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

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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,

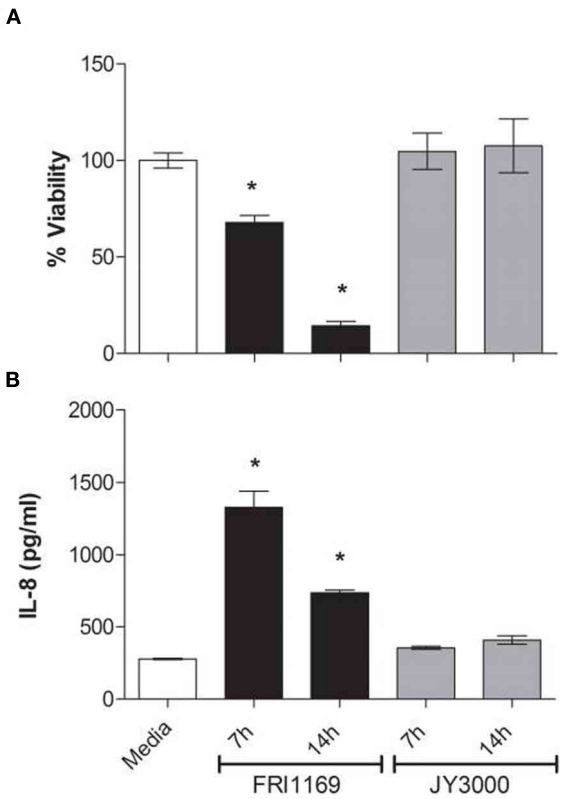

FIGURE 1 | *In vitro* comparison of wildtype FRI1169 and variant JY3000 cytotoxicity. A549 cell cytotoxicity **(A)** and IL-8 response **(B)** after 4 h exposure to *S. aureus* broth culture supernates of FRI1169 (wildtype cytolytic) and JY3000 (biofilm-derived variant, non-cytolytic) grown for 7 or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

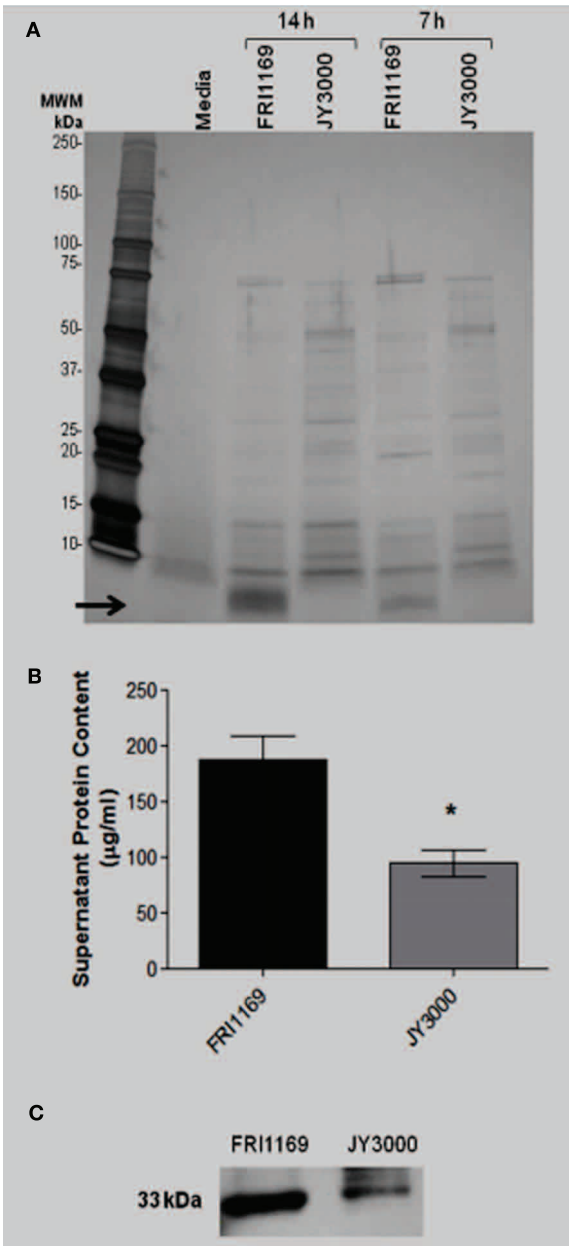

FIGURE 2 | Characterization of exoprotein production by wildtype FRI1169 and variant JY3000. (A) 14 and 7 h broth culture supernates (20 μg of protein loaded per lane) were separated by SDS-PAGE and silver-stained. Media denotes lane electrophoresed with Todd Hewitt media alone. Arrow denotes small molecular 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**).

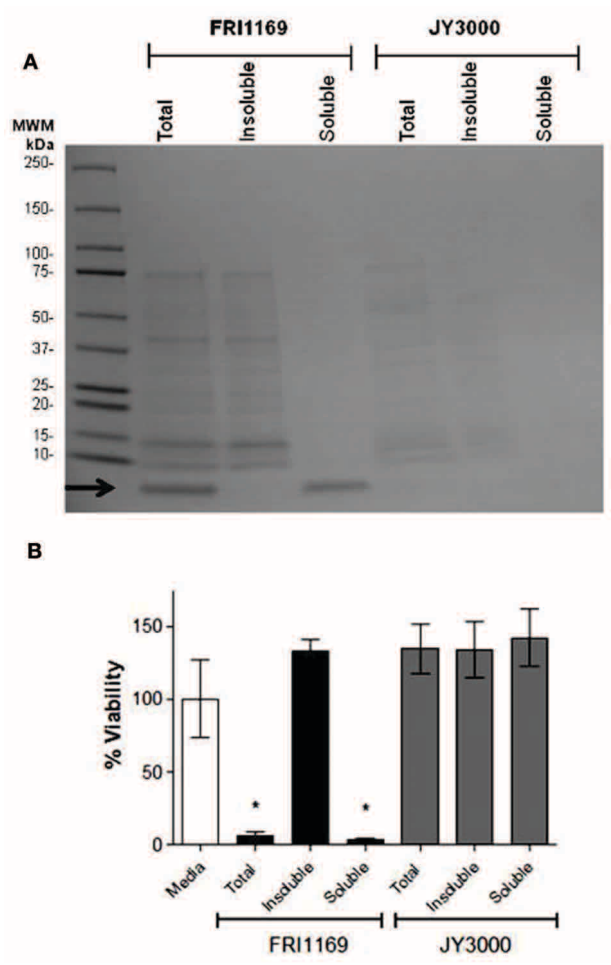

FIGURE 3 | Ethanol (80%) separation of exoprotein and determination of cytotoxicity. (A) 14 and 7 h broth culture supernatant fluids (20 μg of protein was loaded per lane) were treated with 80% (final concentration) ethanol and total, ethanol-insoluble, and ethanol-soluble fractions were separated by 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

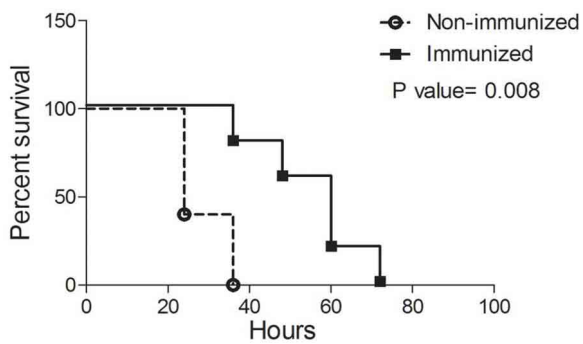

FIGURE 4 | Effect of immunization against PSMs on lethal sepsis/infectious endocarditis. Rabbits ($n = 4$ or 5 per group) were either immunized against a cocktail of PSMα-1, PSMα-4, and PSMγ/δ-toxin (squares) or left unimmunized (circles). Aortic valves were then mechanically damaged, and rabbits were 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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