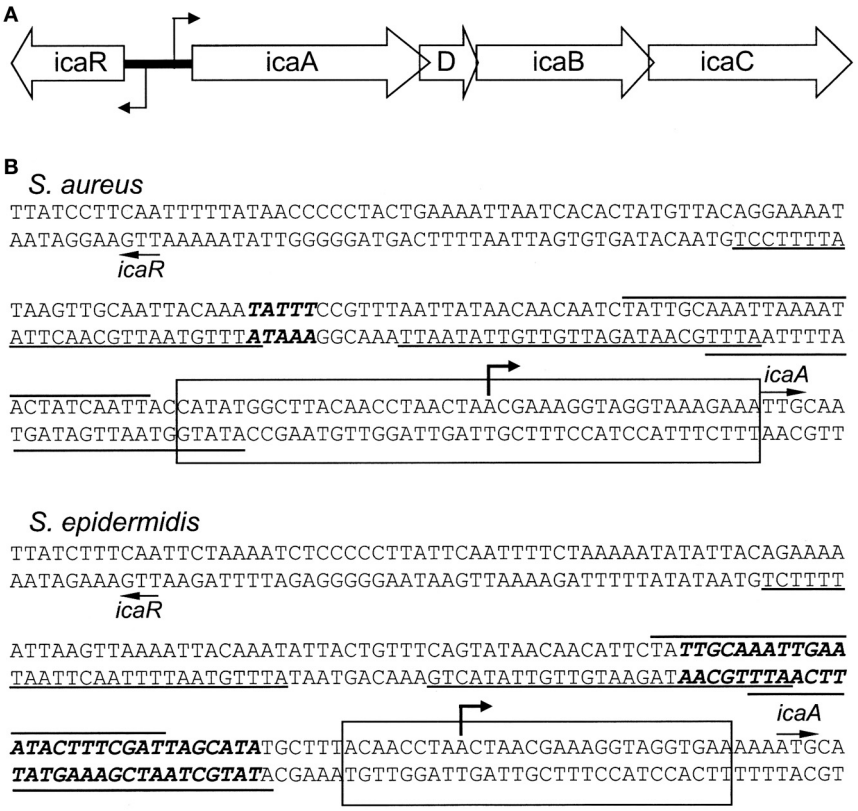

FIGURE 1 | (A) Organization of the intercellular adhesion (*ica*) operon in *S. aureus* and *S. epidermidis*. The bent arrows indicate the transcriptional start sites. **(B)** The *icaR*-to-*icaA* intergenic regions. The start sites of *icaR* and *icaA* are indicated by arrows. The putative binding sites for the SarA protein are underlined or overlined (Tormo et al., 2005). The bent arrow indicates the *icaADBC* transcription start site determined for *S. epidermidis* RP62A; (Heilmann et al., 1996b; Mack et al., 2000). Top: Sequence of the *S. aureus* NCTC 8325 *ica* locus (Gillaspy et al., 2006). The bold, italicized nucleotides indicate base pairs deleted in *S. aureus* MN8m that resulted in PIA/PNAG overproduction (Jefferson et al., 2003). The rectangle indicates the region bound by IcaR in DNase I protection experiments (Jefferson et al., 2003). Bottom: Sequence of *S. epidermidis* RP62A *ica* locus (Heilmann et al., 1996b). The bold, italicized nucleotides represent the highest affinity TcaR binding site (Chang et al., 2010). The rectangle indicates the IcaR binding site (Jeng et al., 2008).

of *icaADBC* may actually be detrimental for the survival of skin isolates of *S. epidermidis* (Rogers et al., 2008).

Subsequent to its discovery in *S. epidermidis*, the *ica* locus was found in *S. aureus* and appears to be present in nearly all isolates of the latter (Cramton et al., 1999). The *S. aureus ica* genes are organized as in *S. epidermidis* and the encoded proteins share from 79% to 89% similarity and 62–78% identity. The cloned *S. aureus* genes could confer biofilm production, PNAG synthesis and *N*-acetylglucosaminyltransferase activity to an *S. aureus ica* mutant and *to S. carnosus*.

The regulation of *icaADBC* expression is mediated by a number of regulatory factors (**Table 1**). These factors include global regulatory proteins such as SarA and σ^B, as well as factors like IcaR and TcaR which seem to regulate relatively few genes. Some factors regulate *icaADBC* expression directly (e.g., IcaR) whereas regulation by other proteins seems to be indirect (e.g., σ^B). Notably, mechanisms governing *ica* expression often vary not only between different species of staphylococci, but also between different strains of the same species. It is also worth noting that different laboratories induce *ica* expression and measure biofilm formation under a variety of different conditions. For example, most studies utilize standard 96 well microtiter plates to assay biofilm production *in vitro*. Some researchers use untreated plates whereas others coat their plates with serum prior to adding bacteria to the wells. Proponents of the latter method argue that serum coating more closely approximates *in vivo* conditions. These variations in assay conditions can complicate comparisons of results from different laboratories. Moreover, no *in vitro* conditions or animal model can replicate the environment of an infected human host. Despite these limitations, the studies cited below have established the importance of the *ica* genes in biofilm formation by staphylococci. Here, we will summarize what is known about various regulators of the *ica* locus.

REGULATORY FACTORS AFFECTING ica EXPRESSION

IcaR

The *ica* locus contains the same five known genes in both *S. aureus* and *S. epidermidis* (**Figure 1A**). IcaADBC are encoded by a single transcript that initiates 29 bp upstream of the *icaA* start codon in *S. epidermidis* strain RP62A (Accession number U43366) (Heilmann et al., 1996b; Mack et al., 2000). The fifth gene, *icaR*, is transcribed divergently from the other *ica* genes. The

Table 1 | Regulatory proteins affecting *icaADBC* expression in staphylococci.

Regulatory factor	Overall effect on icaADBC transcription	Probable mechanism	Reference
Sa IcaR	Negative	Direct binding to *icaADBC* promoter	Conlon et al. (2002)
Se IcaR			Jefferson et al. (2003)
Sa SarA	Positive	Direct binding to *icaADBC* promoter	Valle et al. (2003)
Se SarA			Tormo et al. (2005)
Se SarX	Positive	Direct binding to *icaADBC* promoter	Rowe et al. (2010)
Se SarZ	Positive	Unknown	Wang et al. (2008)
Sa TcaR	Negative	Direct binding to *icaADBC* promoter	Jefferson et al. (2004)
Se TcaR			Chang et al. (2010)
Sa σ^B	Variable depending on study	Indirect	See text
Se σ^B			
Sa Rbf	Positive	Repression of *icaR* through a hypothetical regulator	Cue et al. (2009)
Se Rbf			Rowe (2010)
Se LuxS	Negative	Unknown	Xu et al. (2006)
Sa Spx	Negative	Upregulation of *icaR*	Pamp et al. (2006)
Se Spx	Negative	Unknown, but not through *icaR*	Wang et al. (2010)
Sa SrrAB	Positive	Direct binding to *icaADBC* promoter	Ulrich et al. (2007)
Se Ygs	Positive	Unknown	Wang et al. (2011)
Sa GdpS	Positive	Unknown	Holland et al. (2008)
Se Gdps			Tu Quoc et al. (2007)
Sa CcpA	Positive	Indirect, see text	Seidl et al. (2008)
Se CcpA			Sadykov et al. (2011)

start codons of *icaA* and *icaR* are separated by approximately 163 bp of DNA (Conlon et al., 2002).

IcaR is an approximately 22 kDa protein of the TetR family of transcriptional regulators, (Conlon et al., 2002; Jeng et al., 2008). Amino acid sequence alignments first suggested that *icaR* might encode a transcriptional regulator (Ziebuhr et al., 1999; Rachid et al., 2000a). Conlon et al. (2002) inactivated *icaR* in strain CSF41498, a clinical isolate of *S. epidermidis* that produced a weak biofilm when grown in BHI broth at 37°C. Insertional inactivation of *icaR* significantly increased *icaA* expression, indicating that *icaR* may function as a repressor of *icaADBC*. Transcription of the *icaR* gene was unaffected in *icaR* mutants indicating that *icaR* is not autoregulated, a trait that is conserved in *S. aureus* (Jefferson et al., 2003). In CSF41498, transcription of *icaA* was inducible by high NaCl, high glucose, or ethanol, whereas *icaR* expression was reduced by ethanol, but was unaffected by NaCl or glucose. Expression of *icaA* was unaffected by ethanol in CSF41498 *icaR*::Erm^r, but was increased by NaCl or glucose in the same strain. It was concluded that regulation of *ica* expression by ethanol was *icaR*-dependent, whereas regulation by NaCl-glucose was *icaR*-independent. IcaR provided in *trans* was able to complement the *icaR*::Erm^r mutation and repress transcription of *icaA*. Complementation of *icaR* was modulated by ethanol.

The *icaR* gene has also been shown to be a negative regulator of *icaADBC* in *S. aureus*. The predicted *S. aureus* 8325-4 IcaR protein has 65.6% identity and 90.9% similarity with IcaR from *S. epidermidis* RP62A (Heilmann et al., 1996b; Mack et al., 2000). Jefferson et al. (2003) demonstrated that IcaR can bind to a DNA region immediately 5′ to *icaA* and that a short nucleotide sequence in the *icaA-icaR* intergenic region could affect expression of *icaADBC*. A spontaneous mutant of *S. aureus* MN8, called MN8m, was isolated which exhibited constitutive hyperproduction of PIA/PNAG and enhanced biofilm formation (McKenney et al., 1999; Jefferson et al., 2003). The mutation in MN8m responsible for hyperproduction of PIA/PNAG was determined to be a 5 bp (TATTT) deletion within the *icaA-icaR* intergenic region (**Figure 1B**). The 5 bp deletion increased *icaADBC* transcription but had no effect on *icaR* expression. Substitution of the TATTT sequence with ATAAA resulted in the same phenotype as the original deletion.

DNase I protection experiments did show that recombinant IcaR protected a 42 bp region upstream of the *icaA* gene (**Figure 1B**) (Jefferson et al., 2003). The TATTT sequence, however, played no role in IcaR binding. The latter result seemed to indicate that another DNA binding protein utilizes the TATTT sequence to regulate *icaADBC* expression. It was postulated that deletion of the TATTT sequence might alter an intrinsic bend in *ica* DNA, but this possibility was not directly tested. Interestingly, the TATTT sequence lies between two putative binding sites for SarA protein which is a positive activator of *icaADBC* (Tormo et al., 2005). Thus it is possible that the 5 bp deletion affects SarA binding to the *ica* intergenic region. Precisely how the deletion affects *icaADBC* transcription has not yet been determined, however.

Subsequent work by these same authors demonstrated that *icaR* is a repressor of *ica* transcription and that the protein is functional in MN8 and MN8m (Jefferson et al., 2004). Deletion of *icaR* increased *icaADBC* expression by 100-fold and PNAG production by 10-fold. PIA/PNAG production by strain MN8 requires exogenous glucose, whereas MN8 *icaR* overproduces PNAG in the absence of glucose, leading the authors to conclude that in *S. aureus*, upregulation of PNAG by glucose is at least in

part due to alleviation of IcaR-mediated repression of *icaADBC*. Recall that glucose reportedly did not affect *icaR* expression in *S. epidermidis* CSF41498 (Conlon et al., 2002). Deletion of the *icaR* gene in *S. aureus* 8325-4 also resulted in hyperexpression of *icaADBC* and increased PNAG production (Cue et al., 2009). As described below, several different regulators appear to affect *icaADBC* expression by repression or upregulation of *icaR*.

The crystal structure of IcaR from *S. epidermidis* was recently reported (Jeng et al., 2008). Like other proteins in the TetR family, IcaR is primarily α-helical. Three α helices form an N-terminal DNA binding domain with α helices two and three forming a helix-turn-helix motif. The native IcaR protein exists primarily as a homodimer. Dimerization is mediated by a large helix bundle formed by α helices 4–9 near the C-terminus of IcaR. Electromobility shift assays (EMSAs) performed with recombinant IcaR revealed a 28 bp *ica* operator centered 17/18 nucleotides 5′ to the *icaA* start codon. This agrees well with the localization of the IcaR binding site in the *S. aureus* operator (Jefferson et al., 2003). Two IcaR dimers bind cooperatively to the operator with the two dimers binding to opposite faces of the DNA. It was proposed that the binding of one *icaR* dimer may alter the DNA conformation thereby allowing binding of a second dimer (Jeng et al., 2008).

The ability of certain antibiotics to inhibit IcaR binding to DNA was also investigated, in part, because DNA binding by some members of the TetR family has been shown to be inhibited by certain antibiotics. Tetracycline, which can induce *ica* expression in *S. epidermidis*, did not affect DNA binding by IcaR, however, two aminoglycoside antibiotics, streptomycin and gentamicin, were shown to inhibit IcaR binding to DNA, presumably by directly altering the conformation of the protein (Rachid et al., 2000b; Jeng et al., 2008).

The Sar Protein Family

The Sar (staphylococcal accessory regulator) family of proteins is composed of at least 11 different proteins some of which (e.g., SarA and SarR, SarX) are found in both *S. aureus* and *S. epidermidis*. The various Sar proteins have been categorized as fitting into one of three subfamilies (Cheung et al., 2008). The first subfamily, which includes SarA and SarX, are generally small, about 15 kDa, basic proteins with a single DNA binding domain that probably bind DNA as homodimers. Proteins in the second subfamily have two homologous DNA binding domains and likely bind DNA as monomers. The final subfamily is comprised of proteins that seem closely related to MarR protein (Liu et al., 2001, 2006; Manna and Cheung, 2001; Li et al., 2003; Ballal and Manna, 2009).

SarA. SarA is arguably the most well studied of the various Sar proteins. The SarA protein is a 124 amino acid residue protein that has a calculated PI of around 9. SarA is a dimeric protein with a central core region comprised of a winged-helix DNA binding domain where the helix-turn-helix domain recognizes the major groove and the winged region interacts with the minor groove. Dimerization appears to be mediated by a conserved α-helical region near the N-terminus of the protein. Structure function studies have suggested that multiple SarA dimers may bind a single target sequence and that the association of multiple dimers is fostered by Ca^{++} binding. It has been proposed that a SarA homodimer can bind a target site and recruit additional homodimers to the site (Liu et al., 2006).

The SarA protein can function as either an activator or repressor of transcription (Bayer et al., 1996; Beenken et al., 2003; Tormo et al., 2005; Oscarsson et al., 2006). SarA is a global regulatory protein affecting expression of many genes in *S. aureus* including many genes involved in pathogenesis thus making SarA a major virulence factor. Among the genes under positive regulation by SarA is the *agr* (accessory gene regulator) locus. The *agr* locus contains two divergent promoters that produce two transcripts. One transcript, RNAII, encodes four proteins that constitute a quorum sensing system. The second transcript, RNAIII, is a regulatory RNA and also encodes *δ*-toxin. The *agr* system in general is involved in the switch from synthesis of cell surface proteins during exponential growth to synthesis of toxins and degradative proteins in the postexponential to stationary growth phases. Expression of *agr* can reduce the capacity of *S. aureus* to form biofilms (Vuong et al., 2000; Cafiso et al., 2007; Coelho et al., 2008; Beenken et al., 2010). Due to the fact that SarA is a positive activator of *agr*, and because *agr* can repress biofilm formation, it might be anticipated that mutation of *sarA* would increase biofilm. It appears this is not the case, however, as *sarA* mutants have a reduced capacity to form biofilms (Valle et al., 2003; Handke et al., 2007; Tsang et al., 2008; Beenken et al., 2010). This is perhaps not surprising in that SarA affects biofilm formation by affecting expression of multiple targets. For example, mutation of SarA results in increased expression of proteinases and nucleases, both of which have a negative impact on biofilm (Beenken et al., 2010). SarA also appears to enhance biofilm formation more directly by increasing *ica* expression (Valle et al., 2003).

Valle et al. (2003) screened a library of Tn917 insertion mutants to identify biofilm-defective mutants of *S. aureus*. Some of the mutants had Tn917 insertions within *sarA*. Subsequently, they deleted or insertionally inactivated *sarA* in four unrelated *S. aureus* strains, all of the mutants failed to produce a biofilm. Deletion of *agr* in the wild type strains did not affect biofilm formation, indicating that the effect of the *sarA* mutations was independent of *agr*. A series of experiments was performed to determine if increased protease production accounted for the phenotype of the *sarA* mutants. The authors concluded that enhanced proteolysis could not account for the biofilm deficient phenotype. This is somewhat at odds with some of the studies described above. In the Valle et al. (2003) study, *sarA* mutations did significantly decrease *ica* transcription and PIA/PNAG production, but this study did not determine whether SarA can bind to the ica promoter. Subsequent studies, however, did establish that SarA could bind the *ica* promoter (Tormo et al., 2005).

S. epidermidis also encodes SarA which is 84% identical to SarA from *S. aureus* (Fluckiger et al., 2005). Tormo et al. (2005) deleted *sarA* in two different clinical isolates of *S. epidermidis* and reported that both mutants were deficient in biofilm formation. PIA/PNAG production and transcription of *icaA* were both significantly reduced but not abolished in the *sarA* deleted strains. Transcription of *icaR* was unaffected by deletion of *sarA*. As has been observed for *S. aureus*, proteinase production was increased

in *sarA* mutants, which likely contributed to the mutants' inabilities to form biofilms. Recombinant SarA protein was shown to bind with comparable affinities to the *icaR-icaA* promoter regions of *S. aureus* and *S. epidermidis*. The *icaR-icaA* promoter regions of both species contain multiple SarA consensus binding sites (**Figure 1B**).

It is important to note that protein phosphorylation/dephosphorylation plays an important role in biofilm formation and SarA activity. Two *S. aureus* serine/threonine kinases, Stk1/PknB and SA0077, can both phosphorylate SarA. Phosphorylation by Stk1/PknB seems to increase the affinity of SarA for some promoters and decrease it's affinity for other promoters (Didier et al., 2010). In *S. epidermidis*, Stk is required for biofilm formation and plays a major role in *icaADBC* expression (Liu et al., 2011). The *sarA*, *agr,* and *sigB* genes are all regulated by Stk1/PknB in *S. aureus* (Tamber et al., 2010), thus phosphorylation of SarA and possibly other regulatory proteins, seems likely to significantly affect *ica* expression.

SarX. SarX was first discovered in *S. aureus* by virtue of its homology with other Sar family proteins (Manna and Cheung, 2006). It is a 119 amino acid protein representative of the single domain class of Sar proteins. Manna and Cheung (2006) demonstrated that the SarX protein of *S. aureus* RN6390 binds to the *agr* promoter, repressing synthesis of RNAII and RNAIII, thereby indirectly repressing exoprotein synthesis. Subsequently, Rowe et al. (2010) demonstrated that SarX from *S. epidermidis* strain CSF41498 bound to its cognate *agr* promoter and repressed *agr* transcription.

Reportedly, *sarX* did not affect biofilm formation in *S. aureus* RN6390, but did promote biofilm formation by *S. epidermidis* CSF41498 in an *ica*-dependent manner (Rowe, 2010, C.Y.L. and J.P.O., unpublished data). Expression of *S. epidermidis sarX* on a multicopy plasmid not only complemented a *sarX* mutation, but also enhanced biofilm formation by the wild type strain (Rowe et al., 2010). Expression of *sarX* increased *icaA* transcription as well as PNAG production, but expression of *icaR* was unaffected by *sarX*. A purified maltose binding-SarX fusion protein bound to *ica* promoter DNA generating a ladder of protein-DNA complexes. A similar pattern was previously shown for SarX binding to the *agr* promoter region (Manna and Cheung, 2006). To account for the observed laddering, it has been suggested that either the *ica* and *agr* promoters each contain multiple SarX binding sites or that SarX oligomers form on bound DNA. Thus *sarX* appears to directly affect *icaADBC* transcription, at least in *S. epidermidis* CSF41498. Modulation of *agr* expression by SarX is apparently inadequate to affect biofilm formation in *S. aureus*.

Interestingly, *sarX* is located immediately downstream from the *rbf* gene in both *S. aureus* and *S. epidermidis* (see below) and is under positive regulation by *rbf* in *S. aureus* strains 8325-4 and UAMS-1. The *rbf* gene has been shown to upregulate biofilm formation and *icaADBC* expression and may do so, at least in part, by increasing *sarX* transcription (Lim et al., 2004; Cue et al., 2009).

SarZ. SarZ has also been shown to affect *ica* expression in *S. epidermidis* 1457. Wang et al. (2008) utilized a novel biofilm screening assay, involving separate and consecutive screens, to isolate biofilm-defective mutants with Tn917 insertions. The screen resulted in the isolation of two mutants both of which had Tn917 inserted in *sarZ*. The mutants had defects in primary attachment as well as biofilm accumulation. PIA/PNAG production and *icaADBC* expression were both reduced in *sarZ* mutants. Moreover, *sarZ* was shown to contribute to virulence in both rat and mouse models of biofilm-associated infection. Microarray studies revealed that the *sarZ* regulon is comprised of at least 80 genes thus decreases in *ica* expression may not completely account for the biofilm negative phenotype of *sarZ* mutants. As an example, three genes encoding proteinases were all upregulated in the *sarZ* mutant (Wang et al., 2008). Increased proteinase activity seems likely to account, in part, for the mutant phenotype.

TcaR

TcaR is a member of the MarR family of transcription factors and is encoded by both *S. aureus* and *S. epidermidis*. A role for TcaR in *ica* expression was first revealed by Jefferson et al. (2004) who used DNA affinity chromatography to identify *S. aureus* proteins capable of binding to a DNA fragment containing the *icaA-icaR* promoter region. Topoisomerase IV, SarA and DNA-binding protein II were also recovered in the same experiment. Purified TcaR did not produce a distinct footprint with *ica* DNA, however, and produced a ladder of complexes in EMSA experiments. These results suggested that either there are multiple TcaR binding sites in *ica* DNA or that TcaR oligomerizes once bound to DNA (Jefferson et al., 2004). Of these, the former possibility seems the most probable (Chang et al., 2010).

Northern analysis indicated that inactivation of *tcaR* increased transcription of *icaADBC* fivefold in three different strains of *S. aureus*, indicating that *tcaR* is a negative regulator of *ica*. Surprisingly, deletion of *tcaR* did not affect bacterial binding to polystyrene nor did it affect PIA/PNAG production, whereas, deletion of *icaR* affected both attachment and PIA/PNAG. When coupled with an *icaR* deletion mutation, deletion of *tcaR* increased *ica* expression fivefold over the single *icaR* mutant and 500-fold over the wild type. The *icaR* mutation alone augmented *icaA* transcription approximately 100-fold. Bacterial adherence and PNAG production were also increased in an *icaR tcaR* double mutant, relative to an *icaR* single mutant (Jefferson et al., 2004).

TcaR binds DNA as a dimer and displays non-cooperative binding to the *ica* promoter region (Chang et al., 2010). TcaR from *S. epidermidis* binds to at least one of three consecutive 33 bp pseudopalindromic sequences located immediately upstream of *icaA*. TcaR seems to have the highest affinity for the most proximal binding site which is only a few bps away from the *icaR* binding site. It is not known whether TcaR and IcaR can bind simultaneously to *ica* DNA. A number of antibiotics were shown to inhibit DNA binding by TcaR, this was believed to be due to antibiotic induced changes in the conformation of the TcaR DNA binding domain. Three aminoglycoside antibiotics, kanamycin, gentamicin, and streptomycin, were shown to inhibit DNA binding by TcaR and to promote biofilm formation by *S. epidermidis* RP62A. However other antibiotics, such as β-lactams, disrupted DNA binding but had no significant effect on biofilm formation. It was proposed that low concentrations of some antibiotics, by virtue of their abilities to disrupt DNA binding by TcaR and IcaR,

may derepress *icaADBC* which, in turn, would confer increased antibiotic resistance due to biofilm formation.

σ^B

σ^B is an alternative sigma factor found in staphylococci and other Gram-positive bacteria that plays a key role in the response to environmental stress (Conlon et al., 2004). In *S. aureus*, σ^B is activated by signal transduction in response to high temperature, high osmolarity, antibiotics, or extreme pH. Transcription of the *sigB* operon is driven by three distinct promoters. The first is a σ^A-dependent promoter that produces a transcript encoding *rsbUVW* and *sigB*. The second is a σ^B-dependent promoter that drives synthesis of a shorter transcript lacking *rsbU*. The third promoter is the *mazEF* promoter which drives transcription of a 3.7 kb mRNA encoding the toxin-antitoxin pair, MazEF, as well as RsbUVW-σ^B. Transcription from p_{mazEF} is enhanced by heat shock and exposure to tetracycline or erythromycin (Donegan and Cheung, 2009). Full expression of *sigB* appears to require all three promoters.

The activity of σ^B is controlled by a network of kinases and phosphatases. In the absence of stress, σ^B is inactive due to its association with an anti-σ factor, RsbW. RsbW also functions to phosphorylate and thereby inactivate the anti-anti-σ factor RsbV. Under stress conditions, RsbU, a phosphatase, dephosphorylates RsbV. RsbV can then bind RsbW, disrupting the latter's association with σ^B. The released sigma factor can then associate with the core RNA polymerase (Knobloch et al., 2004).

σ^B has been shown to regulate in excess of 200 genes, including a number of genes involved in biofilm formation (Bischoff et al., 2004; Pane-Farre et al., 2006; Nielsen et al., 2011). Rachid et al. (2000a) reported a role for σ^B in *S. aureus* biofilm formation in a clinical isolate and the laboratory strain RN4220. In these experiments, σ^B was found to be required for induction of *ica* transcription and biofilm formation in response to high NaCl. Cerca et al. (2008) looked at *icaADBC* and *icaR* expression in σ^B-deleted derivatives of *S. aureus* strains SA113 and Newman (Cerca et al., 2008). Surprisingly, σ^B was found to be a positive regulator of both *icaR* and *icaADBC*. This unexpected result was proposed to be possibly due to rather weak repression of *icaADBC* by *icaR* in the strains used in the study.

As described above, Valle et al. (2003) reported that *sarA* was critical for *ica* expression and biofilm formation in multiple, unrelated *S. aureus* clinical isolates. Additionally, they reported that mutation of σ^B in the same strains had no significant effect on *ica* expression or biofilm. Remarkably, inactivation of σ^B in *sarA* mutant strains increased PIA/PNAG production and biofilm formation relative to the single *sarA* mutant strains. The latter occurred even though *icaA* expression in *sarA-sigB* double mutants was significantly less than in *sarA* mutants. It was proposed that σ^B might upregulate expression of a factor involved in turnover of PIA/PNAG.

S. epidermidis also possesses a *sigB* operon similar in size, organization, and function as *sigB* in *S. aureus*. *S. epidermidis* σ^B has been shown to affect biofilm production both *in vivo* and *in vitro* (Knobloch et al., 2004; Handke et al., 2007; Pintens et al., 2008). RsbU was shown to function as a negative regulator of *icaADBC* in *S. epidermidis* strains 1457 and 8400 (Knobloch et al., 2004). This effect was shown to be due to a reduction of σ^B expression that, in turn, increased *icaR* expression. Increased *icaR* expression decreased *icaADBC* expression and biofilm. As was observed for *S. aureus*, high NaCl concentration did not induce biofilm in a *sigB* mutant. The biofilm defect in *sigB* mutants could be overcome, however, by growth in the presence of subinhibitory concentrations of ethanol. The effect of ethanol was due to σ^B-independent repression of *icaR*. The σ^B defect could also be overcome by multiple copies of *icaADBC*. The repression of *icaR* via ethanol has been speculated to involve an unknown intermediate factor. Notably, *sarA* was expressed in *sigB* mutants via the σ^B-independent *sarA* promoters, P1 and P2, thus decreased *icaADBC* expression was apparently not due to loss of SarA.

Upregulation of biofilm by anaerobiosis also involves σ^B. Anaerobic activation of *icaADBC* was σ^B-dependent and was concomitant with σ^B-dependent repression of *icaR*. σ^B appears to play a more important role in *ica* regulation under anaerobic conditions than it does under aerobic conditions, at least in *S. epidermidis* 1457 (Cotter et al., 2009). None of the aforementioned studies found any evidence for direct regulation of *ica* genes by σ^B in either *S. aureus* or *S. epidermidis*. This coupled with the lack of a σ^B consensus promoter sequence in the *ica* intergenic region, implies σ^B regulation is indirect.

The role of σ^B in biofilm expression is further complicated by the fact that σ^B can also activate *ica*-independent biofilm formation. σ^B has also been shown to function in *ica*-independent biofilm formation in *S. aureus* USA300 LAC, a CA-MRSA isolate (Lauderdale et al., 2009). In general, σ^B can promote biofilm by repressing the production of proteases and toxins, an effect that is manifest through decreasing expression of RNAIII as well as through positive activation of *sarA*. In USA300 LAC, loss of σ^B increased the level of RNAIII and mutation of *agr* restored biofilm formation in a *sigB* mutant. The addition of proteinase inhibitors to growth media also restored biofilm in a σ^B deficient strain. Thus another role of σ^B appears to be repression of *agr* that, in turn, represses the production of proteinases that are involved in the disassembly of biofilm.

Rbf

Rbf (regulator of biofilm) is a transcriptional regulatory protein found to play an important role in biofilm formation in both *S. aureus* 8325-4 and *S. epidermidis* CSF 41498 (Lim et al., 2004; Rowe, 2010). Rbf is a member of the AraC/XylS family of transcriptional regulators, a family that bears a highly conserved 100 amino acid region forming a dual, helix-turn-helix DNA binding motif. The dual helix-turn-helix is usually localized in the C-terminal region of a protein (Gallegos et al., 1997). The two helix-turn-helix domains within a monomer are believed to interact with consecutive major grooves of DNA, thus their binding sites are typically longer than they are for classical HTH proteins (Schleif, 2010). There are hundreds of different AraC/XylS-like proteins, many of which have been identified in sequence data bases by virtue of possessing the dual HTH motif (Gallegos et al., 1997). *S. aureus* is predicted to encode at least 6 AraC/XylS-like proteins at least two of which, Rbf and Rsp, regulate biofilm formation (Lim et al., 2004; Lei et al., 2011).

Typical AraC/XylS proteins are relatively small, 250–300 amino acids long, and many have effector binding sites in the N-terminal domains of the proteins. The effector binding sites and DNA binding regions are typically separated by a linker region. The effector binding domain often regulates the DNA binding activity (Gallegos et al., 1997). For example, DNA binding to the *araBAD* promoter by the AraC protein of *E. coli*, is affected by binding of arabinose and some other sugars. In the presence of arabinose, AraC binds to two half-sites, called I1 and I2, which lie just upstream of *araBAD*. In this conformation AraC can interact with RNA polymerase and promote transcription of *araBAD*. In the absence of arabinose, AraC binds to the I1 half-site and to a third half-site, O1, which lays 210 bp upstream of I1. In this conformation a DNA loop is formed and transcription of *araBAD* is repressed (Schleif, 2010).

Although Rbf protein has the conserved DNA binding motif, it is an atypical AraC-like protein. First, it is significantly larger than most other AraC proteins, approximately 716 amino acid residues in *S. aureus* 8325-4. Additionally, the DNA binding motif of Rbf is located near the N-terminus of the protein. Expression of *rbf* is likely to be complex as the promoter-regulatory region contains putative binding sites for σ^B and *saeR* (C.Y.L. unpublished). The *rbf* gene was originally identified by screening of a transposon insertion library of strain 8325-4 for biofilm-defective mutants (Lim et al., 2004). Loss of *rbf* led to a defect in biofilm formation in response to high NaCl and glucose, but did not affect ethanol-induced biofilm. Inactivation of *rbf* did not affect initial attachment of staphylococci to polystyrene but did severely inhibit multicellular aggregation. Extensive macrocellular clumping was observed when Rbf from either *S. aureus* or *S. epidermidis* was expressed from a multicopy plasmid (Lim et al., 2004; Rowe, 2010). Additionally, multicopy *rbf* increased biofilm formation in *S.aureus* via increasing intercellular aggregation. The protein was also found to play a significant role in biofilm formation *in vivo* (Luong et al., 2009).

In a subsequent study, microarray experiments were performed to determine the *rbf*-regulon in a clinical isolate of *S. aureus* strain UAMS-1 (Cue et al., 2009). Expression of Rbf from a multicopy plasmid was found to increase expression of six genes and reduce expression of 35 genes. A number of the *rbf*-regulated genes could, potentially at least, affect biofilm formation. The *tagB* gene, which encodes teichoic acid biosynthesis gene B, is upregulated by *rbf*. Four genes likely to affect cell lysis and DNA release in biofilms, *lytSR, lrgAB* are all repressed by *rbf*. The *lytSR* genes encode a two component system that upregulates *lrgAB*. The *lrgA* gene product function as an antiholin that can inhibit cell lysis and the release of DNA into the environment (Sadykov and Bayles, 2011). Thus by inhibiting *lytSR* expression, Rbf would be predicted to increase the level of extracellular DNA in biofilm. SarX, a protein observed to enhance *icaADBC* expression in *S. epidermidis* (Rowe et al., 2010) is regulated by *rbf* in *S. aureus* UAMS-1. The gene encoding KdpD, a histidine kinase affecting *luxS* expression, is also regulated by *rbf* (Cue et al., 2009).

The microarray experiments performed with UAMS-1 produced two surprising results. The first was that deletion of *rbf* had no effect on gene expression. This was apparently due to the fact that the *rbf* gene in UAMS-1 has a 2 bp insertion near the N-terminal coding region. Thus, although *rbf* is transcribed, no Rbf would be synthesized. The second surprise was that multi-copy *rbf* increased *icaADBC* transcription about five to sixfold. This was surprising due to the fact that Lim et al. (2004) reported that expression of an *icaA*-xylE fusion was unaffected by inactivation of *rbf* in 8325-4. Real time quantitative PCR experiments, as well as PIA/PNAG, assays confirmed that *rbf* does positively regulate *icaADBC* transcription in 8325-4 and UAMS-1. The reason Rbf failed to increase transcription of the *icaA*-XylE fusion is unclear at this time.

The microarray experiments also revealed that *rbf* can reduce *icaR* transcription, a finding confirmed by qRT-PCR experiments. Thus, it appears that *rbf* activates *icaADBC* expression, at least in part, via inhibiting expression of *icaR*. Most AraC/XylS proteins act as activators of transcription and some, such as AraC, also act as repressors (Gallegos et al., 1997). It is possible that *rbf* affects *ica* by direct activation of *icaADBC*, in addition to repression of *icaR*. Experiments to test for direct binding of Rbf to the *ica* promoter have yielded only negative results, suggesting that *rbf* regulation may be indirect (Cue et al., 2009; Rowe, 2010). Recombinant Rbf also failed to bind to the promoter regions of other genes, (i.e., *sarA, sarX, sarZ, spx*, and *srrA*) that regulate *ica* (Rowe, 2010).

Rbf in *S. epidermidis* CSF41498 is 46% homologous and 65% similar to Rbf from *S. aureus* with the highest similarity being in the putative DNA-binding domains (Rowe et al., 2010). Expression of the CSF414498 *rbf* gene in *S. aureus* increased macroscopic cell clumping, biofilm formation and *icaA* expression. Site-specific mutagenesis of the *rbf* DNA-binding domain, resulted in the loss of Rbf -induced cell clumping and biofilm. As was observed for 8325-4, mutation of *rbf* in CSF41498 reduced biofilm formation in response to high NaCl and glucose, but not in response to ethanol. Initial attachment by *S. epidermidis* was unaffected by *rbf*. Interestingly, the cloned *rbf* gene did not fully complement the biofilm defect of CSF41498 *rbf*, leading Rowe (2010) to propose that overexpression of Rbf may have negative effects on biofilm formation. It has been demonstrated that Rbf could bind specifically to the *sarR* promoter of *S. epidermidis* and that *rbf* had a modest effect on *sarR* transcription in stationary phase cultures. Furthermore, it was found that SarR protein could bind specifically to the *ica* promoter. While these data support a model where Rbf could regulate *ica* expression through SarR, mutation of *sarR* had no significant effect on *icaADBC* expression or biofilm formation, thus the significance of the DNA binding studies is unclear at present (Rowe, 2010).

LuxS

LuxS is part of a quorum sensing system found in numerous species of Gram-negative and Gram-positive bacteria, including *S. aureus* and *S. epidermidis* (Doherty et al., 2006; Li et al., 2008). LuxS is required for the synthesis of autoinducer 2 (AI-2) a family of small, diffusible compounds that can penetrate cell membranes. Unlike other quorum-sensing systems *luxS* is not species-specific, rather, AI-2 produced by one species can affect gene expression in multiple bacterial species.

Several groups have determined that *luxS* is a negative regulator of biofilm formation in *S. epidermidis* (Kong et al., 2006; Xu et al., 2006; Li et al., 2008). AI-2 can be found in culture supernatants of *S. epidermidis* and is secreted optimally during log and early stationary phases of growth. AI-2 present in culture supernatants of *S. epidermidis* can activate expression of AI-2 responsive genes in *E. coli* DH5α (Xu et al., 2006).

Deletion of *lux* in *S. epidermidis* strain 1457 was found to enhance biofilm formation. This effect seemed largely due to changes in *ica* expression as transcription of *icaC* increased over fourfold and PIA/PNAG synthesis was enhanced about threefold in the *luxS* strain. Expression of *ica* returned to the wild type level in a complemented strain and when exogenous AI-2 was added to cultures of the *luxS* mutant. Whether *icaR* expression was affected by deletion of *luxS* was not reported. The *luxS* mutant was found to more virulent than the wild type strain in a rat intravascular, central-venous-catheter-associated model, presumably due to increased PIA/PNAG production by the mutant strain (Xu et al., 2006).

While *luxS* appears to be an important *ica* regulator in *S. epidermidis*, the role of *luxS* in *S. aureus* biofilm formation is less clear. Doherty et al. (2006) found no role for *luxS* in the expression of virulence traits in strain RN6390, including biofilm formation. LuxS is reportedly inactivated by serine/threonine phosphorylation in *S. aureus*, but the effects on biofilm formation have not been reported (Cluzel et al., 2010). It has been reported, however, that a furanone derived from a marine algae could promote biofilm formation by *S. aureus* strain Newman and *S. epidermidis* strain 1457. In *S. epidermidis*, enhanced biofilm formation correlated with reduced *luxS* expression and increased PIA/PNAG production. It was not reported whether the furanone affected biofilm formation by the same mechanism in *S. aureus* (Kuehl et al., 2009).

Spx

The Spx protein is a global transcriptional regulator that is itself subject to regulation by the energy-dependent ClpXP proteinase complex in a number of Gram-positive bacteria. Spx appears to function in *S. aureus* in much the same manner as Spx in *Bacillus subtilis*. In the latter organism, Spx acts as both a transcriptional activator and a repressor. Spx binds directly to the α subunit of RNA polymerase thereby potentially blocking the interaction between RNA polymerase and other transcription factors. Spx can also directly affect promoter recognition by RNA polymerase (Nakano et al., 2010). Spx is a redox sensitive regulator that can activate genes, such as those encoding thioredoxin and thioredoxin reductase, important in the cellular response to oxidative stress. The N-terminal region of Spx contains two cysteine residues that form an intramolecular disulfide bond under thiol-oxidizing conditions. Oxidized Spx can associate with RNA polymerase and direct transcription of select *B. subtilis* genes (Nakano et al., 2003, 2005).

In *S. aureus* 8325-4, Spx plays an important role in response to stress as a *spx* mutant is hypersensitive to high and low temperatures, high osmolarity, and oxidative stress. Transcription of *txrB*, the gene encoding thioredoxin reductase, requires *spx* in strain 8325-4. ClpP and Spx also affect biofilm formation. In a *spx* mutant, initial attachment, cell aggregation and biofilm formation were all enhanced. Transcription of *icaADBC* was increased in *spx* mutants while *icaR* transcription was decreased. Thus the normal role of Spx with regard to *ica* expression is to repress *icaADBC* via enhancement of *icaR* transcription. Precisely how Spx enhances *icaR* expression is unclear. It should be noted that, while an *S. aureus spx* null mutant could be constructed, the mutant exhibited growth defects even in the absence of stress (Pamp et al., 2006).

In *S. epidermidis*, *clpP* mutants reportedly accumulate high levels of Spx and exhibit defects in initial attachment and biofilm formation (Wang et al., 2010). The *spx* gene may be essential in *S. epidermidis* as knockout of the gene was not achieved in *S. epidermidis* 1457. However, a knockdown plasmid construct carrying an antisense *spx* RNA could promote biofilm formation, *icaADBC* transcription and PIA production. Unlike the case with *S. aureus*, *spx* did not affect expression of *icaR* nor did *spx* affect initial attachment. *S. aureus* and *S. epidermidis* also differ in that the *S. epidermidis* Spx does not regulate *trxB* (Wang et al., 2010).

SrrAB

SrrAB is a two-component regulatory system responsive to anaerobiosis (Ulrich et al., 2007). An *srrAB* mutant of *S. aureus* strain SA113 exhibited downregulation of *icaA* transcription and PIA/PNAG expression under anaerobic condition. SrrAB did not affect *icaR* expression. Phosphorylated SrrA protein bound to a 100 bp DNA segment immediately upstream of the *icaADBC* promoter. SrrAB is important for anaerobic growth and protection of staphylococci from killing by neutrophils under anaerobic conditions (Ulrich et al., 2007).

Ygs

Ygs is a general stress response protein identified by transposon mutagenesis of *S. epidermidis* strain 1457. Strains with mutation of *ygs* show decreased survival upon exposure to a variety of stressful conditions including high temperature, high osmolarity, pH, and ethanol exposure. Loss of *ygs* disrupted biofilm formation but not primary attachment. The biofilm defect seemed to be due to decreased *icaADBC* expression and PIA/PNAG production in the mutant strains, but expression of *icaR* was unaffected by Ygs. Ygs also played a significant role in biofilm formation *in vivo* and pathogenesis in rats (Wang et al., 2011).

GdpS

Holland et al. (2008) reported that a novel staphylococcal protein, GdpS (GGDEF domain protein from staphylococcus), plays a role in *ica* expression in *S. epidermidis* CSF41498. These authors identified *gdpS* by searching data bases for proteins with homology to diguanylate cyclases that bear a conserved GGDEF domain. These enzymes are responsible for the synthesis of cyclic-dimeric-GMP in many bacterial species. c-di-GMP allosterically activates enzymes involved in exopolymer synthesis. Staphylococci have only a single GGDEF domain protein, GdpS, which reportedly lacks cyclase activity (Holland et al., 2008). Despite this, *gdpS* was shown to enhance biofilm formation, *icaADBC* expression and PIA/PNAG production in media supplemented with NaCl. Expression of *icaR* was unaffected by mutation of *gdpS*. *S. aureus*

also encodes *gdpS* which is important in *icaADBC* expression (Tu Quoc et al., 2007), but like the *S. epidermidis* protein, lacks cyclase activity. It is unclear precisely how *gdpS* regulates *ica* expression.

CcpA, the TCA cycle and ica expression

Vuong et al. (2005) noted that many of the same conditions that induce PIA/PNAG production, i.e., high osmolarity, high temperature, ethanol, etc., are also known to inhibit the tricarboxylic acid, or TCA, cycle. They proposed that *ica* expression may respond to the metabolic state of the cell via alterations in the levels of TCA cycle intermediates. They did show that PIA/PNAG production could be upregulated by exposing cultures of *S. epidermidis* to fluorocitrate, an inhibitor of the TCA cycle enzyme aconitase (citrate (isocitrate) hydroxylase). Subsequently, the same group inactivated the gene coding aconitase (*acnA*) in *S. epidermidis* 1457 to study its effect on biofilm and *ica* expression (Sadykov et al., 2008). TCA activity was blocked and *icaADBC* expression was increased by the *acnA* mutation. Inactivation of the TCA cycle increased the intracellular concentration of the immediate biosynthetic precursor of PIA/PNAG, UDP-*N*-acetylglucosamine. Moreover, transcripts of genes encoding enzymes for the synthesis of UDP-*N*-acetylglucosamine from glucose-6-phosphate were all increased. Thus a major effect of *acnA* inactivation is a rerouting of carbon into *N*-acetylglucosamine biosynthesis.

The level of *icaADBC* transcript increased dramatically as a result of *acnA* inactivation. Surprisingly, the expression of *icaR*, *sarA,* and *sigB* were all increased in the *acnA* mutant. To determine whether any of these regulators affected PIA/PNAG production in response to TCA cycle disruption, the effects of fluorocitrate on PIA/PNAG was determined for *icaR*, *sigB,* and *sarA* mutants. Fluorocitrate increased PIA/PNAG in both the *icaR* and *sigB* mutant strains, indicating that neither of these regulators responds to TCA-induced metabolic changes. Fluorocitrate did not significantly affect PIA/PNAG production in a *sarA* mutant, however, making SarA a candidate for a TCA cycle-responsive regulator (Sadykov et al., 2008).

Sadykov et al. (2008) noted, however, that the aconitase mutant accumulated higher levels of branched chain amino acids than the wild type strain. This result suggested that CodY, a transcriptional regulatory protein that is responsive to branched chain amino acids, could be involved in *icaADBC* regulation. The authors also noted that the carbon catabolite repression protein, CcpA, may respond to higher intracellular levels of fructose-6-phosphate and increase *icaADBC* expression. CodY and CcpA are both regulators of *icaADBC* in *S. aureus* (Majerczyk et al., 2008; Seidl et al., 2008).

As mentioned above, CcpA has been shown to be an activator of *icaADBC* in *S. aureus* (Seidl et al., 2008) and, more recently, in *S. epidermidis* (Sadykov et al., 2011). CcpA is the primary mediator of carbon catabolite repression in staphylococci and is known to function as either a repressor or activator of transcription. Repression of TCA cycle genes is a common response to high concentrations of glucose in culture media, a response that among Gram-positive bacteria is mediated by CcpA. The activity of CcpA is regulated by intracellular levels of glucose-6-phosphate and fructose-1,6-bisphosphate, both of which affect phosphorylation of histidine-containing protein (Hpr). Phosphorylated Hpr can complex with CcpA affecting the interaction of the latter with DNA, typically causing CcpA to act as a repressor (Fujita, 2009).

Glucose can induce biofilm formation by *S. aureus* strain SA113 (Seidl et al., 2008). Induction of biofilm formation by glucose is dependent upon CcpA. Deletion of *ccpA* in both *S. aureus* SA113 and DSM20231 blocked biofilm formation but not initial attachment to polystyrene. CcpA was found to affect *icaA* transcription but was also required for expression of *cidA*. The latter is a putative holin protein that contributes to the release of bacterial DNA in biofilms (Ranjit et al., 2011). Biofilms formed by a SA113 *ccpA* mutant were more susceptible to disruption by exogenous DNase than were biofilms formed by SA113. Transcription of other regulatory genes, *sarA, arlRS, mgrA,* and *rbf*, were unaffected by deletion of *ccpA*. Based in part on the work with *S. epidermidis*, the effect of CcpA on transcription of *citZ* and *citB* was investigated. CitB is the *S. aureus* homolog of AcnA while *citZ* encodes citrate synthase. Both *citB* and *citZ* were repressed by *ccpA* in strain SA113 thereby linking CcpA with TCA cycle regulation. Based upon studies with *Bacillus subtilis* CcpA, a putative binding site for CcpA was found upstream of the *citZ* open reading frame. No such site was found upstream of *citB*. These findings suggested that CcpA may regulate *citZ* directly and *citB* indirectly (Seidl et al., 2008). Thus CcpA appears to play an important role in regulating biofilm in the presence of high glucose. The effect of CcpA on *ica* is likely indirect and a consequence of downregulation of the TCA cycle, in part, through repression of *citB* and *citZ* (Seidl et al., 2008).

CcpA has also been found to coordinate the TCA cycle and biofilm formation in *S. epidermidis* 1457 (Sadykov et al., 2008, 2011). Deletion of *ccpA* resulted in increases in aconitase and citrate synthase activity as well as *acnA* and *citZ* transcripts. CcpA proved critical for biofilm production in glucose-containing media. Deletion of *acnA* resulted in the upregulation of genes involved in PIA/PNAG synthesis including *icaD*, *glmU* (encoding glucosamine-1-phosphate *N*-acetyltransferase), *pfkA* (6-phosphofruktokinase), and *glnA* (glutamine synthetase). The increased expression of *icaD* and *pfkA* were *ccpA*-dependent and were manifest in 2 and 6 h cultures. Expression of *glmU* was similarly regulated except that expression was only evident in 6 h cultures. The *acnA* mutation increased the level of the *glnA* transcript after 6 h, but not after 2 h of incubation and was independent of CcpA. CcpA binding sites were located 5′ to both the *acnA* and *glmU* genes. The authors argued that CcpA both regulates TCA cycle activity and conveys signals associated with the TCA cycle to PIA/PNAG biosynthetic genes (Sadykov et al., 2011).

SUMMARY

It is obvious from the long list of factors that affect *ica* expression that regulation is extremely complex and multifactorial. This seems especially true for *S. aureus* as many isolates do not produce PIA/PNAG *in vitro* even though nearly all *S. aureus* isolates encode *icaADBC*. Moreover, the relative importance of the various factors seems to differ considerably between different strains as well as between species. In some instances, researchers have identified regulatory proteins that act directly on *ica* DNA.

However in other instances regulators appear to act indirectly, via affecting the expression or activity of hypothetical proteins that, in turn, interact with *ica* DNA. Even in cases where proteins have been shown to bind *ica* DNA, precisely how these factors regulate transcription is not completely clear. It seems highly likely that multiple regulatory factors are co-expressed during infection and we have virtually no information on how these factors may interact with DNA and/or other macromolecules to regulate gene expression. Moreover, it remains unknown how PIA/PNAG synthesis is induced during infection.

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A coverslip-based technique for evaluating *Staphylococcus aureus* biofilm formation on human plasma

Jennifer N. Walker and Alexander R. Horswill*

Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Odile Tresse, French National Institute for Agricultural Research/Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering, France
Julie Morrissey, University of Leicester, UK

****Correspondence:***
Alexander R. Horswill, Department of Microbiology, University of Iowa, 540F EMRB, Iowa City, IA 52242, USA.
e-mail: alex-horswill@uiowa.edu

The ability of the opportunistic pathogen, *Staphylococcus aureus*, to form biofilms is increasingly being viewed as an important contributor to chronic infections. *In vitro* methods for analyzing *S. aureus* biofilm formation have focused on bacterial attachment and accumulation on abiotic surfaces, such as in microtiter plate and flow cell assays. Microtiter plates provide a rapid measure of relative biomass levels, while flow cells have limited experimental throughput but are superior for confocal microscopy biofilm visualization. Although these assays have proven effective at identifying mechanisms involved in cell attachment and biofilm accumulation, the significance of these assays *in vivo* remains unclear. Studies have shown that when medical devices are implanted they are coated with host factors, such as matrix proteins, that facilitate *S. aureus* attachment and biofilm formation. To address the challenge of integrating existing biofilm assay features with a biotic surface, we have established an *in vitro* biofilm technique utilizing UV-sterilized coverslips coated with human plasma. The substratum more closely resembles the *in vivo* state and provides a platform for *S. aureus* to establish a robust biofilm. Importantly, these coverslips are amenable to confocal microscopy imaging to provide a visual reference of the biofilm growth stage, effectively merging the benefits of the microtiter and flow cell assays. We confirmed the approach using clinical *S. aureus* isolates and mutants with known biofilm phenotypes. Altogether, this new biofilm assay can be used to assess the function of *S. aureus* virulence factors associated with biofilm formation and for monitoring the efficacy of biofilm treatment modalities.

Keywords: Staphylococcus aureus, MRSA, biofilm, assay

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterial pathogen and a major public health problem in the United States and other countries. *S. aureus* causes a wide range of infections that includes acute, chronic, and toxin mediated disease (Chambers and DeLeo, 2009; DeLeo and Chambers, 2009). The ability of this pathogen to cause such a diverse array of problems is due to its arsenal of virulence factors that include pore-forming toxins, superantigens, matrix-binding surface adhesins, and tissue-degrading enzymes (Lowy, 1998). Like many bacterial pathogens, *S. aureus* can attach to a surface, accumulate biomass, and form a community of cells encased in an extracellular matrix called a biofilm (Gotz, 2002; Kiedrowski and Horswill, 2011). Many chronic infections, such as endocarditis, osteomyelitis, and growth on medical implants have biofilm-like features (Parsek and Singh, 2003; Zimmerli et al., 2004; Costerton, 2005). The formation of biofilms has been shown to protect the bacteria from host immune defenses as well as antibiotic therapy (Zimmerli et al., 2004; Costerton, 2005; Hall-Stoodley and Stoodley, 2005; del Pozo and Patel, 2007), and they remain an ongoing challenge for treating chronic infections. Although numerous factors have been demonstrated to be essential for *S. aureus* biofilm development, much remains unknown about the specific mechanisms employed to form and dissemble biofilm communities (Boles and Horswill, 2011; Kiedrowski and Horswill, 2011).

Many *in vitro* assays have been developed to quantify *S. aureus* biofilm formation (Cassat et al., 2007). Perhaps the most commonly used assay is the direct attachment of the bacteria to uncoated, polystyrene microtiter plates. While this assay is convenient and low-cost, it is becoming evident that many clinical *S. aureus* isolates attach poorly to abiotic surfaces (Lauderdale et al., 2009; Beenken et al., 2010). Studies examining medical implants indicate that they are coated with host matrix proteins upon implantation (Francois et al., 1998, 2000), and these host matrices can serve as a handle that are bound by the numerous proteins coating the *S. aureus* surface (Foster and Hook, 1998), facilitating cell attachment and biofilm accumulation. Matrix proteins can be coated on microtiter plates to more accurately reproduce *in vivo*—like conditions (Cassat et al., 2007), and in side-by-side comparisons, this strategy accentuates *S. aureus* biofilm formation (Beenken et al., 2010). These matrix-coated plates are convenient for biofilm assays, but they are not ideal in the experimental transition to confocal microscopy for obtaining a visual representation of the biofilm structure. Flow cells continue to be the method of choice for *in situ* microscopy, but they are laborious to perform, amenable to only limited experimental throughput, and require large quantities of media that can exhaust valuable reagents.

Although the microtiter and flow cells assays are the most commonly used methods to assess *S. aureus* biofilm formation

(Cassat et al., 2007), other related assays have been developed to examine pathogens such as *Enterococcus faecalis* and *Pseudomonas aeruginosa* during biofilm formation (Goeres et al., 2009; Guiton et al., 2009). These assays utilize a variety of surfaces for bacterial attachment to assess biofilm formation through microscopy, staining of the biomass, or viable plate counts. Our goal in this work was to develop a hybrid biofilm method that drew upon the benefits of both microtiter-based assays and flow cells. Based on success in *Enterococcus faecalis* biofilm studies with coverslips as a substratum (Guiton et al., 2009), we developed a coverslip-based approach amenable to *S. aureus* biofilm studies. Using clinical isolates and strains with characterized mutations, we demonstrated that the new method is robust and mirrors results obtained with other biofilm assays.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

The bacterial strain used in this study was a community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolate of the USA300 pulse field gel group called LAC, hereafter called "LAC WT." As a negative control, we used a sigma factor B (SigB) deficient mutant, hereafter called "LAC Δ*sigB*" (Lauderdale et al., 2009). Additional control strains used in this study include the USA400 clinical isolate MW2 (Baba et al., 2002) and a LAC strain with a complete deletion of the *ica* locus (Lauderdale et al., 2009). The biofilm phenotypes of LAC WT, LAC Δ*ica*, LAC Δ*sigB*, and the construction of the Δ*sigB* and the Δ*ica* mutations, were reported previously (Lauderdale et al., 2009). *S. aureus* strains were maintained in tryptic soy broth (TSB) or brain heart infusion broth (BHI) throughout this work.

PREPARATION OF BIOFILM MICROTITER PLATES

22 mm × 22 mm polyvinyl plastic coverslips (Raylabcon, cat #20 40 5300) were placed in each well of a six well cell culture plate (Corning Costar, cat # 3516). Plates with coverslips were then UV-sterilized in a tissue culture hood for at least 2 h. Heparin was added 1:1 to human plasma prepared from donors at the University of Iowa Inflammation Program to prevent clotting. The prepared human plasma was then diluted in 50 mM sodium bicarbonate solution to a final concentration of 20%. A 2 ml aliquot of either the sodium bicarbonate buffer or the 20% human plasma solution was added to each of the sterilized wells with coverslips and incubated at 4°C overnight. The human plasma was aspirated off the next day, and the wells and coverslips were washed twice with 2 ml sterile water.

BIOFILM CONDITIONS

Biofilms were grown by subculturing 18 h cultures to a starting OD_{600} of 0.2 and immediately diluting them 1:100 in BHI supplemented with 0.4% glucose. A 5 ml aliquot of each strain was added to each of the 3 wells previously coated with human plasma. Biofilms were allowed to shake at 100 rpm at 37°C for 18 h. To harvest the biofilms, the media was aspirated off and the biomass was washed twice with 5 ml of sterile water. The biomass was allowed to air dry and stained for quantification. To assess biomass, biofilms were stained with 500 μl of 0.1% crystal violet for 10 min at room temperature. Excess dye was removed and biofilms were washed with 5 ml of sterile water. Before solubilization, coverslips were imaged on a Chemi Gel Doc 2000 imaging station (Bio-Rad Life Sciences, Hercules, CA). Biofilms were air-dried and the biomass was dissolved in 500 μl 33% acetic acid. A 100 μl aliquot was transferred to a 96 well plate and measured on an Infinite M200 Tecan plate reader at an optical density (OD) of 635. Biomass was calculated by averaging three coverslips per strain, and at least seven separate experiments were performed with similar results.

CONFOCAL MICROSCOPY

All images were captured on a Nikon ECLIPSE C1 confocal laser scanning microscope (Nikon Inc., Melville, NY) using a 40× lens. Images were captured using the laser scanning C1 software from Nikon Inc. For microscopy, biofilms were harvested as described above and stained with SYTO9 (Invitrogen) and TO-PRO3 (Invitrogen) for 10 min in the dark. To prepare the stain, 1 μl of SYTO9 was mixed with 1 μl of TO-PRO3 from the manufacturer's stocks in 1 ml of water. Excess stain was removed by washing with 5 ml of sterile water. Coverslips were mounted on glass slides using mounting media made from 2.4 g polyvinyl alcohol, 12 ml 0.2 M Tris base, 6 g glycerol, and 2.5% DABCO (Sigma-Aldrich, St. Louis, MO) in 25 ml H_2O and kept at 4°C until microscopy was performed. Microscopy was performed with three coverslips per strain with at least four separate experiments.

RESULTS AND DISCUSSION

DEVELOPMENT OF THE COVERSLIP BIOFILM ASSAY

Using studies on *E. faecalis* biofilms as a guide (Guiton et al., 2009), we developed a coverslip biofilm method for *S. aureus*. To facilitate cell adherence and biofilm growth of clinical strains and to develop an assay that more closely resembles an *in vivo* state, the coverslips were coated with human plasma to provide matrix proteins for *S. aureus* attachment. As a testing strain, we used LAC WT, which is known to form biofilms in flow cells (Lauderdale et al., 2009, 2010), but attaches poorly to unconditioned microtiter plates. After 18 h of growth, LAC WT formed thick and confluent biofilm structures on the coverslip coated with 20% human plasma. The coverslips were stained with 0.1% crystal violet, and images revealed dark, confluent staining across the surface indicating robust biofilm formation (**Figure 1A**). To determine the ability of these strains to attach specifically to plastic, LAC WT was grown on coverslips without plasma coating and the staining was markedly reduced (**Figure 1B**). Biofilm formation on these coverslips can be quantified through solubilization of crystal violet and measurement of OD. For the LAC WT biofilm, quantification with plasma coating revealed a significant increase vs. uncoated coverslips (OD_{635} of 1.3 vs 0.35; **Figure 1C**).

As a biofilm negative control, we tested the LAC Δ*sigB* mutant strain, which is deficient in biofilm formation in microtiter assays and flow cells (Lauderdale et al., 2009; Boles et al., 2010). The LAC Δ*sigB* mutant revealed minimal staining across the coverslips coated with 20% human plasma, suggestive of poor biofilm forming capacity (**Figure 1A**), and the low OD (0.15 at OD_{635}) supported this assessment (**Figure 1C**). In previous studies with flow cells (Lauderdale et al., 2009), and plasma-coated microtiter

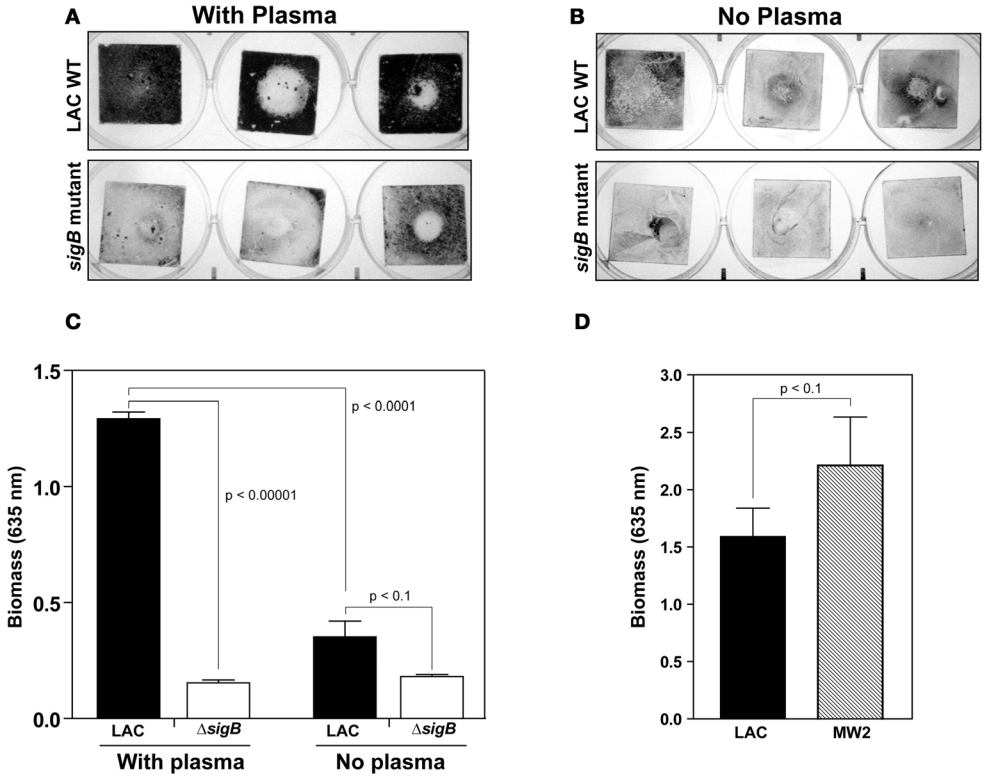

FIGURE 1 | Visualization and quantitation of coverslip biofilms. Biofilms were formed on plastic coverslips coated with 20% human plasma **(A)** or left untreated **(B)** for LAC WT and LAC Δ*sigB* mutant. The coverslips were post-stained with crystal violet and images were taken. **(C)** Average measured biomass for plasma-coated or untreated coverslip biofilms for LAC WT and Δ*sigB* **(C)** or USA400 strain MW2 compared with LAC WT **(D)**. In both **(C)** and **(D)**, biomass was measured at OD_{635} and *P* values were determined by students two-tailed *T*-test.

plates (Mootz and Horswill, 2012), the LAC Δ*sigB* mutant is defective in biofilm formation, which is consistent with our coverslip assay observations. As anticipated, the LAC Δ*sigB* mutant also developed a poor biofilm on uncoated plastic (**Figure 1B**) with a measured OD_{635} of 0.18 (**Figure 1C**). The plasma-only control wells had no bacterial growth or measurable biomass (data not shown).

To confirm the generality of the new biofilm method, the clinical isolate MW2 (USA400 PFGE) was grown on plasma-coated coverslips alongside LAC WT. Post staining revealed that the MW2 strain formed thick biofilms with an average biomass of 2.2 at OD_{635} (**Figure 1D**), which was slightly higher than the LAC WT control. This observation is consistent with previous experiments where MW2 accumulates more biomass than USA300 strains (Kiedrowski et al., 2011). As another test of the new method, an LAC strain derivative with the Δ*ica*::Tet deletion was examined, and this mutation eliminates the ability of *S. aureus* to synthesize the polysaccharide intercellular adhesin (Cramton et al., 1999). In previous flow cell biofilm experiments, we observed that introduction of the Δ*ica* deletion into strain LAC did not generate an *in vitro* biofilm phenotype (Lauderdale et al., 2009). Similar to the previous findings, the LAC Δ*ica* formed robust biofilms similar to LAC WT (data not shown), again confirming that the new coverslip assay provides an effective means to assess *S. aureus* biofilm formation.

CLSM OF COVERSLIP BIOFILMS

An important advantage to the coverslip method is the feasibility of confocal microscopy imaging of the biofilms. To prepare samples for imaging, the biofilms were grown and harvested as described above, and the coverslips were subjected to Live/Dead staining using SYTO9 and TO-PRO3 dyes and a *z* series of images was collected by confocal laser scanning microscopy (CLSM). The image analysis of LAC WT grown on 20% human plasma revealed thick, structured biofilms with profuse staining of live cells and relatively few dead cells (**Figure 2A**). CLSM of LAC WT grown on uncoated coverslips formed considerably thinner biofilms with irregular structures (**Figure 2B**). While the LAC strain can form robust biofilm structures in flow cells on abiotic surfaces (Lauderdale et al., 2009, 2010), the limited amount of biomass accumulating on the uncoated coverslips observed here mimics previous reports with microtiter plates (Beenken et al., 2010), indicating optimal biofilm formation in this assay is strongly dependent on surface conditioning. Previous reports comparing *S. aureus* biofilm structure on uncoated vs. heparin-coated coverslips also noted the strict dependence on surface modification to induce biofilm formation (Shanks et al., 2005). CLSM confirmed LAC Δ*sigB* grown either on plastic or on 20% human plasma-coated coverslips established poor biofilms with little structural architecture (**Figures 2A,B**), supporting previous flow cell observations with *sigB* mutants (Lauderdale et al., 2009).

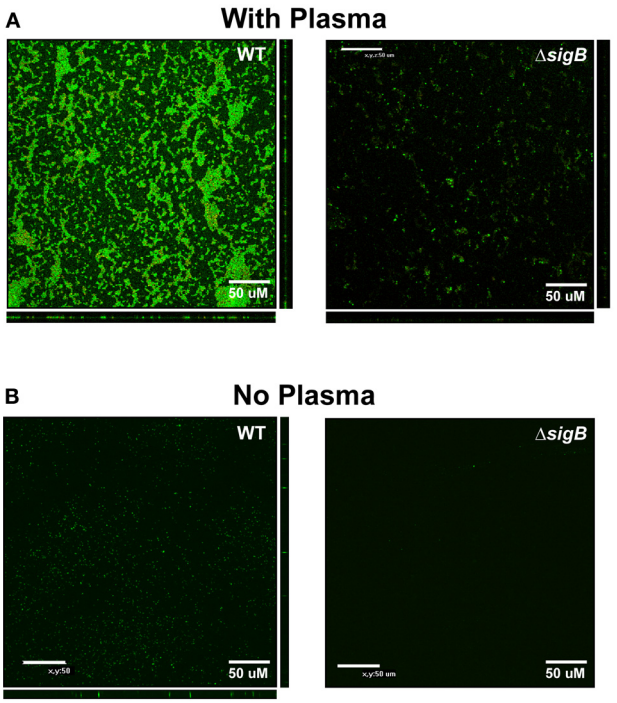

FIGURE 2 | CLSM Images of coverslip biofilms. LAC WT and LAC Δ*sigB* mutant biofilms were grown on human plasma-coated coverslips **(A)** or untreated coverslips **(B)**. The biofilm were post-stained with SYTO9 (green) and TO-PRO3 (red), and a *z* series of images was obtained with CLSM to visualize biofilm capacity. Each image shows a larger top down (XY) view with cross-section views of biofilm depth on the right (YZ) and bottom (XZ). Only top down (XY) images were taken of LAC Δ*sigB* grown on plastic due to the lack of cell adherence. Biofilms did not form in the media only control wells (not shown).

Further analysis of the imaging revealed that only small clumps of live cells were attached to the slide surface.

CONCLUSIONS

The ability of *S. aureus* to attach to surfaces and develop biofilm communities complicates chronic infections, making them difficult to treat. Understanding the mechanisms employed to form biofilms is essential for developing better treatment regimens, and one valuable laboratory tool to define these mechanisms has been the microtiter assay. High-throughput microtiter assays are a convenient and cost effective strategy to screen clinical isolates or mutants for biofilm capacity, but the relevance of the observations and translation *in vivo* is sometimes questioned. Flow cells could be a more biologically relevant mimic of the *in vivo* situation, but these assays have throughput and resource limitations that can hamper experimental progress. The new coverslip assay outlined in this study combines the benefits of microtiter and flow cell assays and also integrates a biotic substratum for optimal *S. aureus* attachment. We established that the new coverslip assay is effective for monitoring biofilm formation by multiple, wild-type MRSA isolates. Mutant strains with known biofilm phenotypes also behaved as anticipated, demonstrating the new coverslip assay is robust and provides results consistent with other assays. The ability to form a biofilm showed a strong dependence on plasma coating of the coverslip, which parallels observations made with microtiter plate assays. The addition of human matrix proteins could facilitate studies on adherence proteins, transcriptional regulators, and other virulence factors that have potential roles in biofilm maturation and dispersal. Transitioning the coverslip biofilms to CLSM was also uncomplicated, providing another means to visualize biofilm phenotypes. Additionally, various surfaces such as glass, polycarbonate, or metals such as stainless steel or aluminum can potentially be substituted for the polyvinyl plastic coverslips to adapt this method to other relevant conditions, although many of these surfaces prevent efficient microscopic analysis. Altogether, the new coverslip method is a straightforward approach to quantifying and imaging *S. aureus* biofilms on biotic surfaces that should facilitate future studies in this important area of research.

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Genetic manipulation of Staphylococci—breaking through the barrier

Ian R. Monk* and Timothy J. Foster

Department of Microbiology, Moyne Institute of Preventive Medicine, School of Genetics and Microbiology, Trinity College Dublin, Dublin, Ireland

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Jodi A. Lindsay, St. George's, University of London, UK
Chia Lee, University of Arkansas for Medical Sciences, USA

****Correspondence:***
Ian R. Monk, Department of Microbiology, Moyne Institute of Preventive Medicine, School of Genetics and Microbiology, Trinity College Dublin, Dublin 2, Ireland.
e-mail: monki@tcd.ie

Most strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* possess a strong restriction barrier that hinders exchange of DNA. Recently, major advances have been made in identifying and characterizing the restriction-modification (RM) systems involved. In particular a novel type IV restriction enzyme that recognizes cytosine methylated DNA has been shown to be the major barrier to transfer of plasmid DNA from *Escherichia coli* into *S. aureus* and *S. epidermidis*. While the conserved type I RM system provides a further barrier. Here we review the recent advances in understanding of restriction systems in staphylococci and highlight how this has been exploited to improve our ability to manipulate genetically previously untransformable strains.

Keywords: transformation, mutation, staphylococcus, allelic exchange, electroporation, aureus, epidermidis, restriction-modification

INTRODUCTION

The principles behind the use of genetic manipulation to identify virulence factors in pathogenic bacteria were articulated by Stanley Falkow as Molecular Koch's Postulates (Falkow, 1988). The basic premise is that by using precise genetic manipulation, the genes encoding putative virulence factors can be inactivated and the mutants tested for loss of virulence in infection models. It is also necessary to demonstrate that complementation restores virulence to wild-type levels. As pointed out by Falkow, "these postulates place a heavy burden on an investigator. They insist that genetic manipulation of the microorganism is a prerequisite for success, and, of course, for some pathogens, such study is not yet possible." (Falkow, 1988).

A major barrier to the genetic manipulation of staphylococci and fulfilling Molecular Koch's Postulates is the inability to transform plasmid DNA into the majority of clinical isolates due to a strong restriction-modification (RM) barrier. Consequently studies have focused on a small number of transformable laboratory strains of *S. aureus* (Voyich et al., 2005; Baba et al., 2008; O'Neill, 2010) and *S. epidermidis* (Heilmann et al., 1996). In this review we will focus on the recent developments in the understanding of RM systems in staphylococci and will show how these findings, combined with the development of new tools for genetic analysis, have advanced our ability to manipulate staphylococci genetically.

RM SYSTEMS OF STAPHYLOCOCCI—A HISTORICAL OVERVIEW

Four different types of RM systems are known but only three are found in staphylococci. Type I RM systems comprise genes that encode a host specificity of DNA (*hsd*) specificity (S) protein, a modification (M) protein and a restriction (R) endonuclease (Murray, 2000). HsdS functions in an $HsdS_1HsdM_2$ complex which recognizes a specific DNA sequence. The complex methylates hemi-methylated DNA and inhibits cleavage by the endonuclease complex $HsdS_1HsdM_2HsdR_2$ which would otherwise assemble on unmethylated DNA (Murray, 2000). Cleavage of unmodified DNA occurs after HsdR-dependent translocation of the complex along the molecule until it collides with a second complex (or DNA secondary structure), which stimulates the formation of double stranded DNA breaks (Simons and Szczelkun, 2011). Type II RM systems are well known to molecular biologists because the restriction endonucleases are widely used as reagents in molecular biology. The cleavage of DNA is sequence-dependent and can be prevented by the DNA methylation status. In *S. aureus* the Sau3AI type II RM system is present in a limited subset of strains (Stobberingh et al., 1977). The type IV system is the simplest form of restriction system with a single protein able to detect the methylation status. Examples from *E. coli* are *mcrA, mcrBC* which recognize 5-hydroxymethylcytosine and *N*-4-methylcytosine, respectively, while and *mrr* recognizes *N*-6-methyladenine as foreign. DNA containing these motifs are restricted by the corresponding enzyme (Kelleher and Raleigh, 1991).

Over 50 years have passed since RM was first recognized in *S. aureus*. In early phage typing studies it was observed that some strains were resistant to infection by phage. However, a strain could be infected if a high phage titer was used (Roundtree, 1956) or the recipient was heat-shocked beforehand (Asheshov and Jevons, 1963), suggesting that the barrier to infection could be overwhelmed or by-passed by transient inactivation. Restriction-deficient mutants of the clonal complex (CC—lineages derived from multi locus sequence typing) (Enright et al., 2000) 8 strain 8325 (SA113) (Iordanescu and Surdeanu, 1976) and the CC51 strain 879 (879R4) (Stobberingh and Winkler, 1977) were isolated which could take up foreign DNA and modify it so that it could be transferred to closely related wild-type strains. Both strains are thus r^-m^+, i.e., defective in restricting foreign DNA but capable of modifying the newly introduced DNA.

In order to facilitate genetic manipulation of *S. aureus* it is necessary to be able to transform *S. aureus* with shuttle plasmids that have been constructed in *E. coli*. Strain 8325-4 (8325 cured of three prophages) was subjected to heavy chemical mutagenesis and then transformed by protoplast transformation with a shuttle plasmid isolated from *E. coli* in order to isolate a mutant that could accept foreign DNA (Kreiswirth et al., 1983). From these experiments a single transformant was obtained. The plasmid was eliminated and then the *S. aureus* clone shown to accept the *E. coli* isolated plasmid at a reasonable frequency. This strain, called RN4220, has been extensively used by staphylococcal researchers ever since. However, it only provides a gateway into a limited set of closely related strains, e.g., in our hands 8325-4 isolated plasmid cannot transform MRSA252 (CC30) and vice versa. Also we cannot transform *S. epidermidis* isolates tested (RP62a or AMC5) with RN4220 isolated plasmid (unpublished data). The genome sequence of RN4220 revealed a nonsense mutation in the *hsdR* gene of a type I RM system among the 110 single nucleotide polymorphisms by which it differs from the parental strain (Nair et al., 2011). It had been previously shown by Waldron and Lindsay (Waldron and Lindsay, 2006) that complementation of RN4220 mutant *hsdR* allele with wild-type *hsdR* expressed from a low copy number plasmid prevented transformation by electroporation with a shuttle plasmid isolated from *E. coli* K-12, inhibited transduction and reduced the frequency of conjugation of a plasmid from *Enterococcus faecalis*. However Veiga and Pinho (2009) were unable to confirm the role of HsdR as the barrier to uptake of foreign DNA when they deleted *hsdR* in 8325-4 and COL. Mild heat shock (56°C for 2 min) prior to electroporation allowed transformation of 8325-4Δ*hsdR* but not the parental 8325-4. These results suggested that an additional heat-sensitive restriction system prevented transformation with plasmid DNA from *E. coli* K-12 (Veiga and Pinho, 2009). Interestingly, the majority of sequenced *S. aureus* isolates contain 2 sets of *hsdMS* genes located on the alpha and beta pathogenicity islands (Waldron and Lindsay, 2006), with *hsdR* located at a third site on the chromosome. This is in direct contrast to *S. epidermidis* and *S. lugdunensis* where the type I RM genes are clustered together (unpublished observation). In some MRSA strains, a third complete *hsdMSR* has also been identified in the staphylococcal cassette chromosome mec element (SCCmec) III, with *hsdMR* found in the SCC*mec* VII (Malachowa and DeLeo, 2010). The functionality of the modification and specificity genes in staphylococci has not been published. In *S. aureus* the sequences of the *hsdM* genes are highly conserved, while the two *hsdS* genes are divergent (Waldron and Lindsay, 2006). *hsdS* sequence variation is localized to the two target recognition domains (TRDs) within the gene, with *hsdS* gene content shown to be lineage specific, e.g., CC30 cluster together as do CC8 strains (Cockfield et al., 2007; Lindsay, 2010).

A major advance in the understanding of staphylococcal RM occurred with the discovery of a novel type IV restriction enzyme, which was shown to be the dominant barrier to prevent the uptake of foreign DNA by *S. aureus* (Corvaglia et al., 2010). Mutants of *hsdR* in UAMS-1 (CC30) and SA564 (CC5) were not or poorly transformable (respectively) with plasmid DNA isolated from *E. coli* K-12 (Corvaglia et al., 2010). UV mutagenesis of SA564*hsdR*$^-$ and subsequent transformation of the pooled survivors with a shuttle plasmid from *E. coli* K-12 resulted in 18 transformants. The genome of the strain that exhibited the highest transformation efficiency was sequenced along with the parental SA564. A frameshift mutation was identified in an ORF that has 98% identity to Sao_2790 of 8325. This gene was subsequently designated *sauUSI* (Xu et al., 2011). Disruption of *sauUSI* in SA564 and UAMS-1 yielded a strain that was transformable with the *E. coli* K-12-derived plasmid. Analysis of *sauUSI* in RN4220 identified a nonsense mutation in the middle of the gene and complementation using a multicopy plasmid carrying wild-type *sauUSI* reduced transformation into RN4220 100-fold (Corvaglia et al., 2010).

We have restored the *sauUSI* gene in the chromosome of RN4220 to wild-type by allelic exchange which resulted in a 10^{-4}-fold reduction in the transformation frequency in RN4220*sauUSI*$^+$ compared to RN4220 (Monk et al., 2012). The SauUSI protein has a very limited similarity to HsdR except for a DNA helicase domain. Deletion of the type I RM specificity genes *hsdS1* and *hsdS2* in SA564 did not yield a transformable strain indicating that SauUSI acts independently of the type I RM system (Corvaglia et al., 2010). The gene upstream of *sauUSI* in strain Newman (called Sae_2385) encodes a protein with homology to a nudix hydrolase that could potentially be involved in removal of toxic nucleotide derivatives. However deletion of this gene did not enhance transformation indicating that it is not important for SauUSI activity (Monk et al., 2012). SauUSI is highly conserved in *S. aureus.* However the CC5 strains N315 and Mu50 contain an allele of *sauUSI* with a nonsense mutation within the middle of the gene. Loss of SauUSI has made the strains permissive to transformation with plasmid DNA isolated from *Enterococcus faecalis* strain JH2-2 (Corvaglia et al., 2010), which could have implications for the enhanced the spread of antibiotic resistance between these organisms in the hospital environment (Zhu et al., 2008). Homologues of *sauUSI* occur in *S. epidermidis and S. pseudintermedius* and also in some enterococci, bacilli and lactobacilli.

The biochemical properties of SauUSI were recently characterized (Xu et al., 2011). The enzyme was shown to be a type IV endonuclease. The motif recognized by SauUSI was identified as methylation of cytosine bases in the motif C/G^{m}CNGC/G. The *E. coli* K-12 strains that are widely used for cloning such as DH5∝, TOP10, XL1-Blue and DH10B methylate both adenine (*dam*) and cytosine (*dcm*) residues. Plasmids isolated from these strains are readily degraded by SauUSI. In order to bypass the type IV restriction barrier in *S. aureus* the plasmid must be isolated from an *E. coli* strain that is defective in cytosine methylation. DNA methylation at cytosine residues is not only limited to *E. coli.* Some type II RM systems use cytosine methylation of target sites to prevent the activity of the cognate restriction enzyme, with these including *S. aureus* lineage CC398 (Bosch et al., 2010), some *Listeria monocytogenes* (Yildirim et al., 2010) and *Lactococcus lactis* strains (O'Driscoll et al., 2005).

The loss of Dam methylation in *E. coli* leads to deregulated mismatch repair and an elevated frequency of transition mutations (Wion and Casadesus, 2006) which means that a *dam* mutant is unsuitable for cloning. However a *dcm* mutant of *E. coli*

does not have an enhanced mutation rate (Palmer and Marinus, 1994). A *dcm* mutant of a high efficiency cloning strain of *E. coli* would be a useful host for constructing recombinant plasmids prior to direct transformation into a wild-type SauUSI proficient strain of *S. aureus*.

ELECTROPORATION OF STAPHYLOCOCCI—BYPASSING THE RESTRICTION BARRIER

While *S. aureus* contains homologues of genes involved in natural competence, and induction of some of the *com*-like genes has been shown upon the over expression of the *comX* homolog *sigH* (Morikawa et al., 2003), there is no experimental evidence that facilitated uptake of foreign DNA can occur. Electroporation is the method of choice for introducing plasmid DNA into *S. aureus*

The most widely used protocol involves the growth of cells into early logarithmic phase followed by washing with a hypertonic buffer (e.g., 500 mM sucrose) to remove salts and to stabilize the cells (Augustin and Gotz, 1990; Kraemer and Iandolo, 1990; Oskouian and Stewart, 1990; Schenk and Laddaga, 1992; Lee, 1995). The cells are concentrated to 1–3 $\times$ 10^{10} CFU/ml and purified plasmid DNA added. A defined electric pulse is discharged through the cells to facilitate the uptake of the DNA. The cells are then grown in broth for a short period of time to allow recovery and for growth to begin prior to plating on media containing an antibiotic that selects for the plasmid-containing transformants. Lofblom et al. (2007) described extensive optimization of electroporation for *Staphylococcus carnosus*. Application of the final protocol with minor modifications to *S. aureus* strain Newman gave a 50-fold improvement over the sucrose-wash protocol (Monk et al., 2012) with transformants being obtained directly with DNA isolated from *E. coli* K-12 strains at a low frequency (between 10^1 and 10^2 CFU/5 μg plasmid DNA). In strain Newman the restriction barriers cause a 10^{-4} reduction in the transformation efficiency when comparing uptake of plasmid DNA isolated from wild-type Newman with that isolated from *E. coli* K-12. Thus improving the efficiency of electroporation allowed the type IV and type I RM systems to be bypassed.

DC10B—A UNIVERSAL HOST FOR CONSTRUCTING PLASMID FOR INTRODUCTION INTO STAPHYLOCOCCI

To improve the transformation of *S. aureus* we created an unmarked *dcm* deletion mutation in the high efficiency cloning strain *E. coli* DH10B to generate strain DC10B (Monk et al., 2012). The absence of cytosine methylation allows plasmid DNA to bypass the type IV restriction barrier. Transformation with plasmid DNA isolated from DC10B occurred in 15 strains from different CCs that we have so far tested. The CCs were chosen to represent a diverse selection of *S. aureus* lineages and to encompass the major MRSA CCs. The only strain we were unable to transform was from CC97 (Monk et al., 2012). Using DC10B we have been able to transform and isolate chromosomal mutations in strains that were previously refractory to genetic manipulation, for example the CC30 strains Cowan and MRSA252 (Monk et al., 2012). The improved transformation protocol has enabled us to transform several strains of *S. lugdunensis* (Heilbronner and Foster, unpublished) and combined with DC10B has allowed the direct transformation of *S. epidermidis*. For both coagulase negative species, a reduced efficiency was found compared to *S. aureus* Newman with a maximum of 10^3 CFU/5 μg plasmid DNA. Deletion of the *sauUSI* homologue (termed *mcrR* for methylated cytosine recognition and restriction) in *S. epidermidis* isolate RP62a (Gill et al., 2005) produced a strain that could accept plasmid DNA from a Dcm^+ *E. coli* K-12 host. This directly demonstrates the importance of the type IV restriction barrier in this species (Monk et al., 2012). In conclusion using plasmid DNA isolated from the DC10B strain of *E. coli* and or an enhanced electroporation protocol will dramatically improve our ability to conduct genetic studies in many different staphylococci.

E. coli STRAINS THAT MODIFY PLASMID DNA FOR STAPHYLOCOCCI

While bypassing the type IV barrier allows us to transform DNA directly into wild-type staphylococci, the efficiency of plasmid transfer is still low and for some applications borderline for selection (e.g., transferring pVW01*ts* into *S. epidermidis* RP62a or direct integration of plasmids at phage *att* sites mediated by integrase [see below]). Bypassing the type I RM barrier would require the decoration of plasmid with the methylation pattern determined by the *hsdMS* genes in the strain to be transformed. There is a paucity of information on the properties of the type I RM systems in *S. aureus* (Waldron and Lindsay, 2006; Sung and Lindsay, 2007). They appear to be involved in the limiting uptake of phage DNA from unrelated staphylococci (Veiga and Pinho, 2009), play an additive role with SauUSI in restricting foreign DNA (Monk et al., 2012) and impede the transfer of DNA between staphylococci (Corvaglia et al., 2010; Lindsay, 2010). In the simplest system where only one *hsdRMS* operon is present (e.g., *S. epidermidis* RP62a) expression of the *hsdMS* genes in *E. coli* DC10B should further improve the efficiency of plasmid transfer. There is a 60-fold reduction in transformation of RP62a with plasmid isolated from DC10B compared to RP62a isolated plasmid, suggesting the presence of a second active RM system (unpublished data). The term plasmid artificial modification (PAM) has been coined to describe pre-methylation of plasmid DNA in an *E. coli* strain which expresses the target strain's modification and specificity genes (Suzuki and Yasui, 2011).

Two groups have described the use of this technology for bifidobacteria. O'Connell Motherway et al. (2009) isolated the modification genes of a two different type II RM systems from *Bifidobacterium breve* UCC2003 and expressed them either from a plasmid or from a chromosomal locus in *E. coli*. An increase in transformation by 1000-fold was observed for the plasmid-borne methylation genes, while a 50-fold improvement was observed for the chromosomally encoded genes compared to DNA from the parental *E. coli* strain. Two type II RM methylase genes from *Bifidobacterium adolescentis* were cloned into an *E. coli* plasmid giving a ~10,000-fold increase in transformation frequency (Yasui et al., 2009). There is only one example of PAM being applied to a type I RM system (Yasui et al., 2009). The expression of the *hsdMS* genes of *Lactococcus lactis* IO-1 from a plasmid in *E. coli* BL21 (DE3) yielded a seven fold improvement in transformation.

We have constructed a strain of *E. coli* that expresses the functional set of *hsdMS* genes from MRSA252 from an intergenic

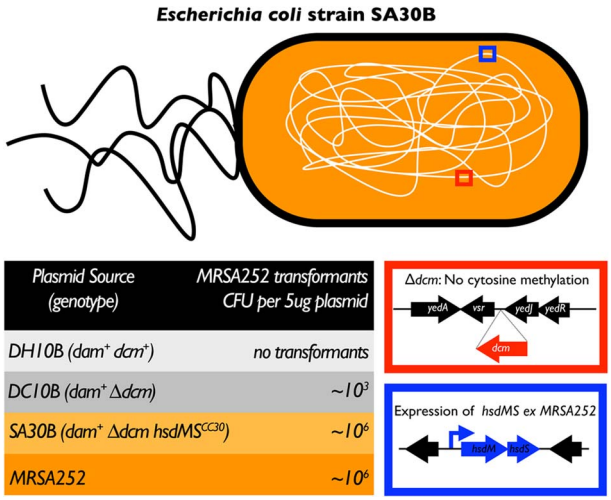

FIGURE 1 | Plasmid artificial modification—application to staphylococci. In a cloning strain of *E. coli* (DH10B), the chromosome was manipulated by recombineering to develop a host for the maximal efficiency of plasmid transfer into the target staphylococcal strain. To bypass the type IV restriction system, the *dcm* gene was deleted resulting in a loss of cytosine methylation (red box), creating strain DC10B. Secondly, the *hsdMS* genes of the target staphylococcal strain, under the control of a strong promoter (blue box) were introduced onto the chromosome of DC10B at a neutral site forming, for example. *E. coli* strain SA30B—with *hsdMS* genes derived from MRSA252 (clonal complex 30). A shuttle plasmid was isolated from each *E. coli* strain described above and *S. aureus* MRSA252. Five micrograms of shuttle plasmid was transformed into MRSA252 with the CFU enumerated after growth on antibiotic-containing selective agar (bottom left table).

location in the chromosome (manuscript in preparation, Monk and Foster) **(Figure 1)**. Plasmid DNA isolated from SA30B (DC10B::*hsdMS*^{MRSA252}) was transformed into MRSA252 at the same frequency as the plasmid isolated from MRSA252 with a 1000-fold improvement in transformation efficiency compared to plasmid isolated from DC10B. We are currently introducing the functional *hsdMS* genes from strains of different CC's of *S. aureus* and from different staphylococcal species into DC10B. These hosts will be invaluable for generating plasmids for genetic manipulation of staphylococcal strains that are currently refractory to transformation and will permit fulfillment of Molecular Koch's Postulates in diverse hosts.

EFFICIENT ALLELIC EXCHANGE

Several plasmids have been developed to facilitate the construction of mutations in the chromosome of staphylococci by allelic exchange. The preferred method employs a temperature sensitive (ts) plasmid, which replicates by the rolling circle mechanism. A ts version of pE194 is the most widely used replicon for allelic exchange in staphylococci (Gryczan et al., 1982). The procedure for creating a mutation is a two-step process **(Figure 2)**. First, the plasmid carrying the mutational cassette is transformed into the target strain at a temperature that is permissive for replication. Then integration by a single crossover (SCO) event at either the upstream or downstream region of homology is selected by growing at the non-permissive temperature for replication while selecting for antibiotic resistance encoded by the plasmid. This forms an integrant and creates a duplication of the locus to be mutated with one copy being wild-type and the other copy carrying the mutation. Excision of the plasmid by a SCO event is stimulated by decreasing the temperature to one permissive for plasmid replication, in the absence of antibiotic selection. This triggers recombination and loss of the plasmid. If the second crossover event occurs at the region of homology used for integration the strain remains wild-type. If recombination occurs at the opposite region of homology the mutant allele is left in the chromosome. Excision at the same site as that used for integration should occur in theory at the same frequency as at the heterologous site giving a mutation frequency of 50%. In practice mutation frequencies as low as 1% (or lower) may occur (Biswas et al., 1993; Bae and Schneewind, 2006). This can make identification of a mutant that lacks a selectable marker a laborious and time consuming process. A number of improvements to allelic exchange have been devised for staphylococci and are detailed below.

pMAD/pORI280

A temperature sensitive shuttle plasmid was created by joining pE194ts to pBR322 with the subsequent addition of a gene encoding a constitutively expressed thermostable ß-galactosidase (Arnaud et al., 2004). While plasmid excision cannot be selected, colonies that lack the plasmid can be identified on plates containing X-gal where they form white colonies. A similar concept has been applied in lactococci with the pORI280 two-plasmid system (Leenhouts et al., 1996). A suicide plasmid missing two of the replication genes and encoding ß-galactosidase is used for allelic exchange, with a second ts plasmid, pVE6007 (with a pWV01ts replicon) supplying the missing *repAD* encoded replication functions *in trans*. By growth at a temperature that is restrictive for replication pVE6007 is lost and the integrants with pORI280 in the chromosome selected with erythromycin. Resolution of integrants occurs after growth in the absence of antibiotic. We have been unable to manipulate *S. aureus* using the pORI280 two-plasmid system even though two groups have previously reported success (Pinho and Errington, 2004; Daly et al., 2010).

pKOR1

Bae and Schneewind (2006) revolutionized the isolation of mutations in *S. aureus* by introducing counter selection into the procedure for allelic exchange. A *secY* antisense transcript which hybridizes to mRNA encoding part of the essential Sec protein secretion system acts as an inhibitor of growth impairing colony formation on agar. Combining tetracycline inducible *secY* expression and Gateway cloning into the pTS1 ts shuttle plasmid (pE194ts replicon) (Greene et al., 1995) generated the vector pKOR1 (Bae and Schneewind, 2006). The initial stages of allelic exchange are the same as for any ts plasmid. Cells where the plasmid has been lost by reverse SCO can be selected directly on agar following induction by anhydrotetracycline of *secY* antisense giving cells lacking pKOR1 a growth advantage. However, conditions used for allelic exchange with pE194ts replicons can produce secondary mutations in *sae, a* locus which encodes a two component signal transduction system (Sun et al., 2010). The elevated temperature of growth (43°C), aeration and low levels of antibiotic

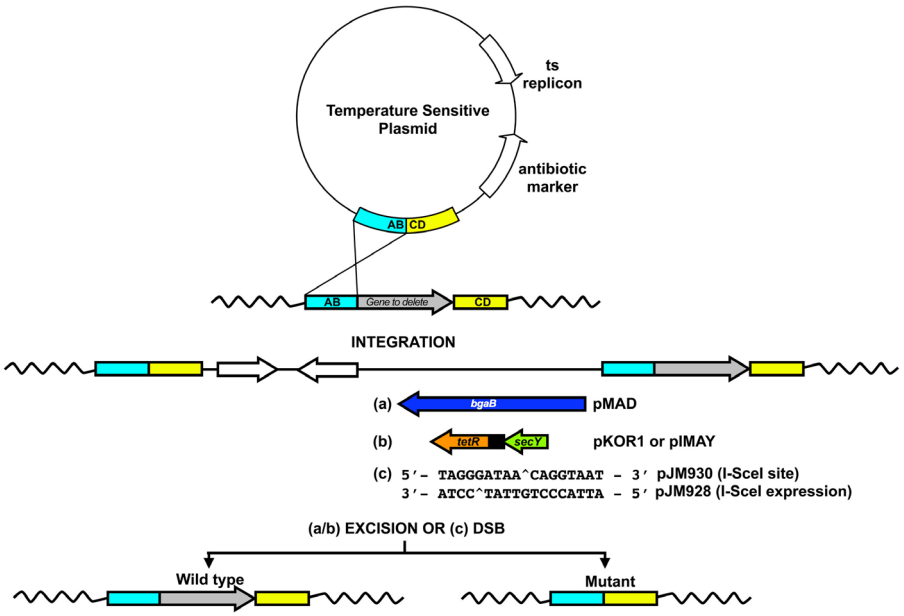

FIGURE 2 | Allelic exchange in staphylococci. The two-step approach (integration/excision) is shown above for the creation of directed mutations. A deletion construct is assembled in the multiple cloning site of a temperature sensitive plasmid and then transformed into the target strain at a temperature permissive for replication. A temperature shift, to one non-permissive for plasmid replication (in the presence of selection for antibiotic resistance encoded by the plasmid), stimulates *integration* through either the up (AB—shown here) or downstream (CD) region of cloned homologous DNA. Decreasing the temperature and removing antibiotic selection stimulates rolling circle replication which leads to vector *excision*. As described in the text, the different allelic exchange plasmids developed for staphylococci contain additional features which aid in discrimination of colonies lacking the plasmid either post excision, e.g., (a) pMAD: constitutive *bgaB* for the hydrolysis of the colourmetric substrate—detected as white colonies on X-gal containing agar, (b) pKOR1/pIMAY: ATc inducible *secY* antisense to repress growth of plasmid-containing strains or (c) I-SceI induced double strained DNA breaks (DSB) which promote homologous recombination.

resistance expressed by the plasmid's chloramphenicol resistance determinant, particularly when at a single copy in the chromosome, can promote the selection of *sae* mutations. *sae* mutations can influence the expression of other genes and alter virulence (Herbert et al., 2010).

pIMAY

To alleviate problems associated with using pKOR1 we have constructed a plasmid vector for allele exchange that has a strongly expressed drug resistance marker and a ts replicon that allows selection of integrants at 37°C (Monk et al., 2012). The pVWO1*ts* replicon on pIMAY is functional in staphylococci (*S. aureus*, *S. epidermidis* and *S. lugdunenesis* have so far been tested) at the permissive temperature (30°C) but the plasmid cannot replicate at the restrictive temperature of 37°C. The replicon used to propagate the plasmid in *E. coli* is low copy number which should improve the stability of cloned staphylococcal DNA. The chloramphenicol resistance (*cat*) gene is expressed from a strong promoter which allows efficient selection as a single copy when integrated into the staphylococcal chromosome. The plasmid carries the inducible *secY* antisense counterselection determinant of pKOR1. Furthermore, we have recently applied a sequence- and ligation-independent cloning (Li and Elledge, 2007) to pIMAY which increases the cloning efficiency (greater than 90% of colonies screened contain inserts) and reduces the costs and time involved in production of deletion constructs. From the start of cloning to mutant confirmation can be conducted in under two weeks (Monk et al., 2012).

I-SceI

Counter selection with *secY* enriches for cells which have lost the integrated plasmid following the second SCO event. However, enhancing the rate of plasmid excision requires initiating DNA replication at the integrated plasmid's origin. An alternative approach utilizes a rare cutting restriction enzyme SceI which recognizes an 18 bp sequence yet to be found in bacteria (Posfai et al., 1999). It was first shown in mammalian cells that the induction of double stranded DNA breaks (DSBs) following expression of SceI induced homologous recombination (Choulika et al., 1995). After codon optimization of *sceI* the enzyme was applied to several Gram-negative and Gram-positive bacteria (Janes and Stibitz, 2006; Szurmant et al., 2007; Flannagan et al., 2008; Blank et al., 2011; Martinez-Garcia and de Lorenzo, 2011). Pagels et al. (2010) developed the SceI system for use in *S. aureus*. The allelic exchange plasmid with the pE194ts replicon carries the SceI restriction cleavage site (pJM930). After integration into the chromosome by SCO at the locus to be deleted, a second plasmid (pJM928) with a compatible pT181ts replicon constitutively expressing SceI was delivered by transduction. The enzyme introduces a DSB within the integrated plasmid. This induces homologous recombination which promotes plasmid excision, which in other bacteria has increased the frequency of wild-type to mutant up to 50% (Posfai

et al., 1999; Janes and Stibitz, 2006). Cells where the plasmid has been excised will not have a DNA break in the chromosome while the excised plasmid carrying the cleavage site will be destroyed. This approach should dramatically improve the efficiency of generating mutations. Ideally an inducible *sceI* gene should be part of the mutational plasmid to avoid the need to use a second plasmid, but this will require a very strong repressor to prevent SceI expression during plasmid propagation and integration.

TEMPERATURE—AN IMPORTANT CONSIDERATION

A recent publication has identified sequential non-selective passage at a reduced temperature (25°C) as a method to improve the frequency of co-integrate resolution (Kato and Sugai, 2011). Their results also highlighted the requirement for the isolation of both SCOs, either through the up or downstream region of homology. We have found that resuspension of a colony and direct plating rather than broth growth at the non-permissive temperature for plasmid replication improves the isolation of both SCOs (Monk et al., 2012). Combining the DC10B strain with pIMAY or other ts plasmids will enhance isolation of mutations in laboratory strains and diverse clinical isolates.

VALIDATION OF MUTATIONS BY COMPLEMENTATION

After construction of a mutant, any change in phenotype should be corroborated by complementation in order to prevent attributing properties to the missing gene that are actually due to secondary mutations. Currently four approaches to complementation can be applied.

SHUTTLE PLASMIDS

A number of shuttle plasmids that replicate both in *E. coli* and staphylococci have been constructed that utilize several different plasmid replicons (pE194, pC194, pT181, pKS1). The effect of plasmid copy number should be considered when attempting to complement a mutation. A gene-dosage effect may lead to high level expression which could potentially be toxic. This could be reduced by using an inducible gene expression system (see below). Plasmid-based complementation can be established quickly compared to other methods. However it can be difficult to use in animal infection experiments where the plasmid is often lost in the absence of antibiotic selection *in vivo* (Cho et al., 2011). It is possible to maintain selection by administering antibiotics into drinking water but this is not ideal (de Azavedo et al., 1985; Bubeck Wardenburg et al., 2006). For an extensive review of plasmids used in staphylococcal research see McNamara (2008).

INDUCIBLE GENE EXPRESSION

Promoters that can be activated by the inducers IPTG (Zhang et al., 2000), xylose (Peschel et al., 1996), cadmium (Charpentier et al., 2004) and anhydrotetracycline (ATc) (Bateman et al., 2001) have been used in staphylococci. The ATc inducible vector pRMC2 was derived from pALC2073 (Bateman et al., 2001) by increasing the level of expression of the TetR repressor to reduce leakiness (Corrigan and Foster, 2009). Recently the laboratory of R. Bertram has constructed and validated a series of improved ATc inducible expression vectors that were derived from pRMC2. By inserting a second *tetO* binding site for TetR downstream of the −10 box of the $P_{xyl/tet}$ promoter, creating pRAB11, a greater level of repression compared to that of pRMC2 was observed (Helle et al., 2011). Mutations in the –10 and –35 boxes of the $P_{xyl/tet}$ promoter in pRAB11 resulted in reduced expression, but achieved a higher level of repression. Finally, a hybrid *tetR* gene (*tetR*-BD) improved both the level of expression when induced and the level of repression when uninduced compared to pRAB11 while introduction *revtetR* (reverse *tetR*—contains 3 amino acid changes which reverse the activity of TetR) yielded a construct comparable to pRAB11, except exhibiting repression in the presence of ATc but induction in the absence (Helle et al., 2011). These vectors should be of great value for experiments requiring controlled expression of a cloned gene *in vitro*.

PHAGE INTEGRASE VECTORS

Integrating vectors that utilize the *att* sites and integrases of lysogenic phages have been developed to eliminate the problems associated with complementation by extrachromosomally replicating plasmids. These allow the integration of a plasmid at a specific phage attachment site in the chromosome directed by a phage integrase (van Mellaert et al., 1998; Haldimann and Wanner, 2001; Lauer et al., 2002; Huff et al., 2010). The integrated plasmid lacks a replicon that functions in *S. aureus* and is stable even in the absence of selective antibiotic. The presence of only a single copy of the plasmid eliminates gene-dosage effects.

The pLL39 phage integrase vector encodes both ϕL54a and ϕ11 *attP* sites and in the presence of the appropriate phage integrase can insert into the matching chromosomal *attB* site (Luong and Lee, 2007). This vector is a refinement of the previously developed and successfully applied pCL83/pCL84 vectors (Lee et al., 1991). To stimulate pLL39 integration, the integrase is encoded on a second extrachromosomally replicating plasmid (Luong and Lee, 2007). Similar two plasmid systems have been created based on the phage related *S. aureus* pathogenicity island (SaPI1) using the SaPI1 *int* and *attS* to direct integration (Charpentier et al., 2004). However until now both pLL39 and the SaPI1 based vector system could only be integrated into the chromosome of RN4220 and must then be moved by generalized transduction into the target strain. Transduction from RN4220 can be problematic because DNA that is located adjacent to the integrated plasmid will also be introduced by the double reciprocal recombination event. This could introduce mutations into the recipient. Also transfer is limited to strains that are closely related to RN4220, similar to plasmid transformation discussed earlier, due to RM. It is also possible that the integration of the plasmid itself could cause a change in virulence of the host. It is of note that the ϕL54a *attB* site is located within the *geh* gene which encodes an extracellular lipase (Lee and Iandolo, 1985). Although this could be controlled for by comparing the complemented mutant strain with the mutant carrying the empty vector, the ideal integrating vector would insert into an *attB* site that is located in an intergenic region. Lei et al. (2012) recently constructed a phage integrase vector, which recognizes an engineered core *attB* sequence based on the ϕL54a. This synthetic *attB* sequence was inserted onto the chromosome of RN4220 at a region characterized with negligible transcriptional activity and allowed the transformation with the *attP* compatible vector (pLL102). The region is highly conserved

among *S. aureus* isolates which should allow the transfer either by phage transduction or by direct electroporation.

We are currently testing two new small (∼3 kb) phage integrase vectors that incorporate phage ϕ11 or ϕ80 *attP* sites as well as the appropriate integrase gene. The plasmids can be propagated in *E. coli* DC10B and transformed directly into the wild type *S. aureus* strain where they will integrate under the direction of the encoded integrase. This eliminates the need for a second plasmid encoding the integrase and improves the efficiency of the process (Monk, Tan, Shah and Foster, unpublished).

GENE RECONSTITUTION

Sometimes genes cloned into multicopy plasmids can be toxic to *E. coli* or the target strain (Pilgrim et al., 2003; Alonzo et al., 2011), expression from a phage integrase vector may occur at a different level compared to wild-type despite the presence of the native promoter (Lauer et al., 2002) or polar effects may need to be ruled out. Reconstitution of the mutated gene to wild-type (Donegan and Cheung, 2009; Diep et al., 2010; Monk et al., 2012) by reverse allelic exchange can be employed. The wild-type gene and flanking DNA are cloned into a ts allelic exchange-promoting plasmid such as pIMAY. A codon change that creates a novel restriction site without altering the amino acid sequence of the encoded protein (http://emboss.bioinformatics.nl/cgi-bin/emboss/silent) is introduced to facilitate identification of the restored gene. This is time consuming but results in a complemented strain that differs from wild-type only by the introduced restriction site.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this article we have described a genetic toolbox that is applicable to many species of Staphylococcus and we have reviewed how recent developments in understanding of RM systems have greatly improved the ability to manipulate these bacteria genetically. Bypassing the host encoded RM systems with plasmid DNA isolated from *E. coli* DC10B expressing staphylococcal HsdMS proteins will enable the rapid construction of mutant strains by allelic exchange and for their complementation.

S. epidermidis strains are much more difficult to work with than *S. aureus* because they are transformable by electroporation at a much lower frequency. Wall teichoic acid has recently been identified as a barrier to transformation in *S. epidermidis* (Holland et al., 2011). Optimization of conditions for generating competent cells for electroporation (Lofblom et al., 2007) will be required to increase the frequency of transformation. Development of a single plasmid phage integrase vector for *S. epidermidis* would require enhanced transformation.

To take advantage of the high transformation efficiencies that can be achieved in *S. aureus*, we propose to apply single stranded DNA recombineering where point mutations, small insertions or deletions can be rapidly constructed in chromosomal genes (Swingle et al., 2010). This technology has been used *E. coli*, *Shigella flexneri, Salmonella typhimurium*, *Pseudomonas syringae* (Swingle et al., 2010), Mycobacteria (van Kessel and Hatfull, 2008) and more recently to lactic acid bacteria (van Pijkeren and Britton, 2012). Expression of the *recT*-encoded single strand DNA annealing protein enhances the incorporation of synthetic oligonucleotides designed to bypass the mismatch repair pathway. We have identified several *recT* homologues that are functional in *S. aureus* and are currently optimizing the procedure (Monk, van Pijkeren, Britton and Foster, unpublished).

Through mining staphylococcal genome sequences, we have observed that both the type I and/or type IV systems are highly conserved in staphylococci. However, some strains (e.g., *S. aureus* RF122, Cowan, JKD6159 and *S. pseudintermedius* ED99) encode additional type I or II RM systems, which can impair transformation (unpublished data). The type IV restriction system can be by-passed using plasmid that lacks cytosine methylation while a type I or a type II system bypass would require the plasmid obtain the methylation profile of the specific RM system.

In summary the improvements in vectors and transformation described above has facilitated genetic manipulation in many strains of *S. aureus* and other staphylococcal species which were previously not amenable to transformation with plasmid DNA (Monk et al., 2012). Also the time taken to construct strains and to perform genetic manipulation has been reduced significantly. This will facilitate more sophisticated genetic manipulation in clinically relevant strains and will contribute directly to rapid advances in knowledge about this important group of organisms

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Comparative genomics of the *Staphylococcus intermedius* group of animal pathogens

Nouri L. Ben Zakour*[1,2]*, Scott A. Beatson*[2]*, Adri H. M. van den Broek*[3]*, Keith L. Thoday*[3] *and J. Ross Fitzgerald*[1]*

[1] *The Roslin Institute and Edinburgh Infectious Diseases, The University of Edinburgh, Edinburgh, UK*
[2] *Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia*
[3] *Dermatology Unit, Division of Veterinary Clinical Sciences, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Edinburgh, UK*

Edited by:
Martin John McGavin, University of Western Ontario, Canada

Reviewed by:
Steven Gill, University of Rochester School of Medicine and Dentistry, USA
Mark Holmes, University of Cambridge, UK

****Correspondence:***
J. Ross Fitzgerald, The Roslin Institute and Edinburgh Infectious Diseases, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Campus, Midlothian, Edinburgh EH25 9RG, UK.
e-mail: ross.fitzgerald@ed.ac.uk

The *Staphylococcus intermedius* group consists of three closely related coagulase-positive bacterial species including *S. intermedius*, *Staphylococcus pseudintermedius*, and *Staphylococcus delphini*. *S. pseudintermedius* is a major skin pathogen of dogs, which occasionally causes severe zoonotic infections of humans. *S. delphini* has been isolated from an array of different animals including horses, mink, and pigeons, whereas *S. intermedius* has been isolated only from pigeons to date. Here we provide a detailed analysis of the *S. pseudintermedius* whole genome sequence in comparison to high quality draft *S. intermedius* and *S. delphini* genomes, and to other sequenced staphylococcal species. The core genome of the SIG was highly conserved with average nucleotide identity (ANI) between the three species of 93.61%, which is very close to the threshold of species delineation (95% ANI), highlighting the close-relatedness of the SIG species. However, considerable variation was identified in the content of mobile genetic elements, cell wall-associated proteins, and iron and sugar transporters, reflecting the distinct ecological niches inhabited. Of note, *S. pseudintermedius* ED99 contained a clustered regularly interspaced short palindromic repeat locus of the Nmeni subtype and *S. intermedius* contained both Nmeni and Mtube subtypes. In contrast to *S. intermedius* and *S. delphini* and most other staphylococci examined to date, *S. pseudintermedius* contained at least nine predicted reverse transcriptase Group II introns. Furthermore, *S. pseudintermedius* ED99 encoded several transposons which were largely responsible for its multi-resistant phenotype. Overall, the study highlights extensive differences in accessory genome content between closely related staphylococcal species inhabiting distinct host niches, providing new avenues for research into pathogenesis and bacterial host-adaptation.

Keywords: *Staphylococcus*, genomics, pathogenesis, host-adaptation, animal, antibiotic resistance

INTRODUCTION

Population genetic analysis has revealed the existence of a group of closely related coagulase-positive staphylococcal species associated with different host-species, collectively known as the *Staphylococcus intermedius* group (SIG; Varaldo et al., 1988; Bannhoer et al., 2007; Sasaki et al., 2007b). The SIG consists of *Staphylococcus pseudintermedius*, an opportunistic pathogen which predominantly colonizes the skin and mucosal surfaces of dogs (Kloos, 1980; Greene and Lammler, 1993), *Staphylococcus delphini* which has been isolated from a wide array of animals, including minks, horses, cows, and pigeons (Sasaki et al., 2007b), and *S. intermedius*, which has only been isolated from pigeons to date (Bannoehr et al., 2007; Sasaki et al., 2007b). Although *S. pseudintermedius* is a component of the canine normal flora, disruption of the normal skin flora or an underlying condition such as atopic dermatitis, can lead to *S. pseudintermedius* infections such as superficial and deep canine pyoderma, and otitis media or externa (Cole et al., 1998). Recently, strains of *S. pseudintermedius*, which are refractory to treatment by most commonly used classes of antibiotic including methicillin have emerged and disseminated widely (Perreten et al., 2010; Ruscher et al., 2010). *S. pseudintermedius* is rarely isolated from humans but can occasionally cause severe zoonotic infections, typically through dog bite wounds (Mahoudeau et al., 1997; Tanner et al., 2000; Pottumarthy et al., 2004). Furthermore, *S. pseudintermedius* has the capacity to produce enterotoxins related to those made by *Staphylococcus aureus* and has been implicated in several food poisoning outbreaks (Khambaty et al., 1994; Becker et al., 2001). Our understanding of the molecular pathogenesis of *S. pseudintermedius* canine pyoderma is very limited (Fitzgerald, 2009). However, the recent announcement of the first genome sequences for *S. pseudintermedius* strains has revealed the complement of genes encoding putative virulence factors (Ben Zakour et al., 2011; Tse et al., 2011), leading to proteomic studies which have identified novel host–pathogen interactions (Bannoehr et al., 2011a,b). An enhanced understanding of the pathogenesis of *S. pseudintermedius* is required in order to facilitate the design of novel therapeutics for the control of bacterial pyoderma infection caused by multi-resistant *S. pseudintermedius*. Furthermore,

the distinct host-tropisms of the three members of the SIG suggest that these closely related species would represent an excellent system for investigating evolutionary events underlying bacterial host-adaptation, a fundamental aspect of bacteriology which has not been well examined to date.

Recently, we published an announcement of the *S. pseudintermedius* ED99 genome, briefly listing several noteworthy features of the genome (Ben Zakour et al., 2011). Here we provide a comprehensive analysis of the ED99 genome in comparison to high quality draft genomes of the closely related species *S. delphini*, and *S. intermedius* generated in the current study, and to publicly available genomes for other staphylococcal species. The resulting data represent an excellent framework for investigations into the pathogenesis of canine pyoderma, and the molecular basis for staphylococcal host-specificity.

MATERIALS AND METHODS

BACTERIAL STRAINS

The previously sequenced *S. pseudintermedius* ED99 (formerly M732/99) was isolated from a clinical case of canine bacterial pyoderma in 1999 in Scotland and was selected to represent one of the common clones identified in a previous population genetic study of *S. pseudintermedius* (Bannoehr et al., 2007; Ben Zakour et al., 2011). *S. delphini* 8086 was isolated from the trachea of a horse in the UK (Bannoehr et al., 2007), and the type strain *S. intermedius* NCTC11048, from the anterior nares of a pigeon in the Czech Republic (Hajek, 1976).

GENOMIC DNA PREPARATION

Genomic DNA was isolated from 1 ml of overnight culture of *S. pseudintermedius* in BHI (Oxoid) at 37°C with shaking at 200 rpm. Genomic DNA extraction was carried out with a bacterial genomic DNA purification kit (Edge Biosystems) according to the manufacturer's instructions, except that prior to incubation at 37°C for 10 min, 125 μg/ml lysostaphin (AMBI L) was included.

GENOME SEQUENCING

Whole genome sequencing of *S. pseudintermedius* ED99 was carried out as previously described (Ben Zakour et al., 2011). Genome sequencing of *S. delphini* 8086 and *S. intermedius* NCTC11048 was carried out using the Illumina 3G Genome Analyzer. For each strain, we generated a total of 4,087,613 and 3,879,139 paired-end reads, respectively, with a fixed length of 36 bp and an average insert size of 200 bp, corresponding to more than 58× and 50× genome coverage, respectively. *De novo* assembly was performed by using the Velvet short reads assembler program (Zerbino and Birney, 2008). For each genome, contigs were mapped against the completed whole genome of *S. pseudintermedius* ED99 using MauveAligner (Rissman et al., 2009) and manually inspected for potential mis-assemblies. To confirm the reliability of the sequences obtained by this *de novo* sequencing approach based only on very short reads, re-sequencing of *S. pseudintermedius* ED99 as an internal control was also performed in parallel to *S. delphini* 8086 and *S. intermedius* NCTC11048. An automatic annotation was then performed by the RAST annotation server to predict CDS and their putative functions (Aziz et al., 2008). Functional categories were assigned by searching all predicted proteins against the COG database (www.ncbi.nlm.nih.gov/COG). The software AlienHunter (Vernikos and Parkhill, 2006) was used to detect atypical genome regions corresponding to putative horizontal gene transfer, insertion sequence (IS) elements were identified by interrogation of the IS database (Siguier et al., 2006), and clustered regularly interspaced short palindromic repeat (CRISPR) elements were characterized by the CRISPRFinder web software (Grissa et al., 2007a). The draft genome sequences of *S. delphini* 8086 and *S. intermedius* NCTC11048 have been deposited in the GenBank WGS database and have Genome Bioproject ID numbers PRJEA87011 and PRJEA87009, respectively.

COMPARATIVE GENOMIC ANALYSIS

Orthologous CDS between *S. pseudintermedius* ED99 (accession number CP002478), *S. aureus* Mu50 (accession number BA000017; Kuroda et al., 2001), *Staphylococcus epidermidis* RP62A (accession number CP000029; Gill et al., 2005), *Staphylococcus haemolyticus* JCSC1435 (accession number AP006716; Takeuchi et al., 2005), *Staphylococcus saprophyticus* ATCC15305 (accession number AP008934; Kuroda et al., 2005), *Staphylococcus carnosus* TM300 (accession number AM295250; Rosenstein et al., 2009), *S. delphini* 8086, and *S. intermedius* NCTC11048, were identified by reciprocal best hits using BLASTP, with a maximum *e*-value of 0.01, a minimum percentage identity of 40% and a minimum percentage sequence coverage of 80%. The average nucleotide identity (ANI) between the complete and draft genome sequences available for all staphylococcal species and the outgroup *Macrococcus caseolyticus* JCSC5402 (accession number AP009484; Baba et al., 2009), was determined by using the *in silico* DNA–DNA hybridization method of the JSpecies software using default parameters (Richter and Rossello-Mora, 2009). The distance matrix based on the ANI values obtained was then used in Splitstree to construct a Neighbor-Joining tree (Huson and Bryant, 2006).

RESULTS AND DISCUSSION

GENERAL FEATURES OF THE SIG GENOMES

We previously sequenced and annotated the complete genome of *S. pseudintermedius* ED99, used in this study as a reference for comparative genomic analysis with other SIG species (Ben Zakour et al., 2011). As previously reported, the ED99 genome was composed of a single circular chromosome of 2,572,216 bp (**Figure 1**) with an average G + C content of 37.6%, contained five ribosomal operons, 58 tRNA loci and encoded for 2401 predicted protein-coding sequences (CDSs; Bannoehr et al., 2011a). Wider analysis for the current study revealed that 44 putative CDS are pseudogenes, and functional information could be assigned to 77.6% of all CDS (1863 CDS), with 13.1% encoding conserved hypothetical proteins (315 CDS), and 9.3% encoding hypothetical proteins without significant homology to proteins of known function.

The draft genome sequence of *S. delphini* 8086 was assembled into 133 contigs and had an approximate size of 2.53 Mb including 2369 predicted CDS. The draft genome sequence of *S. intermedius* NCTC11048 was assembled into 228 contigs, and had an approximate size of 2.78 Mb, significantly larger than the two other members of the SIG, and was predicted to encode 2589

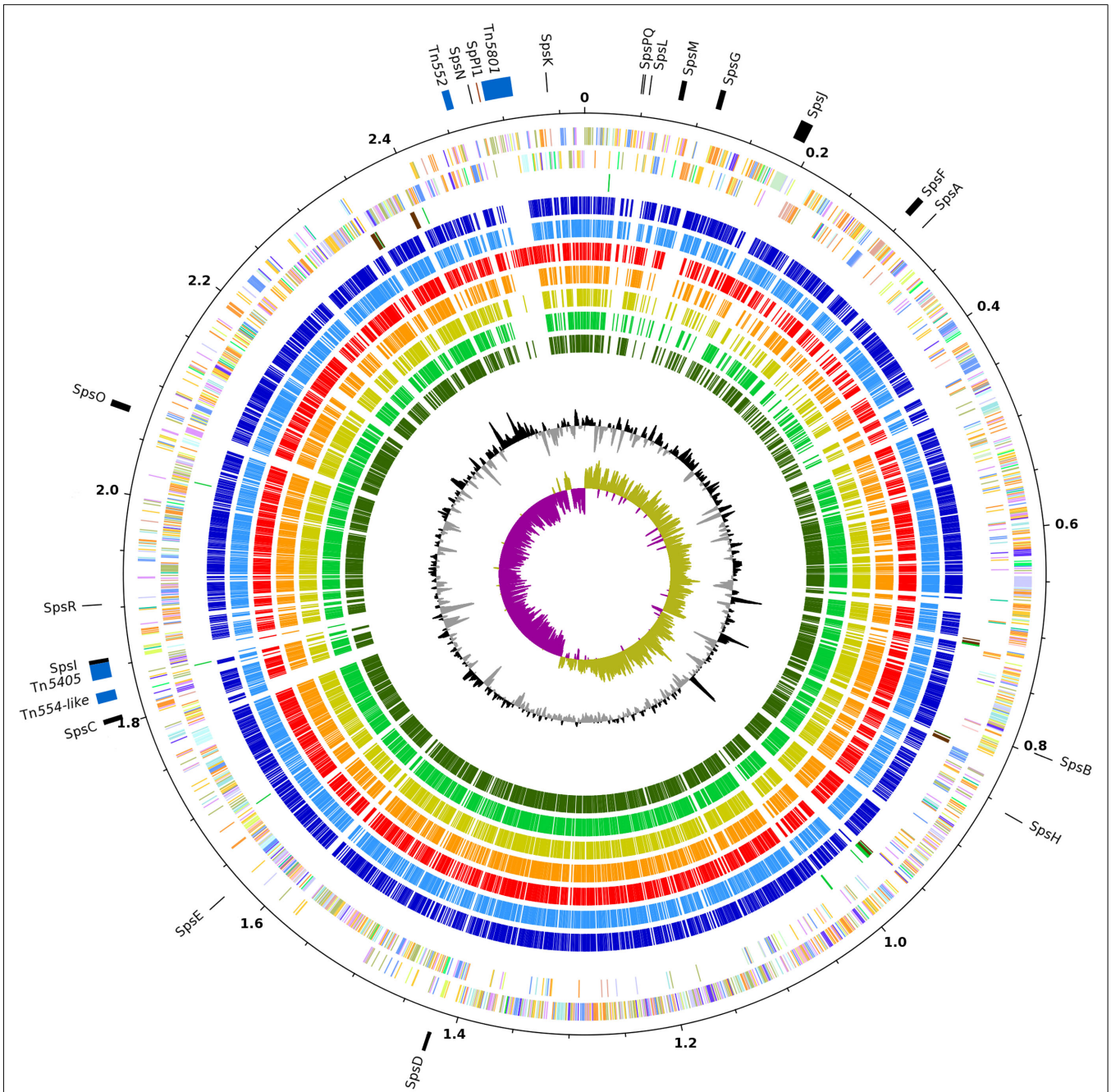

FIGURE 1 | Genome atlas of *S. pseudintermedius* ED99 compared to other staphylococci. Orthologs depicted in other species are identified by reciprocal best hits using BLASTP. Major features of the *S. pseudintermedius* ED99 are indicated on the outermost circle, including MGE (blue), CWA proteins (black), and SpPI1 (maroon) with positions on the reference genome indicated in Mb. From the outer to inner circles, circle 2 and 3, represent *S. pseudintermedius* ED99 genes on plus and minus strands, respectively (colored according to their COG category); circle 4, tRNA (green), rRNA (brown); circle 5, *S. delphini* 8086 (dark blue); circle 6, *S. intermedius* NCTC11048 (blue); circle 7, *S. aureus* Mu50 (red); circle 8, *S. epidermidis* RP62A (orange); circle 9, *S. haemolyticus* JCSC1435 (yellow); circle 10, *S. saprophyticus* ATCC15305 (light green); circle 11, *S. carnosus* TM300 (dark green); circle 12, G + C content plot (black and gray); and circle 13, GC skew plot (purple and khaki).

CDS (**Table 1**). Of note, the average GC content of the three SIG genomes ranges from 37.4 to 38.3%, which is considerably higher than any other staphylococcal species sequenced to date suggesting the existence of distinct selective pressures influencing the SIG genome nucleotide composition.

COMPARATIVE GENOMIC ANALYSIS OF THE *STAPHYLOCOCCUS* GENUS

The genomes of the three SIG species were compared with those of *S. aureus* Mu50, *S. epidermidis* RP62A, *S. haemolyticus* JCSC1435, *S. saprophyticus* ATCC15305, and *S. carnosus* TM300 (**Table 1**). Comparison of the eight selected staphylococcal chromosomes by

Table 1 | General features of the SIG genomes in comparison with other staphylococci.

Feature	*S. pseudintermedius* ED99	*S. delphini* 8086	*S. intermedius* NCTC11048	*S. aureus* Mu50	*S. epidermidis* RP62A	*S. haemolyticus* JCSC1435	*S. saprophyticus* ATCC15305	*S. carnosus* TM300
Length of sequence (bp)	2,572,216	∼2,528,000	∼2,780,000	2,878,529	2,616,530	2,685,015	2,516,575	2,566,424
G + C content (%)								
Total	37.6	38.3	37.4	32.9	32.2	32.8	33.2	34.6
Open reading frames								
Number	2401	∼2369	∼2589	2697	2494	2676	2446	2462
Average size (bp)	894	∼881	∼860	894	867	863	861	894
Percentage	83.4	∼82.6	∼80.1	83.7	82.6	86.0	83.7	85.8
Ribosomal RNAs								
16S	5	NA	NA	5	6	5	6	5
23S	5	NA	NA	5	6	5	6	5
5S	7	NA	NA	6	7	6	8	5
Transfer RNAs	58	NA	NA	60	61	59	60	60
Genomic islands								
IS	21 (12)	1#	12#	26 (13)	64 (18)	82 (60)	9 (2)	0
Transposons	4	0	0	3	5	2	0	0
SCC	0	0	0	1	1	1	2	0
Prophages	0	1#	3#	2	1	2	0	1
Plasmids	1*	NA	0	1	1*	3	2	0
Other islands	2	NA	NA	5	2	5	1	1

*NA, not available; #estimated number. *Cryptic plasmid. Number of intact IS element genes is indicated in parenthesis.*

reciprocal BLASTP revealed a high level of conservation and synteny (**Figure 1**). However, a large chromosomal region (∼465 kb) of low similarity was identified which corresponds to the *oriC* environ, a region of staphylococcal genomes containing many species-specific CDS (Takeuchi et al., 2005). Similar to *S. saprophyticus* ATCC15305 and *S. haemolyticus* JCSC1435, the genome of *S. pseudintermedius* ED99 contains a large chromosomal inversion at the beginning of the *oriC* environ, from the position 33,035 to 2,568,216, and which spans the entire chromosome. Consistent with their close-relatedness, the *oriC* environ exhibits a high level of gene content conservation among the closely related SIG members.

By performing reciprocal best blast hits analysis, we have determined a set of 1214 genes defining the core genome shared by *S. pseudintermedius* ED99, *S. aureus* Mu50, *S. epidermidis* RP62A, *S. haemolyticus* JCSC1435, *S. saprophyticus* ATCC15305, *S. carnosus* TM300, *S. delphini* 8086, and *S. intermedius* NCTC11048 (Table S2 in Supplementary Material). Of the predicted proteins, 12.4% have a general predicted function, 9.1% are of unknown function and 4.3% have no homolog found in the COG database, which is consistent with previous studies (Takeuchi et al., 2005; Rosenstein et al., 2009). Based on the core genome only, the average percentage similarity of *S. pseudintermedius* ED99 proteins compared to other staphylococcal species ranges from 68.8% with *S. carnosus* TM300 to 95.2 and 97.7% with *S. delphini* 8086 and *S. intermedius* NCTC11048, respectively. The evolutionary relatedness of the staphylococcal species was examined by calculation of their ANI. The phylogenetic tree based on pairwise comparison of the ANI confirms the close-relatedness of the SIG compared to the other staphylococcal species and is consistent with previously determined phylogenies (**Figure 2**). Of note, despite sharing distinct ecological niches, *S. pseudintermedius* ED99 and *S. delphini* 8086 share an ANI of 93.61%, which is very close to the threshold of species delineation of 95% ANI, equivalent to the DNA–DNA re-association threshold of 70% (Goris et al., 2007).

DISTRIBUTION OF MOBILE GENETIC ELEMENTS AMONG THE SIG

Although the *S. pseudintermedius* ED99 genome demonstrated a large degree of conservation and synteny with the other staphylococcal genomes, numerous regions of differences (RDs) of greater than 5 kb in size were identified including IS elements, genomic islands, and prophage- and plasmid-related sequences (**Figure 1**). A total of 21 predicted IS elements were identified including 7 identical copies of IS*Sp1* which had 47% nucleotide identity with IS*Sha1* of *S. haemolyticus* and IS*1182* integrated in Tn*5405* which had 100% nucleotide homology with the same element in *S. aureus*. In several cases the closest homologs of *S. pseudintermedius* IS elements were in other genera such as *Clostridium tetani* E88 (46% identity), and *Geobacillus thermodenitrificans* NG80-2 (52% identity). In addition, transposase-related sequences indicating the presence of at least one IS element in *S. delphini* 8086 and 12 IS elements in *S. intermedius* NCTC11048 were identified.

Reverse transcriptase (RT) Group II introns are self-splicing retro-elements composed of an intron RNA domain and a RT gene which are present in 25% of sequenced bacterial genomes, including members of the Firmicutes such as *Bacillus*, *Streptococcus*, and

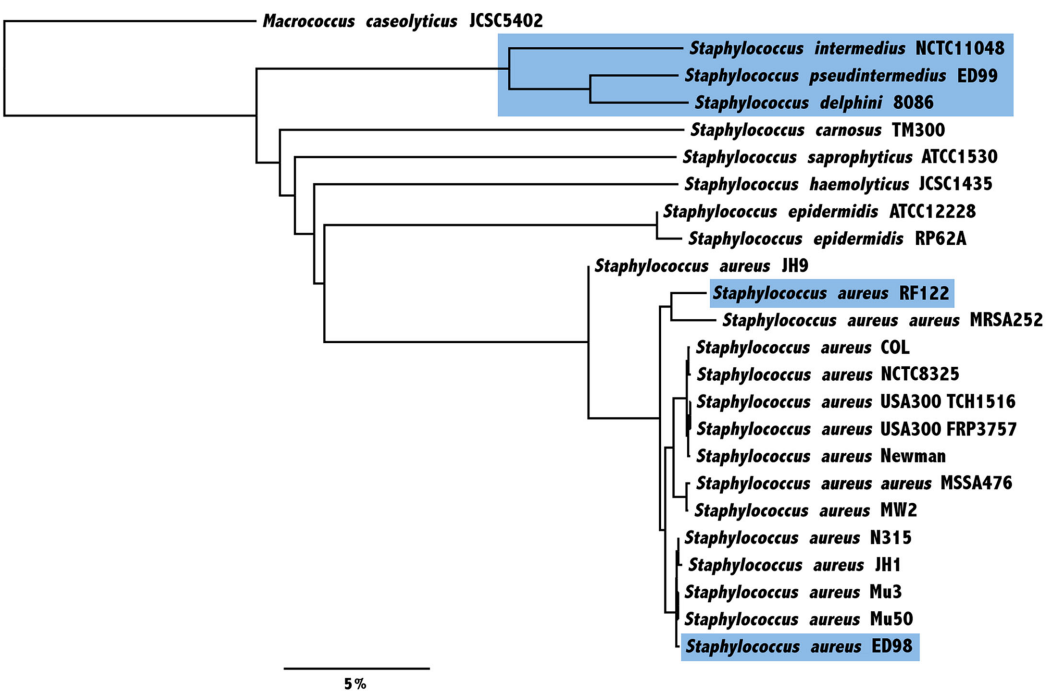

FIGURE 2 | Average nucleotide identity (ANI)-based Neighbor-Joining tree of 23 sequenced strains from eight staphylococcal species in addition to an outgroup *Macrococcus caseolyticus*. The evolutionary relatedness was examined by calculation of ANI, based on core genes only. Species and strains of animal origin are highlighted in blue (scale indicates the % difference ANI).

Lactococcus (Matsuura et al., 1997; Granlund et al., 2001; Tourasse and Kolsto, 2008). RT Group II introns are extremely rare in staphylococcal genomes, and generally present in low number with one or two copies, such as in *S. aureus* JKD6159 (Chua et al., 2011) and *S. pseudintermedius* HKU-10 (Tse et al., 2011). A total of nine intact copies and one pseudogene of the RT gene were identified in the genome of *S. pseudintermedius* ED99. All copies had a predicted intergenic insertion site, and no particular trend in their location or the nature of the genes adjacent to these elements was observed. However, it is possible that the integration of RT Group II introns may influence the expression of downstream genes or promote recombinational events (Tourasse and Kolsto, 2008). Of note, RT Group II introns were not identified in the genomes of *S. delphini* 8086 and *S. intermedius* NCTC11048.

The *S. pseudintermedius* ED99 genome does not contain any predicted complete prophages but contains three RDs containing phage-related genes, and a putative integrated 3.5 kb plasmid with 38% overall identity to plasmid pC221 of *S. aureus* was detected within the *oriC* environ. The genome also contains a novel member of the staphylococcal pathogenicity island family (SpPI1) adjacent to Tn*5801*, which is 4.1 kb in size, and includes attachment sites and genes with 55% identity to the terminase *orf5*, 67% identity to the repressor *orf20*, and 90% identity to the integrase *int* of SaPIbov of *S. aureus* (**Figure 3**). Of note, these three genes belong to the core minimal set of genes required for a functional mobile SaPI (Ubeda et al., 2008). In theory, SpPI1 may represent an ancestral minimal form of SaPI but *orf20* and *int* contain premature stop codons implying that SpPI1 is no longer mobile. Predicted virulence genes were not identified in SpPI1 but two genes encoding hypothetical proteins of unknown function were present. We examined the distribution of SpPI1 by PCR screening of 13 *S. pseudintermedius* strains, which represented diverse sequence types, with various geographical, host and clinical origins, and found that it was present in 11 strains (data not shown).

Staphylococcus pseudintermedius ED99 contains a novel 14 kb genomic island located adjacent to a *tRNA* locus, which is also present in *S. delphini* 8086 (**Figure A1** in Appendix). The island contains two genes with 51 and 32% homology respectively to *orf32* and *orf33* of ΦMu50B, two genes encoding the bi-component leukotoxin Luk-I identified previously *in S. pseudintermedius* SD91 (Prevost et al., 1995), and seven genes related to L-ascorbate transport and utilization not found in other staphylococci sequenced to date. The L-ascorbate utilization operon is organized in a novel combination of the *ula* operon of *Escherichia coli* (Yew and Gerlt, 2002) and shares similarity ranging from 55 to 68% with the ascorbate PTS system IIABC of *Streptococcus* sp. and the other members of the operon (*ulaG*, *ulaD*, *ulaE*, and *ulaF*) found in *Enterococcus* sp. The operon was identified in all 25 representative strains of *S. pseudintermedius*, *S. intermedius*, and *S. delphini*, with the exception of *S. intermedius* NCTC11048, which only contains the bi-component leukotoxin Luk-I (data not shown). In addition, despite being located downstream of a *t*RNA locus, which is a well-known integration site for mobile elements, mobility genes and flanking repeats regions were not identified in the sequence of both islands. Taken together, these characteristics imply an ancient acquisition in a progenitor of the SIG group and active maintenance of the acquired function among the SIG species.

S. PSEUDINTERMEDIUS ED99 AND *S. INTERMEDIUS* NCTC11048 CONTAIN CRISPR LOCI

Clustered regularly interspaced short palindromic repeats, recently described as an adaptative bacterial immune system, were shown to provide protection against viruses in *Streptococcus thermophilus*

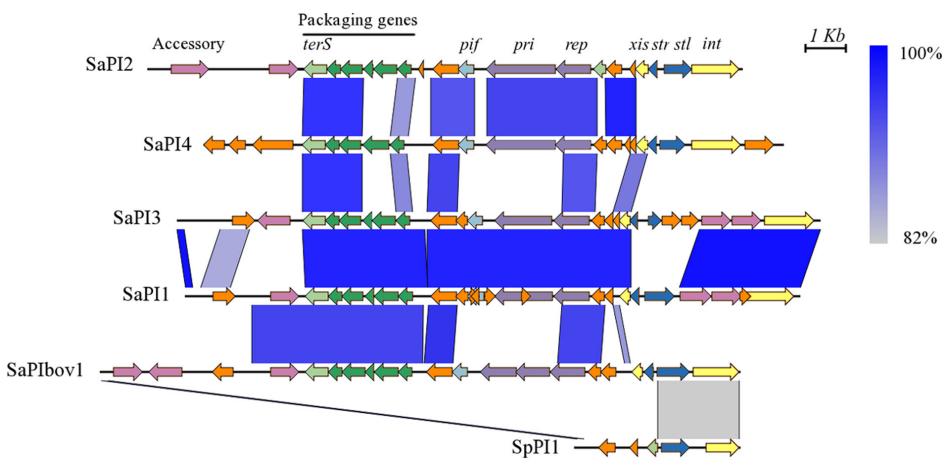

FIGURE 3 | Comparison of SpPI1 with the major staphylococcal pathogenicity islands. The pathogenicity island SpPI1 from *S. pseudintermedius* ED99 is compared to SaPI2 from *S. aureus* RN3984, SaPI4 from *S. aureus* MRSA252, SaPI3 from *S. aureus* COL, SaPI1 from *S. aureus* RN4282, and SaPIbov1 from *S. aureus* RF122. The similarity between regions is indicated by a spectrum of blue to gray, from 100 to 82% similarity. Genes are colored according to their sequence and function, as described previously (Novick et al., 2010): *int* (integrase) and *xis* (excisionase) in yellow; transcription regulators in dark blue; replication genes including the primase gene, *pri*, and the replication initiator gene, *rep* in purple; encapsidation genes in green; terminase small subunit gene (*terS*) in light green; superantigen and other accessory genes in pink; *pif* (related to phage interference) in light blue; and genes encoding hypothetical proteins in orange. Figure produced with EasyFig (Sullivan et al., 2011).

(Barrangou et al., 2007) and prevent conjugative transfer of plasmids in *S. epidermidis* (Marraffini and Sontheimer, 2008). CRISPR are widespread and have been identified in ∼40 and 90% of bacterial and archeal genomes sequenced (Grissa et al., 2007b). Occurrences of CRISPR are rare in staphylococcal species and are so far represented by the Mtube subtype (Haft et al., 2005) in the genome of *S. epidermidis* RP62A (Marraffini and Sontheimer, 2008), *S. lugdunensis* HKU09-01 (Tse et al., 2010) and on a novel SCC*mec*V element carried by livestock-associated methicillin-resistant *S. aureus* (Golding et al., 2010). Both *S. pseudintermedius* and *S. intermedius* genomes harbor a CRISPR locus of the Nmeni subtype, exclusively associated with vertebrate pathogens and commensals (Gill et al., 2005), and for which the closest homologs identified were found in *Streptococcus* M1 GAS and *Ruminococcus lactaris* ATCC 29176 (**Figure 4**). The identity between *S. pseudintermedius* and *S. intermedius* CRISPR-associated genes ranged from 78.7 to 86.7% while the 36-bp-repeat units were almost identical (97%), suggesting that the locus may have been acquired before speciation by a common ancestor to the SIG and then lost by *S. delphini*, although independent acquisition by both species cannot be ruled out. The repeat array of the *S. pseudintermedius* CRISPR locus contains 22 different spacers, with a size ranging from 29 to 31 bp, for which 6 share similarity with sequences of prophages and plasmids such as Φ2638A, pMG1, and pH308197 associated with staphylococcal, enterococcal, and bacillus species, respectively. Of note, eight additional spacers were found to share similarity with prophage-related regions from the *S. intermedius* genome. In addition, a CRISPR locus of the Mtube subtype was also identified in the genome of *S. intermedius*, for which the closest homologs were the CRISPR loci found in *S. epidermidis* RP62A and *S. lugdunensis* HKU09-01 (**Figure 4**). The presence of CRISPR loci in *S. pseudintermedius* and *S. intermedius* correlates with the absence of plasmids, and prophages as previously described (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). However, the role of CRISPRs in immunity to non-phage genomic islands is unclear, as illustrated by the presence of several recently acquired transposons in the *S. pseudintermedius* ED99 genome. Furthermore, other mechanisms such as restriction–modification can limit the transfer of mobile genetic elements (MGE) between bacteria (Kobayashi, 2001). Although, we did not mention it specifically in the paper,

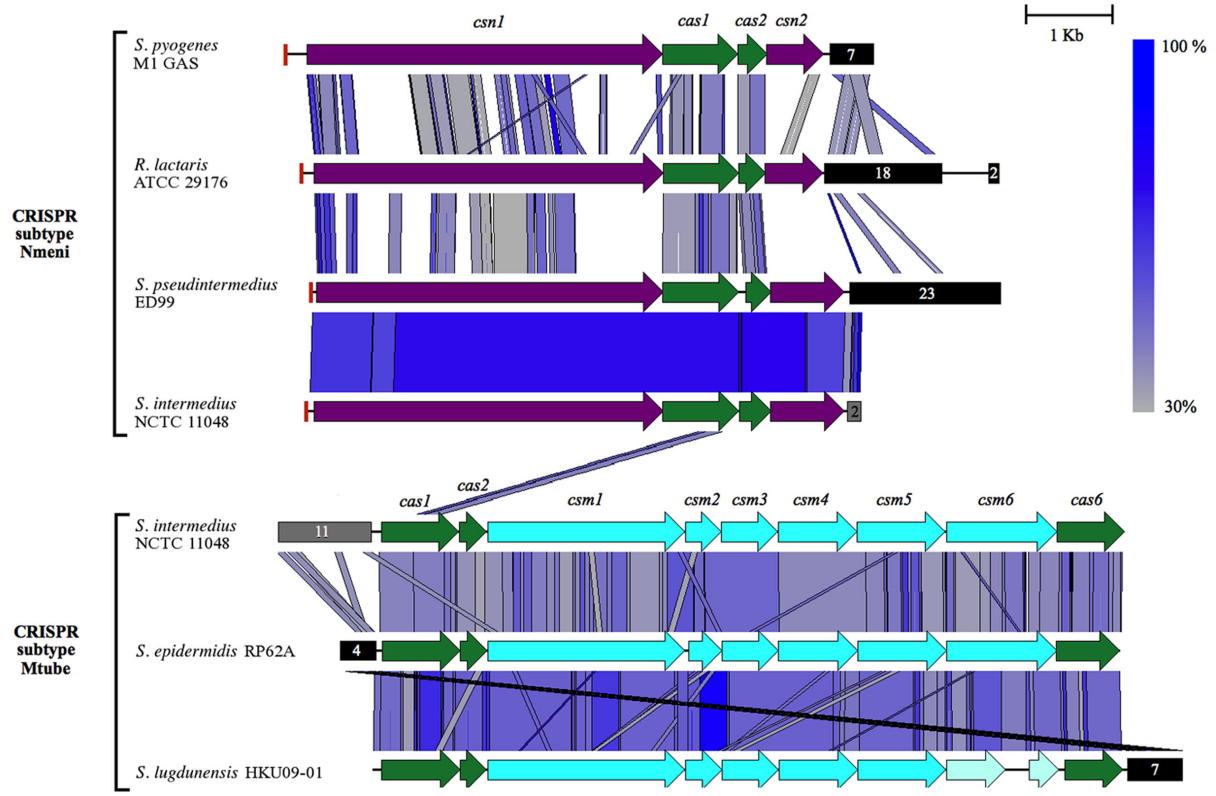

FIGURE 4 | Comparison of the CRISPR loci of *S. pseudintermedius* ED99 and *S. intermedius* NCTC 11048 with related Nmeni and Mtube types. The CRISPR locus subtype Nmeni of *S. pseudintermedius* ED99 and *S. intermedius* NCTC 11048 are compared to those of *Streptococcus* M1 GAS and *Ruminococcus lactaris* ATCC 29176. The CRISPR locus subtype Mtube of *S. intermedius* NCTC 11048 is compared to those of *S. epidermidis* RP62A and *S. lugdunensis* HKU09-01. The similarity between regions is indicated by a spectrum of blue to gray colors depicting a range of 100–30% similarities. Genes of the *csn* family are shown in purple, the *cas* family in green, and the *csm* family in light blue. Of note, the *csm6* gene in *S. lugdunensis* HKU09-01 appears to be a pseudogene. Black boxes represent clusters of direct repeats regions with the number of spacers between the direct repeats indicated. The red box at the beginning of each Nmeni subtype CRISPR locus corresponds to a single copy of the repeats identified in the cluster of downstream direct repeats. The gray boxes indicate the approximate number of direct repeats at the locus in *S. intermedius* NCTC 11048 (due to the unfinished status of the genome). Figure produced with EasyFig (Sullivan et al., 2011).

S. delphini contains several genes related to these functions, which could partly explain the lack of genomic islands in *S. delphini*. In addition, little is known about the bacterial composition of the environmental niche occupied by *S. delphini*. Therefore, hypotheses explaining the lack of genomic islands in *S. delphini* include (i) that less diversity is encountered by this species in its niche, or (ii) most of the other species encountered are distantly related, resulting in less frequent successful horizontal gene transfers.

GENOMIC INSIGHTS INTO THE PATHOGENESIS OF *S. PSEUDINTERMEDIUS* AND SIG INFECTIONS

All SIG species genomes encode a number of predicted toxins including several previously identified such as the enterotoxin SeI-nt (Becker et al., 2001; Hendricks et al., 2002; Futagawa-Saito et al., 2004), hemolysin III, the β-hemolysin (Dziewanowska et al., 1996), the bi-component leukotoxin Luk-I (Prevost et al., 1995) and several exfoliative toxins (Terauchi et al., 2003; Futagawa-Saito et al., 2009; Iyori et al., 2010; **Table 2**). We also identified a putative novel exfoliative toxin specific to *S. pseudintermedius ED99* designated SPETA which has 78 and 76% amino acid identity with the exfoliative toxin A SHETA from *Staphylococcus hyicus* and ETA from *S. aureus*, respectively. The existence of several exfoliative toxin variants made by different *S. pseudintermedius* strains is consistent with the skin tropism of *S. pseudintermedius* and the characteristic skin pathology associated with pyoderma. A wide range of exoenzymes is encoded by all three SIG species, such as the serine protease HtrA, two lipases encoded by the genes *lip* and *geh*, a thermonuclease, a protease ClpX, and a thermolysin (Wladyka et al., 2008). In contrast to other staphylococcal genome sequences, *S. pseudintermedius* ED99 and *S. delphini* 8086 genomes both contain genes encoding a putative sialidase or neuraminidase. The neuraminidase modification of host sugars may contribute to host colonization by providing a carbon source for growth, contributing to biofilm formation, or by enhancing adherence by exposing receptors on the host cell (Sakarya et al., 2010). Overall, the SIG species share a large number of toxins and exoenzymes reflecting their recent common ancestry and possibly their common skin niche.

All three SIG species contained genes encoding proteins with ∼42% identity to the Von Willebrand-binding protein of *S. aureus* which is involved in the formation of abscesses (Cheng et al., 2010). *S. pseudintermedius* ED99 contains a cluster of eight genes encoding predicted glutamyl-endopeptidases which share 56–75% identity with each other, and 29–42% identity with the probable glutamyl-endopeptidase ORF2 encoded by the *etd* pathogenicity island of *S. aureus* TY114 (Yamaguchi et al., 2002). This glutamyl-endopeptidases cluster is also present in *S. intermedius* NCTC 11048 and *S. delphini* 8086 and is not associated with a predicted MGE implying it is part of the core genome of the SIG species.

A total of 18 genes encoding putative cell wall-associated (CWA) proteins, designated SpsA to SpsR, were previously identified in the genome of *S. pseudintermedius* ED99 (Bannoehr et al., 2011a). Of these, nine were also encoded in the *S. intermedius* and *S. delphini* genomes, revealing a considerable number of *S. pseudintermedius*-specific CWA proteins which may be important for its canine host tropism. Recently, it was demonstrated that SpsD and SpsL, mediated binding to several extracellular matrix proteins. Of note, there was enhanced affinity of SpsL for canine in comparison to human fibrinogen implying a role in host-specific interactions (Bannoehr et al., 2011a).

Finally, the control of *S. aureus* virulence gene expression is coordinated by an array of global regulators (Novick, 2003). With the exception of the *S. aureus* regulators (*saeSR*) and the staphylococcal accessory regulators encoded by *sarSTU*, homologs of numerous well-characterized regulators of virulence previously identified in sequenced staphylococcal species were detected in the genomes of the SIG species (**Table 2**).

NICHE ADAPTATION BY THE SIG

Compared to human-specific bacterial species, the SIG species encounter distinct environmental conditions dependent on their host-species with several biophysical parameters influenced, including skin hydration and pH (Montagna, 1967). For instance, in contrast to humans, sweat glands in the skin of animals such as dogs are not involved in thermoregulation (Affolter and Moore, 1994) resulting in lower relative skin hydration (Boelsma et al., 2003; Shimada et al., 2009). In addition, skin pH in dogs has been reported to differ from humans and to vary greatly depending on the anatomical site and breed of dog (Matousek and Campbell, 2002). Furthermore, there are known host-dependent differences in the availability of iron which influence the mechanisms of iron acquisition employed by staphylococci (Pishchany et al., 2010). In relation to these differing environments, the SIG species have a diverse complement of genes encoding proteins potentially involved in osmo-protection and resistance to oxidative stress. For example, *S. pseudintermedius* ED99 has four putative nitroreductase genes, one more than *S. intermedius* NCTC11048 and *S. delphini* 8086. All SIG species harbor several sodium/salt transporters, with the exception of the high-affinity potassium system Kdp which is absent from *S. intermedius* NCTC11048. *S. pseudintermedius* ED99 and *S. delphini* 8086 also contain a second catalase gene known as *katB* which has to date only been identified among staphylococcal species that inhabit high osmotic and oxidative stress niches including, *S. xylosus*, *S. saprophyticus*, and *S. equorum* (Blaiotta et al., 2010). Another source of genetic variation among the SIG species which could be related to their host niche is the transport and metabolism of small molecules such as carbohydrates. *S. pseudintermedius* ED99 contains genes encoding proteins predicted to mediate the transport and utilization of lactose/galactose in contrast to *S. intermedius* NCTC11048 and *S. delphini* 8086 which encode variant sugar transporters likely to have distinct substrates. *S. intermedius* NCTC11048 also encodes a novel ribose ABC transporter while *S. delphini* 8086 harbors two additional PTS systems, predicted to be involved in transport of cellobiose and glucitol/sorbitol, respectively.

In common with other staphylococci, the SIG species have developed several mechanisms for acquisition of iron (**Table 3**). One such mechanism is the production of low-molecular-weight chelating agents called siderophores. For example, the SIG genomes contain the genes *sfaABCD* and *htsABC* involved in the biosynthesis and transport of staphyloferrin A and transport of heme (Hammer and Skaar, 2011). In contrast to other non-*S. aureus* species sequenced, the SIG species can also produce

Table 2 | Distribution of virulence factors identified in eight staphylococcal species.

Product	Gene name	Location in SP	SA	SP	SD	SI	SE	SS	SH	SC
EXOENZYMES										
1-Phosphatidylinositol phosphodiesterase	*plc*	*oriC* environ	+	−	−	−	−	−	−	−
Staphylocoagulase	*coa*		+	+	+	+	−	−	−	−
Triacylglycerol lipase	*lip*		+	+	+	−	+	+	+	+
Lipase	*geh*		+	+	−	−	+	+	−	+
Serine protease	*htrA*		+	+	+	+	+	+	+	+
Cysteine protease	*sspB, C*		+	−	−	−	+	−	−	−
Serine V8 protease	*sspA*		+	−	−	−	+	+	−	−
Glutamyl-endopeptidase			+	+ (8)	+ (13)	+(10)	−	−	−	−
Thermonuclease	*nuc*		+	+ (3)	+ (3)	+ (2)	+	+	+	+
Serine proteases	*spl(s)*		+	−	−	−	−	−	−	−
Staphylokinase	*sak*		+	−	−	−	−	−	−	−
Hyaluronidase	*hysA*		+	−	−	−	−	−	−	−
Zinc metalloproteinase aureolysin	*aur*	*oriC* environ	+	+	+	+	+	+	−	−
Cell wall hydrolase	*lytN*		+	−	−	−	−	−	−	−
Sialidase	*nanB*		−	+	+	−	−	−	−	−
proteases ClpX	*clpX*		+	+	+	+	+	+	+	+
TOXINS										
Exotoxins/superantigen-like proteins	*set(s)*		+	−	−	−	−	−	−	−
α-Hemolysin	*hly*		+d	−	−	−	−	−	−	−
β-Hemolysin	*hlb*		+d	+	+ (2)	+	+	−	−	−
δ-Hemolysin	*hld*		+	+	+	+	+	+	+	+
Hemolysin III			+	+	+	+	+	+	+	+
Leukotoxins	*lukDE*		+d	−	−	−	−	−	−	−
Leukocidins	*lukF, M*		+d	−	−	−	−	−	−	−
	lukF-I, S-I		−	+	+	+	−	−	−	−
Panton–Valentine leukocidin	*lukS, F-PV*		+d	−	−	−	−	−	−	−
Toxic shock syndrome toxin 1	*tst*		+d	−	−	−	−	−	−	−
γ-Hemolysin components	*hlgA, B, C*		+	−	−	−	−	−	−	−
Enterotoxins	*SE(s)*		+	*se-int*	+	+	−	−	−	−
Exfoliative toxins	*eta, etb*	Upstream SpPI1	+	*speta*	−	−	−	−	−	−
			−	*siet*	+	+	−	+	−	−
ADHESINS										
Extracellular matrix binding proteins	*ebhA, B*		+	−	−	−	+	−	−	−
Elastin-binding protein	*ebpS*		+	+	+	+	+	+	+	+
Fibronectin-binding proteins	*fnbA, B*		+	−	−	−	−	−	−	−
Intercellular adhesion proteins	*icaABCD*		+	+	+	+	+	−	−	−
Collagen adhesin precursor	*cna*		+	−	−	−	−	−	−	−
Clumping factors	*clfA, B*		+	−	−	−	−	−	−	−
Ser-Asp rich proteins	*sdr*		+	−	−	−	+	+	+	−
Iron-responsive surface determinant	*isdA-G*		+	−	−	−	−	−	−	−
Other putative cell surface proteins identified in *S. pseudintermedius* ED99										
	spsA	*oriC* environ	−	+	+	+	−	−	−	−
	spsB		−	+	+	+	−	−	−	−
	spsC		+	+	+	+	+	+	+	+
	spsD		−	+	−	−	−	−	−	−
	spsE		+	+	+	+	+	+	+	+
	spsF	*oriC* environ	−	+	−	−	−	−	+	−
	spsG	*oriC* environ	−	+	+	−	−	−	−	−
	spsH		−	+	+	+	−	−	−	−

(Continued)

Table 2 | Continued

Product	Gene name	Location in SP	SA	SP	SD	SI	SE	SS	SH	SC
	spsI		−	+	−	−	−	−	−	−
	spsJ	*oriC* environ	+	+	−	−	−	−	−	−
	spsL		−	+	−	−	−	−	−	−
	spsM		−	+	−	−	−	−	−	−
	spsN		−	+	+	+	−	−	−	−
	spsO		−	+	−	−	−	+	−	−
	spsR		−	+	+	+	−	−	−	−
OTHERS										
Immunoglobulin G (IgG)-binding protein A	*spa*	*oriC* environ	+	*spsP*	−	−	−	−	−	−
		oriC environ	−	*spsQ*	−	+	−	−	−	−
Capsular polysaccharide synthesis proteins	*capA-G*		+	−	−	−	−	+	+	−
Lipoproteins	*lpl(s)*		+	−	−	−	−	−	−	−
IgG-binding protein SBI	*sbi*	*oriC* environ	+	*spsK*	+	+	−	−	−	−
TWO-COMPONENT REGULATORY SYSTEMS										
Accessory gene regulator	*agrA, B, C, D*		+	+	+	+	+	+	+	+
S. aureus exoprotein expression regulator	*saeS, R*	*oriC* environ	+	+	+	+	+	−	−	−
Staphylococcal respiratory response protein	*srrA, B*		+	+	+	+	+	+	+	+
Autolysis-related locus	*arlS, R*		+	+	+	+	+	+	+	+
	lytS, R	*oriC* environ	+	+	+	+	+	+	+	+
SarA PROTEIN FAMILY										
Staphylococcal accessory regulator A	*sarA*		+	+	+	+	+	+	+	+
Staphylococcal accessory regulator R	*sarR*		+	+	+	+	+	+	+	+
Staphylococcal accessory regulator Z	*sarZ*	*oriC* environ	+	+	+	+	+	+	+	+
Staphylococcal accessory regulator T, U	*sarT, U*		+	−	−	−	−	−	−	−
Repressor of toxins	*rot*		+	+	+	+	+	+	+	−

SP, S. pseudintermedius ED99; SD, S. delphini 8086; SI, S. intermedius NCTC11048; SA, S. aureus Mu50; SE, S. epidermidis RP62A; SH, S. haemolyticus JCSC1435; SS, S. saprophyticus ATCC15305; SC, S. carnosus TM300. Number of copies is indicated in parentheses. d: Strain-dependent.

and transport staphylobactin A mediated by the operons *sbnA-I* and *sirABC*. Uptake of ferrous iron is also enabled through the presence of the FeoAB system, which is not shared with *S. saprophyticus* ATCC15305 or *S. haemolyticus* JCSC1435. Further differences with other staphylococcal species include the absence of the siderophore–Fe transport system SstABC, the lipoprotein receptor for Fe^{3+} (FhuD) and the hemoglobin–Fe transport system IsdA-H, found in *S. aureus*. In addition, variation among the three SIG species were observed with the absence of the ferrous iron transporter EfeUOB in *S. pseudintermedius* ED99 and the presence of an inactivating mutation in the iron–manganese *mntABC* operon in *S. delphini* 8086. Overall, in common with other staphylococci, the SIG species have an extensive array of systems for acquiring iron from the host, which may be important in the context of the skin environment where iron sources are particularly limited.

EVOLUTION OF ANTIMICROBIAL RESISTANCE IN *S. PSEUDINTERMEDIUS*

In common with *S. aureus* and *S. haemolyticus*, multi-resistance in *S. pseudintermedius* strains is frequent and includes resistance to tetracycline (Schwarz et al., 1998), macrolides, lincosamides and streptogramins (Eady et al., 1993; Boerlin et al., 2001), aminoglycosides and aminocyclitols (Noble et al., 1996; Boerlin et al., 2001), fluoroquinolones (Intorre et al., 2007), and methicillin (Piriz et al., 1995; Kania et al., 2004; Sasaki et al., 2007a). We determined that *S. pseudintermedius* ED99 is resistant to ampicillin, erythromycin, tetracycline, and trimethoprim whereas *S. delphini* 8086 and *S. intermedius* NCTC11048 are sensitive to all antibiotics cited above (data not shown). Four transposons containing one or more antibiotic resistance genes were identified in the genome of *S. pseudintermedius* ED99 (**Table 4**). Of these, 2 (Tn*552* and Tn*554*-like) found also in *S. haemolyticus* JCSC1435, and in *S. epidermidis* and *S. aureus* strains respectively, encode the *bla* operon, which confers β-lactam resistance. Tn*5405* encodes the aminoglycoside–streptothricin resistance genes *aad6–sat4–aphA-3* and is associated with the macrolide–lincosamide–streptogramin resistance gene *ermB* previously described in *Enterococcus faecium* (Werner et al., 2003) and *S. intermedius* (Boerlin et al., 2001), and the putative conjugative transposon Tn*5801* (25.8 kb) encodes the *tetM* gene responsible for resistance to tetracycline. Tn*5801* was previously identified in *S. aureus* Mu50 and is related to the conjugative transposon Tn*916* of *Enterococcus faecalis* (Flannagan et al., 1994). The existence of transposons which are nearly identical to those found in human-associated staphylococcal species indicates a recent inter-species horizontal transfer of antibiotic resistance. In contrast to the closely related species *S. delphini* and *S. intermedius*, which have limited clinical importance,

Table 3 | Distribution of iron transport related genes identified in eight staphylococcal species.

Product	Gene names	SA	SP	SD	SI	SE	SS	SH	SC
Siderophore staphylobactin production	*sbnABCDEFGHI*	+	+	+	+	−	−	−	−
Siderophore ABC transporter	*sirABC*	+	+	+	+	−	−	−	−
Siderophore production (staphyloferrin A)	*sfaABCD*	+	+	+	+	+	+	+	+
Siderophore ABC transporter (staphyloferrin A)	*htsABC*	+	+	+	+	+	+	+	+
Transcriptional repressor of iron uptake	*fur*	+	+	+	+	+	+	+	+
Iron-regulated ABC transporter (siderophore?)	*sstABCD*	+	−	−	−	+	+	+	+
Ferrichrome ABC transporter	*fhuCBG*	+	+	+	+	−	+	+	+
Lipoprotein receptor for Fe^{3+}	*fhuD*	+	−	−	−	−	+	+	+
Iron-responsive surface determinant (iron uptake)	*isdA-G, srtB*	+	−	−	−	−	−	−	−
Iron–manganese ABC transporter	*mntABC (sitABC)*[c]	+	+	−[a]	+	+	+	−[b]	+
Heme-regulated ABC transporter (heme detoxification)	*hrtAB*	+	+	+	+	+	+	+	+
Ferrous iron transporter	*efeUOB*	+	−	+	+	−	−	+	+
Ferrous iron uptake homolog	*feoAB*	+	+	+	+	+	−	−	+

SP, S. pseudintermedius ED99; SD, S. delphini 8086; SI, S. intermedius NCTC11048; SA, S. aureus Mu50; SE, S. epidermidis RP62A; SH, S. haemolyticus JCSC1435; SS, S. saprophyticus ATCC15305; SC, S. carnosus TM300.

[a] sitA is a pseudogene in S. delphini. [b] sitABC regulator sitR is missing in S. haemolyticus. [c] in S. epidermidis.

Table 4 | Mobile genetic elements associated with antibiotic resistance identified in *S. pseudintermedius* ED99.

Name	Resistance factor(s) encoded	Closest homologs
*Tn*5801	*tetM*	*S. aureus*
*Tn*552	*bla* operon	*S. haemolyticus, S. epidermidis*
*Tn*554-like	*bla* operon	*S. aureus*
*Tn*5405	*aad6–sat4–aphA-3, ermB*	*Streptococcus* sp., *Enterococcus faecium*

S. pseudintermedius encounters considerable antibiotic selective pressures, which have contributed to the spread of MGE encoding antibiotic resistance. Since the 1990s, methicillin-resistant *S. pseudintermedius* strains have emerged through several independent SCCmec acquisition events and disseminated widely (Piriz et al., 1995; Kania et al., 2004; Bannhoer et al., 2007; Sasaki et al., 2007a). Importantly, *S. pseudintermedius* strains containing MGE encoding antibiotic resistance could represent a reservoir for the spread of resistance genes to human commensal skin flora (Guardabassi et al., 2004a,b).

CONCLUSION

The spread of antibiotic-resistant strains of *S. pseudintermedius* and the lack of an effective vaccine means that alternative approaches for controlling canine pyoderma are required. The identification of novel virulence determinants in the genome of *S. pseudintermedius* has provided candidate new targets for therapeutic interventions. In particular, the identification of CWA-associated proteins and toxins which contribute to the pathology associated with pyoderma infection should provide the impetus for detailed characterization of these critical host–pathogen interactions and for investigations into their potential as vaccine components. Furthermore, the discovery that transposons are largely responsible for the multi-resistant phenotype of *S. pseudintermedius* provides important insights into the evolution of antibiotic resistance within the species. Future genome sequencing projects should include representatives of the widespread methicillin-resistant *S. pseudintermedius* clones (Bannoehr et al., 2007). Furthermore, comparative genomic analysis also revealed the presence of CRISPR loci, and an unusually high %GC content of the SIG species, which provides intriguing avenues for basic research into bacterial genome evolution. Finally, several additional species-specific features which likely reflect the distinct ecological niches occupied, were identified such as genes involved in carbohydrate metabolism iron acquisition and resistance to oxidative stress. The identification of the genetic events which led to differentiation of the SIG species and the determinants which correlate with their distinct host-tropisms provide avenues for fundamental studies into the molecular basis of bacterial host-adaptation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2012.00044/abstract

Table S1 | Genes specific to *S. pseudintermedius* ED99.

Table S2 | Core genes identified in *S. pseudintermedius* ED99.

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APPENDIX

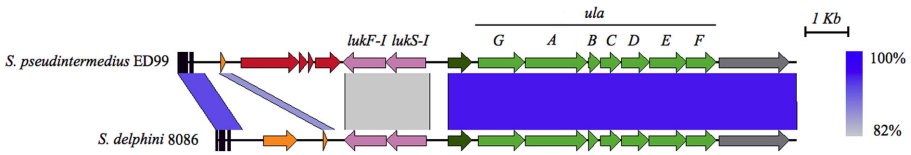

FIGURE A1 | Comparison of the genomic island containing the bi-component leukotoxin Luk-I and the L-ascorbate transport and utilization operon in *S. pseudintermedius* ED99 and *S. delphini* 8086. The similarity between regions is indicated by a spectrum of blue to gray, ranging from 100 to 82% similarity. Genes are colored according to their sequence and function as follow: phage-related genes in red; leukotoxin genes in pink; putative phosphoglucomutase in dark green; L-ascorbate transport and utilization genes in green; putative transcriptional regulator in gray; *tRNA* genes are represented by black boxes; and genes encoding hypothetical proteins in orange.

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Evolutionary blueprint for host- and niche-adaptation in *Staphylococcus aureus* clonal complex CC30

Martin J. McGavin[1]*, Benjamin Arsic[1] and Nicholas N. Nickerson[2]

[1] *Department of Microbiology, Schulich School of Medicine and Dentistry, Siebens Drake Research Institute and Centre for Human Immunology, University of Western Ontario, London, ON, Canada*
[2] *Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada*

Edited by:
David Heinrichs, University of Western Ontario, Canada

Reviewed by:
Steven Gill, University of Rochester School of Medicine and Dentistry, USA
Jodi A. Lindsay, St George's University of London, UK

****Correspondence:***
Martin J. McGavin, Department of Microbiology, Schulich School of Medicine and Dentistry, and Centre for Human Immunology, Western University, London, ON N6A 5C1, Canada.
e-mail: martin.mcgavin@schulich.uwo.ca

Staphylococcus aureus clonal complex CC30 has caused infectious epidemics for more than 60 years, and, therefore, provides a model system to evaluate how evolution has influenced the disease potential of closely related strains. In previous multiple genome comparisons, phylogenetic analyses established three major branches that evolved from a common ancestor. Clade 1, comprised of historic pandemic phage type 80/81 methicillin susceptible *S. aureus* (MSSA), and Clade 2 comprised of contemporary community acquired methicillin resistant *S. aureus* (CA-MRSA) were hyper-virulent in murine infection models. Conversely, Clade 3 strains comprised of contemporary hospital associated MRSA (HA-MRSA) and clinical MSSA exhibited attenuated virulence, due to common single nucleotide polymorphisms (SNP's) that abrogate production of α-hemolysin Hla, and interfere with signaling of the accessory gene regulator *agr*. We have now completed additional *in silico* genome comparisons of 15 additional CC30 genomes in the public domain, to assess the hypothesis that Clade 3 has evolved to favor niche adaptation. In addition to SNP's that influence *agr* and *hla*, other common traits of Clade 3 include tryptophan auxotrophy due to a di-nucleotide deletion within *trpD*, a premature stop codon within *isdH* encoding an immunogenic cell surface protein involved in iron acquisition, loss of a genomic toxin–antitoxin (TA) addiction module, acquisition of *S. aureus* pathogenicity islands SaPI4, and SaPI2 encoding toxic shock syndrome toxin *tst*, and increased copy number of insertion sequence ISSau2, which appears to target transcription terminators. Compared to other Clade 3 MSSA, *S. aureus* MN8, which is associated with Staphylococcal toxic shock syndrome, exhibited a unique ISSau2 insertion, and enhanced production of toxic shock syndrome toxin encoded by SaPI2. Cumulatively, our data support the notion that Clade 3 strains are following an evolutionary blueprint toward niche-adaptation.

Keywords: *Staphylococcus aureus*, evolution, pseudogene, pathogenicity island, insertion sequence, toxin-antitoxin addiction module, pathoadaptation, virulence

INTRODUCTION

Society has become imbued with the Superbug label to define strains of antibiotic resistant bacteria that cause hospital-associated outbreaks of infection (Foster, 2004; Abbott, 2005; Brazier, 2008; Guo et al., 2011). This term, denoting the sudden emergence and spread of new antibiotic resistant strains, could also be applied to an historic global pandemic caused by a penicillin-resistant *S. aureus* clone known as phage type PT80/81, which emerged in Australia, Great Britain, and North America in the early 1950's (Rountree and Beard, 1958; Williams et al., 1959; Wormald, 1961; Tanimoto, 1962). The initial outbreaks occurred in hospitals, especially among newborns and nursing mothers, but quickly spread to the wider community, causing unusually severe invasive skin infections, and fatal sepsis or necrotizing pneumonia in young and healthy individuals (Hassall and Rountree, 1959). Although the pandemic dissipated after 10 years (∼1953–1963), concomitant with the introduction of methicillin, genetically related contemporary strains are prominent in both the community and health-care settings. These consist of clinical methicillin susceptible *S. aureus* (MSSA), the epidemic EMRSA-16 lineage of hospital associated MRSA (HA-MRSA) which has the Type II Staphylococcal cassette chromosome SCC*mec* element, and the hyper-virulent Southwest Pacific (SWP) clone of community associated MRSA (CA-MRSA) which, like other unrelated CA-MRSA, has Type IV SCC*mec*. All of these strains belong to clonal complex CC30 as determined by multi locus sequence typing (MLST) analysis (Robinson et al., 2005).

To better understand the evolutionary development of CC30, we recently employed comparative genome sequencing to evaluate nine CC30 strains (DeLeo et al., 2011), including the reference genome of MRSA 252, representing the EMRSA-16 clone of HA-MRSA (Holden et al., 2004; Lindsay and Holden, 2004). Phylogenetic analyses based on a contiguous 1.4 Mb region of each genome, or with concatenated nucleotide segments, supported the existence of three major branches that evolved from a common ancestor. Clade I consists of the historic PT80/81

pandemic. This clonal type is typically ST30*spa*43, as determined by MLST and staphylococcal Protein A (*spa*) gene typing, and possesses the Panton Valentin Leukotoxin (PVL), that is also characteristic of contemporary CA-MRSA, including the SWP clone, which is ST30*spa*19 and comprises Clade 2. Although temporally separated by nearly 50 years, Clades 1 and 2 share a number of common traits, which in addition to PVL, include abundant production of α-hemolysin Hla, elevated transcription of RNAIII encoded by the accessory gene regulator *agr* locus, and a hypervirulent trait in murine infection models (DeLeo et al., 2011).

Clade 3 is comprised of the EMRSA-16 clone of HA-MRSA, which is typically ST36*spa*16, and contemporary clinical methicillin susceptible *S. aureus*, which are often ST30*spa*33. Although Clade 3 strains exhibited attenuated virulence in murine infection models relative to Clades 1 and 2, these are still associated with a high burden of disease. EMRSA-16, which is known in the United States as USA200, has become one of the most successful HA-MRSA clones (Cox et al., 1995; Enright et al., 2000; Johnson et al., 2001; McDougal et al., 2003; Seybold et al., 2006; Fowler et al., 2007). Others defined an association of CC30 MSSA, frequently ST30*spa33*, with bacteremia, infective endocarditis, and osteomyelitis (Cassat et al., 2005; Fowler et al., 2007; Nienaber et al., 2011). Staphylococcal toxic shock syndrome, which emerged in the late 1970's (Altemeier et al., 1981), is also associated with CC30, and the *tst* gene encoding toxic shock syndrome toxin has a strong clonal association with CC30 nasal carriage and bacteremia isolates (Holtfreter et al., 2007). MSSA that resemble the EMRSA-16 clone were also commonly associated with asymptomatic nasal carriage in the United States (Kuehnert et al., 2006), and other studies concur that CC30 is a major clonal complex associated with nasal carriage (Feil et al., 2003; Melles et al., 2004; Kuehnert et al., 2006; Fowler et al., 2007; Ko et al., 2008; Melles et al., 2008). Therefore, although Clade 3 exhibits attenuated virulence in murine infection models, we proposed that the high burden of disease associated with these hospital associated strains could be due to the high incidence of colonization, affording more opportunity to cause infection.

Several observations support the contention that Clade 3 evolved to favor enhanced colonization, at the expense of attenuated virulence. Foremost, the genome of MRSA 252 has the highest content of pseudogenes compared to other *S. aureus* genomes (Holden et al., 2004), and gene decay is a major force in niche-adaptation of microbial pathogens (Moran and Plague, 2004). Most notable among the pseudogenes was a CAG to TAG transition at Gln_{113} of *hla* encoding α-hemolysin (Hla), which is a major lethal virulence factor of CA-MRSA (Bubeck Wardenburg et al., 2007). This mutation, which creates a premature stop codon, is broadly disseminated in Clade 3, including HA-MRSA and clinical MSSA (DeLeo et al., 2011). Clade 3 strains also possessed a single nucleotide polymorphism (SNP) in *agrC* of the accessory gene regulator *agr* locus, causing a Gly_{55} >Arg change in the AgrC sensor protein, leading to attenuated transcription of the RNAIII product that is needed to produce secreted virulence factors (DeLeo et al., 2011). Consequently, attenuated transcription of *agr*, and inability to produce Hla contribute to the attenuated virulence of Clade 3.

Other defining traits of genomes that are in transition toward niche adaptation include acquisition of mobile genetic elements, and amplification of insertion sequence (IS) elements (Moran and Plague, 2004). However, a common limitation of conducting multiple genome comparisons by mapping short sequence reads from multiple strains on to a known reference genome is that it may not detect large insertions or deletions that differentiate one or more strains from the reference genome. Accordingly, although *tst* encoding toxic shock syndrome toxin is associated with different *S. aureus* pathogenicity island (SaPI) structures, and has a strong clonal association with CC30, *tst* was not present in the MRSA 252 reference genome, and we failed to identify the relevant SaPI through multiple genome comparisons. Herein, we present a detailed analysis of the hypothesis that Clade 3 strains have evolved in favor of niche adaptation, by conducting *in silico* comparisons of 15 additional CC30 genomes that are available in the public domain. Our analysis of pseudogenes, SaPI and IS elements, and gene deletion events, support the hypothesis that Clade 3 is following an evolutionary blueprint towards host- and niche-adaptation.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

A description of CC30 strains that were used in this study for analysis of secreted proteins and PCR assays is provided in **Table 1**. In addition, *S. aureus* RN4220 was obtained from Richard Novick (Novick, 1991). When needed for production of secreted proteins, cultures were grown overnight in tryptic soy broth (TSB; Difco) supplemented with 0.25% glucose, then sub-cultured into 25 ml of fresh TSB in a 125 ml Erlenmeyer flask to an initial optical density of 0.01 ($OD_{600} = 0.01$), and grown for 18 h at 37°C on an orbital shaker at 150 rpm. To assess *yefM-yoeB* addiction module function, cells were grown on brain heart infusion (BHI) agar supplemented with 10 μg/ml erythromycin for plasmid maintenance, and 5 μm cadmium where indicated for induction of the P_{cad} promoter.

GENOME COMPARISONS

Table 2 provides information on 19 CC30 strains for which genome sequence data are available in the public domain. Of these, MRSA 252 (Holden et al., 2004) was used as a reference genome for SNP analysis of multiple CC30 genomes (DeLeo et al., 2011), which in addition to MRSA 252 included three other strains listed in **Table 2**; M1015, WBG10049, and MN8. Of the genomes referred to in **Table 2**, those of TCH60 and MRSA 252 are assembled as a single nucleotide sequence, and all others are in assembly phase. Sequence coverage ranged from 10.5× (WW2703/97) to 36× (MN8). Unpublished genome data from strain UAMS-1 was provided by Dr.'s Mark Smeltzer and Jacques Schrenzel. The Basic Local Alignment Search Tool (BLAST) was used to query these genomes with segments of the annotated genome of MRSA 252 (Holden et al., 2004). Query segments were selected on the basis of SNP's, indels, or mobile genetic elements previously noted in the genome of MRSA 252, that were of discriminatory value in assigning evolutionary variants of CC30 (DeLeo et al., 2011). Genomes were also queried with the integrase (*int*) gene of known *S. aureus* pathogenicity islands (SaPI),

Table 1 | Strains used in this study.

Strain	Description	Clade[a]	Year	MLST	Spa type[b]	Source/Reference
ATCC 12598; Cowan	Pre-pandemic; septic arthritis	ND	1935	ST30	99/t076	ATCC
NRS204; Florey	Pre-pandemic	ND	1940	ST30	251/t318	Barry Kreiswirth
ATCC 25923; Seattle 1945	Pre-pandemic	ND	1945	ST243[c]	43/t021	ATCC
M809	PT80/81 pandemic	1	1961	ST30	43/t021	Robinson et al., 2005
M1015	PT80/81 pandemic	1	1962	ST30	43/t021	Robinson et al., 2005
WBG10049	CA-MRSA; Southwest Pacific Clone	2	1999	ST30	19/t019	Robinson et al., 2005
MN8	Contemporary MSSA; menstrual toxic shock	3	1980	ST30	33/t012	Schlievert and Blomster, 1983
L516	Contemporary MSSA; infective endocarditis	3	1994	ST30	33/t012	Lindsay Nicolle, University of Manitoba
L528	Contemporary MSSA; bacteremia	3	1994	ST30	33/t012	Lindsay Nicolle, University of Manitoba
UAMS-1	Contemporary MSSA; osteomyelitis	3	1995	ST30	33/t012	Gillaspy et al., 1995
PM7	HA-MRSA; EMRSA-16	3	2002	ST36[c]	16/t018	Moore and Lindsay, 2002
PM64	HA-MRSA; EMRSA-16	3	2002	ST36[c]	16/t018	Moore and Lindsay, 2002

[a] *ND, not determined.*

[b] *Provided in both Kreiswirth [Shopsin et al. (1999)] and Ridom (spa.ridom.de) nomenclature.*

[c] *Single-locus MLST variants of ST30.*

to identify contigs that contained SaPI structures. Genome segments containing SaPI's and other mobile genetic elements or genes of interest were analyzed using MacVector version 7.2.3 software (Accelerys).

PCR was employed to assess presence or absence of two unique ISSau2 insertions in different strains. One insertion adjacent to the *saeRS* regulatory locus was detected with primers SAR0758_For 5′-CAATATCGAACGCCACTTGAGC-3′, and SAR0757_Rev 5′-CAGCTATGATTGCAGGTTACCAGC-3′. Another insertion adjacent to the 5S-rRNA-3 site (**Figure 1**) was detected with primers SAR2148_For 5′-TTTCCCTCAACGTCCAGGTGC-3′ and 5S-rRNA-Rev 5′-GCCGAACACAGAAGTTAAGCTCC-3′. PCR was conducted with Roche AmpliTaq Gold DNA polymerase.

ANALYSIS OF SECRETED PROTEINS

Isolates representing different CC30 genotypes were cultured for 18 h in TSB, after which proteins in the cell-free culture supernatant were precipitated with trichloroacetic acid, and subjected to SDS-PAGE as described previously (Nickerson et al., 2010). For detection of Hla, Western blot assays were conducted with PVDF membrane (Pall Corporation), and rabbit anti-Staphylococcal α-toxin primary antibody (Sigma). The secondary antibody was donkey anti-rabbit IgG IR800 conjugate (Rockland Immunochemicals Inc.), and blots were visualized on an Odyssey Infrared Imager from LiCor Biosciences.

Identification of Coomassie-Blue stained proteins was conducted at the London Regional Proteomics Centre at the University of Western Ontario. Protein bands were excised using an Ettan^{TM} Spot Picker, and processed for mass spectrometry using a Waters MASSPrep Automated Digestor as described (Gyenis et al., 2011). Processed samples were spotted on MALDI plates and analyzed on an Applied Biosystems 4700 Proteomics Analyzer. Data were acquired and processed using 4000 Series Explorer and Data Explorer (Applied Biosystems), and the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria, using the MASCOT search engine.

CLONING AND EXPRESSION OF *yefM-YoeB* TOXIN–ANTITOXIN TA-MODULE

Gene segments were amplified by PCR, and cloned in pCN51 (Charpentier et al., 2004), for expression of the *yefM-yoeB* antitoxin-toxin genes together, or *yoeB* toxin on its own, from the cadmium inducible P_{cad} promoter. PCR was conducted using template DNA from *S. aureus* strain M1015, with primers YefM_For 5′-cgc***ggatcc***gttaactaattaaCAA*AGGAGG* GTTTATATGATTATC-3′, and YoeB_Rev 5′-tt***ggcgcgcc***TTAATC ATAATGTGACCATGCCG-3′, generating a 538 nt product containing *yefM-yoeB*. The lower case nucleotides incorporate *BamHI* (***ggatcc***) or *AscI* (***ggcgcgcc***) restriction sites, and in YefM_For also add TAA stop codons in all three open reading frames prior to the *AGGAGG* ribosome binding site of *yefM*. For *yoeB*, primers YoeB_For 5′-cgc***ggatcc***gttaactaattaa *caaaggagggtttat*ATGAGCAATTACACGGTTAAG-3′, and YoeB_Rev were used to generate a 266 nt product. The underlined lower case nucleotides in YoeB_For incorporate the ribosome binding site that precedes *yefM*, such that both constructs have identical P_{cad} promoter and translation initiation signals. PCR products were digested with *BamHI* and *AscI*, and ligated into pCN51 that had been digested with the same enzymes. The ligated plasmids were electroporated into restriction deficient *S. aureus* RN4220, and transformants were selected for growth on BHI agar containing 10 μg/ml erythromycin for maintenance of pCN51.

RESULTS

CC30 STRAINS ARE DIFFERENTIATED BY CONSERVED PSEUDOGENES

Our present analysis reveals that previously emphasized defects in *hla* and *agrC* (DeLeo et al., 2011), co-associate with lesions in *isdH* and *trpD* (**Table 2**). IsdH is a cell surface protein that is

Table 2 | Summary of CC30 strains and genotypic data.

CLADE:													ISSau2[e]				
Isolate[a]	**PRJNA**[b]	**Year**	**Country**	**Genotype**	**SCC*mec***	**PVL**[d]	***agrC***	***hla***	***isdH***	***trpG***	**TA-module**	**SaPI**	***trpS***	***rRNA-3***	***rplQ***	***sbcC***[f]	***saeRS***
CLADE 1:																	
55/2053	55909	1955	England	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
58–424	47005	1958	GA, USA	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P	P	A	A	A
E1410	55915	1962	Denmark	ST30*spa*43	MSSA	A	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
M1015[c]	47009	1962	Australia	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P	P	A	A	A
M876	55917	1961	Australia	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG							
M809	47007	1961	Australia	ST431*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P	P	A	A	A
M899	42993	1961	Australia	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
65–1322	55911	1965	WV, USA	ST30*spa*43	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
68–397	55913	1968	TX, USA	ST30	MSSA	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
C101	43001	1997	England	ST30*spa*43	MSSA	A	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P		A	A	A
CLADE 2:																	
WBG10049[c]	42991	1999	Australia	ST30*spa*19	IV	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P	P	A	A	A
TCH60	CP002110		TX, USA	ST30*spa*19	IV	P	Gly_{55}	CAG_{Gln}	CAA_{Gln}	6TG	P	SaPI1	P	P	A	A	A
CLADE 3:																	
MN8[c]	59529	1980	MN, USA	ST30*spa*33	MSSA	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI2, SaPI4	P	A	P	A	P
UAMS-1		1995	AR, USA	ST30*spa*33	MSSA	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI2, SaPI4	P	A	P	A	A
Btn1260	42987	1999	England	ST30*spa*33	MSSA	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI2, SaPI4	P	A	P	A	A
MRSA 252[c]	57839	1990's	England	ST36*spa*16	II	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI4	P	A	P	P	A
EMRSA-16	48295	1990's	England	ST36*spa*16	II	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI2, SaPI4	P	A	P	P	A
A017934/97	43393	1997	Sweden	ST30*spa*43	IV	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI2, SaPI4	P	A	P	A	A
WW2703/97	42989	1997	Germany	ST30*spa*16	IV	A	Arg_{55}	TAG*	TAA*	5TG	A	SaPI4	P	A	P	A	A

[a] *Data for year, country, MLST, spa, SCCmec and PVL derived from [Robinson et al. (2005)], with exception of TCH60, MN8, and UAMS-1.*

[b] *Public domain whole genome sequencing project nucleic acid sequence accession number.*

[c] *Included in genome comparisons conducted by SOLiD 3 sequencing and SNP analysis [DeLeo et al. (2011)].*

[d] *Defined as present (P) or absent (A), as also for toxin-antitoxin module (TA) and SaPI elements.*

[e] *ISSau2 insertion sites defined in **Figures 1** and **3**.*

[f] *Same distribution profile for ISSau2 insertions at sbcC, rRNA-1 and rRNA-2.*

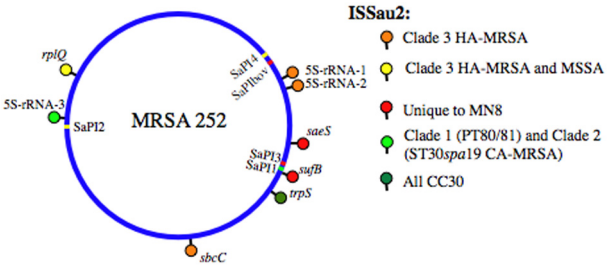

FIGURE 1 | SaPI and ISSau2 distribution in CC30. The location of each element is mapped on the reference genome of MRSA 252 [Holden et al. (2004)]. SaPIs are indicated by colored bars, on the circular blue genome, and are labeled on the interior of the circular genome. SaPI2 and SaPI4 (yellow) are unique to Clade 3. SaPI1 (light green) is present in PT80/81 (Clade 1) and ST30*spa19* CA-MRSA (Clade 2). SaPI3 and SaPIbov (red) are not in CC30, but their location is shown for reference, based on location of *attS* sites. ISSau2 insertions, indicated by colored lollipops above the circular genome, are named after the gene that is adjacent to each insertion. Three different 16S-23S-5S-rRNA loci are abbreviated as 5S-rRNA-1, 5S-rRNA-2, and 5S-rRNA-3.

highly expressed under iron-limiting conditions, and immunization with IsdH protects against *S. aureus* nasal carriage and bovine mastitis (Clarke et al., 2006; Pilpa et al., 2006; Ster et al., 2010). In EMRSA-16 and ST30*spa33* MSSA, *isdH* has a SNP that converts CAA_{Gln} into a TAA stop codon (**Table 2**). An observation that many TSST producing strains are auxotrophic for tryptophan (Kreiswirth et al., 1989; Leung et al., 1993) is accounted for by a TG deletion in a 6×TG segment of *trpD*, in the *trpEGDCFBA* locus. Therefore, *agrC*, *hla*, *isdH*, and *trpD* are defective in contemporary MSSA and EMRSA-16 comprising Clade 3, but are functional in PT80/81 and ST30*spa19* CA-MRSA comprising Clades 1 and 2 (**Table 2**). These latter strains also have PVL, which together with Type IV *SCCmec* is a trait that Clade 2 CA-MRSA shares with other unrelated CA-MRSA, such as USA300 and USA400. Other CC30 strains A01734/94 (ST30*spa43*), and WW2703/97 (ST30*spa16*) in Clade 3 are defined as CA-MRSA due to Type IV *SCCmec*, but they lack PVL, and have the "pseudogene package" (*agrC*, *hla*, *isdH*, and *trpD*) (**Table 2**), which we refer to as a niche-adapted trait.

S. aureus PATHOGENICITY ISLAND (SaPI) CONTENT

Figure 1 summarizes the distribution of insertion sequence ISSau2, and SaPI's in CC30. Irrespective of gene composition or genetic background, a SaPI is defined by a specific integrase *int* that recognizes an *attS* site (Lindsay and Holden, 2004; Novick and Subedi, 2007). Our analysis reveals that the *attS* sites are always located at the 3′-end of a gene, often in association with an operon, and these structures are illustrated in **Figure 2**. As noted previously (DeLeo et al., 2011), SaPI-4 differentiates Clade 3 from Clades 1 and 2. The *attS* of SaPI4 is at the 3′-end of the *rpsF-ssb-rpsR* operon, encoding ribosomal protein S6, a single stranded RNA binding protein, and ribosomal protein S18 (**Figure 2A**). SaPI4 does not have any known virulence factors, but SAR0385 encodes a protein with a signal peptide, identical to ORF011 of *S. aureus* phage ϕ1028 (Kwan et al., 2005). Outside of CC30, SaPI4 is restricted to ovine adapted *S. aureus* strains 011 and 046 (Le Marechal et al., 2011), which have an ortholog of SAR0385 (**Figure 2A**). In bovine adapted *S. aureus* ET3-1 (Herron-Olson et al., 2007), an ortholog of SAR0385 is on SaPIbov, where it is flanked by *tst*, *sec*, and *sel* encoding superantigen toxins (**Figure 2A**). The *attS* of SaPIbov spans the 3′- end of the *xpt-pbuX-guaB-guaA* operon, encoding genes for transport and metabolism of purine nucleotides. The *rpsR* and *guaA* genes, which contain the *attS* sites for SaPI4 and SaPIbov, respectively, are in close proximity (**Figure 1**), and segments of SaPIbov exhibit high similarity to ϕ1028, and SaPI4 (**Figure 2A**), but there are no genomes that have both SaPI's.

In CC30, the toxic shock syndrome toxin *tst* is on SaPI2, where *attS* spans the 3′-end of *groES-groEL* (**Figure 2B**). In CC30, *tst* is the only toxin on SaPI2, but in unrelated HA-MRSA strains N315 and Mu50 (Kuroda et al., 2001), SaPI2 has additional superantigen toxins *sec* and *sel* (**Figure 2B**). In another non-CC30 strain, *tst* co-associates with *sec* and *sel* in SaPI3 (Li et al., 2011), where *att*S overlaps with the 3′-end of *rnr-smpB-ssrA* (**Figure 2C**). This operon encodes a ribonuclease (*rnr*), a non-translated RNA *ssrA*, and its cognate binding protein *smpB*. In the USA400 CA-MRSA, SaPI3 has superantigen toxins *sec* and *sel*, but not *tst*. However, CC30 genomes do not have SaPI3.

The first SaPI identified in *S. aureus* was SaPI1 in strain RN4282 (Lindsay et al., 1998; Ruzin et al., 2001), which has superantigen enterotoxins *sek* and *seq* at the 5′-end, and *tst* at the 3′-end (**Figure 2D**). The genome of RN4282 was not sequenced, but the flanking *attS* sequences of SaPI1 establish the integration site at the 3′-end of *metNPQ*, encoding a putative methionine transporter. Similar SaPI1 structures are in *S. aureus* COL and USA300, although these lack *tst* (**Figure 2D**). In CC30 SaPI1 is present in Clade 1 and 2 strains (**Table 2**), but does not have any obvious virulence factors. Similar SaPI1 structures that lack known virulence factors are in *S. aureus* ED98 (Lowder et al., 2009), which has undergone a recent evolutionary transition from human to poultry host, and in bovine adapted strain LGA251 (Garcia-Alvarez et al., 2011). In summary, although *tst* has been identified on SaPI1, SaPI2, SaPI3, and SaPIbov, depending on the genetic background, our data indicate that in CC30 it exclusively resides on SaPI2, where unlike other SaPI2 structures, *tst* does not co-associate with other superantigen toxins.

ANALYSIS OF ISSau2 CONTENT

CC30 strains are distinguished by their profiles of ISSau2 (**Figure 1**), which is a member of the IS3 family (www-is.biotoul.fr/is.html). In *E. coli*, IS3 is flanked by imperfect inverted 39 nucleotide repeats with terminal 5′-TG and CA-3′ dinucleotides, and has two overlapping reading frames *orfA* and *orfB*, which when produced by default, prohibit transposition (Timmerman and Tu, 1985; Sekine et al., 1997). A –1 translational frame-shift within a poly-A tract in the region of overlap between *orfA* and *orfB* produces a single protein that catalyzes transposition (Prere et al., 1990; Sekine et al., 1994), with duplication of a three base pair target site (Sekine et al., 1994, 1997). ISSau2 has similar features (**Figure 3A**), and targets inverted repeats (**Figures 3B–G**), likely comprising *rho*-independent transcription terminators. An ancestral insertion

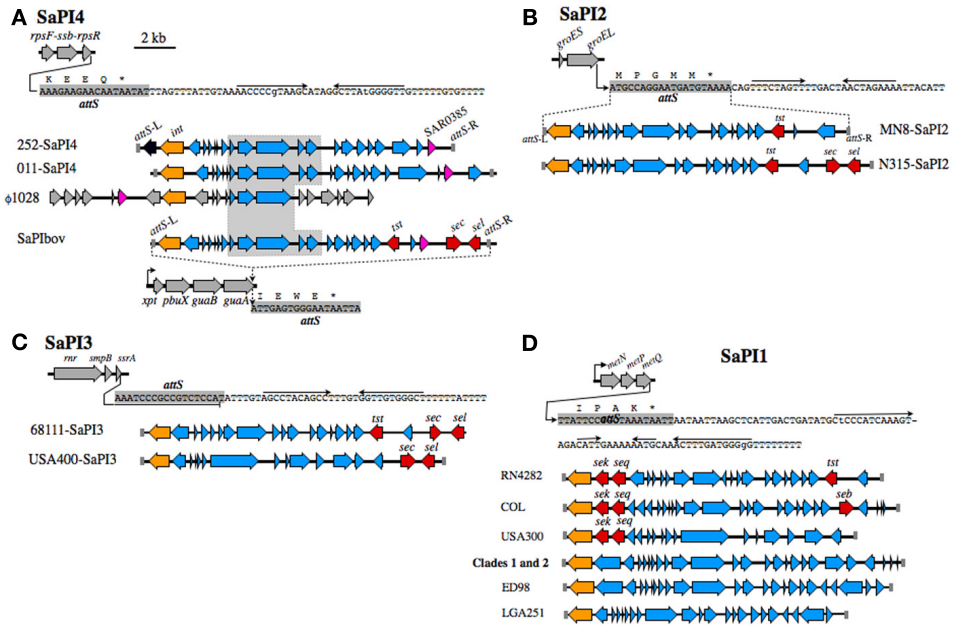

FIGURE 2 | SaPI structures and insertion sites. (A) SaPI4, with an illustration of the *rpsF-ssb-rpsR* operon, and nucleotide sequence extending from the 3′-end of *rpsR*. The *attS* of SaPI4 shaded gray, overlaps the 3′-end of the *rpsR* open reading frame, and letters above the sequence correspond to the C-terminus of RpsR protein. Arrows above the nucleotide sequence indicate inverted repeats, likely comprising a transcriptional terminator. Illustrations below the sequence compare SaPI4 of *S. aureus* MRSA 252 with that of ovine adapted strain 011. The duplicated left and right *attS* sequences are shaded gray, *int* genes are colored orange, while SAR0385 and orthologous genes, encoding a putative secreted protein, are magenta. A central outlined and shaded segment of SaPI4 is highly conserved in *S. aureus* phage ϕ1028, and SaPIbov of bovine adapted *S. aureus* ET3-1. Genes in SaPIbov encoding superantigen toxins are shaded red. The *int* of SaPIbov is shaded orange to emphasize function, although each SaPI family has a distinct *int* and *attS*. Structures of SaPI2 **(B)**, SaPI3 **(C)**, SaPI1 **(D)**, and their genomic insertion sites. Features are labeled as in **A**. SaPI2 of ST30*spa*33 strain MN8 **(B)**, is compared to SaPI2 of a non-related MRSA strain N315. SaPI3 **(C)** is not present in CC30. Structures shown are from strain USA400 [Baba et al. (2002)], which is a CA-MRSA, and strain 68111, which is a triple locus MLST variant of ST30 [Li et al. (2011)]. A clonal complex is comprised of isolates that differ from the ancestral sequence type (ST) at no more than two of seven MLST alleles. Therefore, *S. aureus* 68111 is distantly related to ST30, but is not within CC30. **(D)** shows SaPI1 structures from different strains, in comparison to SaPI1 in Clade 1 PT80/81 strains, and Clade 2 ST30*spa*19 CA-MRSA.

common to CC30 genomes occurs in the intergenic segment separating *trpS* from the *oppAFDBC* oligopeptide permease operon (**Figure 3B**). ST30*spa19* CA-MRSA and PT80/81 have one additional insertion, adjacent to a 16S-23S-5S rRNA operon (**Figures 1** and **3C**). Two other 16S-23S-5S-rRNA loci are targeted in EMRSA-16, which has a third unique insertion adjacent to *sbcDC* (**Figures 1**, and **3D,F**). A fourth insertion adjacent to *rplQ* is also present in ST30*spa33* MSSA (**Figures 1** and **3E**), including strain MN8, a prototypic menstrual toxic shock strain (Schlievert and Blomster, 1983). MN8 is distinguished from other ST30*spa33* strains by two unique insertions (**Figure 1**), one of which is adjacent to *saeRS* (**Figure 3G**), encoding a two-component sensor signal transduction system that is a major regulator of virulence.

Outside of CC30, ISSau2 is restricted to animal adapted *S. aureus*. Ovine adapted ED133 has seven copies (Guinane et al., 2010), bovine strain LGA251 has three (Garcia-Alvarez et al., 2011), and porcine adapted ST398 has one (Schijffelen et al., 2010). The unassembled genomes of ovine strains 011 and 046 also have at least one copy (Le Marechal et al., 2011). In these animal adapted strains, the integration sites for ISSau2 are mutually exclusive of those in CC30.

LOSS OF A TOXIN–ANTITOXIN ADDICTION MODULE IN CC30 EVOLUTION

Toxin-antitoxin (TA) modules encode a stable bactericidal or bacteriostatic toxin, and an unstable antitoxin that forms an inhibitory complex with the toxin. These were first termed addiction modules when discovered on plasmids, since loss of the plasmid during cell division leads to rapid degradation of the unstable antitoxin, followed by activation of the toxin and death of the daughter cells (Meinhart et al., 2003). Most free-living bacteria also have one or more genomic TA modules (Pandey and Gerdes, 2005). An example in *S. aureus* is *mazEF*, where MazF is an RNA'se that induces cell stasis by degradation of mRNA (Fu et al., 2007), and MazE is the antitoxin. Most other *S. aureus* genomes have another uncharacterized TA module, similar to *yefM-yoeB* in *E. coli*, where YoeB is a stasis-inducing RNA'se and YefM is the antitoxin (Kamada and Hanaoka, 2005). In ST30*spa19* CA-MRSA and PT80/81, *yefM-yoeB* is flanked by *frvX* encoding an M42 metallopeptidase/endoglucanase protein family member, and a predicted glutamate synthase, *gltS* (**Figure 4A**). In MRSA 252, the flanking genes *frvX* (SAR2545) and *gltS* (SAR2547) are present, but not *yefM-yoeB*, and this is also characteristic of contemporary CC30 MSSA. Other genomes with this trait are

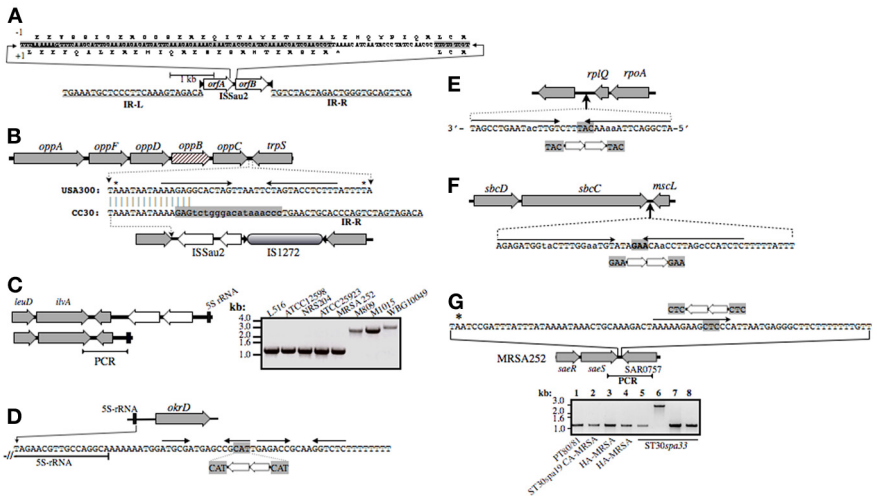

FIGURE 3 | Illustration of ISSau2 (A) and its insertion sites (B–G) in CC30. ISSau2 is comprised of *orfA* and *orfB* **(A)**, flanked by 39 nt inverted repeats IR-L and IR-R, with terminal 5′-TG and 3′-CA dinucleotides **(A)**. The sequence above the illustration spans the 3′-end of *orfA* and 5′-end of *orfB*. The +1 translation of *orfA*, terminating at a stop codon, is shown below the sequence. Above the sequence is a translation that would result from a −1 frame-shift within ***AAAAAAG***. The −1 translation from this point onwards continues throug to the end of *orfB*, and would produce a single 1569 nt trans-frame protein. **(B)** Illustration of *oppAFBDC* and *trpS* genome segment of non-CC30 strain USA300. Beneath this is shown the *oppC-trpS* intergenic sequence of USA300, aligned to that MRSA 252. Asterisks above the USA300 sequence indicate stop codons of *oppC* and *trpS*. Convergent arrows indicate inverted repeats, likely comprising a *rho*-independent transcription terminator stem-loop structure. In all CC30 strains, the left arm of the stem is disrupted by a flanking repeat of IS1272 (shaded gray), which in turn is disrupted by ISSau2. In MRSA 252, *oppB* (cross-hatched) has an in-frame internal deletion that is unique to the EMRSA-16 lineage. **(C)** ISSau2 insertion in the genome of *S. aureus* TCH60 (top), which is ST30*spa19* CA-MRSA (Clade 2), and corresponding segment of MRSA 252 (bottom). PCR with primers spanning the 3′-end of the 5S rRNA and flanking *ilvA* (right panel) reveal that this insertion is in Clade 1 strains (M809, M1015) and another Clade 2 CA-MRSA (WBG10049), but not other CC30, including strains that pre-date the Clade 1 pandemic (ATCC12598, NRS204, ATCC25923). **(D)** ISSau2 insertion adjacent to a 16S-23S-5S rRNA operon, and flanking *okrD* gene, which is unique to Clade 3 HA-MRSA. The sequence below the illustration shows the end of the 5S rRNA transcript, and adjacent *rho*-independent transcriptional terminator, comprised of tandem stem-loops followed by a poly-T segment. ISSau2 disrupts the right arm of the first stem-loop, with duplication of the CAT target site. **(E and F)** show similar disruption of a putative stem-loop structure downstream of *rplQ*, and a likely transcription terminator of the *sbcDC* operon. **(G)** Unique ISSau2 insertion in ST30*spa*33 strain MN8 disrupts stem-loop structure adjacent to *saeRS* regulator. The *saeRS* genome segment of MRSA 252 is shown for reference, and the nucleotide sequence downstream of *saeS* is shown above the illustration. In strain MN8, ISSau2 inserts into the left arm of a putative stem-loop structure, with duplication of the CTC target site. This is confirmed by PCR of a genomic segment spanning *saeS* and adjacent SAR0757, producing a 2.8 kb amplicon in MN8 (Lane 6), and a 1.2 kb product in all other strains including PT80/81 strain M1015 (Lane 1), ST30*spa19* CA-MRSA strain WBG10049 (Lane 2), ST36*spa16* HA-MRSA strains PM7 and PM64 (Lanes 3 and 4), and additional ST30*spa*33 strains UAMS-1 (Lane 5), L516 (Lane 7), and L528 (Lane 8).

restricted to ST398 porcine adapted *S. aureus*, ovine adapted strains 011 and 046, and as yet undefined strains A9635 and 21200.

Although not annotated, *yefM-yoeB* is flanked by long direct repeats LDR-1 and LDR-2 (**Figure 4B**). CC30 genomes that lack *yefM-yoeB* have a single repeat identical to LDR-1, except for two SNP's at the 3′-end that match LDR-2, suggesting that deletion occurred by recombination between LDR-1 and LDR-2. To assess the function of *yoeB*, it was cloned by itself, or paired with *yefM*, in plasmid pCN51 under transcriptional control of P_{cad}. Cells of *S. aureus* RN4220 with either plasmid grew well on BHI agar, but on induction with cadmium, cells with *yoeB* alone did not grow (**Figure 4C**), confirming its function as a toxin, likely through degradation of mRNA to induce cellular stasis.

PRODUCTION OF SECRETED PROTEINS

Clade 3 MSSA (ST30*spa*33) are recovered from a spectrum of conditions, including osteomyelitis (Cassat et al., 2005), infective endocarditis (Nienaber et al., 2011), bacteremia (Xiong et al., 2009), and menstrual toxic shock (Lin et al., 2011). Although these all have the same premature stop codon in *hla*, strains associated with menstrual toxic shock were reported to retain the ability to produce a small amount of Hla (Lin et al., 2011). Further, our data establish that MN8, which is a prototypic menstrual toxic shock strain (Altemeier et al., 1981), is differentiated from other Clade 3 MSSA by unique ISSau2 insertions (**Figures 1**,**3** and **Table 2**), suggesting that it could also have unique phenotypic traits. We, therefore, evaluated production of secreted proteins in the major CC30 clonal types, including PT80/81 (Clade 1), ST30*spa19* CA-MRSA (Clade 2), HA-MRSA (Clade 3), and Clade 3 MSSA recovered from menstrual toxic shock (MN8), osteomyelitis (UAMS-1), infective endocarditis (L516), and bacteremia (L528). Compared to other Clade 3 MSSA, MN8 exhibited more abundant production of secreted proteins (**Figure 5A**), and when compared to UAMS-1 on three separate occasions, it always produced more secreted protein (data not shown). However, irrespective of this difference, Hla was not detected in any of the ST30*spa*33 MSSA in a Western blot (**Figure 5B**).

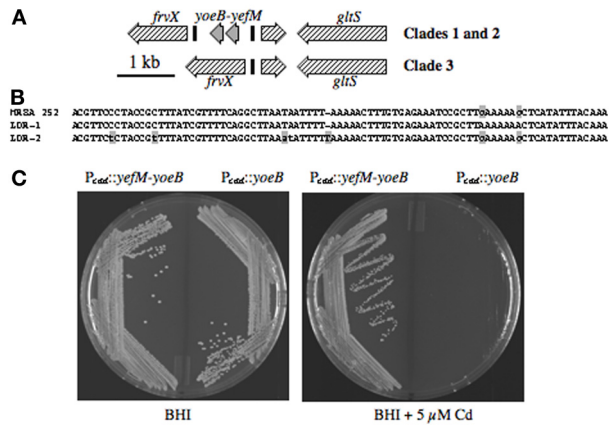

FIGURE 4 | (A) Illustration of *yefM-yoeB* and flanking DNA in CC30. *yefM-yoeB* are flanked by repeats LDR-1 and LDR-2 (black bars) in PT80/81 and ST30*spa19* CA-MRSA, whereas HA-MRSA and contemporary MSSA have only one LDR and lack *yefM-yoeB*. **(B)** Nucleotide sequence of LDR-1 and LDR-2 from strain M1015, and the single LDR of MRSA 252. The lower-case gray shaded nucleotides differentiate LDR-1 and LDR-2. The single LDR of MRSA 252 is a hybrid of LDR-1 and LDR-2, which is suggestive of a recombination event. **(C)** Growth of *S. aureus* RN4220 harboring P_{cad}::*yefM-yoeB* or P_{cad}::*yoeB*, on BHI agar, or BHI supplemented with 5 μM cadmium, to induce P_{cad}.

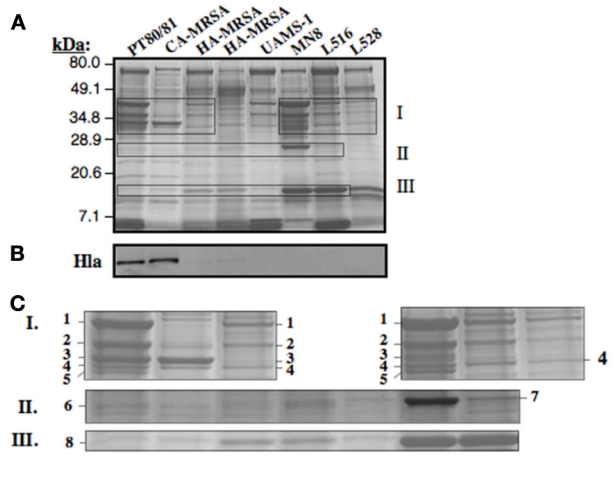

FIGURE 5 | Assessment of CC30 secreted proteins by SDS-PAGE (A) and Western blot for detection of Hla (B). The individual strains are the same as defined in **Figure 3G**. For visualization of secreted proteins by Coomassie Blue staining **(A)**, a total of 3.0 OD_{600} units of cell-free culture supernatant was applied to each lane, while 0.02 OD units was applied in **Figure 3B**. Zones I, II, and III as outlined in the SDS-PAGE gel **(A)** are enlarged in **Figure 3C**. The numbered protein bands were excised from the gel, followed by trypsin digestion and mass spectrometry. The identity of proteins in each band is provided in **Table 3**.

Proteins from zones I, II, and III on the SDS-PAGE (**Figure 5A**) were selected for trypsin digestion and mass spectrometry (**Figure 5C** and **Table 3**). HA-MRSA and ST30*spa*33 MSSA secreted toxic shock syndrome toxin TSST (Zone II, band 7), encoded by *tst* on SaPI2, and except for UAMS-1, they also secreted the SAR0385 gene product encoded on SaPI4 (Zone III, band 8). PT80/81 and CA-MRSA produced Hla (Zone I, band 3 and some carry-over in band 4), but Hla was not identified in the co-migrating protein bands from strain MN8. The PT80/81 strain was unique in abundant production of the LukF component of PVL (Zone I, band 2). All strains produced mature glycerol ester hydrolase/lipase (Zone I, band 1), γ-hemolysin components HglC and HglB (band 2 and carry-over in 3), and glycerolphosphoryl diester phosphodiesterase (band 4). With the exception of CA-MRSA, band 4 also contained the HglA component of γ-hemolysin. Therefore, although our data support the contention that Clade 3 MSSA associated with Staphylococcal toxic shock syndrome may be more virulent due to elevated production of secreted proteins, including γ-hemolysin, we found no evidence to support their production of Hla.

DISCUSSION

Gene gain, gene loss, and gene change are major forces in bacterial genome dynamics (Moran and Plague, 2004; Pallen and Wren, 2007), and we have evaluated these processes in *S. aureus* clonal complex CC30. It was previously established that MRSA 252, representing the EMRSA-16 lineage, has the highest content of pseudogenes compared to other *S. aureus* genomes (Holden et al., 2004; Lindsay and Holden, 2004). A bovine adapted strain ET3-1 was a close second, and several of the accumulated pseudogenes would eliminate production of a number of cell surface proteins and iron acquisition pathways (Herron-Olson et al., 2007). Importantly, the most robust examples of gene decay come from recently emerged pathogens that have changed lifestyle, usually to live in a simpler host-associated niche (Moran and Plague, 2004). As summarized in **Table 2**, defining traits of CC30 Clade 3 include (1), a premature stop codon in *hla* encoding α-Hemolysin; (2), a SNP that causes an Gly_{55}>Arg substitution in the AgrC membrane sensor protein, leading to attenuated transcription of the regulatory RNAIII that is needed to produce secreted virulence factors; (3), *isdH* and *trpD* pseudogenes; (4), acquisition of SaPI4, and in most isolates also SaPI2, which possesses the *tst* gene encoding toxic shock syndrome toxin, and (5), an increase in copy number of ISSau2 relative to Clades 1 and 2. Our analyses have revealed several features that were not previously identified through comparative genome sequencing (DeLeo et al., 2011). These include the presence of SaPII and a functional TA genomic addiction module in Clades 1 and 2, the identification of SaPI2 in Clade 3, and the occurrence of unique ISSau2 insertions in each of the three major Clades. Cumulatively, these findings are concordant with niche adaptation in CC30 Clade 3.

It has long been known that 80% of TSST producing *S. aureus* strains are auxotrophic for tryptophan (Chu et al., 1985; Leung et al., 1993), which we now attribute to a TG deletion in *trpD*. It is believed that amino acid auxotrophy contributes to niche adaptation, because auxotrophic bacteria are restricted to a niche where the appropriate amino acid can be obtained. *Lactococcus lactis* recovered from dairy products are auxotrophic for histidine due to frame-shift mutations in *hisC*, *hisG*, and *hisH*, while strains from non-dairy sources are prototrophic (Delorme et al., 1993). *S. aureus* ET3-1, which is a predominant clonal type associated with bovine mastitis, also has a frame-shift mutation in *hisC*

Table 3 | Identity of proteins excised from SDS-PAGE (Figures. 5A and C)

Zone	Band	Strain	Protein	Accession	C.I.
I	1	PT80/81	lipase precursor	gi|49482552	100
		HA-MRSA	lipase precursor	gi|49482552	100
		MN8	lipase precursor	gi|49482552	100
	2	PT80/81	gamma hemolysin HglC component	gi|4948637	99.99
			gamma hemolysin HglB component	gi|477912	99.19
			LukF-PV	gi|9635192	97.83
		CA-MRSA	gamma hemolysin HglC component	gi|4948637	99.99
			gamma hemolysin HglB component	gi|477912	99.94
		HA-MRSA	gamma hemolysin HglC component	gi|4948637	100
			autolysin protein	gi|32968086	100
			gamma hemolysin HglB component	gi|477912	99.96
		MN8	gamma hemolysin HglC component	gi|4948637	100
			gamma hemolysin HglB component	gi|477912	99.98
			autolysin protein	gi|32968086	83.16
	3	PT80/81	alpha hemolysin precursor	gi|15924153	100
		CA-MRSA	alpha hemolysin precursor	gi|15924153	100
		MN8	gamma hemolysin HglC component	gi|49484636	100
			lipase precursor	gi|49482552	76.21
	4	PT80/81	glycerolphosphoryl diester phosphodiesterase	gi|49483119	100
			gamma hemolysin HglA component	gi|49484635	99.88
		CA-MRSA	glycerolphosphoryl diester phosphodiesterase	gi|49483119	99.99
			alpha hemolysin precursor	gi|15924153	99.99
			nucleotidase lipoprotein	gi|49482539	65.61
		MN8	gamma hemolysin HglA component	gi|49484635	100
			glycerolphosphoryl diester phosphodiesterase	gi|49483119	86.31
		L516	glycerolphosphoryl diester phosphodiesterase	gi|49483119	100
			gamma hemolysin HglA component	gi|49484635	100
		L528	glycerolphosphoryl diester phosphodiesterase	gi|49483119	100
			gamma hemolysin chain II (HglA) component	gi|15925409	97.77
	5	PT80/81	lipase precursor	gi|49482552	100
		MN8	lipase precursor	gi|49482552	100
II	6	PT80/81	Staphopain cysteine protease	gi|3891901	96.7
			alkyl hydroperoxidase subunit C	gi|15923371	29.78
		CA-MRSA	alkyl hydroperoxidase subunit C	gi|15923371	91.75
		HA-MRSA	alkyl hydroperoxidase subunit C	gi|15923371	99.8
	7	HA-MRSA	toxic shock syndrome toxin-1	gi|18535666	99.95
		HA-MRSA	toxic shock syndrome toxin-1	gi|18535666	100
		UAMS-1	toxic shock syndrome toxin-1	gi|18535666	100
		MN8	toxic shock syndrome toxin-1	gi|18535666	100
		L516	toxic shock syndrome toxin-1	gi|18535666	100
III	8	PT80/81	hypothetical protein SAR0622	gi|49482843	99.93
		CA-MRSA	hypothetical protein SAR0622	gi|49482843	99.89
		HA-MRSA	hypothetical protein SAR0385	gi|49482618	98.66
		UAMS-1	hypothetical protein SAR0622	gi|49482843	100
		MN8	hypothetical protein SAR0385	gi|49482618	98.66

(SAB2553), which does not occur in other *S. aureus* genomes. The *trpD* gene, which has a TG insertion in CC30 Clade 3, is part of the *trpEGDCFBA* transcriptional unit, of which *trpBA* encode the subunits of tryptophan synthase, and *trpEGDCF*, encode enzymes necessary for synthesis of indole precursor. *Chlamydia trachomatis* uniformly lack the genes needed to produce indole, but strains that cause ocular vs. genital infections can be differentiated on the basis of the latter being able to produce a functional tryptophan synthase, and it is postulated that tryptophan can be produced by condensation of serine with exogenous indole produced by microflora in the female genital tract (Fehlner-Gardiner et al., 2002; McClarty et al., 2007). Consequently, tryptophan auxotrophy may contribute to tropism of TSST producing CC30 strains for the vaginal mucosa.

Another important factor in evolution of niche adapted strains is an increase in copy number of IS elements, leading to genome deletions and inversions through recombination between adjacent IS elements. An interesting example relevant to our analysis

is a reduction in the numbers of operons encoding 16S-23S rRNA in microbial endosymbionts of insect cells (Andersson and Andersson, 1999; Itoh et al., 2002). This was attributed to IS integration within operons encoding 16S-23S rRNA, followed by recombination to generate deletions (Dale et al., 2003). It is, therefore, striking that all three 16S-23S-5S rRNA loci in the CC30 genome are targeted by ISSau2, with an insertion at rRNA-3 being unique to Clade 1, while insertions at rRNA-1 and rRNA-2 are unique to HA-MRSA in Clade 3 (**Figure 1** and **Table 2**). Our analysis suggests that the insertions adjacent to rRNA operons is due to the propensity of ISSau2 to target inverted repeats, which likely comprise *rho*-independent transcription terminators. ISSau2 is a member of the IS30 family, and two unusual members of the IS30 family in *Mycoplasma fermentans* and *M. bovis*, which are obligate intracellular parasites, also target *rho*-independent transcription terminators, which remain intact and are partially duplicated on transposition (Calcutt et al., 1999; Lysnyansky et al., 2009). Conversely, our data suggest that ISSau2 either disrupts or weakens stem loop structures, as illustrated in **Figures 3D–G**.

Depending on the orientation of ISSau2 with respect to the adjacent gene, this could have important consequences with respect to control of transposition. It is widely accepted that transposition must be maintained at a low level, a commonly cited reason being that excessive transposition is detrimental to the stability of the host genome (Doolittle et al., 1984). Therefore, endogenous transposase promoters are generally weak, and often partially located in the inverted flanking repeats, such that strong promoters can only be created by juxtaposition of inverted repeats due to formation of head-to tail dimers, or circular copies of the IS as noted for the IS30 family (Dalrymple, 1987). IS elements also have mechanisms to attenuate their activation by impinging transcription, following insertion into active host genes. Impinging transcription across the inverted flanking repeats can either sequester translation initiation signals, or disrupt complex formation between the transposase and inverted repeats. These considerations may help to explain the insertion of ISSau2 adjacent to highly transcribed genes, including all three rRNA operons in the CC30 genome, and adjacent to *rplQ* encoding the 50S ribosomal subunit protein L17 (**Figures 1** and **3**). In these situations, ISSau2 is oriented in the antisense orientation with respect to the adjacent gene, such that impinging transcription would also generate antisense RNA to the transposase genes.

In an example that is unique to HA-MRSA in Clade 3 (**Figure 1** and **Table 2**), ISSau2 is inserted in the sense orientation adjacent to *sbcDC* (**Figure 3F**), disrupting the predicted transcriptional terminator. The *sbcDC* genes encode a protein complex that recognizes and cleaves hairpin structures in DNA, has a major role in promoting genome stability and repair of breaks in double stranded DNA, and is induced by the SOS stress response in *S. aureus* (Connelly et al., 1998; Mascarenhas et al., 2006; Chen et al., 2007; Eykelenboom et al., 2008; Darmon et al., 2010). It is noteworthy in this respect that loss of DNA recombinational repair occurs in the initial stages of genome degeneration, as bacteria undergo a transition from an autonomous free-living state to permanent intracellular existence (Dale et al., 2003). This leads to active expansion of IS elements, which in turn promotes deletion or inversion of genome segments *via* IS-mediated recombination. Therefore, based on established evolutionary trends, the ISSau2 insertion adjacent to *sbcDC* may represent an early stage in the pathway toward genome destabilization.

Whether ISSau2 influences expression of adjacent genes is unknown. However, an insertion that is unique to strain MN8, from a case of menstrual Staphylococcal toxic shock, is adjacent to the *saeRS* two-component sensory signal transducer. SaeRS is a major regulator of virulence in *S. aureus* (Geiger et al., 2008; Voyich et al., 2009; Nygaard et al., 2010), and although there is as yet no evidence that ISSau2 influences expression of *saeRS*, we find that relative to ST30*spa33* strains that lack this insertion, MN8 exhibits more abundant production of secreted proteins, including the HglA, HglB, and HglC components of γ-hemolysin, as well as TSST and SAR0385 gene product encoded on SaPI2 and SaPI4, respectively. This is consistent with a recent finding that, with the exception of strain MN8, other ST30*spa33* strains and EMRSA-16 exhibited strongly attenuated transcription of the RNAIII effector component of the *agr* global regulator, due to a common SNP in *agrC* (DeLeo et al., 2011). Although this SNP is also present in strain MN8, transcription of RNAIII was not influenced to the same extent as other strains, suggesting that there was a compensatory mechanism in this strain. Additional work is warranted to determine if this is related to the ISSau2 insertion adjacent to *saeRS*.

The absence of the *yefM-yoeB* TA module (*toxM*) in Clade 3 is also consistent with established evolutionary pathways toward niche adaptation. Most free-living bacteria have multiple genomic TA loci, which are thought to help cope with nutritional stress by inducing a reversible state of dormancy during periods of nutrient depletion (Pedersen et al., 2002; Gerdes et al., 2005), although this has been disputed in *E. coli* (Tsilibaris et al., 2007). However, obligate intracellular pathogens and symbionts experience a less variable environment, and do not have TA modules (Pandey and Gerdes, 2005). Only a few free-living bacteria lack TA modules, the most notable being *Lactococcus lactis*, which is niche-adapted in its association with dairy products. Intriguingly, outside of CC30, the only other *S. aureus* genomes that lack this TA module thus far are restricted to ST398 porcine adapted *S. aureus*, ovine adapted strains 011 and 046, and as yet undefined strains A9635 and 21200. Although we cannot exclude the possibility that this TA module represents a gene acquisition in Clades 1 and 2, rather than a gene deletion in Clade 3, the broad distribution of this element in other *S. aureus* genomes supports the contention that this element is a component of the core genome that is lost in evolutionary development of some strains. However, more work is needed to confirm this hypothesis.

In conclusion, our findings support the notion that Clade 3 is following an evolutionary blueprint toward niche-adaptation, while Clade 2 strains consisting of ST30*spa19* CA-MRSA retain the feral nature of the historic PT80/81 Clade 1. It is important to note that CA-MRSA are defined by Type IV SCC*mec*, which in CC30 is also associated with ST30*spa*43 and ST30*spa*16 strains in Clade 3 (**Table 2**). Therefore, strains of CA-MRSA, which are typically associated with hyper-virulence, are emerging with the niche-adapted trait. Given that CA-MRSA must evolve from MSSA through acquisition of Type IV SCC*mec*, this suggests that the niche-adapted trait is widely disseminated in the human

population, and this is supported by several key observations. First, MSSA that resemble the EMRSA-16 clone of HA-MRSA (Clade 3) were the most common clonal type associated with asymptomatic nasal carriage in the United States (Kuehnert et al., 2006), and several other studies concur that CC30 is a major clonal complex associated with nasal carriage (Feil et al., 2003; Melles et al., 2004, 2008; Kuehnert et al., 2006; Fowler et al., 2007; Ko et al., 2008). Second, *S. aureus* infections are usually caused by the same strain that is associated with nasal carriage, and in our analysis of a panel of 172 CC30 clinical isolates, the occurrence of the *hla* pseudogene, the *agrC* SNP, and *tst* encoded by SaPI2, which are key markers of Clade 3 (**Table 2**), was 70.9%, 72.1%, and 75.6%, respectively, (DeLeo et al., 2011). Third, in a study that assessed nasal carriage isolates from 107 healthy blood donors, 27% were CC30, and 62% of these CC30 strains possessed *tst* (Holtfreter et al., 2007), which is a marker of Clade 3. Moreover, in the same study, *tst* was present in 90% of CC30 bacteremia isolates. In this context, although our studies indicate that the niche adapted trait is associated with attenuated virulence in murine infection models (Holtfreter et al., 2007), a benefit to Clade 3 in having premature stop codons in Hla and IsdH (**Table 2**) is that both proteins are considered as potential vaccine antigens (Clarke et al., 2006; Wardenburg and Schneewind, 2008; Kennedy et al., 2010; Ster et al., 2010), and Clade 3 strains would be immune to this vaccine strategy.

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Staphylococcus aureus temperate bacteriophage: carriage and horizontal gene transfer is lineage associated

Alex J. McCarthy[1]*, Adam A. Witney[2] and Jodi. A. Lindsay[1]

[1] Division of Clinical Sciences, Centre for Infection, St George's University of London, London, UK
[2] Bacterial Microarray Group, Division of Clinical Sciences, St George's University of London, London, UK

Edited by:
Martin John McGavin, University of Western Ontario, Canada

Reviewed by:
Indranil Biswas, University of Kansas, USA
Jose R. Penades, ConsejoSuperior de Investigaciones Científicas, Spain

***Correspondence:**
Alex J. McCarthy, Division of Clinical Sciences, Centre for Infection, St George's University of London, Cranmer Terrace, London SW17 0RE, UK.
e-mail: amccarth@sgul.ac.uk

Staphylococcus aureus is a major cause of human and animal infections. Bacteriophage are a class of mobile genetic element (MGE) that carry virulence genes and disseminate them horizontally, including Panton–Valentine leukocidin (PVL), the immune evasion cluster (IEC) associated with human specificity, and enterotoxin A the major toxin associated with food poisoning. *S. aureus* isolates group into major clonal complex (CC) lineages that largely evolve independently due to possession of different restriction–modification (RM) systems. We aimed to better understand the horizontal and vertical transmission dynamics of virulence and resistance genes by bacteriophage by using (i) bioinformatic approaches to analyze bacteriophage genomes from the first 79 sequenced *S. aureus* isolates and (ii) *S. aureus* microarrays to analyze the distribution of bacteriophage and virulence genes in *S. aureus* isolates from a broader range of lineages. The distribution of eight bacteriophage families was highly variable but lineage associated. Nevertheless, there was evidence of frequent acquisition and loss and not just vertical transmission. Most bacteriophage genes were dispensable, and extensive mosaicism was seen. Surprisingly, virulence genes were tightly associated with specific phage families. This data suggests *S. aureus* bacteriophage evolve rapidly, and the horizontal gene transfer (HGT) of virulence genes encoded by bacteriophage is restricted by bacteriophage family and the lineage of the host bacterium, delaying the evolution of fully resistant and virulent strains.

Keywords: *Staphylococcus aureus*, MRSA, bacteriophage, mobile genetic element, evolution, horizontal gene transfer, transmission, PVL

INTRODUCTION

Staphylococcus aureus is a frequent colonizer of humans and mammals and a prevalent cause of skin and hospital associated infections. Methicillin resistant *S. aureus* (MRSA) is an opportunistic and highly adaptive pathogen that is a major cause of hospital acquired (HA) infections and is emerging to be a common cause of community acquired (CA) and livestock acquired (LA) infections. The emergence of MRSA is a major global concern because resistance to every antibiotic currently available is reported, and therefore the number of antibiotics that are effective in control and treatment of infections is limited (Gould, 2005; Chambers and DeLeo, 2009; Jensen and Lyon, 2009). Of further concern is the emergence of fully vancomycin resistant *S. aureus* (VRSA), reducing our options to prevent and treat severe MRSA infections (Weigel et al., 2003).

The adaptation and evolution of these *S. aureus* is largely due to the acquisition of mobile genetic elements (MGEs) that carry virulence and resistance genes. Such MGEs include bacteriophage, staphylococcal cassette chromosomes (SCCs), plasmids, *S. aureus* pathogenicity islands (SaPIs), and transposons that can move between bacteria by horizontal gene transfer (HGT) mechanisms (Lindsay, 2010; Malachowa and DeLeo, 2010). MRSA clones have emerged through independent acquisitions of SCC*mec* elements harboring *mecA* genes by different *S. aureus* lineages in different countries, whilst fully VRSA have emerged though acquisition of transposons harboring the *vanA* gene (Deurenberg and Stobberingh, 2008). The immune evasion cluster (IEC) locus encodes human-specific immune evasion proteins (Chp, Sak, and Scn) that contribute to adaptation to the human host and are carried on bacteriophage integrated into the chromosome (known as a prophage; van Wamel et al., 2006). The key toxins Panton–Valentine leukotoxin (PVL, encoded by *lukFS-PV* genes) and Staphylococcal enterotoxin A (*sea*) are also found on an integrated prophage (Betley and Mekalanos, 1985; Kaneko et al., 1998). The dissemination of MGEs through *S. aureus* populations is concerning as they can alter the pathogenicity, resistance, and host range of bacteria generating strains that cause novel clinical challenges.

Horizontal transfer of DNA occurs in bacteria by conjugation, transduction, and/or transformation. In *S. aureus* transformation does not occur efficiently, and conjugative plasmids and transposons do not have a wide distribution. Bacteriophage transduction is therefore likely to be the most important HGT mechanism for *S. aureus*. Some bacteriophage can be hitchhiked by SaPIs for transfer (Lindsay et al., 1998), whilst other bacteriophage called generalized transducing bacteriophage, are able to package host bacterial DNA and deliver it to other bacteria. Bacteriophage therefore increase the genome plasticity of *S. aureus*, and are thought to facilitate the adaptation of the pathogen to different conditions during infection (Goerke et al., 2006a,b, 2009). *S. aureus* bacteriophage belonging to the *Siphoviridae* family, can

be classified into families (φ1–φ8) based on the integrase gene (*int*) sequence they possess and the integration site (Lindsay and Holden, 2004).

The major barriers of HGT in bacteria are restriction–modification (RM) systems. *S. aureus* possess two RM systems; a type III RM system that prevents uptake of DNA originating from different bacterial species (Corvaglia et al., 2010) and a type I RM system that prevents uptake of DNA originating from other species but also between *S. aureus* lineages (Waldron and Lindsay, 2006). Therefore, each *S. aureus* lineage has a unique type I RM system that causes lineages to evolve independently, as well as possessing a unique but highly conserved combination of surface genes, secreted genes, and their regulators (Witney et al., 2005; McCarthy and Lindsay, 2010). Other barriers of HGT mechanisms have been described, but are less well characterized in *S. aureus* (Dempsey et al., 2005; Thomas and Nielsen, 2005).

Our overall aim was to investigate the carriage of and genome variation of bacteriophage in order to better understand the horizontal and vertical transmission dynamics in *S. aureus*. In this study we (i) characterize carriage of prophage amongst the first 79 sequenced *S. aureus* genomes and investigate the amount of variation in prophage genomes (ii) investigate the evolutionary relationship of bacteriophage originating from different *S. aureus* lineage backgrounds, and (iii) extend previous microarray analysis to investigate lineage distributions of bacteriophage and virulence genes in 254 S. aureus isolates.

MATERIALS AND METHODS

BACTERIOPHAGE CLASSIFICATION

Bacteriophage are classified into families based on their integration (*int*) gene which also defines the site of prophage integration in the *S. aureus* chromosome (Lindsay and Holden, 2006). Eight different families are prevalent in *S. aureus* and their key features are described in **Table 1**, including alternative nomenclatures. Standard nomenclature includes the phage family followed by the bacterial host name; for example, φ3 (MRSA252) is the φ3 bacteriophage originating from the sequenced MRSA252 genome (Lindsay and Holden, 2006).

STAPHYLOCOCCUS AUREUS GENOMES

Sequence data is currently available for the genomes of 74 *Staphylococcus aureus* isolates on the GenBank database[1]. In addition, genome sequences from an another four *S. isolates* (EMRSA15, LGA251, BB155, MSHR 1132) were made available prior to publication from Matt Holden at The Wellcome Trust Sanger centre, UK[2]. The name and lineage distribution of bacteriophage in all genomes is shown in **Table 2**. The genomes represent 20 different lineages/clonal complexes (CCs), and 26 different sequence types (STs). Only 28 genomes have been fully sequenced, closed and annotated, whilst the remainder are found in contigs.

ISOLATION OF BACTERIOPHAGE GENOMES FROM *S. AUREUS* GENOMES

Carriage of bacteriophage in sequenced genomes was identified using a BLAST search of each bacteriophage integrase (*int*) gene and the flanking regions of insertion sites (**Table 1**). The Artemis Comparison Tool (ACT) was used to compare all full-length *S. aureus* genome sequences against the MRSA252 sequence in order to identify φ2 and φ3 prophage genomes and their coding domain sequences (CDS; Carver et al., 2005). For *S. aureus* genomes that were not completed, contigs that matched φ2 or φ3 bacteriophage were detected by BLAST analysis of the full φ2 (MRSA252) prophage sequence and φ3 (MRSA252) prophage sequence against each genome respectively. Matching contigs were then aligned against the MRSA252 genome in ACT and all CDS in φ2 or φ3 genome were identified. It should be noted that some bacteriophage sequences mapped across more than one contig and therefore such bacteriophage genomes may not be complete. Genomes where full-length φ2 or φ3 sequence mapped over more than three contigs were not been included in analysis. In total, 31 φ2 prophage (10 of which mapped across two contigs) and 48 φ3 (17 of which mapped across two contigs) prophage sequences were isolated from *S. aureus* genome sequences for comparative analysis. All ORFs from each prophage sequence were isolated.

PHYLOGENETIC ANALYSIS

Alignments of all φ2 and all φ3 prophage genomes were generated separately. Firstly, we generated separate pools of φ2 genes and φ3 genes using a strategy we have previously reported for microarray design (Witney et al., 2005). Briefly, one prophage genome was chosen as a reference and all of its genes were deposited in a gene pool. A second prophage genome was then compared against the gene pool and all genes that have a relatively poor BLAST hit score against previous genes were added

[1]www.ncbi.nlm.nih.gov

[2]www.sanger.ac.uk/pathogens

Table 1 | Classification of *S. aureus* bacteriophage.

Bacteriophage family	Representative *int* gene accession number	Insertion site of family in MRSA252 genome	Virulence genes reported in this bacteriophage family
Φ1	SAV0847	SAR0880	*lukFM, eta*
Φ2	SAR1562	SAR1563	*lukFS-PV*
Φ3	SAR2105	SAR2031	*chips, sak, scn, sea, seg, sek, sep*
Φ4	SAS0891	SAR0991	
Φ5	NWMN_1814	SAR1967	
Φ6	SACOL0318	SAR0317	
Φ7	NWMN_0992	SAR1102	*sak*
Φ8	SAB1760	SAR1992	

Table 2 | Distribution of prophage and virulence genes in sequenced *S. aureus* genomes.

Lineage		Strain	Bacteriophage family							
CC	ST		φ1	φ2	φ3	φ4	φ5	φ6	φ7	φ8
1	1	MSSA476*			S, SK, A, G, K					
	1	MW2*		P.R	S, SK, A, G, K					
	1	ATCC 51811/FRI569								
	1	TCH70		P.R	S, SK, K					
5	5	A5937			C, S, SK, P					
	5	A6224			C, S, SK, P					
	5	A9763			C, S, SK, P					
	5	A10102			C, S, SK, P					
	225	04-02981*			C, S, SK, P					
	5	N315*			C, S, SK, P					
	5	CF-Marseille			C, S, SK					
	105	A8796			C, S, SK					
	5	CGS03			C, S, SK					
	105	JH1*			C, S, SK					
	105	JH9*			C, S, SK					
	5	A9719			C, S, SK					
	5	A9781			C, S, SK					
	5	A8115			C, S, SK					
	5	ECT-R2*			C, S, SK					
	5	MR1			C, S, SK, A					
	5	A9299			S, SK, P					
	5	ED98*	E							
	5	Mu3*			S, SK					
	5	Mu50*			S, SK, A					
	5	A6300								
	5	A8117								
7	7	USA300 TCH959*			S, SK, P					
8	8	A9754		P.R	C, S, SK, A					
	8	USA300 TCH1516*		P.R	C, S, SK, A					
	8	930918-3			C, S, SK, A					
	8	A9765			C, S, SK, A					
	8	Newman*			C, S, SK, A					
	8	USA300 FPR3757*		P.R	C, S, SK					
	8	MRSA177		P.R	C, S, SK					
	8	CGS01		P.R	C, S, SK					
	923	MRSA131		P.R	C, S, SK					
	8	NCTC 8325*			C, S, SK					
	8	A5948			C, S, SK					
	8	D30								
	250	COL*								
10	10	H19			C, S, SK, A					
	145	D139			S, SK					
22	22	EMRSA15/5096*			C, S, SK					
30	30	55/2053		P.H	C, S, SK					
	30	65-1322		P.H	C, S, SK					
	30	68-397		P.H	C, S, SK					
	30	M1015		P.H	C, S, SK					
	30	C101		P.H	C, S, SK					
	30	58-424		P.H	C, S, SK, A					

(Continued)

Table 2 | Continued

Lineage		Strain	Bacteriophage family							
CC	ST		φ1	φ2	φ3	φ4	φ5	φ6	φ7	φ8
	30	M876		P.H	C, S, SK, A					
	30	M899		P.H	C, S, SK, A					
	36	MRSA252*			C, S, SK, A					
	36	EMRSA16			C, S, SK, A					
	30	MN8			C, S, SK, A					
	30	A017934/97			S, SK					
	30	WW2703/97			C, S, SK					
	34	C160			C, S, SK					
	30	E1410			C, S, SK					
	30	TCH60*		P.H	C, S, SK					
	30	WBG10049		P.H						
	30	Btn1260								
	30	CGS00								
42	42	C427			S, SK					
45	45	A9635			C, S, SK					
72	72	TCH130								
75	75	MSHR 1132*			S, SK, A					
93	93	JKD6159*		P.R	C, S, SK					
133	133	ED133*					L		Ao	
151	151	RF122/ET3-1*						L		
152	152	BB155*		P.R	S, SK					
239	239	0582/TW20*			S, SK, A					
	239	JKD6008*			S, SK					
	239	JKD6009			S, SK					
	239	ATCC BAA39			S, SK					
398	398	S0385*								
425	425	LGA251*								
431	431	M809		P.H	S, SK					
1173	1173	O11								
	1173	O46								

**Genomes have been completed with full annotation. Bacteriophage presence (gray) and absence (white) is shown. The distribution of characterized virulence genes is shown; P.R, Panton Valentine leukocidin (lukSF-PV) R variant; P.H, Panton Valentine leukocidin (lukSF-PV) H variant; C, chemotaxis inhibitory protein (chp); S, staphylococcal complement inhibitor (scn); SK, staphylokinase (sak); A, enterotoxin A (sea); G, enterotoxin G (seg); K, enterotoxin K (sek); P, enterotoxin P (sep); E, ear; L, leukocidin FM (lukFM); Ao, enterotoxin A ovine variant.*

to the gene pool. The process was repeated for all prophage genomes of a family. We next used BLAST analysis to generate a binary code matrix that reported presence and absence of each unique gene in each prophage genome for φ2 and φ3 bacteriophage families separately. Finally, phylogenetic analysis was performed using MrBayes v3.1.1 (Huelsenbeck and Ronquist, 2001). Analyses were run for 30,000,000 iterations (sampled every 1000 iterations) and convergence of Monte-Carlo Markov chain (MCMC) was assessed by the SD between chains falling below 0.05. The first 25% of sampled trees were discarded so that parameter estimates were only drawn from data generated after chain convergence. Phylogenetic trees were drawn and edited in FigTree v1.3.1[3].

[3]http://tree.bio.ed.ac.uk/software/figtree

MICROARRAY ANALYSIS

A total of 254 human and animal *S. aureus* isolates of UK origin were analyzed using the seven-strain *S. aureus* microarray (SAM-7; Witney et al., 2005). One hundred and sixty-one human carriage and invasive isolates have been previously described and represent the major dominant lineages of *S. aureus* from the Oxford UK community in 1999 (Feil et al., 2003; Lindsay et al., 2006), and an additional 37 isolates represent MRSA from hospitals in London, UK (Edgeworth et al., 2007). The 56 animal isolates have previously been described and originate from cows ($n = 37$), horses ($n = 13$), sheep ($n = 2$), goats ($n = 2$), and a camel ($n = 1$) in the UK (Sung et al., 2008).

The microarray assays were performed as previously described (Lindsay et al., 2006). Briefly, DNA was extracted using genomic-tip 100/G columns (Qiagen) or Bacterial Genomic Prep (Edge Biosystems), and 4 μg of test strain was labeled with Cy3 dye using

DNA polymerase I large fragment (Klenow; Invitrogen), and 4 μg of reference (MRSA252) DNA was labeled using Cy5 dye. The two samples were pooled and hybridized to a *S. aureus* microarray overnight, prior to washing and scanning (Lindsay et al., 2006). Affymetrix 428 scanner was used to scan the microarrays and images were converted to raw data by BlueFuse for Microarrays 2.0 (BlueGnome, Cambridge, UK). All data analysis was performed in GeneSpring 7.0 (Agilent). Raw data from the previous experiments (Lindsay et al., 2006; Edgeworth et al., 2007; Sung et al., 2008) were normalized as a single experiment in GeneSpring using locally weighted scatterplot smoothing (LOWESS). The data we use here is deposited in BμG@Sbase (accession number: E-BUGS-62 and E-BUGS-34) and also ArrayExpress (accession number: E-BUGS-62 and E-BUGS-34). Condition tree clustering using the Spearman correlation was performed as a function in GeneSpring, according to Lindsay et al. (2006).

RESULTS

DISTRIBUTION OF PROPHAGE FAMILIES IN SEQUENCED *S. AUREUS* GENOMES

All eight bacteriophage *int* genes were identified in at least one sequenced *S. aureus* genome (**Table 2**). The frequency of carriage of bacteriophage families in 74 sequenced genomes varied considerably; φ1 (24% carriage), φ2 (53%), φ3 (82%), φ4 (6%), φ5 (22%), φ6 (18%), φ7 (13%), and φ8 (3%).

There was variation in the number of bacteriophage carried in a single isolate; most isolates carried between one and three prophage, but one isolate did not contain a prophage (TCH130) whilst others carried up to four (A6224, A8796, CGS03, CF-Marseille, JH1, JH9, and Newman). There was no association between number of bacteriophage carried and *S. aureus* lineage. No more than one copy of each prophage family was found in each genome sequence.

Certain bacteriophage families were prevalent in particular *S. aureus* lineages, for example φ1 bacteriophage were prevalent in CC5 (15/22 isolates) genomes, whilst φ2 bacteriophage were more common amongst CC1 (3/4 isolates), CC8 (8/12 isolates), and CC30 (14/19 isolates) lineages.

CLUSTERING OF BACTERIOPHAGE POPULATIONS

We assessed the relationship of 31 φ2 prophage and 48 φ3 prophage using Bayesian phylogenetic analyses of 221 and unique 324 genes respectively. Clusters of related prophage appear in both the φ2 and φ3 phylogenies, and Bayesian posterior probabilities (BPP) were consistently high (BPP $\geq$ 0.75; **Figures 1A** and **2A**), meaning that the phylogeny of the tree and relationships of prophage are well supported. We note that we use the phylogenies to make conclusions on the relationship between prophage, and not on the temporal evolution of bacteriophage.

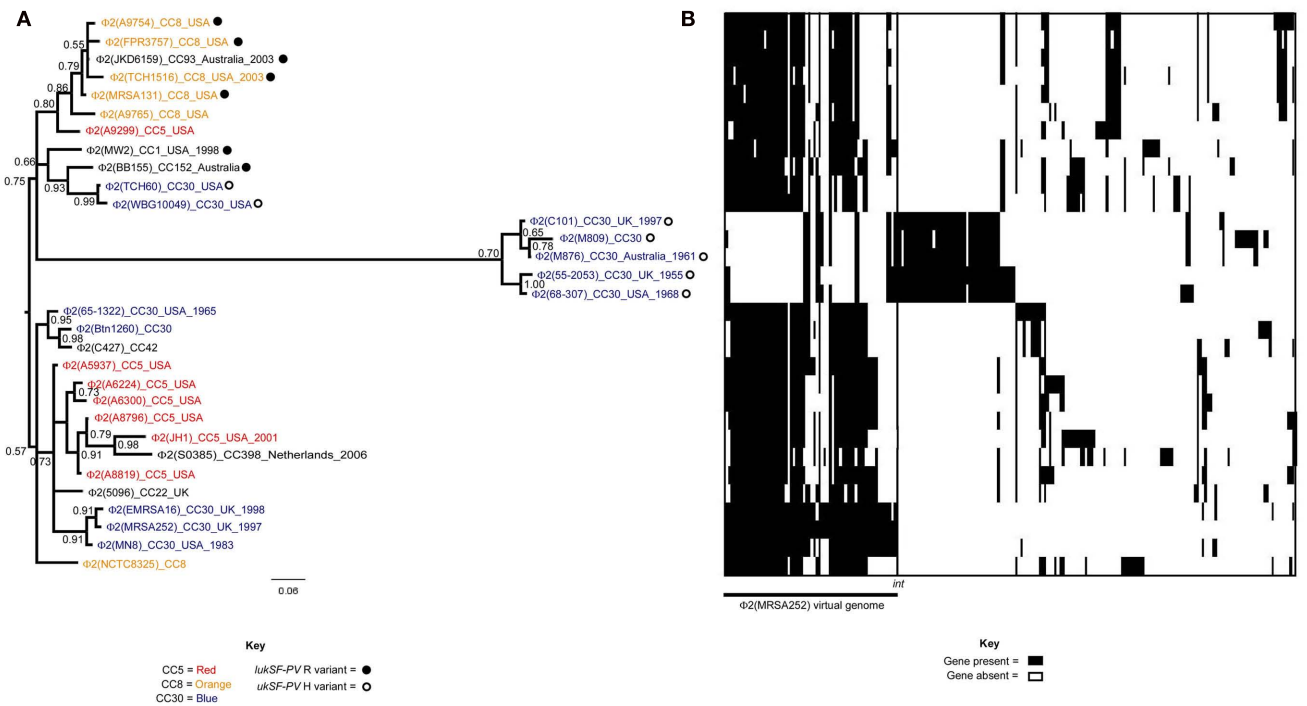

FIGURE 1 | Evolutionary relationships of φ2 *S. aureus* prophage. (A) Phylogenetic analysis of 31 φ2 prophage is shown. Each prophage is named by the family it belongs to φ2, and then the name of the sequenced *S. aureus* genome it originates from (in parentheses). Additional information is giving on the clonal complex (CC) of the *S. aureus* isolate, as well as the country of origin and year of isolation if data was available. Branches are colored by CC lineage if more than one prophage originates from the same CC. The distribution of the *lukSF-PV* R and H variants is additionally mapped onto the phylogeny. **(B)** Presence/absence of 221 φ2 genes is shown for each of the φ2 prophage. Each horizontal line represents the genome of the adjacent φ2 prophage in the phylogeny. Each vertical line represents a φ2 gene. The color in the middle depicts if the gene is present (black) or absent (white). The genes are organized into virtual genomes, and the first genome shown in φ2 (MRSA252).

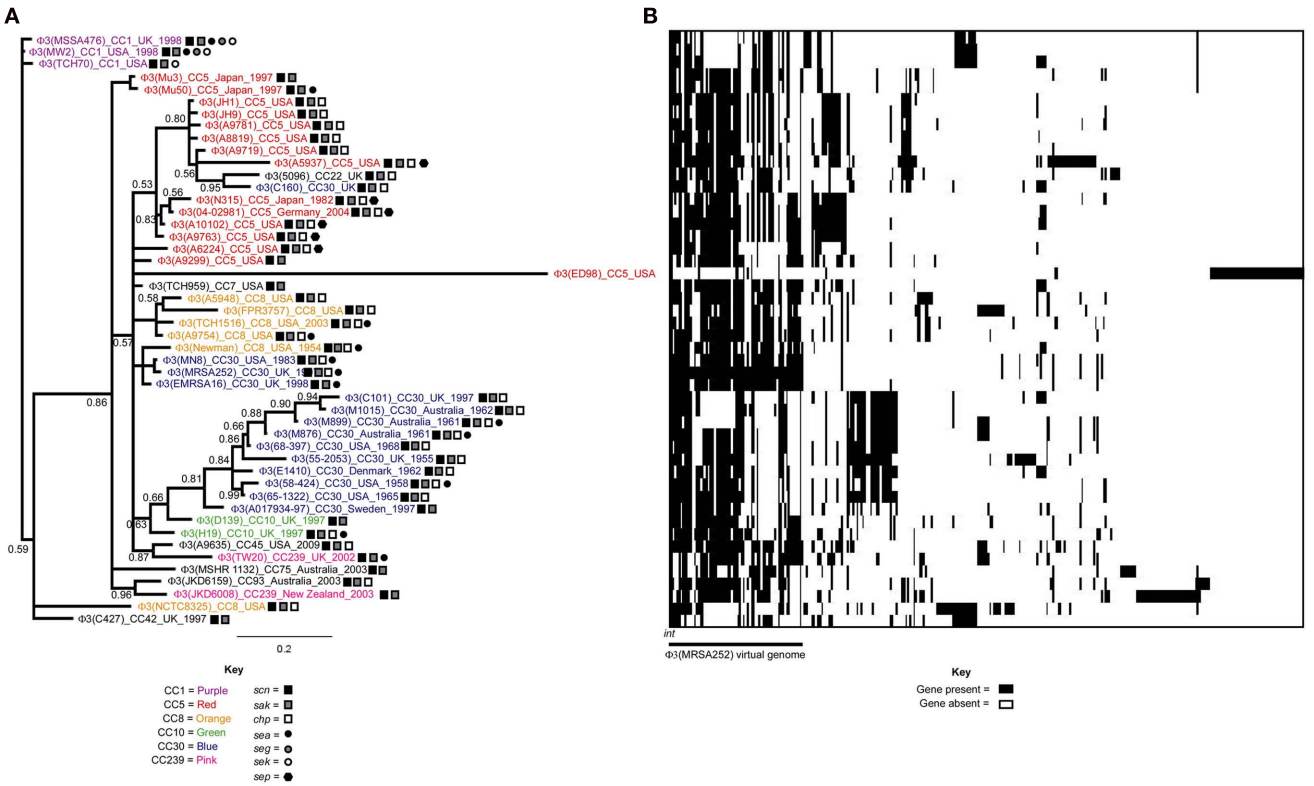

FIGURE 2 | Evolutionary relationships of φ3 *S. aureus* prophage. (A) Phylogenetic analysis of 48 φ2 prophage is shown. Each prophage is named by the family it belongs to φ3, and then the name of the sequenced *S. aureus* genome it originates from (in parentheses). Additional information is giving on the clonal complex (CC) of the *S. aureus* isolate, as well as the country of origin and year of isolation if data was available. Branches are colored by CC lineage if more than one prophage originates from the same CC. The distribution of the virulence genes *chp*, *sak*, *scn*, *sea*, *seg*, *sek*, and *sep* is additionally mapped onto the phylogeny. **(B)** Presence/absence of 324 φ3 genes is shown for each of the φ3 prophage. Each horizontal line represents the genome of the adjacent φ3 prophage in the phylogeny. Each vertical line represents a φ3 gene. The color in the middle depicts if the gene is present (black) or absent (white). The genes are organized into virtual genomes, and the first genome shown in φ3 (MRSA252).

For both the φ2 and φ3 phylogenies prophage generally cluster according to lineage. For example, φ2 prophage from CC5 isolates cluster together (BPP = 0.94), as do φ2 prophage from CC8 isolates (BPP = 1.00; **Figure 1A**). This could be explained by vertical transmission, where the prophage are stable in the chromosome, pass to daughter cells on replication, and slowly evolve with the host genome.

However, there is also evidence of acquisition and loss of prophage. Firstly, the absence of prophage in some isolates of the same lineage suggests loss (**Table 2**). Secondly, some lineages have isolates carrying prophage from separate clusters of bacteriophage; such as the three separate clusters of φ2 found in CC5 (BPPs = 0.80, 0.83, and 0.86; **Figure 2A**). These clusters are typically composed of bacteriophage originating from *S. aureus* isolates in the same geographic region and/or time period. For example, the three distinct clusters of φ3 prophage found in CC5 isolates are from (i) Japan (BPP = 0.86) (ii) USA (BPP = 0.80) and (iii) USA/Germany/Japan (BPP = 0.83; **Figure 2A**). This could be explained by vertical transmission or horizontal transmission with exchange occurring between isolates in temporal and geographical contact. However, an interesting case are CC5 isolates N315 and Mu50, isolated from the same hospital at the same time, and carrying unique φ3 prophage, again arguing for HGT. Thirdly, we found cases where prophage from different *S. aureus* lineages clustered together; arguing that HGT of bacteriophage between lineages does occur but is infrequent. One such example is the close relationship of φ2 (JKD6159) from CC93 with φ2 bacteriophage originating from the CC8 lineage (**Figure 1A**). Likewise, φ3 (5096) of MRSA CC22 is closely related to φ3 (C160) of CC30; both *S. aureus* isolates originate from the UK (**Figure 2A**).

DIVERSIFICATION OF BACTERIOPHAGE GENOMES

The distribution of known virulence genes in prophage originating from sequenced genomes is shown (**Table 2**). In all cases, virulence factors were found strongly associated with only one family of prophage. Notably, PVL was found only on φ2 prophage, while IEC (*chp*, *sak*, and *scn*) and enterotoxin genes were only found on φ3 prophage. There are few or no virulence genes on φ1, φ4, φ5, φ6, φ7, or φ8 prophage, possibly because the identity and function of putative secreted proteins are yet to be characterized.

There was a good distribution between the clustering of prophage and the presence of virulence genes (**Figures 1A** and **2A**). The PVL genes *lukSF-PV* were present in 14 of the 31

φ2 prophage included in our phylogenetic analysis, and these prophage clustered into two well-supported clades (BPP = 0.70 and 0.75; **Figure 1A**). This suggests that PVL-carrying φ2 bacteriophage from CA-MRSA are more closely related to each other than to other φ2 bacteriophage. Two sequence variants of PVL have been previously described, the H variant and the R variant (O'Hara et al., 2008); however the PVL-carrying φ2 prophage did not cluster into these separate clusters (**Figure 1A**). This suggests exchange of *lukSF-PV* variants between prophage clusters. There was also a good association between the clustering of the φ3 bacteriophage and the distribution of virulence factor genes (**Figure 1B**). For example, 4/5 bacteriophage with the virulence gene profile *scn*, *sak*, *chp* and *sep* clustered together (BPP = 0.83). However, there was also evidence that closely related bacteriophage had differing combinations of virulence genes, such as the absence of *lukSF-PV* in φ2 (A9299) and φ2 (A9765), and the acquisition of the staphylococcal enterotoxin A (*sea*) gene by φ3 (Mu50) in comparison to φ3 (Mu3) (**Figures 1A** and **2A**). This suggests that virulence genes can be frequently transferred horizontally between bacteriophage, as well as lost.

The distribution of φ2 genes and φ3 genes amongst φ2 and φ3 bacteriophage are shown in **Figures 1B** and **2B**, respectively. Closely related prophage possessed similar combinations of genes. For example, the φ3 prophage from CC1 isolates formed a single clade and their genomes were highly homologous (**Figure 2B**). In contrast, different clades were more extensively diversified and often differed by the presence/absence of single genes and/or runs of adjacent genes. For example, the two clades of PVL-carrying φ2 prophage possessed different combinations of genes (**Figure 1B**). This data supports the finding that (i) bacteriophage have mosaic genomes, and (ii) that bacteriophage diversification is driven by recombination that causes frequent shuffling of genes.

If virulence genes are strongly associated with specific families this suggests that recombination or exchange of genes does not occur frequently between bacteriophage from different families. We assessed the distribution of φ2 genes amongst φ3 genomes, and vice-versa, and found that 139 genes of a total of 545 genes were common to both the φ2 gene pool and φ3 gene pool (Data not shown). This suggests that genes are exchanged between different bacteriophage families, but that this may be less frequent than exchange within families.

BACTERIOPHAGE DYNAMICS IN LOCALIZED POPULATIONS

The sequenced strains represent selected isolates from around the world, typically virulent and/or multi-drug resistant, and are not representative of locally evolving *S. aureus* populations. We therefore looked for variation in bacteriophage carriage and genomic diversity in a population of 161 carriage and invasive *S. aureus* isolates from the same region of Oxford and the same time period. We used the seven-strain whole genome sequence microarray which included the genes for five phage families. Overall, the carriage of bacteriophage genes in this population showed similar dynamics to the sequenced strain collection. As we extended this study to include hospital MRSA and animal *S. aureus*, the same dynamics were also evident.

The distribution of bacteriophage *int* genes was highly variable amongst *S. aureus* isolates suggesting that bacteriophage loss and movement is frequent (**Figure 3**). Carriage of some bacteriophage families were more frequent in some CC lineages than others. φ1 were associated with lineages CC51 and CC151. φ3 were associated with multiple lineages (CC5, CC6, CC8, CC12, CC22, CC25, CC30, CC45, CC97, and CC239). φ4/6 were associated with lineages CC151, CC239, and CC771. φ5 were associated with the CC771 lineage. Interestingly, φ2 were not associated to any lineage, but were frequent amongst CC30 isolates. φ7 and φ8 were not represented on SAM-7 as they do not appear in the first seven sequenced *S. aureus* genomes. In a few instances bacteriophage carriage was completely associated to lineage; for example φ3 bacteriophage is carried in all CC239 and CC25 isolates (**Figure 1**).

The virulence genes of the IEC (*chp*, *sak*, and *scn*) appeared to be carried by φ3 bacteriophage. φ3 can carry different IEC types, or different combinations of *chp*, *sak*, and *scn*. For example, CC30 isolates carry *chp*, *sak*, and *scn*, whilst CC239 isolates carry *sak* and *scn* but not *chp* (**Figure 1**). This further suggests that HGT of bacteriophage is infrequent between *S. aureus* lineages, as we would expect to see multiple IEC profiles within a lineage if HGT between lineages was frequent.

Overall, the data supported the findings that (i) prophage are highly variable, (ii) particular prophage families are associated with particular lineages, (iii) there is evidence of vertical as well as frequent horizontal transfer of phage between *S. aureus* (iv) HGT occurs within lineages at higher frequency than between lineages, (v) frequent recombination or mosaicism occurs within prophage families, (vi) virulence genes are strongly associated with particular phage families and therefore to particular *S. aureus* lineages.

DISCUSSION

Staphylococcus aureus prophage carry important genes that impact on the ability to colonize the human nose, and to cause disease. It is interesting to consider why the bacterium has evolved to carry these genes on a MGE and not to systematically incorporate them into the stable regions of the chromosome. Presumably there is some cost to the bacterium to keep these elements, yet they are widely distributed and appear to move frequently. Prophage induction and HGT is triggered by stress including antibiotic exposure and oxygen radicals, both expected to be seen by *S. aureus* during infection (Maiques et al., 2006). It is therefore feasible that prophage contribute to the flexibility of the *S. aureus* genome, allowing isolates to rapidly adapt to new niches and environmental threats. Indeed, there are multiple reports of bacteriophage moving into and out of *S. aureus* isolates during colonization or infection (Moore and Lindsay, 2001; Goerke et al., 2004, 2006a,b; McCarthy et al., 2012), and prophage can be induced from clinical *S. aureus* isolates indicating that integrated bacteriophage remain functional (Goerke et al., 2004; Wirtz et al., 2009).

A prophage that is highly prevalent in a *S. aureus* population must be transmitted vertically, or must move frequently and be selected for. φ3 is the major prophage in human *S. aureus* isolates (Sung et al., 2008), and is selected for in human hosts as it encodes human-specific immune evasion proteins Chp, Sak, and Scn (van Wamel et al., 2006). This study shows that φ3 can be lost from the *S. aureus* genome, but has also been acquired on multiple occasions in the sequenced *S. aureus* isolates. Interestingly, φ3 has rearranged its genome frequently, can dispose of nearly all

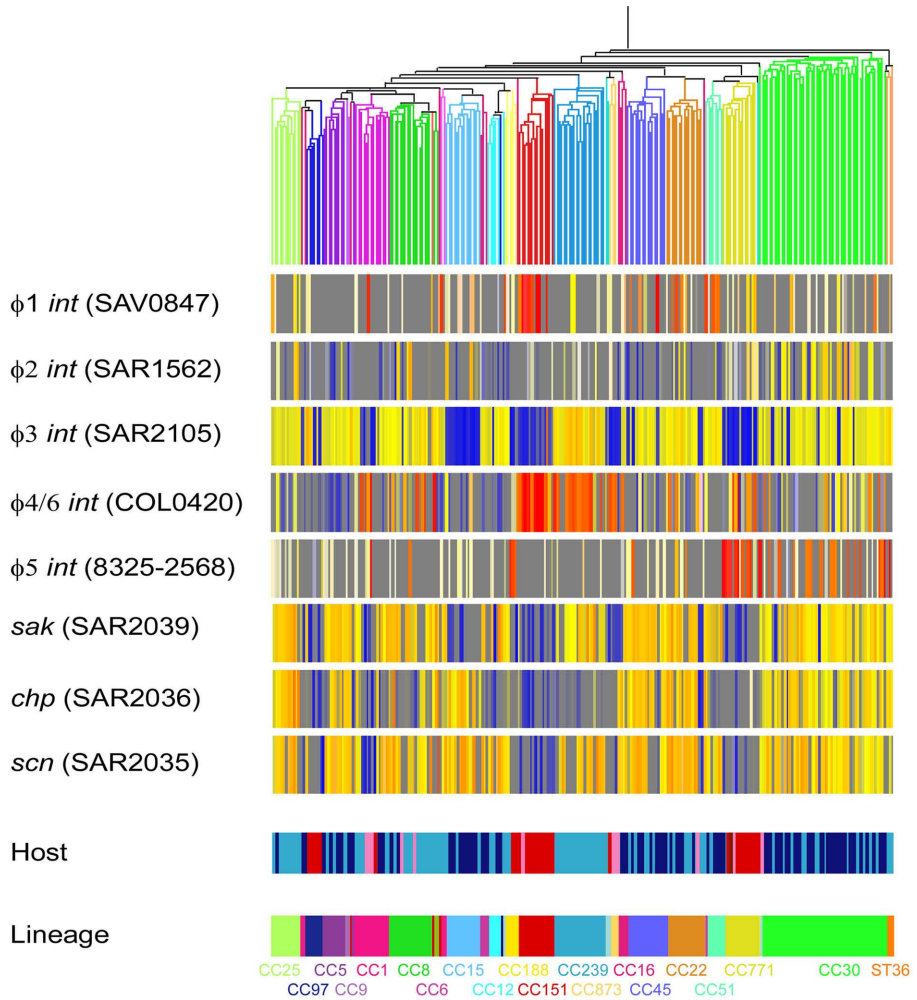

FIGURE 3 | Microarray data from 254 *S. aureus* (198 human isolates and 56 animal isolates). Isolates are clustered by Spearman correlation using 728 CV gene, as reported in Lindsay et al. (2006). Isolates of *S. aureus* are represented by vertical lines, and further information about the origin of each isolate is shown at the bottom of the figure (Host and lineage). Human isolates are colored light blue (invasive) and dark blue (carriage). Animal isolates are colored red (cow), pink (horse), brown (sheep), and light pink (camel). DNA probes that represent bacteriophage *int* genes and immune evasion cluster (IEC) genes are represented by horizontal lines. The color in the middle depicts presence of absence of the gene in an isolate; gene presence is shown as red/yellow and gene absence is shown as blue/gray.

of its genes, but continues to favor the carriage of at least two of the IEC genes. Our data therefore argues for both vertical and frequent horizontal transmission of this bacteriophage. Horizontal transmission of MGEs is limited by the *SauI* RM system and it is anticipated that different *S. aureus* lineages carry bacteriophage at different frequencies (Waldron and Lindsay, 2006). The data presented here supports this conclusion.

Other prophage (φ4, φ5, φ6, φ7, and φ8) were less prevalent amongst both the sequenced *S. aureus* isolates and the isolates included in our microarray analyses. This perhaps suggests their genes are not as important for survival in the major environmental reservoirs, such as host species. Yet, they transfer at high frequency and are maintained in diverse populations, suggesting efficient transfer mechanisms and selection under certain niche environments. Identification of such niche–environment virulence genes could provide novel therapeutic targets to limit and control *S. aureus* populations. It is interesting to consider that PVL, carried only on φ2 prophage, is found at very high frequency in CA-MRSA populations (Vandenesch et al., 2003). This could argue that PVL φ2 prophage are transferred vertically. However, our data suggests substantial variation in φ2 prophage encoding this toxin, suggesting it is transferred and is selected for in CA-MRSA disease causing isolates. The missing link is the data regarding PVL prevalence in CA-MRSA colonizing isolates; if prevalent in colonizing isolates it may play a role in colonization, and if not, it may play an important role in invasive disease. More data from Asian CA-MRSA prophage will also be valuable in determining the role of this toxin.

The strong association of particular virulence factors with particular bacteriophage families is difficult to explain. Our data supports previous studies suggesting that *Staphylococcal* bacteriophage genomes are highly flexible, mosaic, and exchange DNA (Kwan et al., 2005; Goerke et al., 2009; Belcaid et al., 2010). Our

data suggests exchange of genes occurs more often within than between bacteriophage families. Since most strains carry multiple prophage but only one copy from each family, it might be assumed that DNA was exchanged across bacteriophage family boundaries at higher frequency but that does not appear to be the case. It is interesting to question how this genome mosaicism has been generated. It has previously been proposed to be the result of frequent random recombination events followed by selection of the fittest bacteriophage (Hendrix, 2002). Virulence genes may additionally be associated to bacteriophage families if (i) these proteins play a role in the bacteriophage cycle, or (ii) the carriage of a virulence gene compensates the negative affect that a bacteriophage may have on expression of chromosomally encoded virulence genes, for example φ3 bacteriophage integrated into the β-hemolysin (*hlb*) gene.

Because virulence genes are confined to bacteriophage families, and the distribution of bacteriophage appear to be somewhat restrained by lineage, particular virulence genes are associated with particular lineages. This may delay the spread of virulence genes on bacteriophage from one CC lineage to all members of the *S. aureus* population. However, the evolution of new types of MRSA in recent years reminds us that particular lineages are gradually accumulating a wider range of MGEs, and individual isolates can exchange these MGEs within the lineage population. Understanding how this evolution is constrained and recognizing evolutionary leaps can enable the rapid response to emerging clones that will be required to control multi-resistant and virulent isolates in the future.

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