ANTI-VIRULENCE PARADIGM FOR DEVELOPMENT OF STAPHYLOCOCCAL THERAPIES

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Ying-Chi Lin

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Marnie L. Peterson, Adviser

February, 2010

Acknowledgement

The following individuals are gratefully acknowledged for their support during this research project:

First and foremost, I would like to express heartfelt thanks to my adviser, Dr. Marnie Peterson, for her encouragement, guidance, and support throughout my graduate study. I am honored to be her first graduate student.

Dr. Patrick Schlievert, who essentially served as my "co-adviser". His intellect and enthusiasm for science is an inspiration. It has been a privilege to work with him and his lab.

My thesis committee members, Drs. Rory Remmel (final defense), William Elmquist (oral exam), and Timothy Tracy. Thanks for their intellectual input, perspective, and time.

My laboratory colleagues. In particular, Dr. Michele Anderson, who has been very knowledgeable and supportive. Her joining the lab was a blessing to my graduate study. In addition, Matt Schaefers, "the man of the lab", for taking care of the "hard work" and coordinating "happy hours".

The Antibiotic Pharmacodynamic Research Institute, especially its coordinator, Laurie Hovde. It has been nice to have a good neighbor who kindly shared equipments and lent supplies.

The faculty, staff, and fellow students in the ECP department, who have joined to make a diverse yet cooperative big "family". I feel lucky to have been a part of this group.

Last but not least, my family and my fiancé, Wei-Cheng, for their patience and support through these many years.

Abstract

Staphylococcus aureus is a major human pathogen capable of causing various diseases, from skin infections to life-threatening pneumonia and toxic shock syndrome (TSS). *S. aureus* exoproteins contribute significantly to *S. aureus* pathogenesis via causing inflammation, tissue disruption, and immune evasion. Antibiotics treat *S. aureus* disease by eliminating bacteria, but provide no protection from *S. aureus* exoproteins, once released. With the emergence of antibiotic-resistant *S. aureus,* new therapeutic options to treat/prevent *S. aureus*-associated disease are critical. Given most *S. aureus* diseases initiate locally on mucosal surfaces or the skin, it was hypothesized that *S. aureus* exoproteins that have pro-inflammatory and/or cytotoxic effects on epithelial cells, contributing directly to *S. aureus* pathogenesis. Therefore, anti-staphylococcal therapies that inhibit toxin production and/or prevent toxin effects on host cells could reduce or prevent *S. aureus* infections.

A global approach was taken to characterize pro-inflammatory properties of exoproteins from two genetically close TSS *S. aureus* isolates, a pulmonary TSS isolate (MNPE) and a menstrual TSS isolate (CDC587), on epithelial cells. Cytolysins, α- and γ-toxins, superantigens (SAgs), and staphopain (protease) were determined as the most pro-inflammatory (via interleukin-8 [IL-8]) to epithelial cells. MNPE, originating from skin, produced large amounts of α-toxin and SAgs, but little other virulence factors, whereas CDC587, a mucosal strain, produced γ-toxin, small amounts of α-toxin, and large numbers of secreted virulence factors. These findings implied that pro-inflammatory *S. aureus* exoproteins play key roles in environmental selection and

disease severity.

As proof of principle, glycerol monolaurate (GML), a lauric acid monoester known to inhibit *S. aureus* exoprotein production and to have anti-inflammatory effects, was compared with its monoether, dodecylglycerol (DDG), as local anti-virulence agents to prevent *S. aureus* disease using a rabbit wiffleball abscess/TSS model. GML, but not DDG, significantly decreased TSST-1 and local inflammation (via tumor necrosis factor-α [TNF-α]) in the wiffleball and prevented rabbit death from TSS.

In summary, these studies identified key exoproteins important in *S. aureus* mucosal pathogenesis and determined the potential for anti-toxin agents, such as GML, to treat and/or prevent *S. aureus* diseases. These studies also suggest the addition of anti-virulence (toxin) components will improve the effectiveness of antistaphylococcal vaccines and immunotherapies.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Accessory gene regulator (Agr)

Acquired immune deficiency syndrome (AIDS)

Antigen presenting cells (APCs)

Autoinducing peptide (AIP)

Autolysis related locus (Arl)

Capsular polysaccharides (CP)

Center for Disease Control and Prevention (CDC)

Chemotaxis inhibitory protein of *S. aureus* (CHIPS)

Clumping factor (Clf)

Colony-forming units (CFU)

Community-associated methicillin-resistant *S. aureus* (CA-MRSA)

1-O-*rac*-Dodecylglycerol (DDG)

Enzyme-linked immunosorbent assay (ELISA)

Exfoliative toxins (ETs)

Extracellular adherence protein (Eap)

Fibronectin-binding proteins (FnBP)

Food and Drug Administration (FDA)

Glycerol monolaurate (GML)

Human vaginal epithelial cells (HVECs)

Healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA)

Institutional Animal Care and Use Committee (IACUC)

Intensive care unit (ICU)

Interleukin 8 (IL-8)

Intravenous immunoglobulin (IVIG)

International unit (IU)

Isoelectric focusing (IEF)

Keratinocyte serum-free media (KSFM)

Lactate dehydrogenase (LDH)

Major histocompatibility complex (MHC)

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)

Molecular weight (MW)

Methicillin-sensitive *S. aureus* (MSSA)

Panton-Valentine leukocidin (PVL)

Penicillin-binding protein (PBP)

Phenol-soluble modulin (PSM)

Phosphate buffered saline (PBS)

Polymorphonuclear leukocytes (PMNs)

Polyvinylidene fluoride (PVDF)

Pulsed-field gel electrophoresis (PFGE)

Red blood cells (RBCs)

Reverse phase high performance liquid chromatography *(*rHPLC)

Revolutions per minutes (rpm)

Skin and soft-tissue infections (SSTIs)

Sterile distilled water (SDW)

Simian immunodeficiency virus (SIV)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Standard error of the mean (SEM)

Staphylococcal accessory element (Sae)

Staphylococcal accessory regulator (Sar)

staphylococcal chromosomal cassettes (SCC)

Staphylococcal enterotoxin (SE); Staphylococcal enterotoxin-like (SEl)

Staphylococcal inhibitor of complement (SCIN)

Staphylococcal respiratory response (SrrAB)

Staphylococcal superantigen-like proteins (SSLs)

Superantigen (SAg)

T-cell receptor (TCR)

Toxic shock syndrome (TSS); Toxic shock syndrome toxin 1 (TSST-1)

Tumor necrosis factor-α (TNF-α)

Chapter I.

Introduction

1.1. Overview

Staphylococcus aureus is a Gram-positive bacterium capable of infecting virtually every tissue of the body and causing infections ranging from minor skin infections to life threatening infections such as bacteremia, endocarditis, necrotizing pneumonia, and toxic shock syndrome (TSS). *S. aureus* is a major cause of healthcare-associated infections accounting for about 500,000 infections in all hospitalized patients in U.S. hospitals every year (62% of total hospitalized patients) being associated with *S. aureus* infections [1]. The increasing prevalence of antibiotic-resistant *S. aureus*, most well-known as methicillin-resistant *S. aureus* (MRSA), have emerged as a serious public health issue both in the hospital and community settings. MRSA isolates account for more than 60% of nosocomial *S. aureus* infections in the intensive care units [2] and more than 70% of *S. aureus* skin and soft-tissue infections in the community [3]. Patients infected with MRSA are likely to have longer durations of hospital stays, higher hospital charges, and higher mortality rates than patients infected with methicillin-susceptible *S. aureus* (MSSA) [4,5]. Invasive (severe) MRSA infections alone are estimated to cause approximately 19,000 deaths in the U.S. annually, which is similar to the total number of deaths due to acquired immune deficiency syndrome (AIDS), tuberculosis, and viral hepatitis combined [6,7].

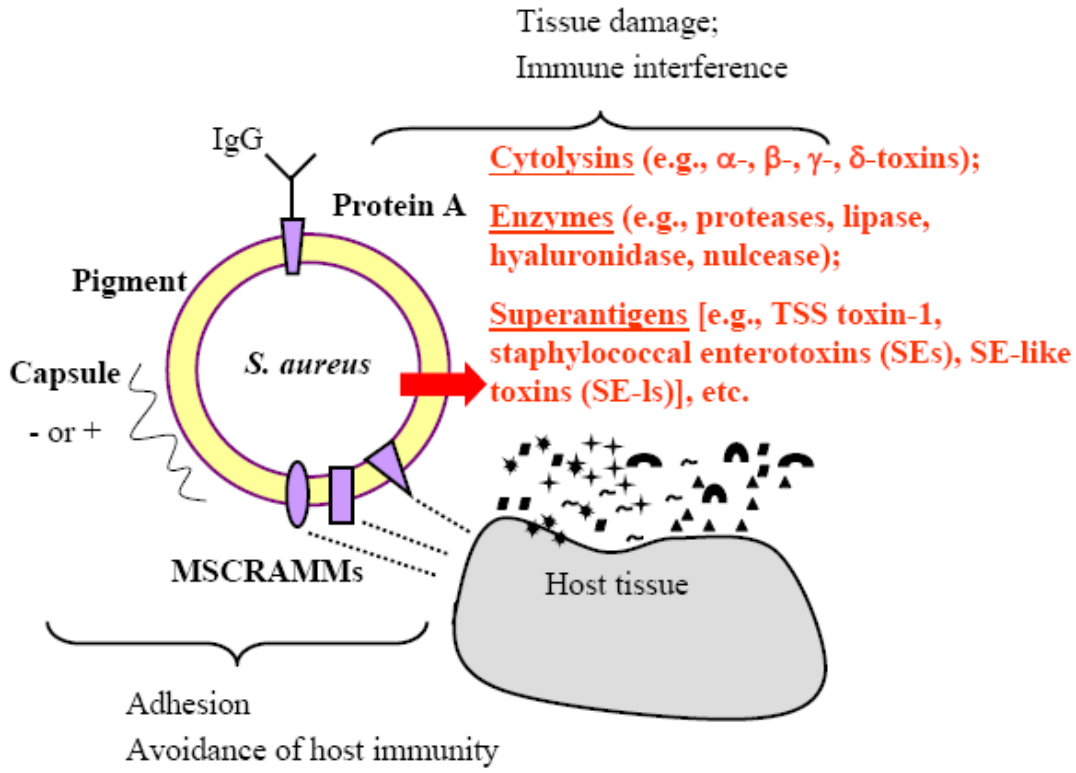
S. aureus produces an array of virulence factors to facilitate its pathogenesis (Fig 1.1). In particular, a group of *S. aureus* exoproteins known as superantigens mediate toxic shock syndrome (TSS). However, only recently have researchers started to recognize the overall importance of staphylococcal exoproteins such as cytolysins and

superantigens in the initiation and progression of infections via direct tissue damage to mucosal membranes and skin [8,9]. Antibiotics of choice for treating *S. aureus*-related illnesses, β-lactams for MSSA and vancomycin for MRSA, kill the organism by lysis of the cell wall or inhibit cell wall biosynthesis, but are not able to inhibit *S. aureus* exoprotein production or neutralize the existing toxins' effects on host cells. In fact, β-lactams may even induce production of cytolysins and other virulence-related exoproteins when inadequately used for treating MRSA, which potentially worsens clinical outcomes [10].

Given the emergence of MRSA and the high mortality associated with *S. aureus* infections, there is a demand for novel antistaphylococcal approaches to manage the increasing burden of *S. aureus* on human health. Due to the important roles of *S. aureus* exotoxins in pathogenesis, a strategy of targeting *S. aureus* virulence factors (exotoxins) to prevent and/or decrease the morbidity and mortality associated with *S. aureus* diseases was proposed. The approach of my thesis research included characterizing the *S. aureus* exoproteins that were most pro-inflammatory and cytotoxic to host epithelial cells, to determine the biologically important exotoxins to target, and then determine the effects of a fatty acid monoester and monoether on exotoxin production and/or the corresponding host inflammatory responses on the progression of *S. aureus* diseases using an *in vivo* animal model.

Current knowledge of the relationship between *S. aureus* disease and secreted virulence factors as well as antistaphylococcal treatments, both currently available and experimental, are reviewed below.

Figure 1.1 *Staphylococcus aureus* virulence factors. *S. aureus* cell wall-associated virulence factors, including microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule, pigment, and protein A, mainly contribute to bacterial cell attachment to host tissues and help avoid phagocytosis by host immune cells. Secreted virulence factors such as cytolysins, enzymes, and superantigens are responsible for damaging host tissues and altering host immune defense.



Modified from figure 12-3 from Medical Microbiology, 4th Edition by Samuel Baron

1.2. *Staphylococcus aureus*-Associated Diseases

1.2.1. Healthcare-associated and community-associated *S. aureus* infections

S. aureus is the most prevalent cause of nosocomial infections in the U.S., accounting for approximately 20% of infections in the hospitals [11]. Due to the frequent exposure to antibiotics in the health care setting, the proportion of methicillin-resistant *S. aureus* (MRSA) in the hospitals steadily increased, from about 36% in 1992 to more than 60% in 2003 in intensive care unit (ICU) patients [2]. Healthcare-associated MRSA (HA-MRSA) primarily affects people who have a compromised immune system and people with prior surgery or implanted medical devices, and typically cause septicemia, pneumonia, and device-associated infections. HA-MRSA isolates are most frequently identified as pulsed-filed gel electrophoresis (PFGE) USA100 and USA200, and most of them carry staphylococcal chromosomal cassettes (SCC*mec*) type I to III, which contain *mecA*, the methicillin-resistant gene that encodes penicillin-binding protein 2a (PBP2a), and multiple other antibiotic resistance genes [12]. These HA-MRSA isolates are typically resistant to multiple antibiotics, including penicillins, β-lactams, erythromycin, clindamycin, fluoroquinolones, and sometimes aminoglycosides.

S. aureus is also a common cause of skin and soft-tissue infections (SSTIs) in the community. MRSA has been traditionally associated with hospitals; however, since the late 1990s there have been increasing reports of MRSA infections in people with no contact with the health care system or pre-defined risk factors for HA-MRSA. These MRSA isolates were therefore referred to as community-associated MRSA (CA-MRSA). The prevalence of MRSA in the community is only approximately 1% according to a national nasal colonization survey by Center for Disease Control and Prevention (CDC)

[13]. However, CA-MRSA strains are virulent and emerging as a major cause of severe SSTIs and necrotizing pneumonia (less frequent) in the community [12]. A study by Moran and colleagues where cultures from adult patients with purulent SSTIs were collected in 11 emergency departments across the U.S. in 2004 indicated that *S. aureus* was responsible for 76% of the SSTIs and 78% of these *S. aureus* isolated were MRSA (about 60% of all infections) [14]. Epidemiological evidence indicated that CA-MRSA isolates are distinct from HA-MRSA isolates in many ways [12]. CA-MRSA isolates are most frequently identified as PFGE USA300 and USA400. Most of them carry SCC*mec* type IV or V and are susceptible to most antibiotics except β-lactams and erythromycin [12]. However, these CA-MRSA isolates have started to spread into hospital settings; therefore, USA300 and USA400 are emerging as major causes of HA-MRSA infections [1,6,15,16]. Furthermore, it is no surprise that the SCC*mec* pattern difference in HA-MRSA and CA-MRSA are also becoming less distinct due to the clonal blending [16,17].

In addition to the difference in demographic distribution and antibiotic susceptibilities, HA-MRSA and CA-MRSA isolates have distinct exotoxin profiles, which have been associated with their genetic backgrounds. For example, Paton-Valentine leukocidin (PVL) is epidemiologically associated with CA-MRSA isolates [17]. Certain superantigens, i.e. staphylococcal enterotoxin B (SEB) and SEC, have also been associated with CA-MRSA; however, there are large variations in the prevalence of these superantigens in the epidemiological studies [12,17,18].

The differences between HA-MRSA and CA-MRSA are summarized in Table 1.1. Generally speaking, MRSA strains are not considered to be more virulent than their

methicillin susceptible *S. aureus* (MSSA) counterparts. However, it is possible that the acquisition of antibiotic resistant mechanisms indirectly influences the secretion of toxins in certain strains, which may result in more virulent MRSA isolates.

Table 1.1 General differences between healthcare-acquired and community-associated methicillin resistant *S. aureus* (A historical perspective)

	Healthcare-associated	Community-associated
Genotypes [a]	USA100, USA200[e]	USA300, USA400
SCC*mec* [b]	I-III (34-67 kbp)	IV, V (21-24kbp)
Antimicrobial Resistance [c]	multi-drug resistant (penicillins, β-lactams, erythromycin, clindamycin, and fluoroquinolones resistance; ± aminoglycosides)	only resistant to penicillins, β-lactams, erythromycin; ± fluoroquinolones and clindamycin (inducible)
PVL [d]	-	+
Risk factors	People who have had surgery or medical devices; elderly; people with weakened immune system; and patients undergoing kidney dialysis	close person-to-person contact
Infections	Urinary tract infection; surgical site infection; pneumonia	Skin and soft-tissue infections; necrotizing pneumonia (rare)

References: [12,17]

[a] Genotyping based on PFGE pattern is a method developed by Center for Disease Control and Prevention (CDC) [19].

[b] Staphylococcal chromosomal cassette, which contains the methicillin-resistant gene, *mecA*.

[c] <80% susceptible to the antibiotic are considered as resistant; 80-90% susceptible are considered "±"

[d] Panton-Valentine leukocidin

[e] USA200 is also the most common (methicillin sensitive) *S. aureus* in the community.

1.2.2. Staphylococcal Toxic Shock Syndrome (TSS)

Superantigens (SAgs) are a group of *S. aureus* exotoxins capable of inducing human diseases independent of the presence of the organism at that specific anatomical location. Greater than 20 different SAgs have been identified from *S. aureus* isolates, including staphylococcal enterotoxins (SEs), enterotoxin-like proteins (SE-ls), and TSST-1. More than 60% of clinical *S. aureus* isolates carry at least one superantigen [20,21]. These SAgs are able to activate T lymphocytes and antigen presenting cells (APCs) such as macrophages or dendritic cells nonspecifically, by cross-linking Vβ regions of the T-cell receptor (TCR) and major histocompatibility complex (MHC) class II molecules of the APCs in a non-antigen specific manner (Figure 1.2) [22]. As a result, SAg are able to activate 5-30% of T-cells (compared to 0.01% by a normal antigen), which induces a massive release of cytokines and chemokines from both T cells and APCs and causes the symptoms observed in staphylococcal TSS [22]. Clinical features of TSS include fever (≥38.9˚C), rash, late desquamation of the palms of the hands and feet (1-2 weeks after disease onset), hypotension, and multi-organ dysfunction, which is acute and potentially life-threatening (Table 1.2).

Based on the site of infection, TSS can be divided into two categories, menstrual and non-menstrual TSS. Menstrual TSS usually occurs within 2 days after the initiation of menstruation or within 2 days after menstruation, and it is associated with tampon usage in women vaginally colonized by SAg-producing *S. aureus*. TSST-1 is responsible for more than 90% of menstrual TSS [23]. Non-menstrual TSS occurs as a complication of *S. aureus* infections after surgical procedures, burns, or post-influenza pneumonia. TSST-1 is responsible for approximately half of non-menstrual TSS cases, and

staphylococcal enterotoxin C (SEC) and SEB are responsible for the majority of the remaining cases [9,23]. The prevalence of the TSST-1 gene (*tst*) is about 25% in all clinical isolates, but is carried by more than 80% of USA200 isolates, where as, the prevalence of SEB and SEC is only about 10% in clinical isolates, with USA400 isolates producing either SEB or SEC [24].

Absence or an insufficient amount of neutralizing antibodies is a risk factor for TSS; therefore, children and young women are especially at risk of developing TSS. The incidence of menstrual TSS is estimated to be approximately 1 per 100,000 women of menstrual ages (15-44 years old) in the U.S. [25]. A surveillance study conducted by Schlievert and colleagues in the Minneapolis-St. Paul area during 2000-2003 suggested a local increase of TSS (including both menstrual and non-menstrual TSS), which rose from 0.9 (in 2000) to 3.4 (in 2003) cases per 100,000 women of menstrual age per year [26]. Based on a study comparing SAg profiles of *S. aureus* vaginal colonizing isolates from 1980 and 1981 to 2003-2005 in the Minneapolis-St. Paul area, the increased incidence of TSS in the above study was most likely due to the increase in the prevalence of vaginal *S. aureus* (from 12% to 23%) or non-menstrual associated TSS cases, instead of an increasing proportion of TSST-1^{+} isolates in vaginal colonization strains [21]. A French surveillance study collecting of 55 TSS cases over 30 months during 2003 to 2006 suggested that non-menstrual staphylococcal TSS are associated with more severe neurological disorders and higher mortality rates, and non-menstrual staphylococcal TSS may be more prevalent than menstrual TSS [27]. Since the diagnosis of TSS relies on clinical symptoms, which may vary due to patient sensitivity and/or other under underlying conditions, the prevalence of TSS may be under-reported.

Figure 1.2 Superantigen mechanism of action. Superantigens bind to major histocompatibility complex (MHC) class II molecules of antigen presenting cells (i.e. macrophages) and Vβ region of T-cell receptor in a non-antigen specific manner, which leads to massive release of cytokines and chemokines, as well as, the clonal expansion of certain clonal types of T-cells.

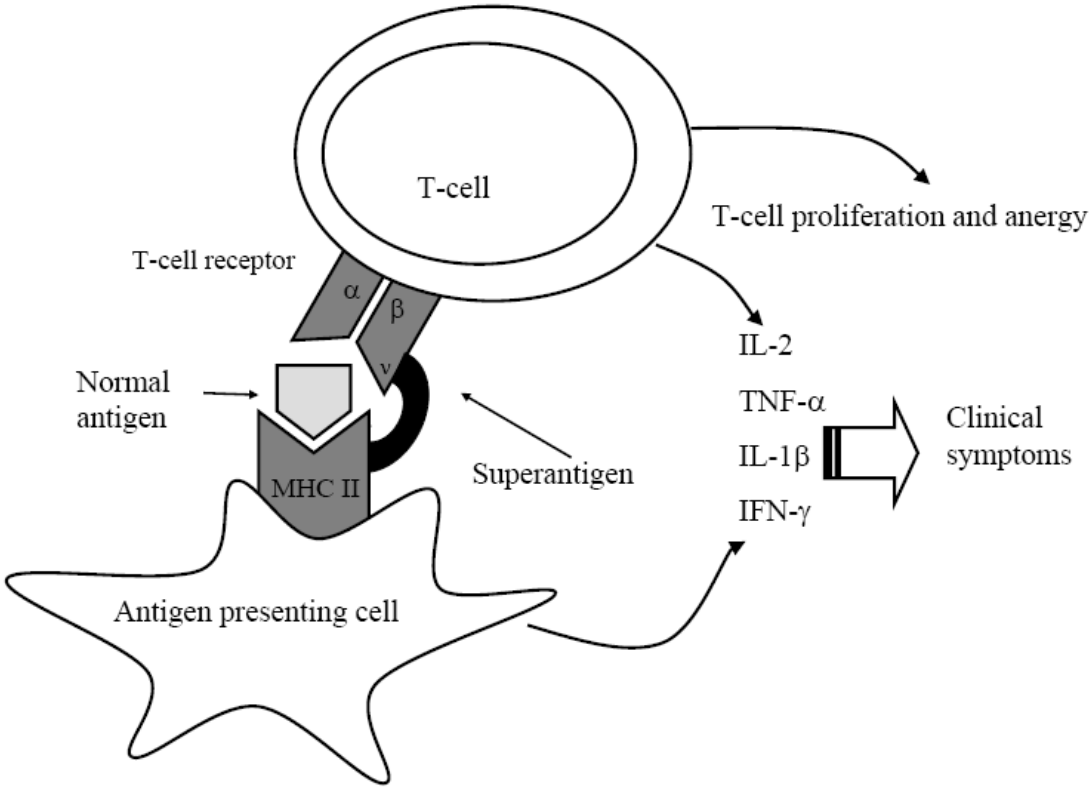


Table 1.2 CDC case definition of staphylococcal toxic shock syndrome (TSS)

Criteria	Definition
Clinical	
Fever	Temperature greater than or equal to 102.0°F (≥ 38.9°C)
Rash	Diffuse macular erythroderma
Desquamation	1-2 weeks after onset of illness, particularly on the palms and soles
Hypotension	Systolic blood pressure ≤ 90 mm Hg for adults or < 5th percentile by age for children aged < 16 years; orthostatic drop in diastolic blood pressure ≥15 mm Hg from lying to sitting, orthostatic syncope, or orthostatic dizziness
Multisystem involvement (≥ 3 organ systems)	
Gastrointestinal	Vomiting or diarrhea at onset of illness
Muscular	Severe myalgia or creatine phosphokinase level at least twice the upper limit of normal
Mucosal	Mucous membrane: vaginal, oropharyngeal, or conjunctival hyperemia
Renal	Blood urea nitrogen or creatinine at least twice the upper limit of normal for laboratory or urinary sediment with pyuria (≥ 5 leukocytes per high-power field) in the absence of urinary tract infection
Hepatic	Total bilirubin, alanine aminotransferase enzyme, or spirate aminotransferase enzyme levels at least twice the upper limit of normal for laboratory
Hematologic	Platelets < 100,000/mm^3
Central nervous system	Disorientation or alterations in consciousness without focal neurologic signs when fever and hypotension are absent

(cont'd next page)

Table 1.2 CDC case definition of Staphylococcal toxic shock syndrome (TSS) (Cont'd)

Criteria	Definition
Laboratory	
Culture	If obtained, negative results on blood, throat, or cerebrospinal fluid cultures (blood culture may be positive for *Staphylococcus aureus*)
Titer	If obtained, no rise in titer to Rocky Mountain spotted fever, leptospirosis, or measles
Case classification	
Probable	A case which meets the laboratory criteria and in which four of the five clinical findings described above are present
Confirmed	A case which meets the laboratory criteria and in which all five of the clinical findings described above are present, including desquamation, unless the patient dies before desquamation occurs

Source: http://www.cdc.gov/ncphi/disss/nndss/print/toxic_shock_syndrome_current.htm

1.3. *S. aureus* Pathogenesis

S. aureus is equipped with an array of functional, diverse and redundant cell wall-associated and secreted virulence factors to facilitate its pathogenesis, mainly by attaching to host tissue, avoidance of host immune clearance, and invading or destroying host cells to gain access to nutrients and to disseminate. Most *S. aureus* infections initiate at mucosal surfaces or skin, which proceed to invasive diseases, the exception being those seeded into the blood stream by invasive medical devices. Therefore, mucosal surfaces and the skin are where most *S. aureus* virulence factors are produced. The biological functions and putative roles of these staphylococcal virulence factors in *S. aureus* pathogenesis are discussed below.

1.3.1. Cell wall-associated virulence factors

S. aureus cell wall-associated virulence factors include capsular polysaccharides (CP), staphyloxanthin (carotenoid pigment), and a group of proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).

CP are produced by about 90% of clinical isolates, and two serotypes, CP5 and CP8, account for approximately 75% of isolates recovered from humans [28]. The main function of capsules in staphylococcal virulence is to impede phagocytosis by neutrophils, but it has also being shown to enhance bacterial colonization and persistence on mucosal surfaces [28]. *S. aureus* golden pigment, staphyloxanthin, also functions to resist killing by neutrophils (reactive oxidant-based phagocytosis) [29]. Inhibition of staphyloxanthin biosynthesis allows *S. aureus* to become more vulnerable

to the innate immune clearance, and resulted in a significant reduction of *S. aureus* bacterial load in the kidney in a mouse model with intraperitoneal bacteria challenge [29].

MSCRAMMs such as clumping factors (Clf), fibronectin-binding proteins (FnBP), collagen adhesin, and protein A play important roles in microbial adhesion to host proteins (i.e. fibronectin, fibrinogen, and collagen) and establish the first step of an infection. These proteins also prevent the organism from recognition by the host immune system [24]. For example, Clf and FnBP are able to cause platelet activation, which results in clotting. Protein A binds to the Fc portion of immunoglobulin to prevent opsonization.

1.3.2. Secreted virulence factors

In contrast to the protective/passive role of cell wall-associated virulence factors, secreted *S. aureus* virulence factors play active roles in disarming host immunity by interfering with host immune system and disrupting host cells and tissues to release nutrients and facilitate bacteria dissemination. The secreted virulence factors are comprised of four main categories: SAgs, pore-forming toxins, various exoenzymes, and miscellaneous proteins. Table 1.3 lists *S. aureus* secreted virulence factors that may play important roles in *S. aureus* pathogenesis.

The global characterization and determination of the key secreted virulence factors important to the initiation and dissemination of *S. aureus* mucosal infections is a key focus of this thesis.

Table 1.3 Secreted *S. aureus* virulence factors

Secreted virulence factor	Putative function
Toxic shock syndrome toxin 1 (TSST-1); Staphylococcal enterotoxins (SEs); SE-like toxins (SEls)	Activate T-cells and macrophages
Cytolysins (α-, β-, γ-, δ-toxins); Phenol-soluble modulin-like peptides (PSMs); Leukocidins (PVL, LukD/E)	Induce apoptosis (at low conc.) and lysis of various cell types, including erythrocytes, lymphocytes, monocytes, epithelial cells, etc.; target specificity varies
Lipase	Inactivate fatty acids
Hyaluronidase	Degradation of hyaluronic acid
Serine proteases; Cysteine proteases (including staphopain); Aureolysin	Inactivate neutrophil proteolytic activity; inactivate antimicrobial peptides
Staphylokinase (Sak)	Plasminogen activation; Inactivate antimicrobial peptides
Exfoliative toxins (ETs)	Act as serine proteases; Activate T-cells
Chemotaxis inhibitory protein of *S. aureus* (CHIPS); Staphylococcal inhibitor of complement (SCIN)	Inhibit complement
Staphylococcal superantigen-like proteins (SSLs); Exotoxins; Extracellular adherence protein (Eap)	Inhibit complement C5 and IgA; Inhibit neutrophil migration

1.3.2.1. Superantigens (SAgs)

As mentioned previously, SAgs are a large family of toxins, capable of activating T-cells and APCs in a non-antigen specific manner, resulting the secretion of high levels of cytokines and chemokines. Each SAg activates different T-cell Vβ subtypes, and some of them are naturally low in abundance. The skewed distribution of T-cell subtypes induced by SAgs may alter immune tolerance status and trigger autoimmune disorders [30]. SAgs can also serve as allergens to stimulate IgE production (via T-cell dependent B-cell activation), mast cell activation, and local inflammation [31]. Additionally, SAgs have also been shown to induce steroid resistance via activating mitogen-activated protein kinase cascades and inducing expression of glucocorticoid receptor GRβ [32,33]. Therefore, SAgs have also been associated with autoimmune diseases such as Kawasaki syndrome, rheumatoid arthritis, psoriasis, and atopic dermatitis, rhinitis, and asthma [31]. Table 1.4 is a short list of diseases that have been associated with SAgs. For most of these diseases, the mechanism of SAg involvement has not been characterized. However, there is no doubt that these toxins function (in many ways) to disturb host immune response to ensure the survival and persistence of *S. aureus* in host niches.

SAgs may also have direct effects on other cells to facilitate *S. aureus* pathogenesis on mucosal surfaces or skin. TSST-1 was determined to have dose-dependent pro-inflammatory and cytotoxic effects on endothelial cells, which may facilitate capillary leakage and cause TSS [34]. TSST-1 is also pro-inflammatory to epithelial cells, which stimulates recruitment of neutrophils that may lead to tissue destruction and increased permeability of the mucosal tissue [35]. Mice respond

superantigenicity to TSST-1, yet do not develop TSS [36]. This indicates the importance of SAgs in acute *S. aureus* pathogenesis independent of their superantigenicity. TSST-1 is responsible for almost all menstrual TSS as it is able to penetrate the vaginal mucosa better than other superantigens, and due to it own proinflammatory and cytotoxic effects on the vaginal mucosa, which is composed of a thick (10 to 30 cells) epithelial layer.

SAgs are also pyrogenic and capable of enhancing sensitivity of rabbits to endotoxin (lipopolysaccharides). Therefore, they have been suggested to play a role in sepsis where a person may become infected with Gram-negative and *S. aureus* organisms [37]. Some SAgs, namely staphylococcal enterotoxins (SEs), can also induce emesis and diarrhea when ingested and SEs are a common cause of food poisoning, where SE-induced inflammation in abdominal viscera has been suggested to induce the symptoms [37]. However, no intestinal epithelial receptor for SEs has been identified.

Table 1.4 Clinical human diseases associated with superantigen(s)

Disease	References
Toxic shock syndrome	[38]
Food poisoning	[38]
Sepsis	[38,39]
Recalcitrant, erythematous, desquamating disorder (in AIDS patients)	[40]
Neonatal TSS-like exanthematous disease	[41]
Sudden infant death syndrome	[42]
Kawasaki disease	[43,44]
Purpura Fulminans	[45]
Nasal polyp	[46]
Allergic asthma	[47]
Atopic dermatitis	[48,49]
Psoriasis	[50]
Eczema	[47]

1.3.2.2. Cytolytic toxins

S. aureus secrete a large number of cytolytic toxins, including cytolysins (α-, β-, γ-, and δ-toxins), leukocidin family toxins (Leukocidin, LukD/E, LukM, and Panton-Valentine leukocidin, PVL), and phenol-soluble modulins (PSMs). Although these toxins are structurally diverse and have various target specificity (i.e. erythrocytes, leukocytes, and epithelial cells), their function on host cells is similar. They form pores in the membranes of target cells and cause leakage (osmotic swelling) and inflammation of the cells at low doses, and cell lysis at high doses.

Alpha-toxin (α-toxin) is the most well characterized cytolysin. The gene encoding α-toxin, *hla*, is carried by virtually all clinical isolates; however, some strains, mostly vaginal TSS isolates, do not produce grossly detectable α-toxin due to a silencing point mutation in the gene structure (pseudogene), which prevents it from being translated [37,51,52]. α-Toxin is secreted as a water-soluble monomer by *S. aureus* and engages surface receptors of sensitive host cells (such as erythrocytes, lymphocytes, macrophages, and epithelial cells), where they assemble into cylindrical heptamers (1-2 nm wide) in cell membranes [37]. The structure of the matured *β*-barrel pore-forming toxin is shown in Figure 1.3. α-Toxin has wide-spectrum of activity against various cells, including erythrocytes, leukocytes (i.e. monocytes, macrophages and polymorphonuclear [PMN] cells), platelets, epithelial cells, and fibroblasts [53]. The toxin has been shown to be hemolytic, dermonecrotic, neurotoxic, and lethal when injected intravenously. α-Toxin was also indicated to induce apoptosis in various cell types, including endothelial cells, peripheral blood mononuclear cells, and T-cells

[54-56]. However, the significance of α-toxin in human disease has not been conclusively established; α-toxin is highly dermonecrotic in human and is thus linked to furuncles [37]. Bubeck Wardenburg and Schneewind [8] successfully demonstrated the role of α-toxin in necrotic pneumonia in a murine model. The mortality rate of mice challenged with high dose (~10^8 colony forming units [CFU]) of CA-MRSA isolates were shown to be correlated with the concentration of α-toxin secreted by the strains. In addition, mice with α-toxin-specific antibodies were protected from lung tissue damage and death from pneumonia despite no significant change (~ 1-log decrease) in bacterial load in the lung tissues. Brosnahan and colleagues [9] demonstrated that the pro-inflammatory activity of low concentrations (5 μg/ml) of α-toxin to epithelial cells can increase mucosal permeability and significantly facilitate TSST-1 penetration through the multi-layered vaginal mucosa in a porcine vaginal tissue model that physiologically resembles human vaginal mucosa [9].

β-Toxin (sphingomyelinase C) is the only cytolysin with a known enzymatic mode of action, which is to specifically hydrolyze its target, sphingomyelin [57,58]. Therefore, the sensitivity of human cells to the toxicity of β-toxin depends upon the distribution of sphingomyelin on the membrane. β-Toxin is cytotoxic to monocytes, erythrocytes, neutrophils, and lymphocytes (especially proliferating T-cells) [57]. β-Toxin was shown to be a major factor in *S. aureus* contributing to toxicity of (immortalized) human T-cells [59]. A recent study by Tajima et al. indicates that the toxin may also contribute to *S. aureus* immune invasion by inhibiting the production of endothelial interleukin-8 (IL-8), which activates neutrophil transmigration [60].

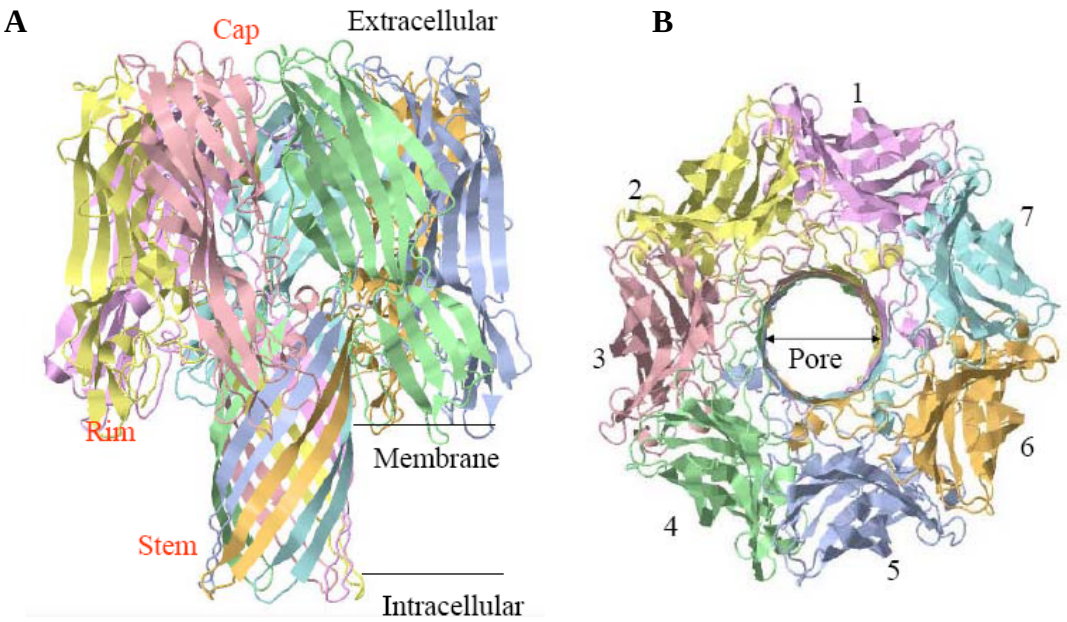
Cytolysin γ-toxin and the leukocidin family, including PVL, leukocidin E/D, leukocidin M/F-PV, are bi-component toxins. Each of these toxins is composed of two separately secreted proteins, referred to as fast-eluting (F) and slow-eluting (S) components. Amino acid sequences of the S-components (HlgA, HlgC, LukM, LukE, LukS-PV) and F-components (HlgB, LukD, LukF-PV) of these bi-component toxins share approximately 70% (ranging 55-81%) sequence identity within groups (S or F) and 30% between groups (S vs. F) [53,61]. These subcomponents (S or F proteins) alone do not have biological activity, but they can assemble with one anther (S+F) to into heptamers (with stoichiometry of 3:4 or 4:3) or hexamers (3:3). These β-barrel structures are similar to α-toxin (although the pore is up to 10% larger than α-toxin). γ-Toxin (composed of HlgA or HlgC, and HlgB) lyses both leukocytes and erythrocytes, while PVL (composed of LukS-PV and LukF-PV) is more toxic to leukocytes than red blood cells [61]. Other combinations of bi-component toxins have various levels of hemolytic and leukocytic activity. These toxins were also shown to induce different levels of inflammatory reactions when injected into the rabbit eye vitreous humor [62] and rabbit skin [63]. γ-Toxin is produced by more than 90% of clinical isolates, whereas PVL (<5%) and other leukocidins such as LukE/D (~30%) are much less prevalent [24]. LukM/F-PV is seldom isolated from human clinical isolates [53].

Although only detected in less than 5 % of clinical isolates, PVL is epidemiologically associated with CA-MRSA isolates [64]. However, whether or not PVL is responsible for the virulence of CA-MRSA isolates is still in debate due to conflicting results in animal models. Labandeira-Rey et al. indicated that PVL is

sufficient to cause necrotizing pneumonia by comparing clinical pneumonia strains, isogenic PVL-positive and PVL-negative research *S. aureus* strains, and purified PVL in a mouse pneumonia model [65]. Voyich and colleagues, however, concluded that PVL is not an important virulence factor based on their observation comparing PVL-positive strains to genetically close PVL-negative isolates and their own isogenic PVL knockout strains in murine sepsis and abscess models; the PVL-positive and PVL-negative strains were comparable at the levels of mortality and skin damage [66]. Given that mouse leukocytes are not as sensitive to PVL as rabbits and humans [67], it is possible that the discrepancy observed from the above studies was actually due to other functionally similar virulence factors produced by the selected strains and not PVL. Presently, whether or not PVL, as well as other cytolysins, play clinically relevant roles in *S. aureus* etiology in humans remains to be determined.

δ-Toxin is a single 26-amino-acid α-helix peptide, which is capable of lysing human erythrocytes, neutrophils, as well as various mammalian cells via its amphipathic (surfactant) activity [37]. δ-Toxin also serves as the effector protein of the accessory gene regulator (Agr), which is a *S. aureus* global virulence regulatory system (which will be discussed below). The toxin is produced by 97% of *S. aureus* isolates. The role of δ-toxin in *S. aureus* pathogenesis remains unclear. However, a recent study by Wang et al. suggested that δ-toxin and other PSMs, may be partially responsible for the virulence of CA-MRSA isolates [68]. The authors determined that CA-MRSA isolates produce high amounts of PSMs (including δ-toxin) and isogenic PSM deletion strains were less virulent than their parental strains in mouse bacteremia and skin infection models [68].

Figure 1.3 Ribbon diagrams of the α-toxin heptamer structure (A) Top down view, (B) side view. The β-barrel pore-forming structure is the assembly of 7 soluble monomer secreted by *S. aureus* on the cell membranes. The α-toxin is the archetype of leukocidin family pore-forming toxins. [Protein data bank (PDB) ID: 7AHL]



Reference: [69]

1.3.2.3. Exoenzymes

S. aureus secrete multiple extracellular enzymes, including a glycerol ester hydrolase (lipase), a nuclease, a hyaluronidase, staphylokinase, and multiple proteases, including serine proteases, cysteine proteases, aureolysin (metalloenzymes), and staphopain, which presumably function to disrupt host tissues and/or inactivate host antimicrobial mechanisms such as lipids, defensins, antibodies, and complement mediators and thereby facilitate bacterial dissemination. The clinical significance of these enzymes has not been well-characterized.

Staphylokinase activates plasminogen into plasmin, a broad-spectrum proteolytic enzyme, to avoid opsonization (via cleavage of human IgG and C3b from bacterial surface) and to facilitate bacterial penetration, and staphylokinase also destroys the antibacterial effect of α-defensins (secreted by neutrophils) to avoid host innate immune [70,71]. Other multiple extracellular enzymes secreted by *S. aureus*, including serine-, cysteine-proteases and metalloenzymes, also have a wide range of pathogenic potentials. These enzymes are insensitive to most human plasma protease inhibitors and are even able to inactivate some of them (e.g., staphopain A and V8 protease inactive plasma α_1-proteinase inhibitor, a potent neutrophil elastase inhibitor, and result in deregulation of neutrophil-derived proteolytic activity) [72]. V8 protease (a serine protease) and aureolysin can cleave human immunoglobulin, as well as FnBP and other bacterial cell surface proteins [73]. Staphopain A possesses elastolytic activity against elastin in connective tissues, which is not an observed activity for V8 protease or aureolysin. However, these proteases require proteolytical processing by one another to be activated;

therefore, it is difficult to demonstrate unambiguously the importance of any particular proteases as a virulence factor [72].

Exfoliative toxins (ETA and ETB) are also a type of serine proteases (although not as prevalent as other extracellular proteases). Different from other proteases, ETs have been indicated as major toxins responsible for bullous impetigo and staphylococcal scalded skin syndrome [74]. These toxins target a particular protein on the skin known as desmoglein 1 and results in the separation of epidermal tissues layers.

1.3.2.4. Miscellaneous proteins

S. aureus also secrete various proteins, including chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal inhibitor of complement (SCIN), extracellular adherence protein (Eap), and staphylococcal superantigen-like (SSL) proteins. Biochemical functions of these newly identified proteins indicate that they may play some roles in *S. aureus* pathogenesis, especially innate immune evasion; however, the *in vivo* roles of these proteins during infections are still largely un-characterized.

SCIN is a C3 convertase (complement) inhibitor, blocking the formation of C3b on the surface of the bacterium and inhibiting phagocytosis by human neutrophils [75], while CHIPS binds to the C5a receptor and formyl peptide receptor on the neutrophils to inhibit complement and neutrophil migration [24].

Eap is a MHC class II analogous protein. This protein putatively plays multiple roles in *S. aureus* pathogenesis, including facilitating adherence and internalization of *S. aureus* to epithelial cells, endothelial cells, and fibroblasts, blocking neutrophil

recruitment by binding to host adhesion protein intercellular adhesive molecule 1 (ICAM-1), inducing T-cell apoptosis, and inhibiting T-cell proliferation [76].

More than 10 SSLs have been identified, based on their structure similarity to SAgs. SSLs do not have superantigenicity but rather display a wide array of activity targeting key elements in the innate immune system. For example, SSL7 binds to IgA and complement factor C5. SSL5 and SSL11 are inhibitors of neutrophil adherence [77]. However, most of them remain to be characterized.

1.3.3. *S. aureus* virulence regulatory systems

The diversity and functional redundancy of *S. aureus* virulence factors play important roles in the fitness and disease causation of *S. aureus* in the host. As a result, eliminating one virulence factor does not usually result in a significant reduction of *S. aureus* virulence and thereby makes analysis of the roles of individual virulence factors in *S. aureus* pathogenesis difficult. SAgs (in TSS and food poisoning), exfoliative toxins (in staphylococcal scalded skin syndrome) and cytolysins are a few toxins that have been determined to be sufficient to provoke staphylococcal disease. To enable *S. aureus* to produce virulence factors only during certain growth or environmental conditions, proper regulation of virulence factors is important. Mutations in virulence regulatory systems, which coordinate virulence factor production in response to environmental signals, results in significantly less virulent strains [78].

Most cell wall-associated *S. aureus* virulence factors are expressed during the exponential growth phase *in vitro*, while secreted *S. aureus* virulence factors are

typically expressed during late-logarithmic to stationary growth phase. The regulation of these virulence factors are controlled by multiple global regulator systems, including two-component systems, DNA-binding proteins, and quorum-sensing systems based on bacterial density and environmental factors such as oxygen and carbon dioxide concentrations, temperature, and pH [79].

The accessory gene regulator (Agr) is one of the most well-studied staphylococcal virulence regulator systems. Agr is a two compartment quorum sensing system, which controls the production of most *S. aureus* virulence factors. Agr is transcribed by two adjacent promoters, promoter 2 (RNAII) and 3 (RNAIII). RNAII encodes Agr structural proteins, AgrBDCA, and RNAIII encodes δ-toxin, and RNAIII is the effector molecule of Agr. Agr B (a transmembrane protein) and AgrD (a propepetide) constitute a quorum-sensing system. AgrC is the transmembrane sensor histidine kinase (a receptor for the mature form of AgrD, autoinducing peptide [AIP]) and AgrA function as the response regulator. Agr controls the expression of most virulence factors via transcriptional and translational regulation. When bacteria reach late logarithmic and stationary phases, an accumulation of AIP increases transcription of RNAIII, which in turn increases the production of secreted exotoxins and decreases the expression of MSCRAMMs [80]. Agr has been indicated to play a role in *S. aureus* intracellular replication and release from epithelial cells [81].

Another well-characterized pleiotropic virulence regulator is SarA of the staphylococcal accessory regulator (*sar*) family. SarA is a DNA-binding protein, which regulates the transcription of *agr* by binding to *agr* P2 and P3 promoter. SarA

transcriptional regulation is complex, mediated by three promoters: SarA can autoinduce its own gene expression by binding to promoter P1, while P3 is dependent on σ^B, a regulator of *S. aureus* stress response. P2 appears to be silent *in vitro* but is activated *in vivo*. Environmental factors that affect SarA are not clear yet. Other members in the family include Sar R, -T, -U, -S, and Rot (repressor of toxins), which were shown to directly or indirectly interact with *SarA* (and other Sar family members) and *agr* to regulate virulence factors [78].

Other *S. aureus* global regulators include the staphylococcal accessory element (*sae*), autolysis related locus (*arl*), and the staphylococcal respiratory response (*srrAB*) system. *Sae* activates γ-toxin, and the regulation appears to be independent of *agr* and *SarA* [82]. *Arl* down-regulates *agr* and up-regulates *sarA*. *Arl's* ability to down-regulate virulence factors such as α-toxin (*hla*), serine protease (*ssp*), and protein A (*spa*) is *agr* and *SarA* dependent [83]. *SrrAB*, on the other hand, inhibits the expression of *agr* RNAIII, TSST-1(*tst*), and protein A (*spa*) under microaerobic conditions (1-2% O_2) [84]. These *S. aureus* virulence factor regulatory systems orchestrate delicately to control the expression of *S. aureus* virulence factors.

1.4. Antistaphylococcal Therapies

As reviewed above, diseases associated with *S. aureus* are mostly attributed to the accumulation of *S. aureus* toxic by-products (i.e. toxins) at the infection site or throughout the body (systemic). However, current antistaphylococcal regimens (antibiotics) focus mainly on the elimination of the infecting organism from the body, but not on the bacteria/host interactions (local and/or systemic) as a way to prevent or reduce the severity of disease. The section below briefly reviews current antistaphylococcal therapies, focusing on their known effects on *S. aureus* virulence, as well as experimental vaccines and immunotherapies under investigation to prevent and/or treat *S. aureus* disease.

1.4.1 Antibiotics

Antibiotics target multiple pathways that are essential for bacterial survival including bacterial cell-wall synthesis (i.e. β-lactams, glycopeptides), folic acid metabolism (sulfonamides), and bacterial protein synthesis (i.e. macrolides, lincosamides, and aminoglycosides). As an extension of its ability to adapt to ecological niches, *S. aureus* is excellent in developing resistance mechanisms to antibiotics, either by acquiring genes encoding enzymes that inactivate the antibiotics (penicillinases and aminoglycoside-modification enzymes), altering target molecules (PBP2a of MRSA and D-ala-D-Lac of peptidoglycan precursors of vancomycin-resistant *S. aureus*), limiting access of the antibiotics to their targets via thickening of the cell wall structures (vancomycin and possibly daptomycin), and/or increasing active efflux of the antibiotic

from inside the cell (fluoroquinolones and tetracycline), to ensure its survival [85].

β-lactams are first-line therapy for *S. aureus* infections. β-lactam antibiotics bind to proteins known as penicillin-binding-proteins (PBP) and inhibit cell wall biosynthesis. However, sub-growth inhibitory concentrations of β-lactams were shown to induce significantly the expression of α-toxin by *S. aureus* up to 30-fold *in vitro* [10]. This observation implies that inadequate use of β-lactams, for example in the case of MRSA infections, may enhance tissue damage associated with the infection by increased production of α-toxin. In contrast, vancomycin, the current drug of choice for MRSA infections, has no effect on virulence factors [10]. Vancomycin inhibits bacterial cell wall biosynthesis by forming complexes with D-ala-D-ala portion of peptide precursor unit to prevent the cross-linking of the cell wall peptidoglycan. Given vancomycin binds directly to the cell wall compared to inhibition of cross-linking enzymes β-lactams, there is no cross-resistance between the two antibiotics [86]. Both β-lactams and vancomycin are more effective in actively growing bacteria.

Antibiotics that inhibit protein synthesis are known to have some degree of anti-virulence effects. Macrolides reversibly bind to 23S ribosomal RNA (rRNA) of the 50S subunit of bacterial ribosome inhibiting RNA-dependent protein synthesis. Using α-toxin expression as a surrogate, clindamycin (a lincosamide) at sub-growth inhibitory concentrations was able to inhibit almost completely the translation of α-toxin, while subinhibitory concentrations of erythromycin (a macrolide) or aminoglycosides only partial reduced α-toxin expression [87,88]. Another study monitoring the expression of PVL showed similar conclusions [10]. Linezolid (an oxazolidinone), a new synthetic

protein inhibitor that binds to the 50S ribosome to prevent the formation of the 70S initiation complex, is also capable of repressing toxin production similar to clindamycin, and has been reported to treat a staphylococcal TSS case with good therapeutic outcome [10,89]. In addition, tetracyclines and macrolides have been known for their anti-inflammatory effects *in vitro* and *in vivo*, independent to their antimicrobial effect [90,91]. Doxycycline (a tetracycline) was shown to inhibit SAg-induced T-cell proliferation and cytokine and chemokine production (by PMNs) *in vitro* [92]. Therefore, these antibiotics, especially tetracyclines, may be beneficial in the treatment of TSS and SSTIs caused by CA-MRSA.

1.4.2 Anti-toxin (virulence) Agents

A few therapeutic agents are known to have anti-toxin effects for *S. aureus* exotoxins, including intravenous immunoglobulin (IVIG), monoester glycerol monolaurate (GML) (in development as a topical antimicrobial agent), and soluble Vβ peptides that block the binding of SEB on T-cell receptors.

Intravenous immunoglobulin (IVIG) contains a cocktail of pooled human antibodies derived from hundreds of donors, which are able to neutralize various bacterial toxins as well as modulate immune responses. IVIG was shown to significantly improve clinical manifestations within hours of administration in patients suffering from TSS (although most evidence was from group A streptococcal TSS), which was presumably due to the combination of its toxin neutralization and immunomodulation effects [93-96]. However, no strong evidence supports the benefit of IVIG as adjunctive

therapy for severe sepsis and septic shock. Meta-analysis studies indicate that IVIG decreases the mortality associated with sepsis and septic shock, but the clinical benefit may be minor due to lack of significant clinical improvement, high cost, and limited supply [97,98].

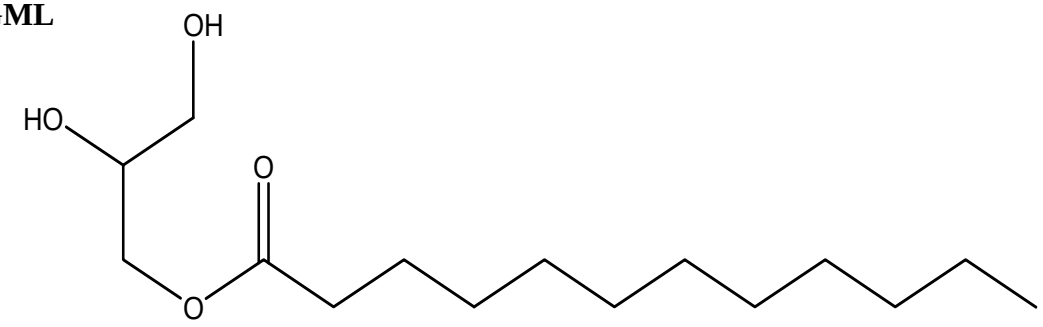
Glycerol monolaurate (GML) is a naturally derived lauric acid glycerol monoester commonly used in the food and cosmetic industries as an emulsifier and preservative. GML was originally recognized by its ability to inhibit the growth of *S. aureus*, block the induction of β-lactamases, and delay the production of *S. aureus* exoproteins, such as TSST-1 and α-toxin via interfering with bacterial cell membrane signal transduction [99-101]. More recently, studies have shown that GML has immunomodulation effects on mammalian cells via membrane stabilization and thereby protect these cells and experimental animals from toxic effects (i.e. osmosis change and cell lysis) due to various bacterial toxins [102]. GML prevents human and rabbit erythrocytes from lysis by bacterial hemolysins including purified α- and β-toxins, inhibits superantigen-stimulated proliferation of lymphocytes, and reduces the production of proinflammatory cytokines and chemokines by epithelial cells in response to *S. aureus* and purified TSST-1 [102,103]. GML, as vaginal gel, prevents lethality in rabbits challenged vaginally with purified TSST-1 [102]. The compound, however, is not stable in the presence of *S. aureus* and can be hydrolyzed by *S. aureus* esterase (lipase) into glycerol and lauric acid [99,101]. In an effort to overcome the limitation of inactivation, compounds with ether linkage have been suggested as potential alternatives to GML. Fatty ether compounds, such as 1-O-Dodecyl-*rac*-glycerol (DDG) (Figure 1.4),

inhibit TSST-1 production as well as *S. aureus* growth, and they are more stable than ester compounds (such as GML) to chemical and enzymatic hydrolysis [104,105]; however, their anti-toxin effects have not been fully characterized. Given that these compounds are inexpensive and easily accessible, they may provide clinical benefit as topical applications to prevent *S. aureus* infections. The characterization of the anti-toxin and immunomodulatory effects of GML and DDG is a major focus of this thesis study.

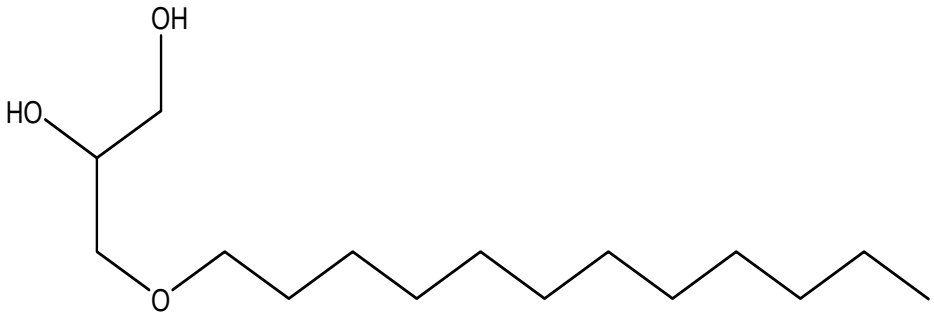
Based on the concern of SAgs, especially SEB, as bioterror weapons, high affinity Vβ antagonists that block the binding of SEB on T-cell receptors have been investigated as treatment for SAg intoxication [106]. The engineered protein competes with SEB for the particular TCR binding site to prevent SEB-mediated T-cell activation and lethality in rabbit intravenously administered with SEB.

Figure 1.4 Structures of glycerol monolaurate; GML (A) and dodecylglycerol; DDG (B). GML is a monoester of lauric acid (C-12), while DDG is the monoether counterpart of GML.

(A) GML



(B) DDG



1.4.3 Vaccines and immunotherapies

Currently, there is no U.S. Food and Drug Administration (FDA) approved vaccine or immunotherapy to prevent or treat *S. aureus* disease.

Antistaphylococcal vaccines and immunotherapies have been focused on the elimination of bacterial cells via targeting cell wall-associated staphylococcal components (Table 1.5) [107]. In theory, these vaccines would elicit functional antibodies that would bind to the target antigen(s) on the bacterial surface and trigger complement activation, which in turn augments the process of uptake and killing of bacteria by phagocytic cells such as neutrophils and macrophages. Despite promising experimental results in animal models (mostly in rodent models), this approach has not successfully provided effective clinical outcomes in phase II and phase III human clinical trials.

A later study testing passively immunization with antibodies to CP and ClfA in a mouse model of mastitis indicated that administration of either antibody alone significantly reduced the bacterial load, but induced the presence of stable unencapsulated *S. aureus* mutants and small colony variants, which are less susceptible to antibiotics. The emergence of the mutants was abrogated when both antibodies to capsular polysaccharide (CP) and clumping factor (ClfA) were administered together [108]. Another study of murine bacteremia and wound infection models indicated the expression of CP and ClfA are variable among strains and the *in vivo* environment [109].

In contrast, active and passive immunization with α-toxin was shown to prevent mice from lethal staphylococcal pneumonia, but did not decrease the bacterial load in

the lung tissue [8]. These studies suggested that a multi-antigen approach is likely necessary to ensure broad coverage against the versatile pathogen, *S. aureus*, and future approaches should include immunization against secreted virulence factors. One such approach is described in the future direction section of this thesis.

Table 1.5 Experimental staphylococcal vaccines and immunotherapies in clinical trials.

Bacterial target	Putative role in virulence	Proposed strategy (product/company)	Trial conclusion
Capsule polysaccharide (CP5 and CP8)	Avoidance of phagocytosis	Active immunization (StaphVAX/Nabi)	Vaccine efficacy (57%) only last up to 40 weeks after immunization in end-stage renal disease (ESRD) hemodialysis patients (n=1804). However, no significant protection was detected against bacteria in a confirmatory follow-up trial (n=3600).
		Human polyclonal antiserum (Altastaph/Nabi)	No reduction in preventing *S. aureus* bacteremia in very low-birth weight (<1500gm) neonates (n=206). No treatment effect in children ≥7 years with *S. aureus* bacteremia and persistent fever (n=40).
Clumping factor A (ClfA)	Attachment to fibrinogen and biomaterial surfaces	selected IVIG (INH-A21; Veronate/Inhibitex)	No prevention in mortality or the rates of late-onset sepsis in infants (n=1983).
		Humanized monoclonal antibody (Tefibazumab; Aurexis/Inhibitex)	No difference in composite clinical endpoints (a relapse, a complication, or death of bacteria) in hospitalized adult patients with bacteremia as an add-on therapy to standard therapy (n=63).

Table 1.5 Experimental staphylococcal vaccines and immunotherapies in clinical trials. (Cont'd)

Bacterial target	**Putative role in virulence**	**Proposed strategy (product/company)**	**Trial conclusion**
ATP-binding cassette transporter	Nutrient uptake and cell attachment	Human-derived single-chain variable antibody fragment (Aurograb/NeuTec)	Lack of efficacy as add-on therapy to vancomycin to treat deep-seated MRSA infections in phase II trial (Development terminated).
Lipoteichoic acid [a]	Bacteria cell wall component	Humanized monoclonal antibody (Pagibaximab/Biosynexus)	Reduced staphylococcal sepsis rate in very low-birth weight neonates (n=88).
Iron-regulated surface determinant B [a]	Iron uptake	Active immunization (V710/Merck)	Safety and efficacy in prevention of serious *S. aureus* infections in adult patients within 90 days after selective cardiothoracic surgery (n=76). A second trial similar trial in 198 patients with ESRD on chronic hemodialysis is in progress.

[a] There are still active clinical trials investigating the product.

Reference: [107]

1.5. Summary

S. aureus secrete an array of virulence factors, including cytolysins, SAgs, exoenzymes, and complement inhibitor proteins. Biochemical properties of these toxins indicate that most of them damage more than one cell type and directly or indirectly impede host immune function. Therefore, there is no doubt that these exoproteins secreted by *S. aureus* play important roles in its pathogenesis. However, only a few of them (i.e. SAgs and ETs) have been clearly shown to cause *S. aureus* disease.

The majority of anti-staphylococcal agents have predominately focused on the elimination of bacteria cells (antimicrobials). This strategy has neglected the contribution of secreted proteins to disease for decades. With the emergence of antibiotic-resistant *S. aureus* possessing multiple secreted virulence factors, the development of alternative approaches to ameliorate *S. aureus* disease is necessary. To date, most emphasis has been on vaccine and immunotherapy development targeted at cell wall-associated virulence factors. Based on previous successes with other bacterial pathogens, most companies have designed their vaccines by targeting one single cell wall-associated virulence factor. Their failure has been in part attributed to the functional redundancy of *S. aureus* virulence factors. *S. aureus* can easily down-regulate the targeted virulence factor and compensate its function using other virulence factors so that there is no significant change in pathogenicity. Multiple component strategies are therefore proposed. Recent studies with *S. aureus* exotoxins (i.e. α-toxin and superantigens) as vaccine targets, have shown promising protection from severe lung damage in a mouse pneumonia model, and indicate that secreted *S. aureus* toxins should

be considered as components of *S. aureus* vaccines or immunotherapies. However, due to the limited understanding of the overall roles these proteins play in *S. aureus* pathogenesis, there is still a debate on which protein(s) would provide clinically significant protection against *S. aureus* infections.

A few clonal types account for most *S. aureus*-associated diseases (both MSSA and MRSA) in the U.S. and around the world, indicating the fitness of these clonal types of *S. aureus* to their ecological niches. Proteomic studies comparing the differentially produced exoproteins by *S. aureus* USA300 and USA400 strains have provided valuable insights on the virulence of these two epidemic CA-MRSA clonal types. However, there is no study evaluating USA200 isolates, which are the predominant clonal type responsible for TSS. Although many of these proteomic studies determined the immunogenicity of *S. aureus* virulence factors *in vivo*, it is difficult to evaluate the role of these proteins in *S. aureus* pathogenesis from these studies.

Most *S. aureus* diseases initiate from breaches of the skin or mucosal surfaces, and secreted *S. aureus* exoproteins directly or indirectly contribute to a large part of the tissue damage, which leads to the dissemination of bacteria and its toxins. Therefore, studies on the interactions between *S. aureus* exoproteins and mucosal membranes should largely enhance the understanding of *S. aureus* pathogenesis. The knowledge gained from such studies will contribute significantly to the development of more effective antistaphylococcal agents to treat and/or prevent *S. aureus*-associated diseases.

1.6. Aims of This Work

My hypothesis is that *S. aureus* exoproteins, such as superantigens and cytolysins, play important roles in *S. aureus* mucosal pathogenesis by having direct (pro-inflammatory and/or cytotoxic) effects on epithelial cells, which enhances superantigens and/or bacterial cells penetration across mucosa and skin.

Given TSS is an important *S. aureus* disease caused by *S. aureus* and the inflammation caused by the cytolysin, α-toxin, has been shown to facilitate TSST-1 penetration across vaginal mucosa, the specific aims of this work are:

- To characterize globally the key exoproteins secreted by two TSS *S. aureus* isolates representing two different types of TSS, menstrual and pulmonary TSS, via their pro-inflammatory and cytotoxic effects on human vaginal epithelial cells.
- To assess the relationship between *S. aureus* toxin production, local inflammation, and mortality in a rabbit TSS model by comparing the effects of GML, a compound known to block α-toxin and TSST-1 production at sub-growth inhibitory concentrations and to modulate host innate immune response to exotoxins, to DDG, the lauric acid monoether counterpart of GML.

There are two short term goals of this work: (1) to identify *S. aureus* exoproteins that are pro-inflammatory and cytotoxic to epithelial cells, and (2) to demonstrate that minimizing local pro-inflammatory effects of *S. aureus* toxins (α-toxin and TSST-1), by either inhibiting toxin production or modulating host response, can prevent *S. aureus* TSS pathogenesis. A long term goal is to utilize these characterized proteins as targets to design effective prevention and/or treatment strategies for *S. aureus* disease.

Chapter II.

Pro-inflammatory exoprotein characterization of toxic shock syndrome *Staphylococcus aureus*

This chapter is submitted to Biochemistry for reviewing

2.1 Introduction

Staphylococcus aureus is the most common cause of ventilator-associated pneumonia, surgical site infections, and catheter-associated bloodstream infections in the healthcare setting as well as a frequent cause of skin and pulmonary infections in the community [110]. About 30% of people are colonized by *S. aureus* in their nose and skin, which are the most common sites of colonization, and up to 35% of the colonizing strains are methicillin-resistant *S. aureus* (MRSA) [13]. *S. aureus* carriers have three-fold higher risk for *S. aureus* infections than non-carriers, with a majority (80%) of the infections being caused by the patient's own endogenous strains on mucosal surfaces and skin [111]. Pulsed-field gel electrophoresis (PFGE) clonal type USA200 is the most widely disseminated methicillin-sensitive *S. aureus* (MSSA) colonizer of the nose, a cause of invasive nosocomial (healthcare-associated) infections, and the major cause of staphylococcal toxic shock syndrome (TSS) [112,113].

S. aureus initiates illnesses at mucosal surfaces or skin where numerous exotoxins and other secreted virulence factors participate. Staphylococcal superantigens (SAgs), especially TSS toxin-1 (TSST-1), mediate severe diseases such as TSS, an acute onset systemic disease with multiple organ involvement. TSS has been associated with vaginal colonization of *S. aureus* that produce superantigens, especially TSST-1, during menstruation, or as a complication of influenza with secondary *S. aureus* infections. Approximately 25% of nasal isolates and 5% of vaginal mucosal isolates possess, *tst*, the gene that encodes TSST-1, and more than 80% of these isolates are USA200 [112,114].

In addition to superantigens, the cytolysins, notably α-toxin (α-hemolysin;

encoded by gene *hla*), have also been shown to play important roles in *S. aureus* illnesses. α-Toxin was recently determined to cause significant lung tissue damage in a murine staphylococcal pneumonia model, a system that does not assess superantigen effects [8]. α-Toxin is highly pro-inflammatory and cytolytic to various mammalian cells, including erythrocytes, monocytes, endothelial cells, and epithelial cells [115]. Our research group has previously demonstrated that α-toxin facilitates TSST-1 penetration of porcine vaginal mucosa by induction of pro-inflammatory responses within the mucosal barrier [9]. However, 75% of menstrual TSS vaginal isolates are considered unable to produce α-toxin due to a defective α-toxin gene, *hla*$^{-}$, which has a nonsense point mutation (CAG [Glu] to TAG [stop codon]) at codon 113 [52]. These menstrual TSS strains were also noted to produce lower quantities of lipase, nuclease, and overall hemolysins than isolates from skin [51].

Proteomic approaches have been used to facilitate our understanding of *S. aureus* pathogenesis and to identify potential targets for therapeutic development. However, most proteomic studies to date focused on MRSA strains associated with community-associated infections, USA300 and USA400, or laboratory strains [116,117]. Although important, these studies mainly focused on identifying proteins differentially expressed by strains, but provided limited information about the relative biological contribution of these exoproteins to *S. aureus* pathogenesis.

2.2 Objectives

Given the prior knowledge that inflammation and direct disruption of the mucosa facilitates superantigen penetration through mucosal surfaces, we designed this study, combining biological testing and proteomic approaches to identify key exoproteins produced by *S. aureus* that are responsible for pro-inflammatory and cytotoxic effects to epithelial cells. Two representative *S. aureus* USA200 isolates, which cause distinguishable types of TSS, menstrual TSS and post-influenza pulmonary TSS, were selected for characterization of their exotoxin profiles which likely contribute to their respective diseases.

2.3 Materials and Methods

Bacteria. Two USA200 isolates, MNPE and CDC587, were used in this study. Both strains are methicillin-susceptible *S. aureus* (MSSA). *S. aureus* MNPE was isolated from a post-influenza pulmonary TSS case in 1987, most likely originating from a skin source [118], and CDC587 is a typical menstrual vaginal TSS strain isolated from a patient in 1980 [119]. The strains were maintained in Dr. Schlievert's laboratory (lyophilized; low passage). Polymerase chain reaction (PCR) [21] revealed that MNPE carries the superantigen genes for TSST-1, SEA, SEC, SEl-G, SEI, SEl-K, SEl-L, SEl-M, SEl-N, SEl-O, and SEl-P, while CDC587 also carries the genes for SEA, SEC, SEl-G, SEI, SEl-K, SEl-L, SEl-M, SEl-N, SEl-O, and SEl-P. The major difference between the two strains is that CDC587 possesses a defective α-toxin gene (*hla*$^-$), while MNPE carries a wild-type α-toxin (*hla*$^+$). Both strains have the γ-toxin gene (*hlg*$^+$). Two additional menstrual vaginal TSS MSSA USA200 isolates, MN8 and Harrisburg, which also carry *hla* with the stop codon (*hla*$^-$), were used to confirm and quantify α-toxin production in some experiments.

Porcine *ex vivo* vaginal mucosal experiments. Porcine vaginal mucosal tissue was used in initial experiments of *S. aureus* toxicity, as it was previously reported to be histologically and structurally similar to human vaginal mucosa [120]. Specimens of normal porcine vaginal mucosa were excised from pigs (0.3-3 years old) at slaughter and transported to the laboratory in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 international unit [IU]/ml), streptomycin (100 μg/ml) and

amphotericin B (2.5 μg/ml). The culture media and reagents were all from Gibco, Invitrogen (Carlsbad, CA).Tissue was utilized within 3 h of excision. Tissue samples of uniform size were obtained from porcine vaginal tissue using a 5 mm biopsy punch. Tissue plugs were washed in serum-free and antibiotic-free media 3 times. The plugs were then placed mucosal side up on 0.4 μm cell culture inserts (Becton Dickinson Labware, Franklin lakes, NJ) in 6-well tissue culture plates containing 10% FCS and antibiotic-free RPMI 1640 (Invitrogen). The mucosal surface of each was continually exposed to air. The mucosal surface of each was challenged with 10^7 colony-forming units (CFU) of bacteria suspended in RPMI. At 24 h, samples were fixed in 10% phosphate-buffered formalin for histological examination by hemotoxylin and eosin staining.

Exoprotein preparation and separation. Bacteria were grown overnight in beef heart medium supplemented with 1% glucose-phosphate buffer (2 liters, made in-house) in ambient air with aeration (200 rpm/min) at 37 ˚C, which favors exotoxin production [79]. Bacterial cells were removed by centrifugation (3,220g, 10 min at 4 ˚C). Proteins in the supernates were precipitated overnight at room temperature by ammonium sulfate (80% final concentration). Precipitates were collected by centrifugation (3,220g, 15 min at 4 ˚C) and re-solubilized in water. Excess salts and proteins with molecular weights smaller than 14 kDa were removed by dialyzing samples against sterile distilled water (SDW) (Spectra/Por 4 RC dialysis tubing, Spectrum, Rancho Dominguez, CA). Insoluble materials were removed by centrifugation at 8000g for 30 min. Samples (75-100 ml)

were mixed with Sephadex G75 (pre-washed with alcohol, Sigma, St. Louis, MO) to generate a thick gel. Thin-layer isoelectric focusing (IEF) in pH gradients of 3-10 (Ampholytes 2.5 ml/gel; Bio-rad, Hercules, CA) were used to separate proteins into 15 fractions. The IEF were run at 1000V, 8W, and 20mA for 24 h. The fractions were eluted with water and passed through syringes stuffed with fiberglass (8 μm) to remove Sephadex. pH of the fractions was measured following IEF separation, and then samples were dialyzed against SDW for 3 days to remove ampholyte buffers prior to lyophilization. The lyophilized protein fractions were dissolved in SDW and the protein concentrations were estimated by the Bradford method (Bio-rad) [121]. A secondary IEF (pH gradient 7-9; Sigma) was applied to further separate fractions 12-15 from the primary IEF. IEF of the MNPE fractions was separated into 15 fractions, while CDC587 IEF was separated into 12 fractions according to the visual protein patterns observed in the gel matrix (fractions 4-15).

Protein compositions of the fractionated proteins (about 250 ng loaded) were determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 100V for 1 h) and silver-staining (Invitrogen). According to the protein patterns on the SDS gels, selected fractions were further resolved by reverse phase high performance liquid chromatography (rHPLC). The rHPLC was done on a Hewlett-Packard AminoQuant 1090L HPLC system with an Agilent Zorbax SB-CN (4.6 x 150 mm; 5 μm) column. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water. Solvent B was 0.1% (v/v) TFA in acetonitrile (ACN). The flow rate was regulated at 1 ml/min. Gradient conditions were as followed: 0.0% B for 2 min, and

then the percentage of B was increased to 60% over 30 min. Eluents were monitored at 215 nm. A typical load was 0.1 mg/ml of protein in water. All peaks obtained via rHPLC were collected, lyophilized to remove acetonitrile, and re-solubilized in SDW or phosphate-buffered saline (PBS) for further analyses.

Mammalian cell bioassays. Immortalized human vaginal epithelial cells (HVECs; ATCC CRL-2616) were maintained as described previously [122]. For all HVEC experiments, confluent monolayers of HVECs in 96-well plates were switched to antibiotic-free keratinocyte serum-free media (KSFM; Invitrogen) the day before experimentation. Protein fractions (2 μg/fraction in 100 μl KSFM, unless otherwise noted) were added to the cell culture medium and incubated with HVECs at 37˚C in the presence of 7% CO_2 for 19-24 h. At the end the experiments, the media were collected and stored at -20˚C until cytokine measurement. Interleukin-8 (IL-8) concentrations in the media were analyzed by enzyme-linked immunosorbent assay (ELISA) using human Quantikine kit (R and D Systems, Minneapolis, MN). This cytokine was used as a measure of pro-inflammatory activity since IL-8 only target for chemo-attraction is polymorphonuclear leukocytes (PMNs), the dominant mediators of acute inflammation. Cell viability was estimated by CellTiter 96 aqueous one solution cell proliferation assay (Promega, Madison, WI) according to manufacturer's instructions. All experiments were performed in duplicates.

Protein analysis via mass spectrometry. Proteins (about 3 μg) obtained from the

rHPLC samples were digested by trypsin, and peptide fragments were detected by tandem mass spectrum (MS/MS). Briefly, protein samples dissolved in water were subjected to in-solution trypsin digestion by trypsin 12 ng/ul (Promega, Madison, WI) in 25 mM ammonium bicarbonate/5 mM calcium chloride at 37°C for 10 h. The digestion was terminated with the addition of formic acid to a final 0.1% v/v. Digested peptides were injected in a Michrom BioResources Paradigm 2D capillary LC system (Michrom Bioresources, Inc., Auburn, CA) online with LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), a linear ion trap. Briefly, samples were loaded on a Paradigm Platinum Peptide Nanotrap (Michrom Bioresources, Inc) precolumn (0.15 × 50 mm, 400-μL volume) and subsequently switched in-line with microcapillary columns (75 μm internal diameter, 12 cm in length) at a flow rate of approximately 350 nl/min. The capillary column was packed in-house with Magic C18 AQ reversed-phase material (5 μm, 200 Å pore size C18 particles; Michrom BioResources, Inc). Peptides were eluted with a linear gradient with 100% solvent A (95:5 water: ACN, 0.1% formic acid), to a final solvent B (5:95 water: ACN, 0.1% formic acid). An electrospray voltage of 2250V was applied distal to the analytic column. The LTQ was operated in the positive-ion mode using data-dependent acquisition methods initiated by a survey MS scan which was followed by MS/MS (collision energy of 35%) on the 4 most abundant ions detected in the survey scan. M/Z values selected in the survey scan for MS/MS were excluded for sub-sequential MS/MS with a dynamic exclusion from further data-dependent MS/MS for 30 sec. The signal intensity threshold for an ion to be selected for MS/MS was set to a lower limit of 1000.

All MS/MS samples were analyzed using Sequest (ThermoFinnigan; version 27, rev. 13) and X! Tandem (www.thegpm.org; version 2007.01.01.1) searching the staphylococcus_NCBI_952306_CTM database assuming trypsin digestion. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 0.80 Da. The Sequest search parameters were: carbamidomethyl modification of cysteine and differential for oxidation of methionine with 2 trypsin miscleavaged sites allowed. Scaffold (version Scaffold-02_02_03, Proteome Software Inc, Portland, OR) and ProteinPilot software version 3.0 was used to validate MS/MS-based peptide and protein identifications. Protein identifications were accepted if they had unused ProtScore equal or greater than 4 and at least 2 identified peptides with at least 99% probability.

γ-Toxin cloning and purification. γ-Toxin gene *hlg* from *S. aureus* was cloned into plasmid pCE104 [123], which contains pUC18, and heat-shock transformed into *E. coli* XL-2-blue (Stratagene, Cedar Creek, TX). The *E. coli* was grown in Luria-Bertani (LB) broth supplemented with 50 μg/ml ampicillin at 37 ˚C overnight with 300 rpm shaking. *E. coli* cells were harvested from 1 liter suspension culture by centrifugation at 3,220g for 15 min at 4 ˚C. The pellets were washed once with 50 ml PBS and the bacterial cells were lysed with 25 ml of Tris-EDTA buffer with 1mg/ml lysozyme (Sigma). Cell debris was removed by centrifugation at 8000g for 30 min at 4 ˚C. Supernates were dialyzed overnight against SDW. Precipitates (if formed after dialysis) were removed by additional centrifugation.

Generation of rabbit anti-α-toxin and anti-γ-toxin sera. Dutch belted rabbits were immunized with 10 μg of the highly-purified α-toxin (from MNPE rHPLC peak 6) or γ-toxin extracted from *E. coli* (crude cell lysates dialyzed against water) with Freund's incomplete adjuvant at times 0, day 14, and day 28. All animal experiments were performed in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Rabbit blood was obtained on day 35, and the anti-α-toxin and anti-γ-toxin antisera were separated from blood and filter-sterilized with 0.2 μm filters. The antisera were used in hemolysin bioassay experiments for neutralization and for Western immunoblotting.

Hemolysin bioassays. Hemolytic activity was estimated on slides containing human or rabbit erythrocytes as described previously [102]. Briefly, rabbit or human erythrocytes were washed three times with PBS. Microscope slides were coated with 4 ml of 0.8% agarose in PBS mixed with 0.3 or 0.6% erythrocytes (final concentration). Samples (2.5 μg of total proteins) were added into wells (4 mm diameter) on the erythrocyte slides and incubated at 37 ˚C for 4 h. In the α-toxin and γ-toxin neutralization assays, overnight culture supernates (5 μl) were mixed with 3-fold anti-α-toxin or anti-γ-toxin sera or PBS (15 μl) in the wells. The slides were incubated at 37 ˚C overnight. Zones of lysis (clear zones) represent the amount of hemolysins (not neutralized) in the sample.

α-Toxin Western immunoblotting. *S. aureus* overnight culture supernates were treated with 4 volumes of absolute ethanol to precipitate proteins and the resultant precipitated

proteins re-solubilized as 20-fold concentrated samples [102]. The samples (10 μl each) were mixed 1:1 with sample loading buffer and electrophoresed on 12% SDS-PAGE (100V for 1 h), transferred onto polyvinylidene fluoride (PVDF; Bio-rad) membranes (100mA for 1.5 h), and immunoblotted with the anti-α-toxin serum (1:2500 dilution). Proteins were detected by chemiluminescence with SuperSignal West Dura Extended Duration substrate (Pierce) on films (Thermo Scientific).

Rabbit model for α-toxin lethality. Ethics statement: All animal experiments were performed in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Studies were performed in rabbits to determine the *in vivo* biological function of wild-type α-toxin (MNPE) versus mutant α-toxin (CDC587), where approximately 1 μg of α-toxin administered intravenously induces death in a rabbit within 15 min. This is a well-characterized activity of α-toxin [115], the only known staphylococcal protein with such activity. Rabbits (3/group; 12 total rabbits) were injected intravenously with 1 μg purified α-toxin, 2 ml of 0.2 μm filter-sterilized supernate from MNPE, 2 ml of 1× concentrated supernate from CDC587, or 2 ml of 10× concentrated supernate from CDC587. The CDC587 supernate was concentrated 10× by air-drying to 1/10 the original volume, followed by dialysis 24 h against PBS to remove excess salts. Animals were monitored up to 1 h for lethality. At the end of the experiment, animals were euthanized by barbiturate overdose (intravenous injection).

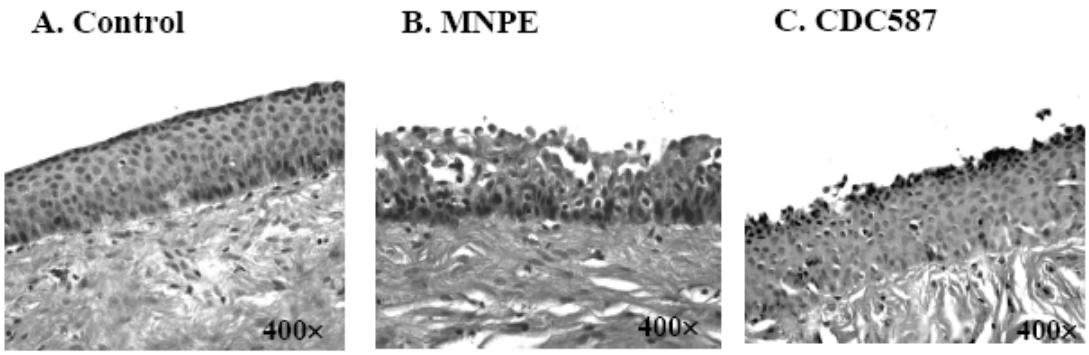
Calculations. Pro-inflammatory activity of an individual fraction was calculated by dividing IL-8 induction by the amount of the protein used in the assay (IL-8 induction/μg of protein). The total of pro-inflammatory activity in the fraction (IL-8/μg of protein × total protein in the fraction) is compared to the sum of the total pro-inflammatory activity from all the fractions (in the same IEF experiment).

2.4 Results

2.4.1 Effects of *S. aureus* (MNPE and CDC587) on *ex vivo* porcine vaginal mucosa.

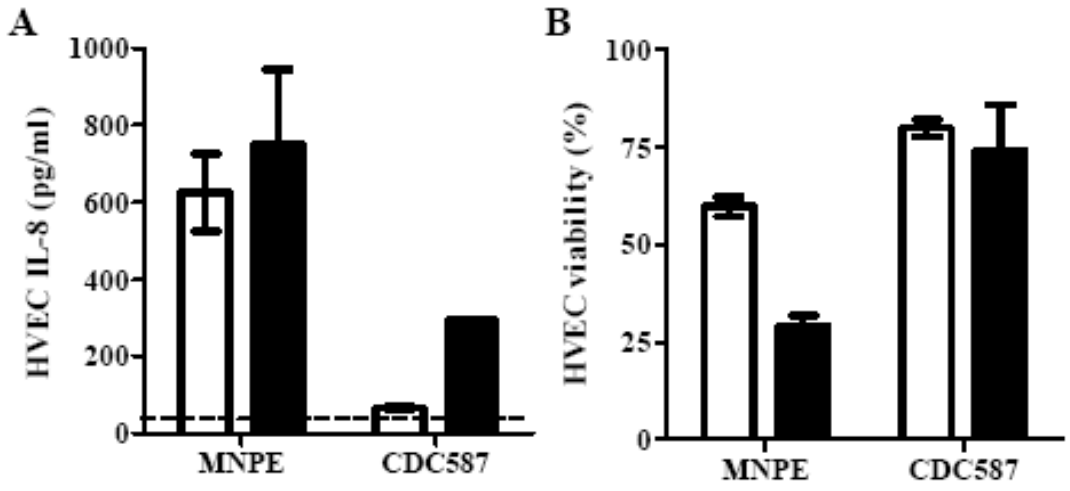
Nearly all *S. aureus* strains induce disease beginning on mucosal and skin surfaces, unless traumatically implanted into the bloodstream. Studies have been initiated in attempt to identify factors that facilitate disease causation, typically focusing on specific virulence factors. In an effort to take a more global approach, we assessed the virulence of two common USA200 *S. aureus* strains on a mucosal surface, beginning with porcine vaginal mucosa inoculated with *S. aureus* MNPE and CDC587 for 24 h. The surface of the porcine vaginal mucosa, like its human counterpart, is lined with stratified squamous epithelium. The surface epithelium of untreated control tissue remained intact, whereas the infected tissue showed signs of disruption (Fig 2.1). The surface epithelium damage in the MNPE infected tissue was several layers deep, and the epithelium exhibited signs of breakdown, whereas the damage due to CDC587 was minor and remained localized to the outermost layers of the infected tissue. Our prior studies suggest that these virulence factors are likely to be secreted exoproteins, rather than cell-surface associated factors (proteins) [35]. Therefore, this histological analysis suggests that MNPE produced more vaginal tissue-damaging virulence factors than CDC587.

Figure 2.1 Effects of *S. aureus* on *ex vivo* porcine vaginal epithelium. The tissue was inoculated with 10^7 CFU *S. aureus* for 24 h and stained with hematoxylin and eosin. (A) Control, (B) *S. aureus* MNPE, (C) *S. aureus* CDC587.



2.4.2 Differences in *S. aureus* (MNPE and CDC587) exoprotein profiles and induction of IL-8 from human vaginal epithelial cells. To examine our hypothesis that exoproteins contributed to the differences of *S. aureus* MNPE and CDC587 damage to vaginal mucosal tissue, total exoproteins (ammonium sulfate-precipitated) in overnight culture supernates from MNPE and CDC587 were compared for their pro-inflammatory and cytotoxic effects on HVECs. IL-8, as a measure of pro-inflammatory potential, was increased approximately 19-fold (752.8 pg/ml) and 7-fold (294.8 pg/ml), compared to media controls, for MNPE and CDC587 total exoprotein (20 μg/ml; 2 μg of total protein/well), respectively (Fig. 2.2A). The corresponding survival of the HVECs to MNPE and CDC587 exoproteins (20 μg/ml) was 29% and 74%, respectively (Fig. 2.2B). Therefore, MNPE exoproteins were both more pro-inflammatory and more cytotoxic to HVECs than CDC587 exoproteins.

Figure 2.2 Effects of *S. aureus* exoproteins on human vaginal epithelial cells (HVECs). HVEC IL-8 production (A) and viability (B) caused by different concentrations of *S. aureus* MNPE and CDC587 exoproteins. Open bars represent 2 μg/ml; closed bars represent 20 μg/ml; Dashed line represents the IL-8 level in the media control; Error bars represent the ranges of the test values.



2.4.3 Distributions of biological activity for *S. aureus* (MNPE and CDC587) exoproteins. In an effort to identify the major MNPE and CDC587 exoproteins that were contributing to the increased production of IL-8 and cytotoxicity, ammonium sulfate-precipitated exoproteins from overnight culture supernates were separated by thin-layer IEF. The initial IEF (pH 3-10) separated MNPE and CDC587 exoproteins into 15 fractions, and fractions 12 to 15 were determined to have the majority of biological activity (Fig. 2.3). MNPE fractions 12 to 15, corresponding pH 6.3 -9.4, (20 μg/ml, 2 μg total protein/well) increased IL-8 production from HVECs by 5-15 fold, which accounted for more than 90% of the total pro-inflammatory activity (Fig. 3A). Corresponding CDC587 fractions 12-15, pH 6.4 – 10.1, 20 μg/ml, were less pro-inflammatory to HVECs (1-2 fold increase of IL-8) than MNPE fractions. These fractions from CDC587 contained 73% of the total pro-inflammatory activity for CDC587 exoproteins (Fig. 2.3B).

Since the majority of biological activity localized to a neutral to basic pH, second thin-layer IEFs of (pH 7-9) were applied to further separate proteins within the 12-15 fractions. The distributions of pro-inflammatory and cytotoxic activity of the fractionated MNPE and CDC587 samples by the second IEF are depicted in Fig. 2.4A and 2.4B, respectively. At the concentration of 20 μg/ml (2 μg total protein), all MNPE fractions were highly cytotoxic (>50% cytotoxicity; data not shown). Therefore, the relative pro-inflammatory responses of the fractions were compared at 2 μg/ml (0.2 μg total protein). MNPE fractions 10-15 (from the pH 7-9 IEF) contained most of the biological activity: fractions 10-13 (pH 7.8-8.4) were highly pro-inflammatory and

contained 82% of total inflammatory activity, while fractions 14-15 (pH 8.5-9.2) were cytotoxic and less inflammatory (contained 0.7% of the total pro-inflammatory activity) (Fig. 2.4A). At 20 μg/ml (2 μg total protein) CDC587 fractions from the pH 7-9 IEF were not highly inflammatory, inducing approximately 2-fold increases in IL-8 production. Fractions 11-15 (pH 7.6-9.0) from CDC587 were highly cytotoxic (18-51% cell viability), but not highly pro-inflammatory to HVECs (Fig. 2.4B). These fractions accounted for 87% of the total pro-inflammatory activity of all pH 7-9 IEF CDC587 fractions. The CDC587 fractions were neither pro-inflammatory nor cytotoxic (>50% cell survival) at the concentration of 2 μg/ml (data not shown).

Rabbit red blood cells are highly sensitive to both α- and γ-toxin, while human red blood cells are more sensitive to γ-toxin than α-toxin (> 50-fold different) [124]. In general, MNPE fractions from the pH 7-9 IEF were more hemolytic (another measure of cytotoxic activity) to rabbit erythrocytes than human erythrocytes (Fig. 2.4C), consistent with the activity of α-toxin. In contrast, CDC587 fractions pH 7-9 IEF (at the same total protein concentration) were hemolytic to both rabbit and human erythrocytes at similar levels, suggestive of the presence possibly of both α- and γ-toxins, although overall they were not as hemolytic as MNPE pH 7-9 IEF fractions (Fig. 2.4D). These observations suggest that MNPE and CDC587 produce different amounts and types of hemolysins (MNPE α-toxin vs. CDC587 possibly α- and γ-toxins), which in part may explain why MNPE fractions were more pro-inflammatory and cytotoxic than CDC587 fractions. Fractions from the pH 7-9 IEF were grouped and further resolved by rHPLC (Appendix A1) based on their protein patterns appearing on silver-stained SDS-PAGE gels (data not

shown). The grouping was to maximize the amount of proteins recovered from rHPLC. Only rHPLC peaks with more than 10 μg total proteins were tested for biological activity and evaluated for protein identification by mass spectrometry. These were 6 peaks from MNPE pH 7-9 IEF fractions being tested: peaks 1 to 3 from fractions 4-7 (pH 6.8-7.5, 3 out of 7 total peaks recovered [3/7]), peaks 4 and 5 from fractions 10-12 (pH 7.8-8.2, 2/2), and peak 6 from fractions 13-15 (pH 8.4-9.2, 1/2) (Fig. 2.5); and there were 5 peaks from CDC587 pH 7-9 IEF fractions being tested: peaks 1 and 2 from fractions 7-10 (pH 6.1-7.2, 2/5), and peaks 3 to 5 from fractions 13-15 (pH 8.1-9.0, 3/4) (Fig. 2.6). One peak from CDC587 pH 7-9 fractions 7-10 was determined to contain 20 μg total protein by Bradford protein assay but could not be detected by silver-stained SDS-PAGE. Therefore, the peak was also excluded from further assays. None of the peaks recovered from CDC587 fractions 11-12 (pH 7.6-8.0) were pro-inflammatory to HVECs at the concentration of 20 μg/ml. Therefore, these peaks were not shown.

All peaks were individually examined by mass spectrometry, and most proteins were detected in multiple samples. MNPE peaks contained 3 major proteins, α-toxin, SEC, and TSST-1 (Fig. 2.5A). MNPE peaks 2, 5, and 6 were highly pro-inflammatory to HVECs at concentrations higher than 2 μg/ml, while peak 3 was highly pro-inflammatory at a concentration of 20 μg/ml (Fig. 2.5B). Peak 3 had relatively lower protein contains compared to other peaks (as indicated in Fig. 2.5A), which indicate the pro-inflammatory activity may be in part contributed by non-protein contents. At the concentration of 20 μg/ml, peaks 3, 5, and 6 were relatively more cytotoxic to HVECs than other peaks, although all of them had > 50% cell survival (Fig. 2.5C). In contrast,

CDC587 peaks contained more varieties of proteins than MNPE peaks (Fig. 2.6A). Peaks 3 and 4 were highly pro-inflammatory at the concentration ≥ 2 μg/ml, whereas peak 5 was only highly pro-inflammatory at the concentration of 20 μg/ml (Fig. 2.6B). These peaks tended to be more cytotoxic than others peaks in the group, but all of them had >50% cell survival (Fig. 2.6C).

Figure 2.3 Distribution of biological activity in *S. aureus* pH 3-10 IEF fractions 1 to 15. HVECs were incubated with 20 μg/ml of fractionated exoproteins from MNPE (A) or CDC587 (B). Bars represent HVEC viability, and the line represents relative IL-8 secreted in the supernates compared to media control (fold-change).

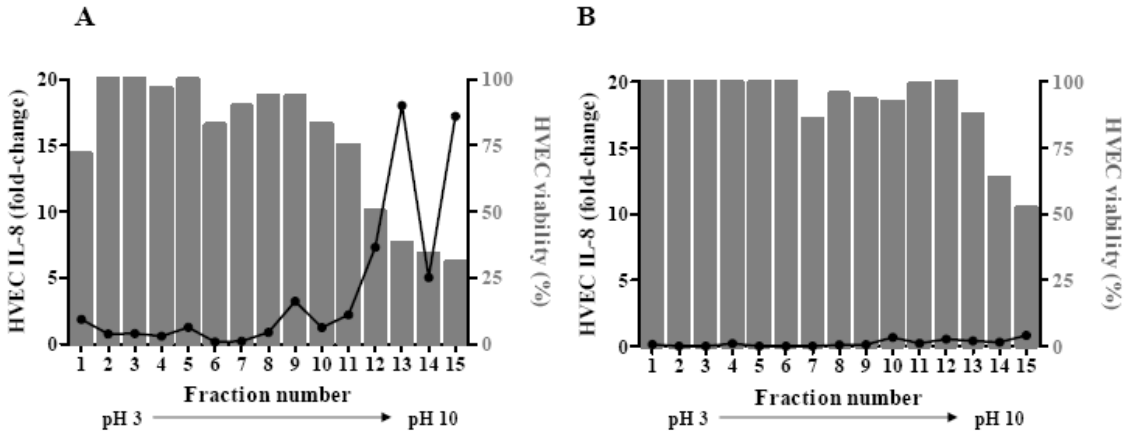


Figure 2.4 Distribution of biological activity from *S. aureus* pH 7-9 IEF fractions 4-15. HVECs were incubated with 2 μg/ml of MNPE proteins (A) or 20 μg/ml of CDC587 proteins (B) fractionated from IEF pH 7-9. Bars represent HVEC viability, and the line represents relative IL-8 secreted in the supernates compared to growth control. Hemolytic ability of 2.5 μg fractionated MNPE (C) and CDC587 (D) to human (closed bar) and rabbit (open bar) red blood cells.

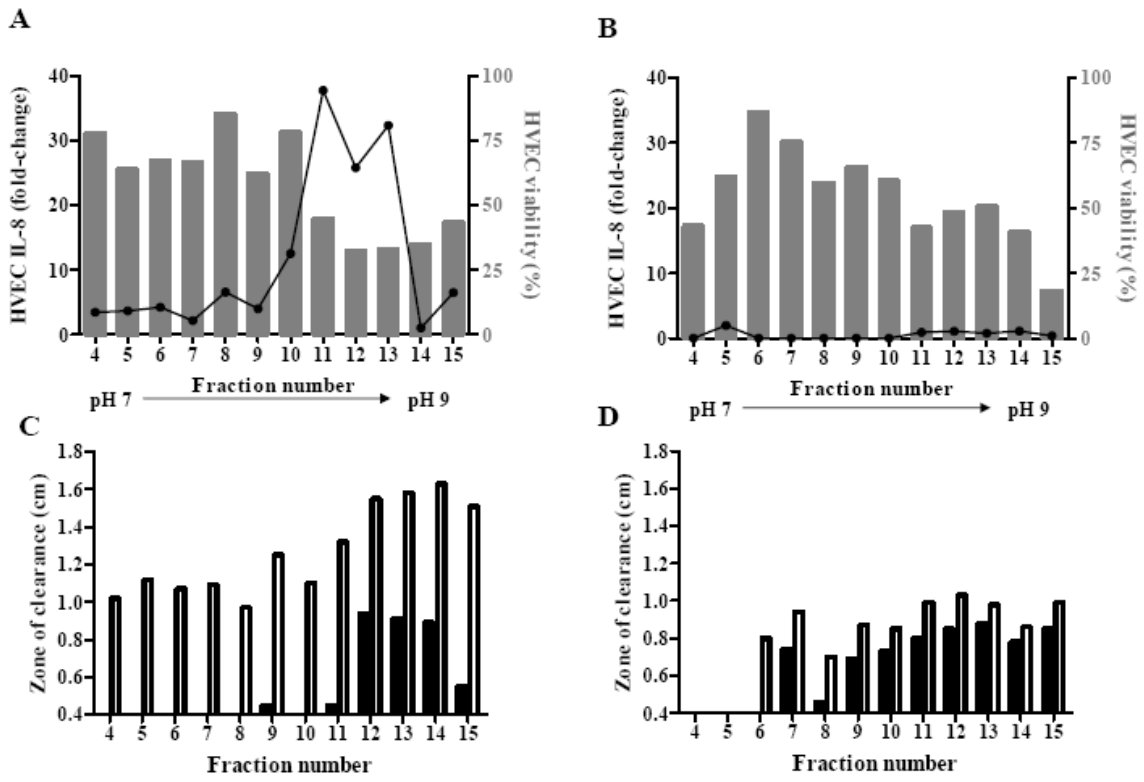


Figure 2.5 Proteins and biological activity in peaks of MNPE pH 7-9 IEF fractions after rHPLC separation. (A) Peaks from rHPLC (approximately 250 ng) separated by 12% SDS-PAGE. Proteins on the gel were identified according to molecular mass and mass spectrometry of the rHPLC peaks. (B) IL-8 production by HVECs treated with MNPE rHPLC peaks. (C) Viability of HVECs treated with MNPE rHPLC peaks. (open bar, 0.2 μg/ml; gray bar, 2 μg/ml; closed bar, 20 μg/ml). Error bars represent the ranges of the test values.

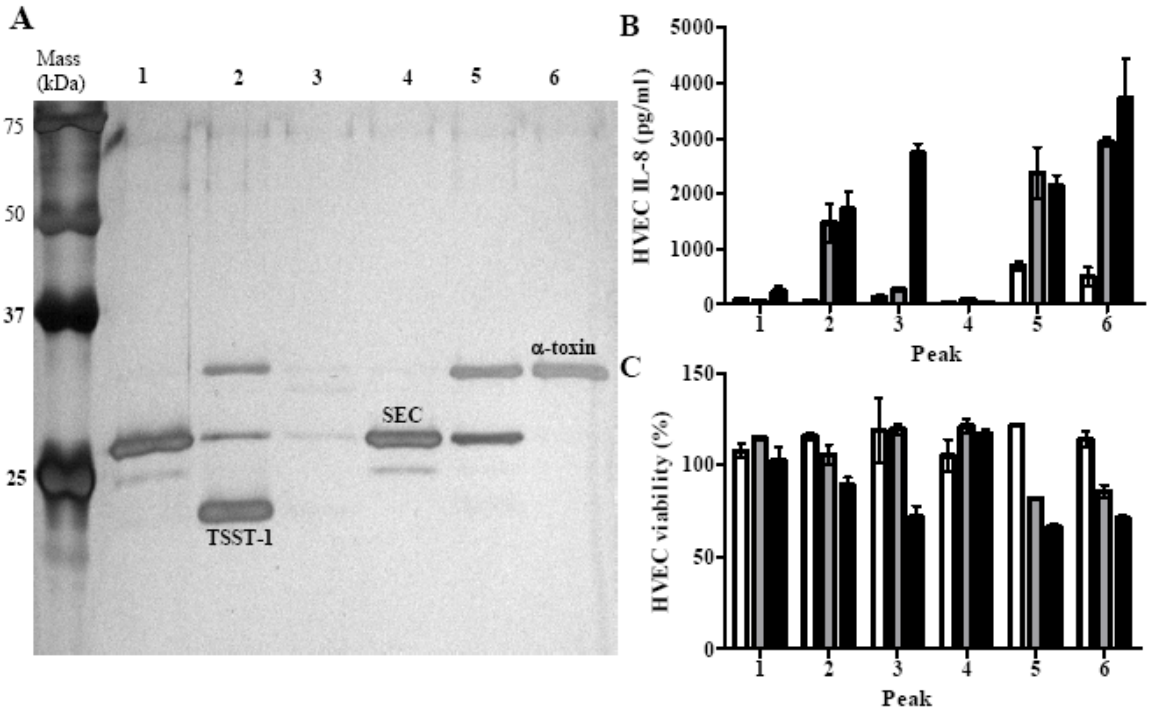
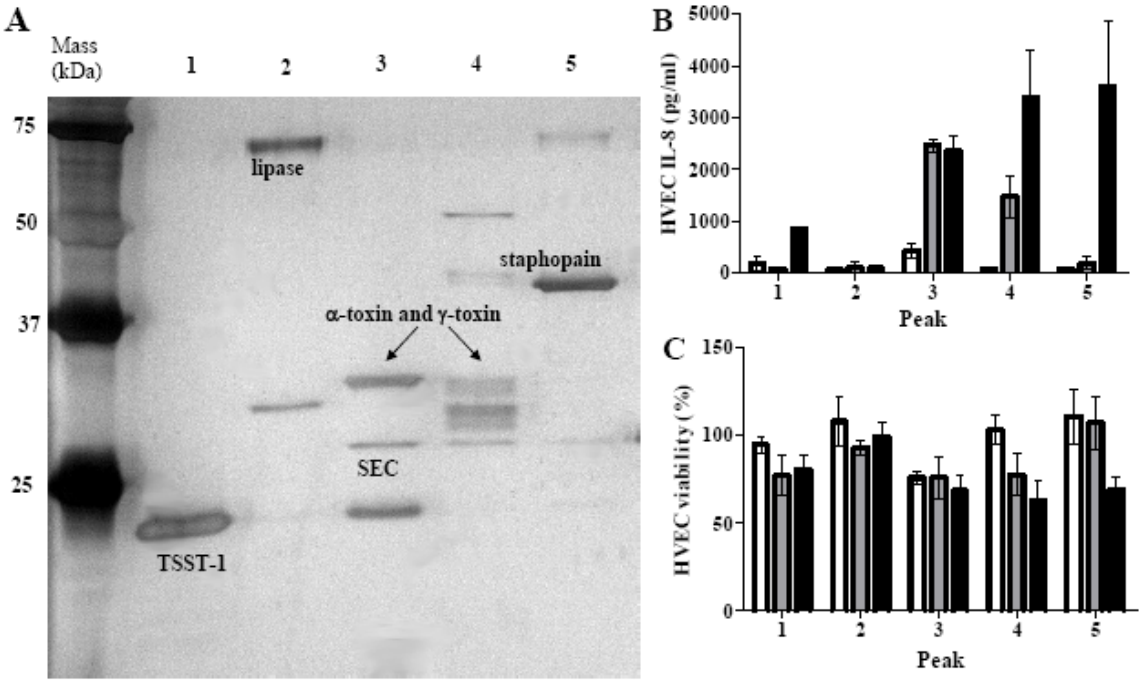


Figure 2.6 Proteins and biological activity of CDC587 rHPLC peaks from pH 7-9 IEF fractions. (A) Peaks from rHPLC (approximately 250 ng) separated by 12% SDS-PAGE. Proteins on the gel were identified according to molecular mass and mass spectrometry of the rHPLC peaks. (B) IL-8 production by HVECs treated with CDC587 rHPLC peaks. (C) Viability of HVECs treated with CDC587 rHPLC peaks. (open bar, 0.2 μg/ml; gray bar, 2 μg/ml; closed bar, 20 μg/ml). Error bars represent the ranges of the test values.



2.4.4 Identification of USA200 *S. aureus* exoproteins associated with virulence via mass spectrometry. Proteins in the rHPLC peaks were treated with in-solution digestion and identified via mass spectrometry analyses. Multiple proteins were detected in each sample, and most proteins were detected in multiple samples. Tables 2.1 and 2.2 list the total virulence-associated proteins (exoenzymes and exotoxins) detected for the MNPE and CDC587 peaks, respectively.

Fewer total proteins were identified from MNPE peaks than CDC587 peaks. There were 9 virulence-associated proteins detected from MNPE peaks, including those hypothesized to be involved in staphylococcal pathogenesis, such as lipase, nuclease, staphylococcal complement inhibitor, staphylokinase, staphopain, serine protease, α-toxin, TSST-1, SEC, and possibly other enterotoxin(s) (Table 2.1). The density of the bands at specific molecular weights on SDS-PAGE gel suggested that TSST-1, SEC, and α-toxin were made in higher concentrations than other exoproteins (Fig. 2.5A). In contrast, there were 24 virulence-associated proteins identified from CDC587 peaks (Table 2.2). In addition to those virulence-associated exoproteins identified in MNPE peaks, there were chemotaxis inhibitor protein CHIPS, MHC II-like molecule, and 1-phosphatidylinostitol phosphodiesterase, signal transduction protein TRAP, secretory antigen ssaA-like protein, and staphylococcal accessory regulator T. There were two subtypes of lipases, lip1 and lip 2, detected in the fractions. Cell wall-associated virulence proteins including iron-regulated hemo-iron binding protein, fibronectin-binding protein, and a surface protein with 5'-nucleotidase were also detected. Exotoxins including phenol soluble modulin, γ-toxin, TSST-1, SEC, and

α-toxin were also identified from CDC587 (Table 2.2).

α-Toxin (highly purified in MNPE peak 6) was therefore identified as the single most pro-inflammatory and cytotoxic protein purified (Fig 2.5). SEC (highly purified in MNPE peaks 1 and 4) was found to be less pro-inflammatory than TSST-1 (highly purified in CDC587 peak 1) to HVECs at the concentration up to 20 μg/ml (Fig 2.6). Pro-inflammatory activity of CDC587 peaks 3 and 4 were likely induced by the mixtures of α-toxin and γ-toxin. Pro-inflammatory activity of CDC587 peak 5 was likely due to staphopain (Fig. 2.6).

Table 2.1 Virulence factors recovered from *S. aureus* MNPE

Protein (gene)	Accession	**MW**	**pI**
Staphylococcal complement inhibitor (*scn*)	gi|88195845	13,052	9.3
Staphylokinase precursor (*sak*)	gi|49484186	18,474	6.8
Thermonuclease precursor (*nuc*)	gi|88194577	25,120	9.3
Toxic shock syndrome toxin-1 (*tst*)	gi|15927587	26,447	8.8
Enterotoxin type C3 (*sec3*)	gi|15927585	30,671	8.2
α-toxin precursor (*hla*)	gi|15926746	35,975	8.7
Staphopain A (*scpA*)	gi|49484150	44,048	9.6
Probable serine protease	gi|82751315	45,764	9.2
Lipase (*lip*)	gi|88196625	76,675	7.1

Table 2.2 Virulence factors recovered from CDC587

Protein (gene)	Accession	**MW**	**pI**
Phenol soluble modulin	gi|49483337	4,496	4.8
Staphylococcal complement inhibitor (*scn*)	gi|88195845	13,052	9.3
Staphylococcal accessory regulator A (*sarA*)	gi|88194390	14,718	7.8
MHC class II analog protein	gi|88194675	15,838	9.3
Chemotaxis inhibitory protein (*chp*)	gi|88195846	17,040	9.6
Staphylokinase precursor (*sak*)	gi|49484186	18,474	6.8
Signal transduction protein TRAP (*traP*)	gi|49484076	19,601	6.2
Thermonuclease precursor (*nuc*)	gi|88194577	25,120	9.3
Exotoxin 1, Superantigen family protein 7, (*set1*)	gi|49482656	26,051	6.9
Exotoxin	gi|49482662	26,378	8.7
Toxic shock syndrome toxin-1 (*tst*)	gi|15927587	26,447	8.8
Secretory antigen SsaA-like protein	gi|88194436	28,187	6.1
Staphylococcal enterotoxin type C (*sec*)	gi|15927585	30,671	8.2
γ-toxin component A (*hlgA*)	gi|49484635	34,958	9.6
γ-toxin component C (*hlgC*)	gi|49484636	35,642	9.3
α-toxin precursor (*hla*)	gi|15926746	35,975	8.7
γ-toxin component B (*hlgB*)	gi|49484637	36,812	9.3
1-Phosphatidylinositol phosphodiesterase (*plc*)	gi|49482345	37,114	7.1
Staphopain A (*scpA*)	gi|49484150	44,048	9.6
Iron-regulated binding protein (*isdB*)	gi|49483291	72,999	9.0
Lipase precursor (*lip1*)	gi|49484866	76,601	7.8
Lipase precursor (*lip2*)	gi|49482552	76,691	9.0
5'-Nucleotidase (*sasH*)	gi|49482276	85,133	9.2
Fibronectin-binding protein precursor (*fnbA*)	gi|49484704	105,691	4.6

2.4.5 Evidence of functionally active α-toxin produced by CDC587. Previous data are conflicting on whether or not functional α-toxin can be produced by USA200 strains with a stop codon in the *hla* structural gene (*hla*$^-$). The mass spectrometry analyses of the CDC587 fractions indicated the presence of peptides that corresponded to the amino acid sequence of α-toxin, despite the presence of the stop codon in the chromosome. (Appendix A2) Biological assays were used to confirm the presence of a functionally active α-toxin produced by CDC587 and two additional USA200 strains with *hla*$^-$, MN8 and Harrisburg.

By chemiluminescence detection and Western immunoblotting for α-toxin, we were able to detect 1 ng of purified α-toxin (Fig. 2.7A). In 20-fold concentrated overnight culture supernates, weak bands with molecular masses the same as α-toxin were visible in CDC587, MN8, and Harrisburg, indicating a small amount of α-toxin was produced by these strains (Fig. 2.7B). MNPE was estimated to produce 50-100 fold more α-toxin than CDC587, MN8, and Harrisburg.

To determine whether or not these small amounts of α-toxin are potentially clinical relevant, we performed bioassays to test classic biological activity of α-toxin: rabbit red blood cell hemolytic activity and rapid lethal activity when given intravenously in rabbits. MNPE culture supernates were more hemolytic to rabbit erythrocytes than CDC587, MN8, and Harrisburg supernates (Fig. 2.8A). Highly-specific anti-α-toxin serum neutralized 80% of the hemolytic activity of MNPE supernates but only decreased the hemolytic activity of supernates from CDC587, MN8, and Harrisburg by 66, 75, and 48%, respectively (Fig. 2.8B). Anti-γ-toxin serum, in

contrast, only neutralized 16% of MNPE hemolytic activity but neutralize 45, 42, and 27% of hemolytic activity in CDC587, MN8, and Harrisburg culture supernates, respectively (Fig. 2.8C). These results confirmed that small amounts of biologically active α-toxin were produced by these strains that possess the defective *hla* gene. In addition, CDC587, MN8, and Harrisburg produced relatively more γ-toxin than MNPE. Rabbits challenged with either unconcentrated MNPE supernates or 10-fold concentrated CDC587 supernates intravenously died within 30 min of injection (3/3 per group), comparable to rabbits that received 1 μg of highly-purified α-toxin. In contrast, unconcentrated supernates from CDC587 did not induce lethality.

Figure 2.7 Confirmation of α-toxin production by Western blotting. Anti-α-toxin antiserum was prepared from a rabbit immunized with MNPE rHPLC peak 6. (A) standard curve (1 μg to 0.1ng) using MNPE rHPLC peak 6 protein. The lower limit of detection was 1 ng. (B) Twenty-fold concentrated overnight culture supernates of MNPE, CDC587, MN8, and Harrisburg. The lower bands were likely degraded α-toxin by proteases in the culture supernates.

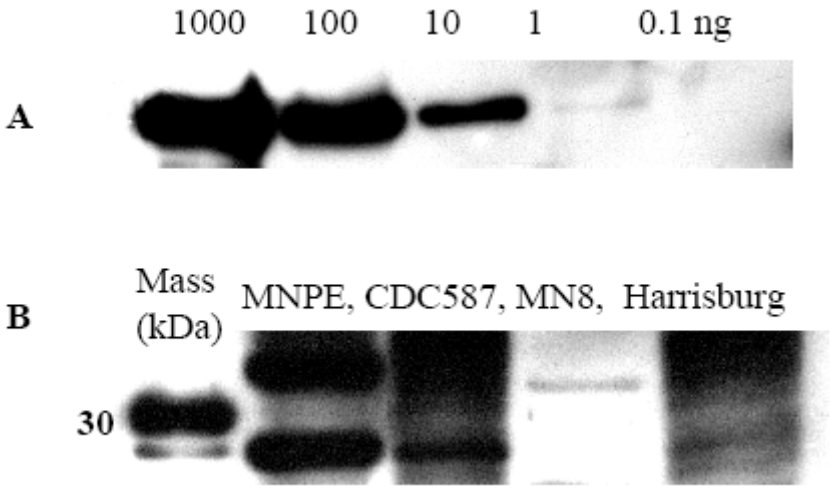
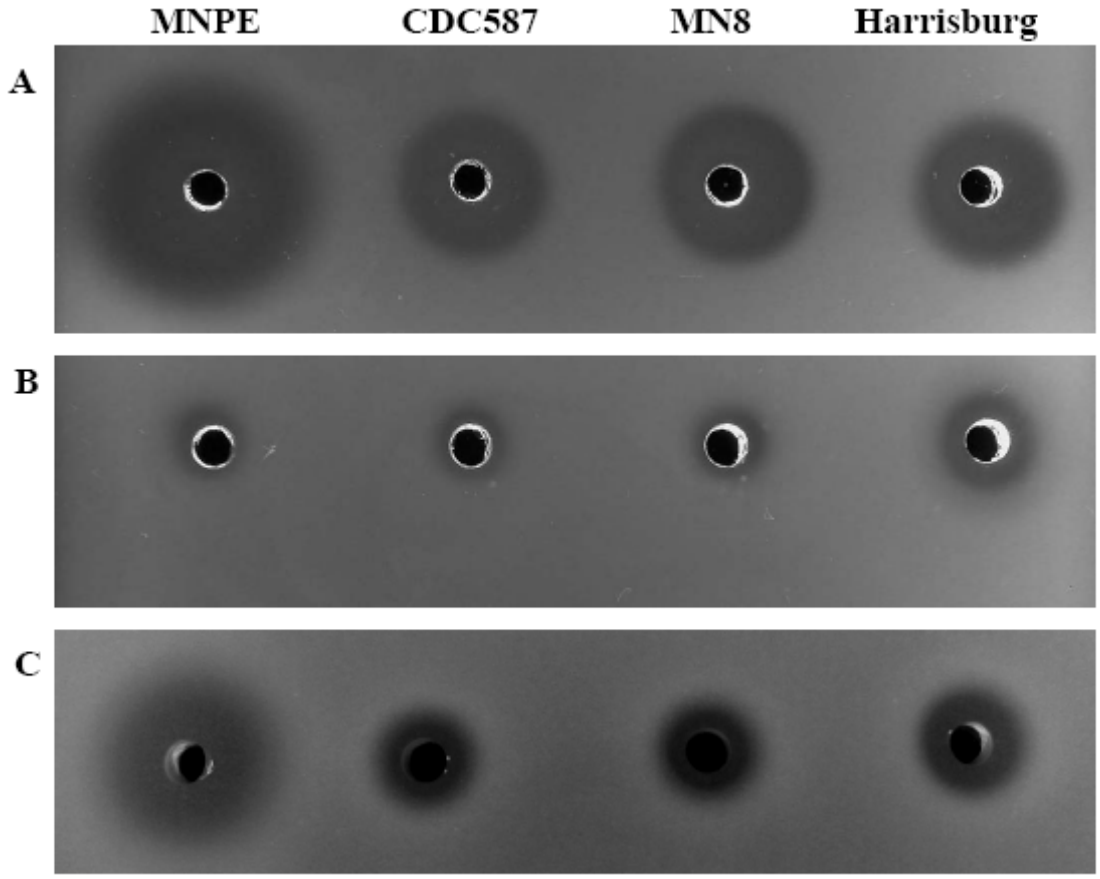


Figure 2.8 Hemolysis to rabbit red blood cell by TSS *S. aureus* exoproteins and neutralization with α-toxin and γ-toxin antisera. Overnight culture supernates were mixed with 3-fold (A) PBS, (B) anti-α-toxin-sera (C) anti- γ-toxin sera in the wells (4 mm diameter) for 24 h at 37ºC.



2.5 Discussion

S. aureus is a highly versatile microorganism, capable of colonizing human mucosal and skin surfaces, as well as producing exotoxins and other factors that cause local tissue damage and systemic diseases such as TSS; the organisms themselves can also cause systemic diseases through traumatic implantation into the circulation [125]. In an effort to estimate the overall contributions of all secreted *S. aureus* exoproteins, individually and in combination, to cause inflammation that opens mucosal barriers or damage epithelial tissue integrity directly, we provided global characterization of pro-inflammatory and cytotoxic proteins from two MSSA USA200 TSS strains. These strains are representative of the most common strains of *S. aureus* that colonize humans and are associated with TSS [112,113]; the USA200 designation is based on pulsed-field gel electrophoresis profile as defined by the Centers for Disease Control and Prevention.

By serial separations based on biochemical properties of the proteins and mass spectrometry, we have separated and identified major staphylococcal exoproteins responsible for pro-inflammatory responses and cytotoxicity to epithelial cells. CDC587 was found to produce more virulence factors than MNPE, but most of these virulence factors were produced at low levels. Cytolysins (α-toxin and γ-toxin), superantigens (i.e. TSST-1 and SEC), staphylokinase, and staphopain A were identified from both isolates. α-toxin, γ-toxin, TSST-1, and potentially staphopain were identified as major staphylococcal exoproteins that contribute to pro-inflammatory and cytotoxicity activity from epithelial cells by the USA200 isolates, and α-toxin was the main exoprotein responsible for the greater cytotoxicity and pro-inflammatory activity to HVECs in

MNPE culture supernates, compared to CDC587. This study also determined that despite both MNPE and CDC587 containing genes for numerous SAgs, including TSST-1, SEA, SEC and SEl-G, -K, -M, -O, -P; only two SAgs, TSST-1 and SEC, were produced in high enough concentrations to be purified and possess biological activity to epithelial cells. As previously reported, these two SAgs, TSST-1 and SEC, together with SEB are associated most cases of TSS.

Our group has previously shown the synergic effects of α-toxin and TSST-1 to promote pro-inflammatory responses and cytotoxicity from epithelial cells, which allows penetration of TSST-1 systemically to cause TSS [9]. α-Toxin was also determined to contribute to the majority of tissue damage in a murine pneumonia model, which is not sensitive to superantigens [8]. Our studies therefore support the previous findings that α-toxin plays important roles in *S. aureus* illnesses.

Interestingly, despite the nonsense mutation in α-toxin, CDC587 and related organisms still produced small amounts of α-toxin, indicating translational read-through in these *S. aureus* strains. Our studies have provided three lines of evidence that these strains produce functional α-toxin, albeit at a low-level: 1) mass spectrometry; 2) Western immunoblotting and antibody neutralization of red cell lysis; and 3) *in vivo* toxicity in rabbits. Stop codon read-through mechanism(s) in *S. aureus* have previously not been characterized. Nonetheless, read-through of an amber stop codon (UAG) has been characterized in certain *E. coli* strains, where the read-through is sufficient for bacteria to produce enough essential proteins such that normal growth occurs [126].

In the present study, MNPE, a strain isolated from a lethal pneumonia TSS case

and likely originating from a skin source, was found to produce a large amount of α-toxin and superantigens but not many other secreted virulence factors, whereas CDC587, a stereotypical menstrual vaginal mucosal TSS isolate, produced a wide variety of secreted virulence factors. Our group has previously shown that menstrual vaginal mucosal TSS *S. aureus* isolates produce significantly less α-toxin than skin organisms [51,52]. It is possible that a strain that produces less α-toxin, such as CDC587, was more suitable for mucosal colonization, whereas high-level α-toxin production by MNPE made the organism more suitable for skin colonization. Therefore, we hypothesize that MNPE requires wild-type α-toxin for survival on intact skin, whereas mucosal isolates such as CDC587 can easily colonize mucosal surfaces without wild-type α-toxin production. The basis for this difference was the difficulty MNPE-related organisms' encounter when attempting to colonize skin, such that high levels of α-toxin are required to cause dermonecrosis with consequent production of a colonization site (furuncle). Thus, organisms such as MNPE devote large amounts of energy to production of α-toxin and TSST-1, two toxins that appear essential to the organism. In contrast, high-level production of α-toxin on mucosal surfaces would be predicted to result in exceptionally high-fatality due to sepsis and/or high-level exotoxin penetration into the circulation; this may have accounted for MNPE causing a fatal case of post-influenza TSS. Also, the emerging skin strains of MRSA, including USA300 and USA400 organisms, have been accompanied by high-level production of cytolysins, presumably necessary for production of the large numbers of abscesses associated with the infections [8,127]. These same organisms are associated with highly fatal necrotizing

pneumonia in humans, consistent with their lack of fitness to mucosal surfaces. Even though USA200 organisms such as CDC587 have the ability to cause mucosal TSS, far more individuals are colonized with the organism than develop TSS. It appears that this organism is adapted to mucosal environment (just as MNPE is adapted to the skin environment) by producing those virulence factors that favor colonization without serious disease.

Various mechanisms of genetic adaptation have been described in other organisms. For example, *Pseudomonas aeruginosa* developed genetic mutations, which result in a mucoid phenotype, to maintain chronic lung infections in patients with cystic fibrosis [128]. The combinations of plasminogen-binding M protein and plasminogen activator streptokinase genetic variants have been associated with the fitness of *Streptococcus pyogenes* isolates on the skin or throat [129]. Josefsson and colleagues have shown that *S. aureus* tends to adapt the transcription levels of virulence factors based on surrounding tissues in a mouse septic arthritis model [130]. *S. aureus* isolates may also adapt to their environment niches (skin or mucosal surfaces) by down-regulation of certain virulence proteins that lead to excessive immune responses. An example of this may be the presence of the stop codon in the α-toxin gene. Thus, CDC587 and related isolates have adapted an unusual read-through mechanism for production of only low-levels of the toxin. The paucity of production of secreted virulence factors by strain MNPE, compared to production of large numbers by CDC587 may be another example of *S. aureus* niche-specific adaption.

In addition to α-toxin and superantigens, γ-toxin and the protease staphopain

were also indicated to be pro-inflammatory molecules to epithelial cells. γ-toxin, α-toxin, and Panton Valentine leukocidin (PVL), a cytolysin epidemiologically associated with severe skin infections in the community, all belong to the heptamer pore-forming cytolysin family. γ-toxin and PVL are hetero-chain heptamers, whereas α-toxin is a homo-chain heptamer; these structural differences may account for their observed activity differences [131]. Similar to α-toxin, γ-toxin has been suggested to play a role in a rabbit *S. aureus* keratitis model [132]. Our data indicate that menstrual TSS isolates such as CDC587 and related isolates produce relatively more γ-toxin than MNPE. However, the role of γ-toxin in *S. aureus* TSS pathogenesis has not been well characterized. Staphopain is a cysteine protease that has been shown to induce vascular leakage in guinea pig skin, which potentially facilitates septic shock [133]. Our data indicate that this protease may be pro-inflammatory to epithelial cells and thereby important for *S. aureus* pathogenesis on mucosal surfaces. Other secreted virulence factors were identified in our studies, including staphylococcal inhibitor of complement, which inhibits complement; staphylokinase, which inhibits host antimicrobial peptide α-defensins; lipases, and nucleases. These secreted proteins were not shown to be pro-inflammatory to epithelial cells in our study.

This study was a global characterization of exoproteins important for inducing cytokines from epithelial calls and cell death. We identified several exoproteins that may play important roles in establish *S. aureus* illness on mucosal surfaces. The differences in the secreted virulence factor profile between these two genetically related strains also suggest that production of virulence factors can vary considerably based on host niche.

Identification of the important role of these exoproteins as performed in this study is an important step towards development of vaccines, prophylactics, and enhanced therapeutics designed to control *S. aureus* diseases, including TSS.

CHAPTER III

Glycerol Monolaurate and Dodecylglycerol Effects on *Staphylococcus aureus* and Toxic Shock Syndrome Toxin-1 *In vitro* and *In vivo*

This chapter was published in *PLoS One*. 2009 4(10):e7499 by Lin, Y. C. et al.

3.1 Introduction

Staphylococcus aureus is an important cause of skin and mucosal infections both in hospital and community settings [134,135]. Superantigens, especially toxic shock syndrome toxin 1 (TSST-1), are also responsible for systemic exotoxemias such as toxic shock syndrome (TSS), an acute onset and potentially life-threatening illness. The most recognized cases of TSS are associated with tampon usage in menstruating women; however, TSS is also associated with *S. aureus* infections at surgical or skin infection sites [135,136]. Bacterial contamination of wound dressings, in particular occlusive dressings, has been suggested as the source of infection in some TSS cases [137].

Based on pulsed-field gel electrophoresis (PFGE), *S. aureus* strains can be grouped into several clonal types. In the United States, USA200 PFGE type were the most common methicillin susceptible *S. aureus* (MSSA) isolates recovered from national nasal colonization studies [112]. USA200 clonal type, which is genetically similar to the epidemic hospital strain EMRSA16 in the United Kingdom, is also the major clonal type associated with TSS, presumably due to the high prevalence of these isolates to possess *tst* (the gene for TSST-1). USA300 and USA400 clonal types have been associated recently with necrotizing pneumonia and necrotizing fasciitis in community settings [17]. Methicillin resistant *S. aureus* (MRSA) USA300 has emerged as one of the major causes of invasive staphylococcal infections in both community and hospital settings [14,17].

Given that most *S. aureus* infections are initiated at mucosal and skin sites, topical anti-staphylococcal agents, that can be incorporated into wound dressings,

disposable medical devices, or tampons to inhibit toxin production and/or *S. aureus* growth and thus prevent *S. aureus* infections or TSS, would have clinical value.

Glycerol monolaurate (GML) (2,3-dihydroxypropyl dodecanoate) is a lauric acid glycerol ester commonly used in the food and cosmetic industries as an emulsifier and preservative and is generally recognized as safe by the Food and Drug Administration for topical use at doses up to 100 mg/ml. GML interferes with membrane signal transduction and thereby inhibits the growth of *S. aureus* and delays the production of *S. aureus* exoproteins, such as TSST-1 and α-toxin [99-101]. GML also reduces the production of proinflammatory cytokines and chemokines by mammalian cells in response to *S. aureus* and purified TSST-1 (100 μg/ml), and prevents lethality in rabbits challenged vaginally with TSST-1 [102,103]. Given these properties, GML has been tested as a tampon additive and reduces staphylococcal exotoxin production and vaginal inflammation *in vivo* [138]. In addition, GML (5% gel) has been demonstrated recently to inhibit innate inflammatory responses [139] and to maintain vaginal health by normalize vaginal microflora [140]. The compound, however, is not stable in the presence of *S. aureus* and can be hydrolyzed by *S. aureus* esterase (lipase) into glycerol and lauric acid [99,101]. To overcome the limitation of inactivation, compounds with ether linkage have been suggested as potential alternatives to GML. Many of these ether compounds inhibit TSST-1 production in addition to *S. aureus* growth, and they are more stable than ester compounds (such as GML) to chemical and enzymatic hydrolysis [104,105]. 1-O-Dodecyl-*rac*-glycerol (DDG) (3-(dodecyloxy)propane-1,2-diol) is the corresponding alkylglycerol ether to GML. DDG inhibits the growth of *Enterococcus*

faecium and *Streptococcus mutans* primarily by stimulating autolysin activity and interfering with cell wall synthesis [105,141-144]. DDG simultaneously inhibits *S. aureus* growth and TSST-1 production, but the mechanisms of action has not been characterized [104].

3.2 Objectives

Given the structural similarity of GML and DDG and its supposed stability to lipase degradation over GML, DDG was hypothesized to be more potent than GML at inhibiting *S. aureus* growth and TSST-1 production, and therefore a better antistaphylococcal agent candidate than GML. Our goal was to compare the efficacy of these two compounds on *S. aureus* growth, and TSST-1 production *in vitro* and *in vivo*. We also studied *in vivo* the interactions between host innate immune responses and the compounds during *S. aureus* infection.

3.3 Materials and Methods

***S. aureus* isolates.** Fifty-four clinical isolates were tested to assess the ability of GML versus DDG to inhibit the growth of *S. aureus*. These included 10 menstrual vaginal TSST-1^+ MSSA isolates within the pulsed-field gel electrophoresis (PFGE) type USA200 as defined by the CDC [2]. These isolates were from TSS patients across the United States. Ten TSST-1^+ MRSA isolates were included within PFGE type USA200, and all of these isolates were from Minnesota, with 6 from patients with TSS. Five isolates were USA400 MRSA, and 5 isolates were USA400 MSSA. All USA400 isolates were from patients with necrotizing pneumonia, purpura fulminans, or non-menstrual TSS [18]. Three of the USA400 MRSA and three of the USA400-related MSSA isolates made the superantigen staphylococcal enterotoxin C (SEC), and two in each group made SEB. All ten isolates were positive for Panton-Valentine leukocidin (PVL). Four isolates were categorized as USA300 MRSA and were positive for the superantigen enterotoxin-like Q and made PVL [2]. Since GML and DDG are likely to be used topically, *S. aureus* derived from both skin and mucous membranes were also evaluated. Vaginal isolates (N=10) were obtained from healthy women during menstruation, and 10 skin strains were obtained from patients with atopic dermatitis. These 20 isolates were not further characterized with respect to exotoxin production or methicillin susceptibility. Collectively, these 54 clinical isolates were isolated from 1995 to 2007 and are maintained in the Schlievert and Peterson laboratories in the lyophilized state as low passage cultures.

S. aureus MN8 is a USA200 MSSA clinical isolate whose growth and exotoxin

responses to GML have been reported previously [100,101]. Therefore, this strain was chosen to evaluate exotoxin inhibitory ability of the compounds and used in the rabbit Wiffle ball infection study.

Antimicrobial compounds. DDG (racemic 1-*O*-dodecylglycerol; ≥99% purity; CAS registry number: 1561-07-5; 3-(dodecyloxy)propane-1,2-diol; Alexis Corporation, Läufeltinger, Switzerland), and glycerol monolaurate (GML) (Monomuls 90-L12; ≥90% purity; CAS registry number: 142-18-7; 2,3-dihydroxypropyl dodecanoate; Cognis, Cincinnati, Ohio) were prepared as high concentration stocks. GML was dissolved in ethanol, and DDG was dissolved in dimethyl sulfoxide (DMSO), as recommended by the manufacturers.

Determination of compound degradation by bacterial lipase. Overnight *S. aureus* (MN8) culture supernates (20 μl) were filtered to remove bacteria and placed into wells on agarose slides incorporated with either GML or DDG (500 μg/ml) and incubated for 5 h at 37°C and degradation of compound was assessed visually by measuring zone of clearing. The method for preparing these slides was adapted from Schlievert et al. [101].

Culture conditions. Bacteria were cultured overnight in Todd-Hewitt (TH) Bacto broth (Becton Dickinson and Company, Sparks, MD) at 37°C with 200 revolutions per minute (rpm) shaking. Experiments were performed with approximate starting inoculum of 1 x 10^7 colony-forming units (CFU)/ml with various concentrations of GML and DDG in 1

ml of TH broth. Samples (50 μl) were serially diluted with phosphate buffered saline (PBS; Cellgro-Mediatech Inc., Herndon, VA), and spirally plated onto sheep blood agar (Becton Dickinson). Plates were incubated at 37°C overnight and CFU counted with aCOLyte Supercount computer software (Microbiology International, Frederick, Maryland). The lower limit of accuracy was 400 CFU/ml, approximately 2.6 $\log_{10}$ CFU/ml. For *S. aureus* MN8 experiments (6h and 24h), an additional 300 μl of samples were collected and frozen for TSST-1 quantification. Bactericidal activity was defined as a 99.9% (3-log10 reduction in CFU/ml) reduction in bacterial density at 18-24 h compared to the initial inoculum. The term bacteriostatic was used when bacterial growth compared to the initial inoculum was either not observed or reduced by less than 99.9% [145].

TSST-1 Western blotting. Proteins in the 300 μl bacterial culture supernates were concentrated by precipitation with four volumes of 100% ethanol and re-suspended in 60 μl of sterile distilled water. Rabbit Wiffle ball supernate samples were not concentrated. For Western blotting, samples [1:1 mixed with Laemmli sample buffer (Bio-Rad Laboratory, Hercules, CA)] were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% acrylamide) [146]. After transfer to polyvinylidene fluoride membranes (Bio-Rad), the membranes were sequentially incubated with primary anti-TSST-1 (Toxin Technology. Inc., Sarasota, FL), secondary anti-rabbit IgG-alkaline phosphatase (Sigma-Aldrich), and 5-bromo 4-chloro 3-indolyl phosphate/ nitroblue tetrazolium (Sigma-Aldrich) for development [147]. The relative

band density was determined with ImageJ software (version 1.40g; http://rsb.info.nih.gov/ij/).

Cytotoxicity of the compounds to human vaginal cells. Immortalized human vaginal epithelial cells (ATCC CRL-2616) were used to determine mammalian cell cytotoxicity of GML and DDG. The cells were maintained in Keratinocyte-Serum Free medium (KSFM, GIBCO-BRL, Grand Island, NY), supplemented with recommended supplements and antibiotics/antifungal (100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml Fungizone). Cells were seeded into 96-well plates and grown to confluency. Cells were changed to antibiotic/antifungal-free KSFM the day before experimentation. Cells were co-incubated with compounds for 6 h at 37˚C in a humidified incubator with 7% CO_2. CytoTox-One homogenous membrane integrity assay (Promega) was used to measure the release of lactate dehydrogenase (LDH) from damaged cells as an indicator of membrane integrity. Assays were performed according to the manufacturer's instructions. Absorbances at 560 nm (excitation) and 590 nm (emission) wavelengths were measured by SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate. Median lethal doses (LD_{50}) of the compounds were the intercepts of 50% cell survival and the regression line of the two points adjacent to the values.

Rabbit Wiffle ball infection model. Ethics statement: All animal experiments were performed in accordance with protocols approved by the University of Minnesota

Institutional Animal Care and Use Committee (IACUC). The rabbit Wiffle ball infection model has been previously described [148,149]. Briefly, golf-ball-sized Wiffle balls were implanted subcutaneously in the flanks of Dutch-belted rabbits (either sex, 1.5 to 2.5 kg). The animals were allowed to recover for 6-8 weeks. On day 0 of experimentation, the animals (n=5) received 0.3 ml of 100 mg/ml GML or DDG (or solvent control) by injection directly into the Wiffle ball (final concentration of 1 mg/ml in the 30 ml Wiffle ball). A 1 mg/ml (0.1%) final concentration for DDG and GML was chosen and hypothesized to be non-toxic and efficacious as we previously determined that a GML (5%) containing gel inserted vaginally every day in monkeys for 6 months was not toxic [103]; and initial *in vitro* results indicated this concentration of GML and DDG would be bactericidal against *S. aureus* and inhibit TSST-1 production. The same treatments were administered every other day (days 2, 4, and 6). Overnight *S. aureus* MN8 cultures were grown in TH broth, washed once with PBS, and re-suspended to the desired concentration in PBS. Immediately after injecting the compounds, 1×10^{10} CFU *S. aureus* MN8 (in a volume of 1.0 ml) was injected into each animal's Wiffle ball (30 ml), bringing the local concentration to approximately 3×10^{8} CFU/ml. Animals were monitored daily for signs of TSS, including fever (with use of rectal thermometers), diarrhea, weight loss, and moribundity (as an indication of imminent death). A small volume of fluid (0.3 ml) was drawn from each Wiffle ball daily for bacterial counts, TSST-1 measurement, and TNF-α determination. (Appendix A3) Animals were euthanized by barbiturate overdose (intravenous injection) on day 7.

Tumor necrosis factor α (TNF-α) ELISA. TNF-α was used as a biomarker of inflammation at the infection site. Purified recombinant rabbit TNF-α, capture antibody, primary detection antibody (goat anti-rabbit TNF-α), secondary anti-rabbit antibody (biotin mouse anti-rabbit TNF-α), and assay reagents were commercially available from Becton Dickinson. Rabbit Wiffle ball fluids were diluted a minimum of 1:2 with assay buffer to eliminate viscosity and nonspecific effects. Lower limit of detection of this assay was approximately 200 pg/ml.

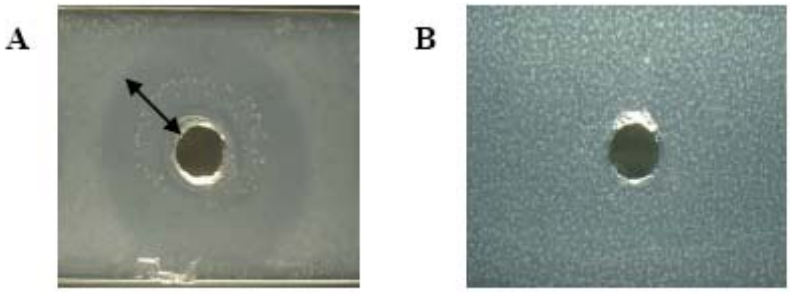
Statistical methods. Paired *t*-tests were performed to compare the differences in bacterial densities between GML and DDG (50 and 100 μg/ml) against the 54 clinical *S. aureus* isolates. Un-paired *t*-tests were used to compare the susceptibility of MRSA and MSSA. Total *S. aureus* CFU/ml and TSST-1 levels among groups were compared using one-way analysis of variance (ANOVA) with Bonferroni method to adjust *p* values for multiple comparisons. Fisher's exact test was used to compare rabbit survival between treatment groups. A $p \leq 0.05$ was considered statistically significant. Computations and graphing were performed using Prism version 5 (GraphPad Software, Inc. La Jolla, CA).

3.4 Results

3.4.1 Stability against *S. aureus* enzymes. GML- and DDG-containing agarose slides were exposed to *S. aureus* overnight cultures to determine the stability of the compounds to lipase contained in the culture supernates. A clear zone was observed on the GML 500 μg/ml agarose slide, but not the DDG 500 μg/ml slide (Fig. 3.1). The solubility limit of GML in aqueous solutions at 37 ºC is approximately 100 μg/ml, and thus the zone of clearance, reflecting GML degradation by lipase, can be observed in the presence of a turbid background. The observation indicated that DDG was resistant to degradation by *S. aureus* MN8 lipase, while GML was not resistant to lipase.

Figure 3.1 Stability of the compounds to *Staphylococcus aureus* (MN8) lipase.

(A) Glycerol monolaurate (GML). (B) Dodecylglycerol (DDG). Clear zone indicates that the compound was degraded. Arrow denotes of the radius of the clear zone on the slide.



3.4.2 *In vitro* growth inhibition. The differences in susceptibility to GML and DDG among *S. aureus* strains were evaluated broadly with a large collection of clinically relevant isolates (MSSA USA200, MRSA USA200, MRSA USA300, MSSA USA400, MRSA USA400, vaginal isolates from healthy women, and isolates from persons with atopic dermatitis). Growth inhibitory effects of GML (50, 100, and 500 μg/ml) and DDG (25, 50, and 100 μg/ml) were examined at 18h. In general, GML was bacteriostatic at concentrations of 50 μg/ml and 100 μg/ml, and was bactericidal (3 log decrease in CFU/ml from the starting inoculum of $1x10^7$ CFU/ml) at the concentration of 500 μg/ml (Fig. 3.2). On the other hand, DDG had a bacteriostatic effect on most strain categories at the concentration of 25 μg/ml (one dilution lower than GML). As the concentrations of DDG increased from 25 to 50 and 100 μg/ml, bacterial densities decreased an additional 1-2 log CFU/ml (Fig. 3.3). Overall, DDG was consistently more effective in preventing bacteria growth among all *S. aureus* strain categories, including vaginal and atopic dermatitis strains, than GML at concentrations of 50 and 100 μg/ml ($p<0.01$ for comparisons of GML and DDG against all 54 strains at both concentrations). There was no significant difference between MSSA and MRSA in response to GML ($p=0.79$ and 0.12 for GML 50 and 100 μg/ml, respectively); However, MRSA appears to be more susceptible to DDG than MSSA at the concentrations 50 and 100 μg/ml ($p=0.01$ and $p<0.01$, respectively). Some clonal variability was noted among strains, where USA400 (MSSA and MRSA) strains were relatively more resistant to DDG and the high dose of GML (500 μg/ml) than other clonal types (USA300 and USA200) tested. MRSA USA300 strains were the most susceptible clonal type to DDG.

Figure 3.2 Glycerol monolaurate (GML) inhibition of *Staphylococcus aureus*. GML concentrations 50, 100, and 500 μg/ml were tested versus *S. aureus* isolates from different PFGE types, USA400 MRSA (A), USA400 MSSA (B), USA300 MRSA (C), USA200 MRSA (D), USA200 MSSA (E), atopic dermatitis strains (F), vaginal strains from healthy women (G), for 18 h at 37˚C with shaking. The dashed line indicates the starting inocula. Each square (■) indicates one isolate. The bars represent the mean ± SEM of bacterial density in the group.

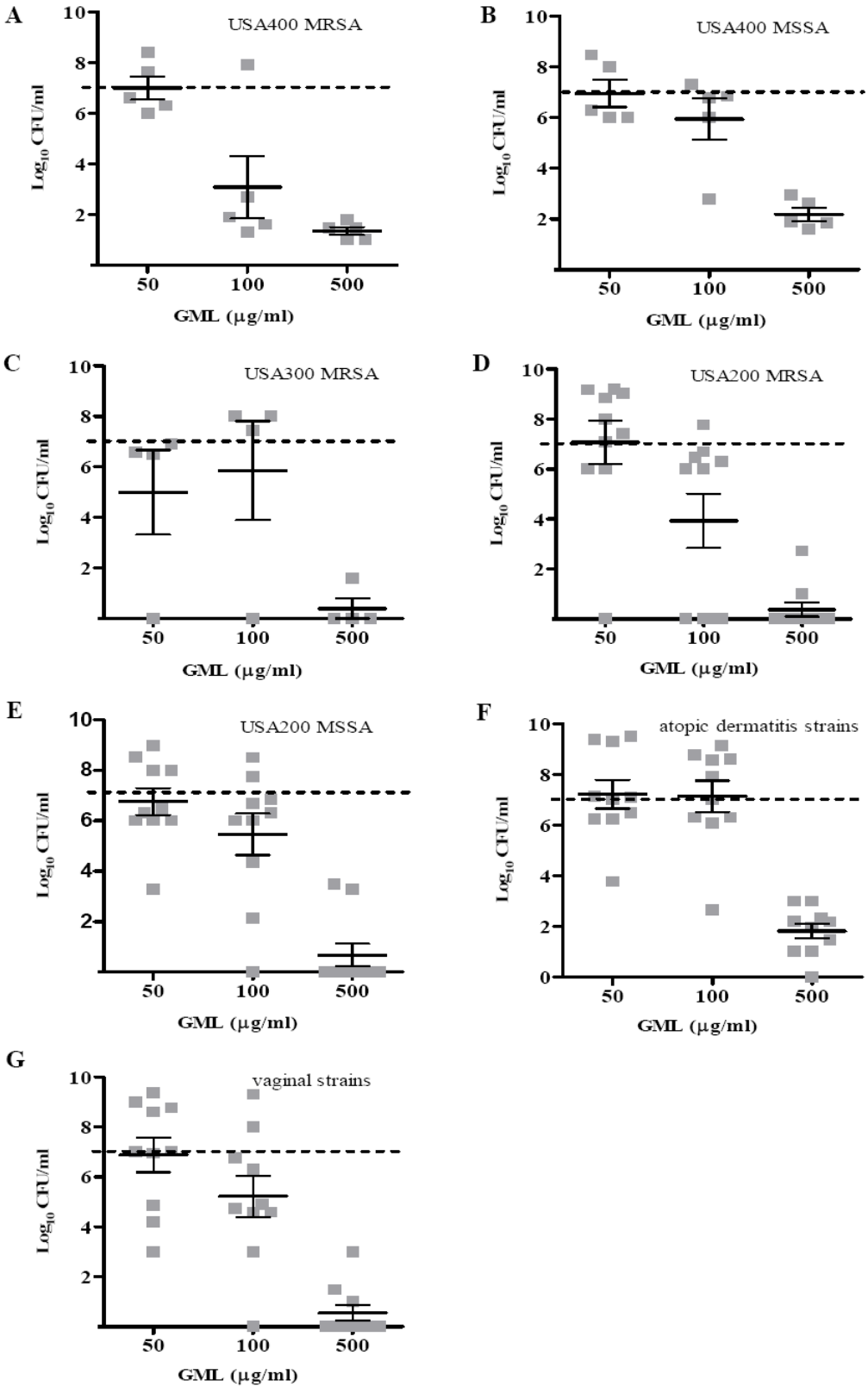
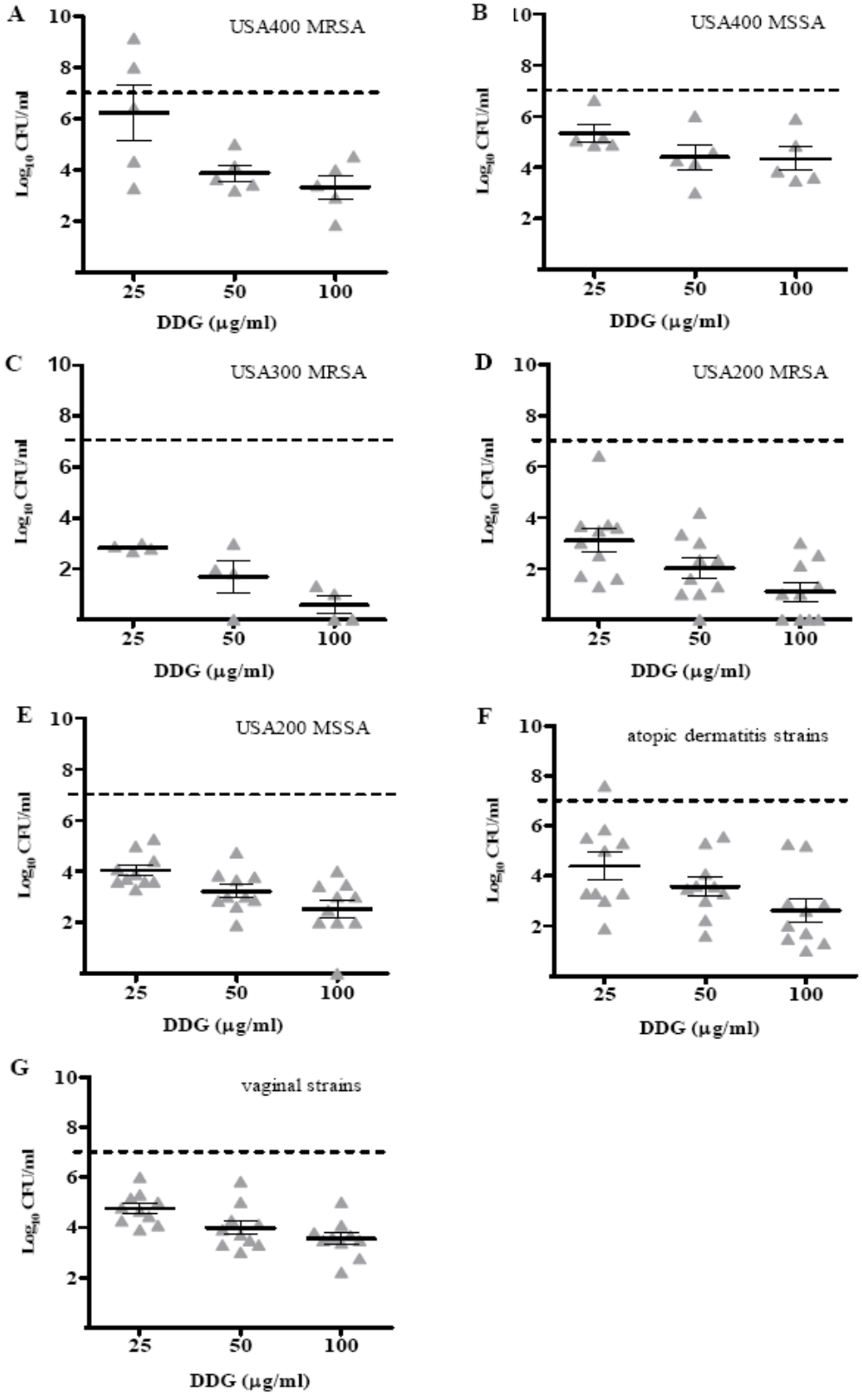

A
USA400 MRSA
B
USA400 MSSA
C
USA300 MRSA
D
USA200 MRSA
E
USA200 MSSA
F
atopic dermatitis strains
G
vaginal strains
Log10 CFU/ml
GML (μg/ml)
50
100
500

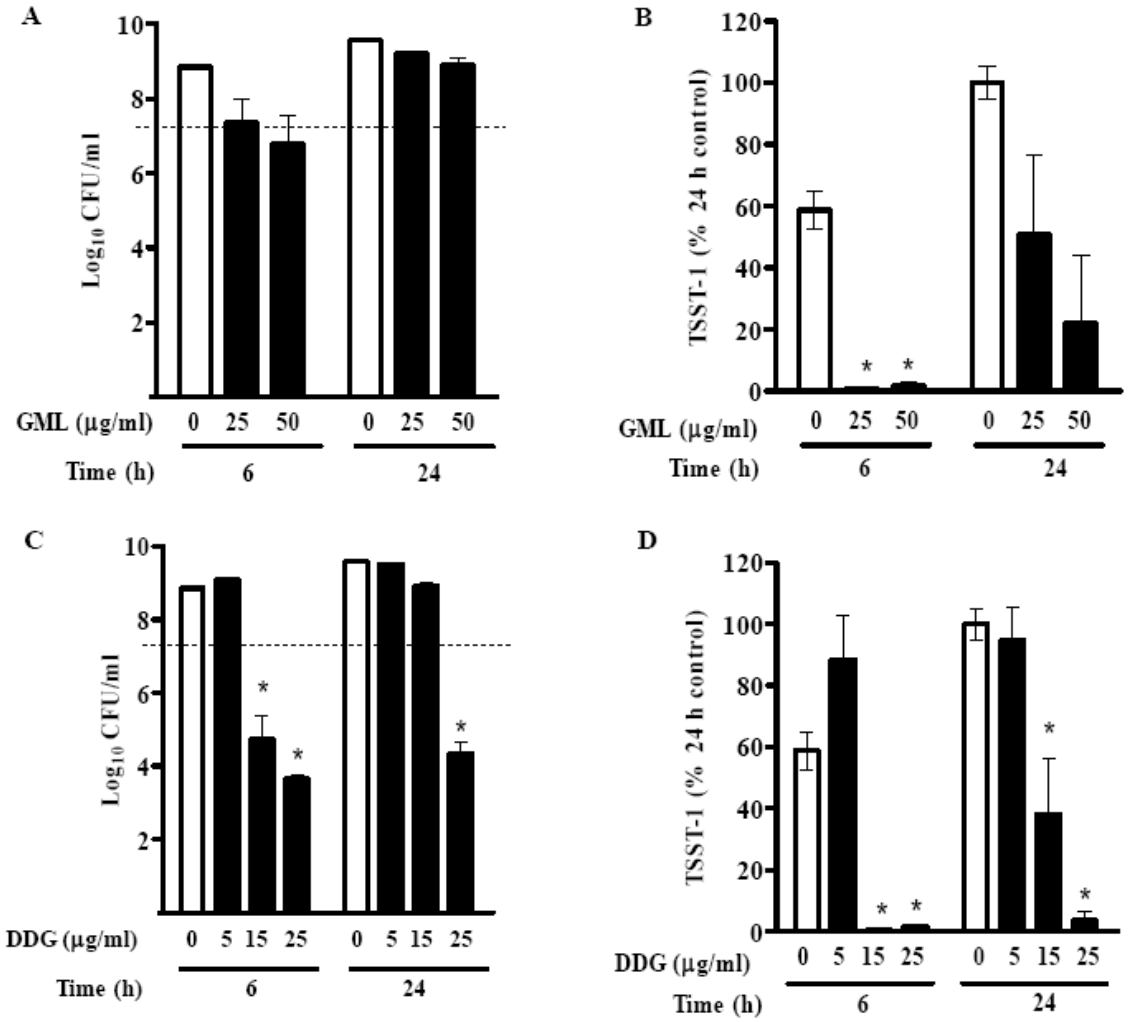
Figure 3.3 Dodecylglycerol (DDG) inhibition of *Staphylococcus aureus*.

DDG concentrations 25, 50, and 100 μg/ml were tested versus *S. aureus* isolates from different PFGE types, USA400 MRSA (A), USA400 MSSA (B), USA300 MRSA (C), USA200 MRSA (D), USA200 MSSA (E), atopic dermatitis strains (F), vaginal strains from healthy women (G), for 18 h at 37˚C with shaking. The dashed line indicates the starting inocula. Each triangle (▲) indicates one isolate. The bars represent the mean ± SEM of bacterial density in the group.


A
USA400 MRSA
B
USA400 MSSA
C
USA300 MRSA
D
USA200 MRSA
E
USA200 MSSA
F
atopic dermatitis strains
G
vaginal strains
Log10 CFU/ml
DDG (µg/ml)
25
50
100
2
4
6
8
10

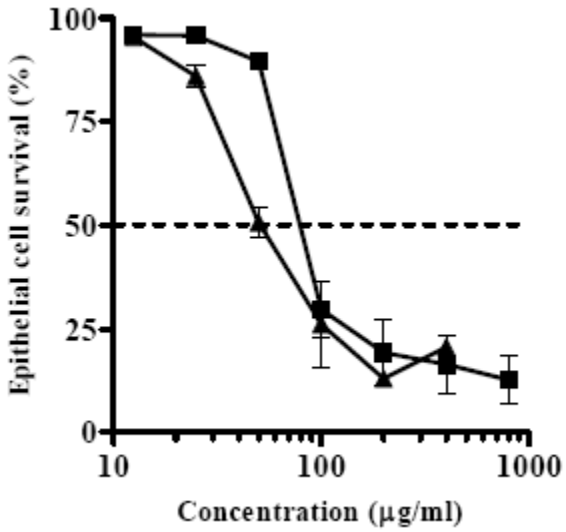
3.4.3 *In vitro* TSST-1 suppression. To evaluate toxin inhibitory effects of the compounds at sub-growth-inhibition concentrations, *S. aureus* MN8 was tested for growth inhibition and the corresponding TSST-1 production by GML (25 and 50 μg/ml) and DDG (5, 15, and 25 μg/ml) at 6 and 24h. GML (25 and 50 μg/ml) inhibited bacterial growth at 6 h, however, this bacteriostatic effect was no longer seen by 24 h (Fig. 3.4A). TSST-1 level was significantly reduced by GML at 25 and 50 μg/ml at 6h (> 99 % reduction), and this effect persisted through 24 h (49 % and 78% reduction, respectively) (Fig. 3.4B). DDG (5 μg/ml) did not inhibit the growth of MN8, and did not inhibit TSST-1 production (Fig. 3.4C). However, DDG (15 μg/ml) inhibited the growth of MN8 at 6 h but not 24 h, and TSST-1 production was inhibited by > 99 % at 6 h and 61 % at 24 h (Fig. 3.4D).

Figure 3.4 Effects of GML and DDG on *Staphylococcus aureus* Toxic Shock Syndrome Toxin-1 (TSST-1) production. (A) *S. aureus* MN8 was exposed to GML 0, 25 and 50 μg/ml for 6 and 24 h, and bacterial densities at 6 and 24 h were determined by plate counts. (B) The corresponding concentrations of TSST-1 of the above GML experiment. (C) *S. aureus* MN8 was exposed to DDG 0, 5, 15, and 25 μg/ml for 6 and 24 h. (D) The corresponding concentrations of TSST-1 from above DDG experiments. TSST-1 concentrations are presented as percent of the TSST-1 concentrations in 24 h control samples. Results are mean ± SEM. The dashed line indicates the starting inocula. *, $p < 0.05$.


A
B
C
D
Log10 CFU/ml
TSST-1 (% 24 h control)
GML (μg/ml)
DDG (μg/ml)
Time (h)
0 25 50
0 5 15 25
6
24

3.4.4 Mammalian cell toxicity. Since GML and DDG are most likely to be utilized in topical applications, they will be in contact with epithelial cells. Therefore, we determined the toxicity (median lethal dose, LD_{50}) of GML and DDG to immortalized human vaginal epithelial cells (HVECs) using an assay to measure the membrane integrity (lactate dehydrogenase [LDH] release) following 6h incubations. The LD_{50} of GML for a monolayer of confluent HVECs was 83 μg/ml (95% confidence interval [CI]: 69 – 99 μg/ml), while the LD_{50} of DDG for HVECs was 50 μg/ml (95% CI: 43 – 62 μg/ml) (Fig. 3.5). These results indicated that DDG was statistically more toxic to HVECs than GML. However, since the LD_{50} concentrations of DDG were lower than its bacterial growth inhibition concentrations *in vitro*, the compound may still be useful as an antistaphylococcal agent.

Figure 3.5 Cytotoxicity of GML and DDG to Human Vaginal Epithelial Cells (HVECs). HVECs were exposed to GML (■) and DDG (▲) for 6 h. Cytotoxicity was accessed by measuring the release of LDH. Error bars are SEM. The dashed line indicates median cell survival (LD_{50}). Symbols:■, GML; ▲, DDG



3.4.5 *In vivo* rabbit Wiffle ball infection model. Both GML and DDG demonstrated *in vitro* potential as topical anti-staphylococcal agents, thus their efficacy *in vivo* was evaluated in a rabbit Wiffle ball infection model with compound (1mg/ml, every-other-day) injected directly into the site of infection [see Materials and Methods]. In this model for toxic shock syndrome (TSS), bacteria are localized in the Wiffle ball both in suspension and as biofilms formed along the Wiffle ball surface; Superantigens can penetrate the Wiffle ball encapsulation tissue into the blood circulation to cause systemic effects, including TSS [148,150]. The survival curves for these experiments are shown in Fig. 3.6A. All rabbits in the control group (N = 5) died by day 4 (3 on day 2 and 2 on day 4) following inoculation of the Wiffle balls with *S. aureus* (MN8) 3 x 10^8 CFU/ml. Two of 5 rabbits in DDG group and 4 of 5 rabbits in GML group were alive by the end of the 7 day study. One rabbit in DDG group died on day 2, 1 on day 4, and 1 on day 7; one rabbit in GML group died on day 2. The survival of rabbits receiving GML (4/5) was statistically better than the control (0/5) group ($p < 0.05$, Fisher exact).

The Wiffle ball infection model provides the opportunity to study the interactions among bacteria, host innate immune responses, and therapeutic compounds by taking repeated samples at the infection site over time. We had difficulty obtaining fluids from Wiffle balls in two rabbits (one of the rabbits in GML and one in DDG group).Thus, the sample analyses, including bacterial counts, TNF-α (as a biomarker for TSS), and TSST-1 levels, were based on available sample points. All Wiffle ball sample fluids uniformly contained *S. aureus*, but *Pasteurella multocida*, was recovered from a sample taken on day 2 of a rabbit in the DDG group. That rabbit died at day 4.

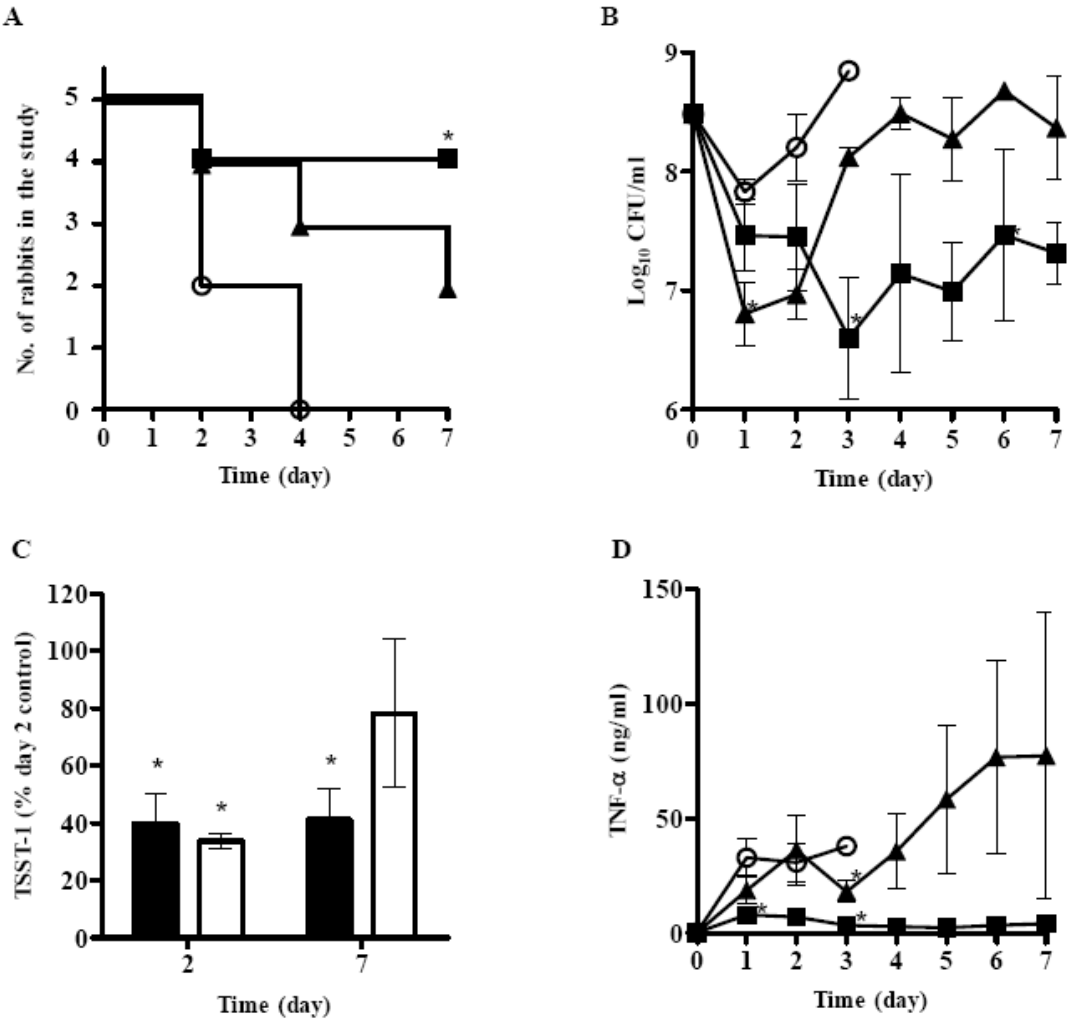
One day after initial dosing, bacterial counts in the DDG treatment group were 0.65 and 1 log lower than those in the GML treatment and the control groups, respectively (Fig. 3.6B). However, in spite of the repeated dosing, bacterial counts in DDG group increased over time. In contrast, GML suppressed bacterial growth throughout the 7 day study period. Neither compound was able to achieve > 3 log reduction in bacterial load within the Wiffle ball at the concentration tested.

TSST-1 concentrations within the Wiffle ball cavities of GML and DDG treated rabbits were lower than the control rabbits (Fig. 3.6C). On day 2, TSST-1 was significantly inhibited by 60% and 66% of the control rabbits for GML and DDG, respectively ($p < 0.001$, both treatments). On day 7, TSST-1 in the GML group remained significantly lower than TSST-1 in control rabbits on day 2 (58% reduction, $p<0.05$), while the level in DDG group was not significantly lower than controls on day 2 (22% reduction).

Baseline TNF-α levels in the Wiffle ball fluids of all rabbits were below the lower limit of detection (200 pg/ml) prior to infection (Fig. 3.6D). One day after bacterial challenge, rabbits treated with GML showed significantly lower concentrations of TNF-α in the Wiffle ball cavities than rabbits in control and DDG treatment groups: TNF-α average concentrations increased to 32.8 ng/ml (range: 11.6 – 61.9 ng/ml), 7.9 ng/ml (range: 1.8-17.7 ng/ml), and 18.6 ng/ml (range: 3.1 - 95.9 ng/ml) for control, GML, and DDG groups, respectively. TNF-α concentrations of rabbits in the GML group remained low throughout the period of study (4.1 ng/ml; range: 3.0 – 5.5 ng/ml). On the other hand, there was wide variation in TNF-α in the DDG group. TNF-α in one

rabbit decreased from 26.9 ng/ml on day 1 to 14.7 ng/ml on day 7, but other rabbits in this group had increasing TNF-α levels throughout the experiment. Overall, there is no strong evidence indicating DDG's ability to modulate innate immune responses.

Figure 3.6 Antistaphylococcal effects of GML and DDG in a rabbit Wiffle ball infection model. Rabbits (n = 5 in each group) were infected with 3 x 10^8 CFU/ml *S. aureus* MN8, and compounds (final concentration 1 mg/ml) were instilled into the Wiffle balls every-other-day and rabbits monitored up to 7 days. Survival of the rabbits (A), bacterial counts (B), TSST-1 production (C), and TNF-α levels (D) in the Wiffle balls. TSST-1 presented as percent of day 2 TSST-1 concentrations of the control rabbits (GML, close bars; DDG, open bars). Error bars are SEM. Symbols: ○, control; ■, GML; ▲, DDG; *, $p < 0.05$.


A
No. of rabbits in the study
Time (day)
B
Log10 CFU/ml
Time (day)
C
TSST-1 (% day 2 control)
Time (day)
D
TNF-α (ng/ml)
Time (day)

3.5 Discussion

TSS is a serious complication of *S. aureus* infection, and the superantigen, TSST-1, is responsible for nearly all menstrual TSS cases and at least half of non-menstrual cases [23]. Many surfactants, including fatty acids linked through ester, ether, amide, or amine bonds, appear to inhibit *S. aureus* growth and toxin production [104]. However, fatty acid esters and amides are susceptible to *S. aureus* enzyme degradation, and amines are irritable to mucous membranes [151]. Therefore, fatty acid ethers were considered to be better candidates as topical anti-staphylococcal agents. Lauric acid (a 12 carbon-containing fatty acid) was determined to be the most potent saturated fatty acid when C8 to C18 -containing fatty acids were tested against gram-positive bacteria [152]. Its ester derivative, GML, has shown excellent potential for being incorporated into tampons to reduce risk of TSS [138]. However, similar to other fatty acid esters, GML is susceptible to *S. aureus* lipase degradation. DDG, the corresponding ether to GML, was therefore compared to GML as an anti-staphylococcal candidate to reduce the risk of TSS. The studies presented in this manuscript determined that GML and DDG inhibit *S. aureus* growth and toxin production, although by apparent different modes of action. In addition, a difference in strain (clonal type) specific susceptibility to both GML and DDG was observed. Overall, the studies indicated that GML is a potentially better anti-staphylococcal agent than DDG for its ability to inhibit exoprotein production regardless of effects on bacterial growth, to reduce mortality in the rabbit Wiffle ball infection model, and to cause less cytotoxicity to epithelial cells than DDG.

A range of minimum inhibitory concentrations (MICs) for GML against *S. aureus* have been reported. The MICs of GML against 29 strains of *S. aureus* in a complex medium were reported to be between 10 to 20 μg/ml with 10^3 to 10^4 CFU/ml inocula [153]. Kabara and colleagues reported the MIC of GML against *S. aureus* was 25 μg/ml with approximate 10^7 CFU/ml inocula in trypticase soy broth [152]. Preuss et al. reported 63 μg/ml with approximate 10^5 to 10^6 CFU/ml inocula in nutrient broth [154]. Kelsey and colleagues reported the MIC of GML against three strains of *S. aureus* was 25-50 μg/ml [155]. The variability in MIC is potentially due to culture conditions, inoculum size, and the *S. aureus* strains tested [153]. By testing the compounds against a large collection of clinical relevant strains, we confirmed that there are differences in sensitivity to GML among bacterial strains, even within the same clonal type. The differences in GML sensitivity may not be solely explained by different levels of lipase produced by the strains since the differences among *S. aureus* clonal types can also be observed in the DDG group, which is not degraded by *S. aureus* lipase. We also observed that USA400 strains do not produce more lipase than USA200 strains (data not shown), despite being more resistant to the compounds. The mechanism(s) behind the differences among *S. aureus* clonal types in response to DDG and GML may be related to cell surface hydrophobicity [156], however, this hypothesis will need to be investigated in future studies.

Although structurally similar, DDG and GML interact with *S. aureus* differently. Glycerol esters are commonly found in bacterial membranes, and cells have mechanisms to maintain membrane integrity in their presence. This is likely to occur also in the

presence of GML. In contrast, glycerol monoethers are uncommon in bacterial membranes, and thus, the bacteria may be expected to have greater difficulty in maintenance of membrane integrity in the presence of DDG. As noted in our studies, GML antimicrobial effects were dose dependent and required higher concentrations for bactericidal activity, while DDG was predominantly bacteriostatic, but active at lower concentrations than GML. Similar mechanisms may also explain the differences between GML and DDG on toxin inhibition. GML blocks toxin induction by interfering with bacterial signal transduction on bacterial cell membranes [100]. In our study, GML (25-50 μg/ml) was able to inhibit TSST-1 production independent of *S. aureus* growth inhibition properties, which is in agreement with previously described results by Schlievert et al. (20 μg/ml*)* [101], and Holland et al. (17 μg/ml) [153]. DDG was also reported to inhibit TSST-1 production by McNamara and colleagues [104]. However, our results suggest that for DDG, and likely other glycerol monoethers, inhibition of toxin production is dependent on bacterial growth inhibition, which is different from that of GML.

GML has a good safety profile on skin and mucosal surfaces. The compound was considered to have negative ocular irritation and have a LD_{50} of >20g/kg for rats when dosed orally for 10 weeks [157]. In fact, GML (5% gel) was safe for chronic vaginal administration in monkeys over a 6 month test period [103]. In unpublished studies with year-long passage of *S. aureus* MN8 on sub-growth-inhibitory concentrations of GML, we observed no increase in resistance to GML's antimicrobial and anti-exotoxin effects. On the other hand, the safety of DDG *in vivo* has not been well studied. In one study,

mice given 1g/kg of DDG orally per day over 4 weeks indicated no signs of toxicity, and DDG was quickly absorbed and eliminated into urine [158]. Since increased doses of DDG do not enhance bacterial growth inhibition in our study, and DDG may be more irritable to mucosal surfaces than GML, minimal effective dose of DDG should be used.

GML has been reported to stabilize the membrane of eukaryotic cells, modulate the production of pro-inflammatory cytokines and thereby prevent the toxicity of bacterial exotoxins on eukaryotic cells [102]. We have previously suggested the benefits of GML as a dual-acting anti-infective, 1) with effects on the microbes to prevent growth and/or exotoxin production, and 2) with anti-inflammatory and membrane stabilizing effects on the host epithelial cells, which reduces the disruption in the mucosal permeability barriers caused by induction of pro-inflammatory cytokines and chemokines following infection [139]. This latter anti-inflammatory and membrane stabilizing property, although counterintuitive, may be equally important or more important than the antimicrobial effect. We reported recently that a GML (5%) containing gel prevented SIV transmission across monkey cervical and vaginal mucosa, despite mucosal surface GML concentrations being below virucidal concentrations [139]. Additionally, histological studies demonstrated an inhibitory effect on innate immunity. In our study, GML also decreased local pro-inflammatory cytokine production (as measured by TNF-α) despite bacterial densities of approximately 1 x 10^7 CFU/ml over 7 days.

Production of TSST-1 is induced by elevated oxygen and carbon dioxide levels, neutral pH, presence of proteins, and increased temperature (37˚C) [79]. Introduction of

oxygen into the typically anaerobic vaginal environment may account for the tampon association with TSS [159,160]. Although abscesses are typically perceived to be anaerobic, Todd and colleagues demonstrated that *S. aureus* abscesses are aerobic, and appear to provide TSST-1 stimulating environmental conditions, similar to those occurring vaginally in the presence of tampons [161]. The Wiffle ball infection model as used in this study has aspects of both types of *S. aureus* infections included in an aerobic encapsulated abscess, which has internal surfaces similar to the vaginal mucosa. As bacteria were encapsulated in the Wiffle ball, the model provided an ideal environment for real-time monitoring of the interactions among the host innate immune response, bacteria, and treatments (DDG and GML) at the infection site.

Based on the collective results of this study, GML is proposed as more effective anti-staphylococcal topical anti-infective candidate than DDG, despite its potential degradation by *S. aureus* lipase.

CHAPTER IV.

Overall Conclusions and Future Directions

The battle between pathogenic bacteria and the human host is never ending. The discovery and continued development of antibiotics have kept humans primarily on the winning side for the last few decades. However, versatile organisms, such as *S. aureus,* are constantly evolving to their environment to become resistant to many antibiotics and, in some cases, more virulent.

S. aureus not only contributes to acute diseases such as infections and TSS, but also contributes to chronic inflammation and autoimmune diseases [13,111]. Skin and the mucosal epithelium is the first line of defense against microbe invasion. This is also where most, if not all, toxic *S. aureus* components are produced, including cytolysins, SAgs, and extracellular enzymes. Prior studies have indicated that cytolysins facilitate SAgs penetration across mucosal membranes via inflammation and/or via direct cytotoxicity to epithelial cells, which increased membrane permeability and/or disrupted the tissue barrier [9]. Based on these findings, I hypothesized that secreted *S. aureus* virulence proteins induce inflammation and/or cytotoxicity in epithelial cells to facilitate the penetration of its toxins and/or itself. By inhibiting the effect of these proteins on epithelial cells, I propose that *S. aureus* mucosal pathogenesis can be prevented or alleviated. Based on this hypothesis I propose the representative *S. aureus* mucosal pathogenesis model presented in Figure 4.1. The model describes the known relationship between *S. aureus* exoprotein-induced inflammation and *S. aureus* disease progression on mucosal membrane, as well as the possible mechanisms of anti-toxin (virulence) therapies to prevent and/or delay the pathogenic process. Given bacterial toxins such as SAgs penetrate damaged mucosal membranes better than whole bacterial cells, TSS

models of infection, including immortalized vaginal epithelial cells, *ex vivo* porcine vaginal mucosa and a rabbit Wiffle ball abscess/TSS model, were chosen as models of *S. aureus* disease to study the interactions between secreted *S. aureus* virulence factors (exoproteins) and *S. aureus* disease progression from mucosal membranes.

In the presented study, cytolysins (α- and γ-toxins), TSST-1, and staphopain (a cysteine protease) were demonstrated to be the major pro-inflammatory and cytotoxic proteins secreted by TSS *S. aureus* isolates. This is consistent with these exoproteins playing a role in *S. aureus* pathogenesis, which has been reported in the literature. α-toxin and γ-toxin induce inflammation and/or cytotoxicity to disrupt the membrane barrier and increase permeability to SAgs [9]. TSST-1 activates the innate immune function of epithelial cells by inducing the secretion of chemokines and cytokines [35]. Staphopain has been shown to induce vascular leakage in guinea pig skin, presumably due to its plasminogen activator activity [133]. In addition to direct impacts on mucosal membrane/skin, these proteins can impede host immune response resulting in delayed bacterial clearance. α- and γ-toxins directly damage host immune cells such as PMNs and lymphocytes; TSST-1 (and other superantigens) disrupt normal T-cell proliferation; and staphopain cleaves antimicrobial peptides and retards phagocytosis [162]. Importantly, these exoproteins are all produced *in vivo* during infections, and are immunogenic and highly preserved across *S. aureus* isolates [117,163,164]. These properties indicate that these proteins may also be potential targets for antistaphylococcal vaccines and/or immunotherapies. Blocking the production of these toxins, their effect on cells, and/or inflammation can theoretically decrease the damage

by these proteins at the local site, enable host immune system to clear the invading organism, and thereby prevent the progression of the *S. aureus* disease.

Exoproteins secreted by *S. aureus* not only contribute to *S. aureus* pathogenesis but also to the fitness of the organism allowing for persistence and dissemination from its host niches. MNPE (presumably a *S. aureus* isolate of skin origin) produced large amounts of α-toxin and SAgs, but not other virulence factors, whereas, menstrual TSS isolate CDC587 (presumably a mucosal isolate) produced SAgs and a large number of other virulence factors, but a significantly reduced amount of α-toxin. These two isolates are genetically similar; therefore, the differences in exoprotein production were hypothesized to be due to the adaptation of these *S. aureus* isolates to their environmental niches. Compared to the mucosal membrane, the skin contains a thick layer of stratum corneum and antimicrobial lipids, which hinders the colonization of *S. aureus* and its ability to obtain nutrients. High concentrations of α-toxin and SAgs may therefore provide survival advantages for *S. aureus* to survive on the skin. In contrast, a high production of α-toxin on the mucosal surface may induce too much inflammation, which induces bacterial clearance by the host immune cells or the host succumbs to the bacteria infection (cytokine storm). However, a small amount of α-toxin may be necessary for the survival of *S. aureus* on the mucosal membrane; therefore, these strains may have develop a "read through" mechanism to significantly down-regulate α-toxin and still maintain the ability to produce other virulence factors to facilitate colonization on mucosal surfaces. This ability to adapt to the environment may in part be the reason that USA200 is the most dominant nasal colonization clonal type in the U.S. [13]. This

theory that α-toxin facilitates skin colonization is in part supported by the finding that α-toxin is produced in higher amounts by USA300, the predominant CA-MRSA that causes skin and soft tissue infections, than USA400, a relatively less prevalent CA-MRSA strain [8,117,127,165].

Previous studies often attributed differentiated virulence factor production to transcriptional regulation systems such as Agr, Sae or SarA [127]. The read through mechanism, which allows the strains to produce a small amount of α-toxin from a silenced α-toxin gene (pseudogene), represents another virulence regulatory system that has not been characterized previously in *S. aureus*. Further characterization of the mechanism involved may significantly improve our understanding of *S. aureus* physiology as well as pathogenesis. Genomic studies of *S. aureus* MRSA252, an isolate genetically related to USA200 isolates, have revealed that numerous pseudogenes, similar to the silenced α-toxin gene in CDC587, exist in this clonal type at the frequency 3-fold higher (66 verses ~20) than other sequenced isolates (i.e. USA100, USA300, and USA400) [166]; no hypothesis of the functional mechanism behind these pseudogenes or their potential roles in bacterial genome was proposed. The findings from this work provide a potential theory to explain the evolutionary puzzle.

In this study, I proposed that *S. aureus* mucosal pathogenesis can be prevented or alleviated by inhibiting the effect of proinflammatory or cytotoxic proteins on epithelial cells. To prove the principal of my theory, GML, a naturally derived compound with anti-toxin, anti-inflammatory, and antimicrobial effect, was characterized for its ability to prevent or alleviate *S. aureus* infection. GML blocked *S. aureus* TSST-1 production

and modulated local inflammation, even when bacterial density was still high enough to cause inflammation. Furthermore, it significantly decreased the mortality of rabbits in a Wiffle ball infection abscess/TSS model. In contrast, a structurally similar compound, DDG, which did not modulate local inflammation, decreased the mortality, but was not significantly greater than control. The mortality reduction was presumably due to its initial antimicrobial effect, which delayed toxin production. Dr. Schlievert also demonstrated previously that chitosan malate, another compound that inhibits bacterial exoprotein production, can also prevent rabbit death from TSS (both staphylococcal and streptococcal TSS) and necrotizing fasciitis [149]. Therefore, these results confirmed my original hypothesis that anti-toxin and/or anti-inflammation, even just locally, can decrease the severity of *S. aureus* infections. These compounds have potential for being developed as (add-on) topical antimicrobial agents, wound dressings, or coatings of medical devices to treat or prevent *S. aureus* infections initiated from skin or mucosal surfaces.

GML was recently determined to reduce bacteria and superantigen concentrations as well as reduce vaginal inflammation in menstruating women when applied as a tampon coating, which may prevent the incidence of menstrual TSS associated with tampon use [167]. In fact, the immunomodulation effects and partial antimicrobial effects, of GML to help maintain healthy vaginal mucosal membrane and may provide clinical benefits in addition to TSS prevention. Similarly, GML (as a vaginal gel) was shown to prevent monkeys from vaginal transmission of simian immunodeficiency virus (SIV) infection (a monkey version of HIV) via reductions in

IL-8 and MIP-3α, which attract SIV-targeted cells to the infection site [139]. GML, tested as a vaginal gel, was also shown to decrease *Candida* and *Gardnerella vaginalis* in women with vulvocandidiasis and bacterial vaginosis, respectively, but have no effect on vaginal pH and normal vaginal micro flora (i.e. *Lactobacilli*) [140]. The long-term safety of GML has been established. GML has been determined be safe following over 6 months of application in monkeys without disturbing mucosal microflora and mucosal integrity [103]. These studies imply that the anti-toxin (virulence) approaches proposed in this thesis study may also facilitate the development of vaginal microbicides to prevent vaginal infections and/or HIV infections.

Although not directly evaluated in this work, antibodies to neutralize toxins (via vaccination or passive immunization) to prevent their toxic effects on host cells should provide similar (or better) protection. Antibodies against α-toxin have been shown to decrease lung tissue damage and mortality in a mice pneumonia model challenged with *S. aureus* [168]. Similarly, passive immunization with antibodies to SEB also protected monkeys from aerosolized SEB intoxication [169]. Although the use of *S. aureus* exoprotein vaccine to prevent *S. aureus* mucosal pathogenesis is largely undetermined, these results indicate that protective mucosal and/or systemic immune response to *S. aureus* exoproteins can be developed.

In summary, this work identified proteins (α- and γ-toxins, SAgs, and staphopains), which may be important in *S. aureus* mucosal pathogenesis, and revealed a novel theory (read through mechanisms of mutated genes) which facilitates *S. aureus* adaptation to host niches, and confirmed the clinical potential of anti-toxin strategies to

prevent or treat *S. aureus* diseases initiating at mucosal/skin surfaces. The role of the above proteins should be further evaluated, and these proteins should be considered as part of future antistaphylococcal vaccines or immunotherapies.

Based on the work presented above, the following future research directions are proposed:

I. Evaluation of γ-toxin in staphylococcal (TSS) pathogenesis and as a vaccine target.

In this study, cytolysins, including γ-toxin, were determined to be an important exotoxin secreted by *S. aureus* with pro-inflammatory and cytotoxic effects on epithelial cells. Previously, I proposed toxin neutralization as a therapeutic strategy for prevention or reducing the severity of *S. aureus* infections; however, the overall role γ-toxin contributes to *S. aureus* infection progression and feasibility as a toxin target is not well understood. Therefore, I have begun the initial steps to explore these questions as a continuum of my thesis study.

Menstrual TSS isolates are associated with γ-production and lack of α-toxin production due to a mutation [119,170]. Approximately two-third of menstrual TSS isolates carry a functional γ-toxin gene, and have a "silenced" α-toxin gene, which only allows for the production of a very small amount of α-toxin (as determined by this study). Therefore, the clinical relevance of γ-toxin in staphylococcal TSS (and other *S. aureus* diseases), especially in menstrual TSS isolates with significantly decreased

α-toxin production, needs to be further evaluated.

In addition, sequence similarity among sub-components of γ-toxin and other bi-component cytolytic toxins (i.e. PVL) implies that immunization with γ-toxin may provide cross-protection against other related cytolytic toxins that are less prevalent than γ-toxin. γ-toxin has been reported as immunogenic as antibody against γ-toxin has been detected from patients chronically infected with *S. aureus*.

Therefore, I plan to determine the role of γ-toxin in *S. aureus* TSS pathogenesis and to evaluate whether or not immunization with γ-toxin can prevent menstrual TSS in a rabbit vaginal TSS model, by comparing the morbidity and mortality in rabbits immunized with or without γ-toxin after vaginal *S. aureus* challenge.

Given γ-toxin protein purified from *S. aureus* isolates may be contaminated with α-toxin, which is known to have synergic inflammatory effect with γ-toxin, recombinant γ-toxin from *E. coli* will be used in this study.

To date, I have successfully cloned *hlg*, the gene for γ-toxin, into *E. coli* and confirmed the bioactivity of the recombinant γ-toxin via lysis of red blood cells. Polyclonal antibodies to γ-toxin have also been obtained by vaccinating a rabbit three times over a 28 day period with recombinant γ-toxin (per the method reported in Chapter II). The anti-γ-toxin polycolonal antibody was able to neutralize γ-toxin produced by *S. aureus* TSS strains (See Fig 2.8). Therefore, I expect no difficulties in completing this evaluation in the future.

II. Continued characterization of α-toxin read through mechanism(s) in *S. aureus* and its role in *S. aureus* pathogenesis

Menstrual TSS *S. aureus* isolates with mutant α-toxin structure gene, such as CDC587, MN8, and Harrisburg, were originally considered to not produce any α-toxin. However, in the presented study, it was determined that a small amount, about 1/50 to 1/100 of wild type α-toxin-producing *S. aureus* isolates, was produced by these strains. The size of the protein shown in the α-toxin Western blot (Fig. 2.7) suggested that a full length was produced by these strains. Additionally, this α-toxin was shown to be biologically active via the hemolysis assays (Fig. 2.8). I confirmed the silence mutation of the α-toxin via genetic sequencing, therefore, the production of α-toxin detected was not due to reactivation by mutation events (i.e. another point mutation) as previously suggested [52]. The read through phenomenon observed here represents a previously uncharacterized post-transcriptional regulation mechanism(s) in *S. aureus*.

A nonsense stop codon read through phenomenon has been described previously in *E. coli*. It was known that mutations in the ribosome, transfer RNA or release factor all allow stop codon read through. The efficiency of the read through in part depends on the type of stop codon (UAA, UAG, or UGA) and the position of the mutation [171,172]. Glutamine was determined to substitute the stop codon in the amber (UAG) stop codon read through [173]. This read through mechanism allows the organism to translate sufficient amounts of protein for normal growth, even in the presence of a nonsense mutation in essential genes [126]. These observations imply that the mechanism allows the organism to have versatile phenotypical change, which may provide survival

advantage. In addition to this study, nonsense mutations of essential membrane proteins have been observed in *S. aureus* strains of animal origin (P. Schlievert, personal communication). Therefore, the post-transcription regulation may represent an important mechanism in *S. aureus* survival and adaption in environmental niches. Characterization of this mechanism in *S. aureus* should improve our understanding of the adaptation of *S. aureus* as such a versatile pathogen.

To date, I have started to determine the potential amino acid replacing the stop codon at position 113 in the α-toxin protein by mass spectrometry. However, I was not able to obtain enough signal to determine the replaced amino acid, presumably due to the small amount of α-toxin presented in the fractionated sample. Therefore, I plan to apply cloning techniques to enforce the production of more "mutant" α-toxin by *S. aureus*, and then use mass spectrometry and/or protein sequencing to confirm the substituted amino acid. The genetically modified strains will also allow me to test their virulence *in vitro* and *in vivo* related to the parental strain. In parallel, I plan to collaborate with bacteria geneticists in the Microbiology department to explore the potential read through mechanism(s) via comparing the homologue of *S. aureus* ribosome genes to known *E. coli* read through mutations.

Although this study is not directly related to antistaphylococcal therapy development, the knowledge gained from this exploratory study would contribute to the overall understanding of *S. aureus* pathogenesis.

III. Evaluation of multi-component exotoxin *S. aureus* vaccine and immunotherapy

in preventing *S. aureus* disease.

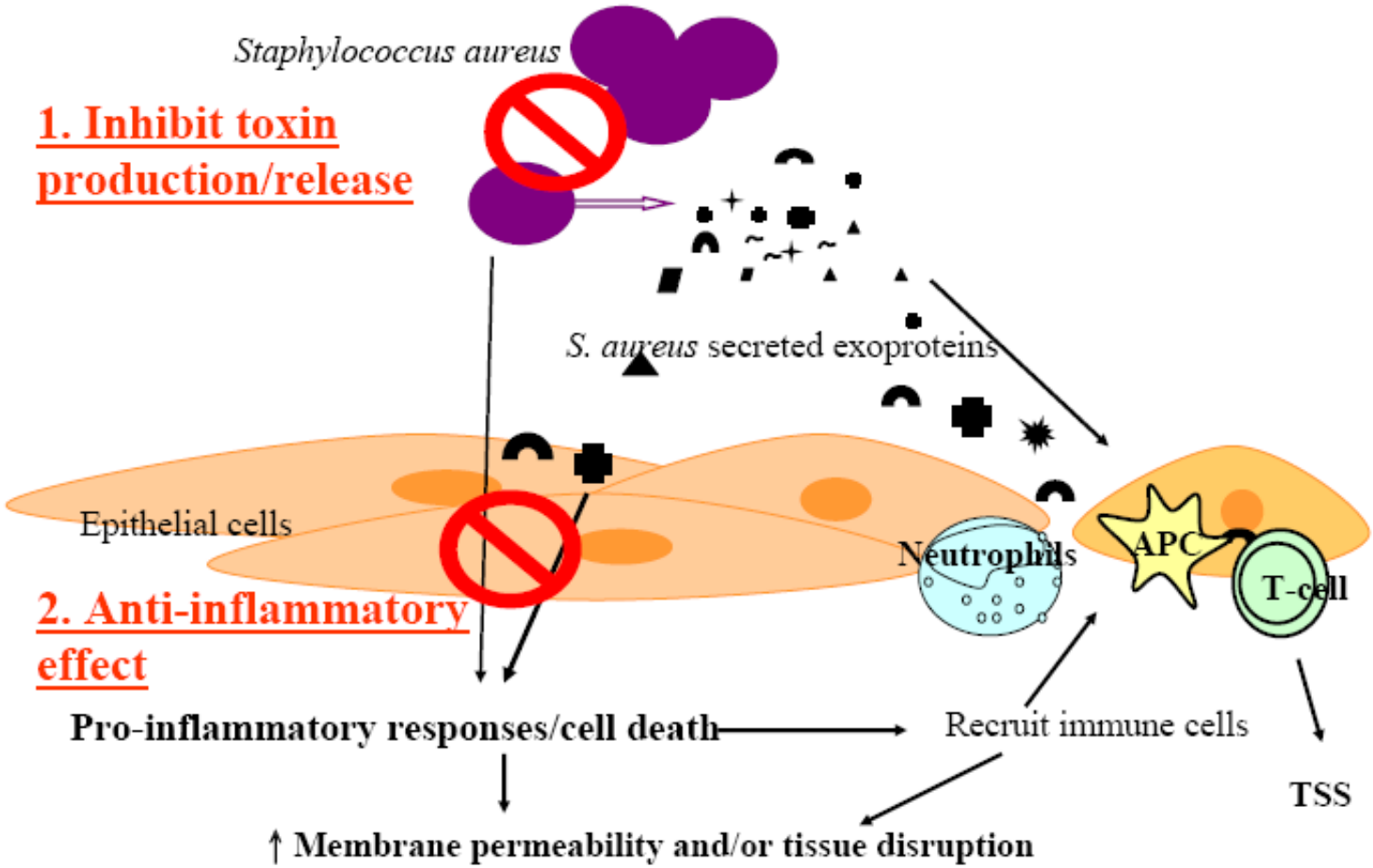
Targeting bacterial cell wall components was a successful vaccination approach against *Haemophilus influenzae* and *Streptococcus pneumoniae*, and has significantly decreased lethal meningitis in children under 5 years of age [174,175]. However, this approach of targeting capsular polysaccharides (CP) or cell wall components, either as active or passive vaccination, has been disappointing in *S. aureus* vaccine development (see Table 1.5). As mentioned above, CP and ClfA, two *S. aureus* cell wall components that have been evaluated as *S. aureus* vaccine targets, are variably expressed among strains *in vivo* [109], and passive immunization with antibodies to either CP or ClfA alone selected for stable unencapsulated *S. aureus* mutants and small colony variants [108]. Therefore, targeting multi-components is necessary against a versatile pathogen like *S. aureus*. In contrast to *H. influenzae* and *S. pneumoniae*, exotoxins of *S. aureus* (i.e. α-toxin and SAgs) have been determined to play important roles in *S. aureus* pathogenesis and are sufficient to induce disease without the presence of the bacteria. Vaccination against cytotoxic bacterial toxins has been successful in the past at preventing toxin-mediated bacterial diseases, such as diphtheria (caused by toxin produced by *Corynebacterium diphtheriae*) and tetanus (caused by toxin produced by *Clostridium tetani*). Therefore, these toxic proteins should be considered in *S. aureus* vaccine development to ensure the effectiveness of the vaccination.

In the present study, I also demonstrated that preventing toxins and/or their effects on host cells significantly reduced the severity of the *S. aureus* infection and identified that cytolysins, α- and γ-toxins, SAgs, and staphopain as potential *S. aureus*

vaccine targets. Therefore, I hypothesize that neutralizing *S. aureus* toxic exoproteins such as cytolysins and SAgs simultaneously via vaccination can decrease *S. aureus* virulence. Vaccination with cytolysins and SAgs may provide several levels of protection: First, antibodies (i.e. secretory IgA [sIgA]) in the mucosa neutralize *S. aureus* toxic exoproteins before they damage host epithelial cells, which protects the integrity of mucosal barrier. Secondly, antibodies (i.e. IgG) in the serum neutralize toxins to prevent their ability to bind T cells and APCs, and the IgG facilitates bacterial clearance by host immune mechanisms. Thirdly, antibodies against SAgs can prevent the toxin's function to alter proper immune response, which not only directly prevents TSS but also allows the host to derive anti-staphylococcal immunity. SAgs impede proper humoral response (antibody generation) to the bacteria and also deplete certain subtypes of T-cells (via T-cell anergy). This anti-toxin approach may be especially important as passive immunization in high risk populations of *S. aureus* infections, i.e. premature neonates, elderly, and immunocompromised patients, who already lack suitable ability to develop proper immune response.

Therefore, I plan to develop a step-wise approach to investigate the effectiveness of vaccination against these components using a rabbit TSS model and infecting rabbits vaginally with a lethal dose of bacteria; rabbits are chosen because mice are not sensitive to *S. aureus* exoproteins such as SAgs and cytolysins. The most important exotoxin components selected from this preliminary study will then be further evaluated in combination with or without *S. aureus* cell wall-associated component(s). The result of this study will provide insights for future antistaphylococcal treatments and preventions.

Figure 4.1 The model for anti-virulence staphylococcal therapies. *S aureus* and its exoproteins induce pro-inflammatory activity and/or cytotoxicity to epithelial cells, which increase membrane permeability and cause tissue disruption that allow toxins and/or the bacterial cells to penetrate membrane barrier. *S. aureus* exoproteins such as superantigens can therefore gain access to host immune cells (i.e. neutrophils, macrophages, and T-cells) attracted to the infection site and facilitate *S. aureus* immune invasion. Anti-virulence (toxin) therapies may block the toxin to prevent *S. aureus* diseases via three main mechanisms: 1. inhibit toxin production; 2. inhibit inflammation; and/or 3. neutralize the toxins.


Staphylococcus aureus
1. Inhibit toxin production/release
S. aureus secreted exoproteins
Epithelial cells
Neutrophils
APC
T-cell
2. Anti-inflammatory effect
Pro-inflammatory responses/cell death
Recruit immune cells
TSS
↑ Membrane permeability and/or tissue disruption

CHAPTER V.

Other Publications Related to This Work

Vaginal *Staphylococcus aureus* superantigen profile shift from 1980 and 1981 to 2003, 2004, and 2005. Schlievert PM, Case LC, Strandberg KL, Tripp TJ, **Lin YC**, Peterson ML. (J Clin Microbiol. 2007 45(8):2704-7)

We determined vaginal *Staphylococcus aureus* superantigens. Staphylococci were quantified from tampons/diaphragms in 2003 to 2005, with counts compared to those determined in 1980 and 1981. In 2003 to 2005, more women were colonized than in 1980 and 1981 (23 versus 12%). Enterotoxins G and I and enterotoxin-like superantigens M and N declined, but enterotoxin-like superantigens K, L, and Q increased.

Secreted Virulence Factor Comparison Between Methicillin-Resistant and Methicillin-Sensitive *Staphylococcus aureus*, and its Relevance to Atopic Dermatitis.

Schlievert PM, Strandberg KL, **Lin YC**, Peterson ML, Leung DY (J Allergy and Clin Immunol. 2010 125(1):39-49)

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains have emerged as serious health threats in the last 15 years. They are associated with large numbers of atopic dermatitis skin and soft tissue infections, but also originating from skin and mucous membranes, have the capacity to produce sepsis and highly fatal pulmonary infections characterized as necrotizing pneumonia, purpura fulminans, and post-viral TSS. This review is a discussion of the emergence of three major CA-MRSA organisms, designated CA-MRSA USA400, followed by USA300, and most recently USA200. CA-MRSA USA300 and USA400 isolates and their methicillin-sensitive counterparts (CA-MSSA) typically produce highly inflammatory cytolysins α-toxin, γ-toxin, δ-toxin (as representative of the phenol soluble modulin family of cytolysins), and Panton Valentine leukocidin. USA300 isolates produce the superantigens enterotoxin-like Q and a highly pyrogenic deletion variant of toxic shock syndrome toxin-1 (TSST-1), whereas USA400 isolates produce the superantigens staphylococcal enterotoxin (SE) B or SEC. USA200 CA-MRSA isolates produce small amounts of cytolysins but produce high levels of TSST-1. In contrast, their MSSA counterparts produce various cytolysins, apparently in part dependent on niche occupied in the host, and levels of TSST-1 expressed. Significant differences seen in production of secreted virulence factors by CA-MRSA versus hospital-associated MRSA and CA-MSSA strains appear to be due to the need to specialize as the result of energy drains from both virulence factor production and methicillin-resistance.

Glycerol Monolaurate Inhibits *Candida,* and *Gardnerella vaginalis In Vitro* and *In Vivo* but not *Lactobacillus*. Strandberg KL, Peterson ML, **Lin YC**, Pack MC, Chase DJ, Schlievert PM (Antimicrob Agents Chemother. 2010 54(2)597-601)

We investigated glycerol monolaurate (GML) effects on *Lactobacillus, Candida*, and *Gardnerella vaginalis* human vaginal microflora. Our previous work demonstrated that 6 months of GML vaginally does not alter lactobacilli counts in monkeys. *Candida* and *G. vaginalis* are commonly associated with vaginal infections in women, many becoming chronic or recurrent. *In vitro* growth-inhibition studies determined effects of GML (0 to 500 μg/ml) against multiple *Candida* species and *G. vaginalis*. A randomized, double-blind study investigated effects of GML on vaginal microflora *Lactobacillus, Candida,* and *G. vaginalis* in colonized or infected women (n=38). Women self-administered intra-vaginal gels containing 0% (n=16), 0.5% (n=13), or 5% (n=9) GML every 12 h for 2 days. Vaginal swabs were collected before, immediately after first gel administration, and 12 h after final gel administration. Swabs were quantified for *Lactobacillus, Candida, G. vaginalis,* and GML. *In vitro* GML concentrations of 500 μg/ml were candicidal for all species tested, while concentrations ≥5 μg/ml were bactericidal for *G. vaginalis.* Control and GML gels vaginally in women did not alter vaginal pH or *Lactobacillus* counts. Control gels reduced *G. vaginalis* counts but not *Candida* counts, whereas GML gels reduced both *Candida* and *G. vaginalis*. No adverse events were reported by participating women. GML is antimicrobial for *Candida* and *G. vaginalis in vitro*. Vaginal GML gels in women do not affect *Lactobacillus* negatively, but significantly reduce *Candida* and *G. vaginalis*. GML gels may improve overall vaginal health.

References

1. Klein E, Smith DL, Laxminarayan R (2007) Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. Emerg Infect Dis 13: 1840-1846.
2. Klevens RM, Edwards JR, Tenover FC, McDonald LC, Horan T, et al. (2006) Changes in the epidemiology of methicillin-resistant *Staphylococcus aureus* in intensive care units in US hospitals, 1992-2003. Clin Infect Dis 42: 389-391.
3. King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, et al. (2006) Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med 144: 309-317.
4. Reed SD, Friedman JY, Engemann JJ, Griffiths RI, Anstrom KJ, et al. (2005) Costs and outcomes among hemodialysis-dependent patients with methicillin-resistant or methicillin-susceptible *Staphylococcus aureus* bacteremia. Infect Control Hosp Epidemiol 26: 175-183.
5. Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, et al. (2005) The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges. Infect Control Hosp Epidemiol 26: 166-174.
6. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, et al. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 298: 1763-1771.
7. Boucher HW, Corey GR (2008) Epidemiology of methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis 46 Suppl 5: S344-349.
8. Bubeck Wardenburg J, Schneewind O (2008) Vaccine protection against *Staphylococcus aureus* pneumonia. J Exp Med 205: 287-294.
9. Brosnahan AJ, Mantz MJ, Squier CA, Peterson ML, Schlievert PM (2009) Cytolysins augment superantigen penetration of stratified mucosa. J Immunol 182: 2364-2373.
10. Stevens DL, Ma Y, Salmi DB, McIndoo E, Wallace RJ, et al. (2007) Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. J Infect Dis 195: 202-211.
11. Styers D, Sheehan DJ, Hogan P, Sahm DF (2006) Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann Clin Microbiol Antimicrob 5: 2.
12. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, et al. (2003) Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA 290: 2976-2984.
13. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, et al. (2008) Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. J Infect Dis 197: 1226-1234.
14. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, et al. (2006)

Methicillin-resistant S. aureus infections among patients in the emergency department. N Engl J Med 355: 666-674.
15. Saiman L, O'Keefe M, Graham PL, 3rd, Wu F, Said-Salim B, et al. (2003) Hospital transmission of community-acquired methicillin-resistant *Staphylococcus aureus* among postpartum women. Clin Infect Dis 37: 1313-1319.
16. Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, et al. (2006) Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin Infect Dis 42: 647-656.
17. Limbago B, Fosheim GE, Schoonover V, Crane CE, Nadle J, et al. (2009) Characterization of methicillin-resistant *Staphylococcus aureus* isolates collected in 2005 and 2006 from patients with invasive disease: a population-based analysis. J Clin Microbiol 47: 1344-1351.
18. Fey PD, Said-Salim B, Rupp ME, Hinrichs SH, Boxrud DJ, et al. (2003) Comparative molecular analysis of community- or hospital-acquired methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 47: 196-203.
19. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, et al. (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol 41: 5113-5120.
20. Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, et al. (2003) Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J Clin Microbiol 41: 1434-1439.
21. Schlievert PM, Case LC, Strandberg KL, Tripp TJ, Lin YC, et al. (2007) Vaginal *Staphylococcus aureus* superantigen profile shift from 1980 and 1981 to 2003, 2004, and 2005. J Clin Microbiol 45: 2704-2707.
22. Bernal A, Proft T, Fraser JD, Posnett DN (1999) Superantigens in human disease. J Clin Immunol 19: 149-157.
23. McCormick JK, Yarwood JM, Schlievert PM (2001) Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol 55: 77-104.
24. Foster TJ (2005) Immune evasion by staphylococci. Nat Rev Microbiol 3: 948-958.
25. Hajjeh RA, Reingold A, Weil A, Shutt K, Schuchat A, et al. (1999) Toxic shock syndrome in the United States: surveillance update, 1979 1996. Emerg Infect Dis 5: 807-810.
26. Schlievert PM, Tripp TJ, Peterson ML (2004) Reemergence of staphylococcal toxic shock syndrome in Minneapolis-St. Paul, Minnesota, during the 2000-2003 surveillance period. J Clin Microbiol 42: 2875-2876.
27. Descloux E, Perpoint T, Ferry T, Lina G, Bes M, et al. (2008) One in five mortality in non-menstrual toxic shock syndrome versus no mortality in menstrual cases in a balanced French series of 55 cases. Eur J Clin Microbiol Infect Dis 27: 37-43.
28. O'Riordan K, Lee JC (2004) *Staphylococcus aureus* capsular polysaccharides. Clin Microbiol Rev 17: 218-234.

29. Song Y, Liu CI, Lin FY, No JH, Hensler M, et al. (2009) Inhibition of staphyloxanthin virulence factor biosynthesis in *Staphylococcus aureus*: *in vitro*, *in vivo*, and crystallographic results. J Med Chem 52: 3869-3880.
30. Larkin EA, Carman RJ, Krakauer T, Stiles BG (2009) *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. Curr Med Chem 16: 4003-4019.
31. Yarwood JM, Leung DY, Schlievert PM (2000) Evidence for the involvement of bacterial superantigens in psoriasis, atopic dermatitis, and Kawasaki syndrome. FEMS Microbiol Lett 192: 1-7.
32. Li LB, Goleva E, Hall CF, Ou LS, Leung DY (2004) Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK-ERK) pathway. J Allergy Clin Immunol 114: 1059-1069.
33. Li LB, Leung DY, Hall CF, Goleva E (2006) Divergent expression and function of glucocorticoid receptor beta in human monocytes and T cells. J Leukoc Biol 79: 818-827.
34. Lee PK, Vercellotti GM, Deringer JR, Schlievert PM (1991) Effects of staphylococcal toxic shock syndrome toxin 1 on aortic endothelial cells. J Infect Dis 164: 711-719.
35. Peterson ML, Ault K, Kremer MJ, Klingelhutz AJ, Davis CC, et al. (2005) The innate immune system is activated by stimulation of vaginal epithelial cells with *Staphylococcus aureus* and toxic shock syndrome toxin 1. Infect Immun 73: 2164-2174.
36. Norton SD, Schlievert PM, Novick RP, Jenkins MK (1990) Molecular requirements for T cell activation by the staphylococcal toxic shock syndrome toxin-1. J Immunol 144: 2089-2095.
37. Dinges MM, Orwin PM, Schlievert PM (2000) Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 13: 16-34, table of contents.
38. Schlievert PM (1993) Role of superantigens in human disease. J Infect Dis 167: 997-1002.
39. Holtfreter S, Roschack K, Eichler P, Eske K, Holtfreter B, et al. (2006) *Staphylococcus aureus* carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. J Infect Dis 193: 1275-1278.
40. Cone LA, Woodard DR, Byrd RG, Schulz K, Kopp SM, et al. (1992) A recalcitrant, erythematous, desquamating disorder associated with toxin-producing staphylococci in patients with AIDS. J Infect Dis 165: 638-643.
41. Takahashi N, Kato H, Imanishi K, Miwa K, Yamanami S, et al. (2000) Immunopathophysiological aspects of an emerging neonatal infectious disease induced by a bacterial superantigen. J Clin Invest 106: 1409-1415.
42. Newbould MJ, Malam J, McIllmurray JM, Morris JA, Telford DR, et al. (1989) Immunohistological localisation of staphylococcal toxic shock syndrome toxin (TSST-1) antigen in sudden infant death syndrome. J Clin Pathol 42: 935-939.
43. Curtis N, Zheng R, Lamb JR, Levin M (1995) Evidence for a superantigen mediated process in Kawasaki disease. Arch Dis Child 72: 308-311.

44. Leung DY, Meissner HC, Fulton DR, Murray DL, Kotzin BL, et al. (1993) Toxic shock syndrome toxin-secreting *Staphylococcus aureus* in Kawasaki syndrome. Lancet 342: 1385-1388.
45. Kravitz GR, Dries DJ, Peterson ML, Schlievert PM (2005) Purpura fulminans due to *Staphylococcus aureus*. Clin Infect Dis 40: 941-947.
46. Bachert C, Gevaert P, Holtappels G, Johansson SG, van Cauwenberge P (2001) Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. J Allergy Clin Immunol 107: 607-614.
47. Semic-Jusufagic A, Bachert C, Gevaert P, Holtappels G, Lowe L, et al. (2007) *Staphylococcus aureus* sensitization and allergic disease in early childhood: population-based birth cohort study. J Allergy Clin Immunol 119: 930-936.
48. Ou LS, Goleva E, Hall C, Leung DY (2004) T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. J Allergy Clin Immunol 113: 756-763.
49. Bunikowski R, Mielke ME, Skarabis H, Worm M, Anagnostopoulos I, et al. (2000) Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. J Allergy Clin Immunol 105: 814-819.
50. Leung DY, Travers JB, Giorno R, Norris DA, Skinner R, et al. (1995) Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. J Clin Invest 96: 2106-2112.
51. Schlievert PM, Osterholm MT, Kelly JA, Nishimura RD (1982) Toxin and enzyme characterization of *Staphylococcus aureus* isolates from patients with and without toxic shock syndrome. Ann Intern Med 96: 937-940.
52. O'Reilly M, Kreiswirth B, Foster TJ (1990) Cryptic alpha-toxin gene in toxic shock syndrome and septicaemia strains of *Staphylococcus aureus*. Mol Microbiol 4: 1947-1955.
53. Menestrina G, Dalla Serra M, Comai M, Coraiola M, Viero G, et al. (2003) Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus*. FEBS Lett 552: 54-60.
54. Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, et al. (2001) alpha-Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. J Cell Biol 155: 637-648.
55. Haslinger B, Strangfeld K, Peters G, Schulze-Osthoff K, Sinha B (2003) *Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway. Cell Microbiol 5: 729-741.
56. Menzies BE, Kourteva I (2000) *Staphylococcus aureus* alpha-toxin induces apoptosis in endothelial cells. FEMS Immunol Med Microbiol 29: 39-45.
57. Huseby M, Shi K, Brown CK, Digre J, Mengistu F, et al. (2007) Structure and biological activities of beta toxin from *Staphylococcus aureus*. J Bacteriol 189: 8719-8726.
58. Rogolsky M (1979) Nonenteric toxins of *Staphylococcus aureus*. Microbiol Rev 43: 320-360.

59. Collins J, Buckling A, Massey RC (2008) Identification of factors contributing to T-cell toxicity of *Staphylococcus aureus* clinical isolates. J Clin Microbiol 46: 2112-2114.
60. Tajima A, Iwase T, Shinji H, Seki K, Mizunoe Y (2009) Inhibition of endothelial interleukin-8 production and neutrophil transmigration by *Staphylococcus aureus* beta-hemolysin. Infect Immun 77: 327-334.
61. Kaneko J, Kamio Y (2004) Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem 68: 981-1003.
62. Siqueira JA, Speeg-Schatz C, Freitas FI, Sahel J, Monteil H, et al. (1997) Channel-forming leucotoxins from *Staphylococcus aureus* cause severe inflammatory reactions in a rabbit eye model. J Med Microbiol 46: 486-494.
63. Gravet A, Colin DA, Keller D, Girardot R, Monteil H, et al. (1998) Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. FEBS Lett 436: 202-208.
64. Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, et al. (1999) Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 29: 1128-1132.
65. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, et al. (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. Science 315: 1130-1133.
66. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, et al. (2006) Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis 194: 1761-1770.
67. Schwartzman WA, Beenhouwer DO, Schaberg DR (2007) How relevant were the models used to measure the impact of Panton-Valentine leukocidin in human staphylococcal infections? J Infect Dis 195: 1726-1727; author reply 1727-1728.
68. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510-1514.
69. Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, et al. (1996) Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science 274: 1859-1866.
70. Bokarewa MI, Jin T, Tarkowski A (2006) *Staphylococcus aureus*: Staphylokinase. Int J Biochem Cell Biol 38: 504-509.
71. Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA (2005) Anti-opsonic properties of staphylokinase. Microbes Infect 7: 476-484.
72. Dubin G (2002) Extracellular proteases of Staphylococcus spp. Biol Chem 383: 1075-1086.
73. McGavin MJ, Zahradka C, Rice K, Scott JE (1997) Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease. Infect Immun 65: 2621-2628.
74. Amagai M, Matsuyoshi N, Wang ZH, Andl C, Stanley JR (2000) Toxin in bullous

impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. Nat Med 6: 1275-1277.
75. Rooijakkers SH, Ruyken M, Roos A, Daha MR, Presanis JS, et al. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol 6: 920-927.
76. Harraghy N, Hussain M, Haggar A, Chavakis T, Sinha B, et al. (2003) The adhesive and immunomodulating properties of the multifunctional *Staphylococcus aureus* protein Eap. Microbiology 149: 2701-2707.
77. Fraser JD, Proft T (2008) The bacterial superantigen and superantigen-like proteins. Immunol Rev 225: 226-243.
78. Pragman AA, Schlievert PM (2004) Virulence regulation in *Staphylococcus aureus*: the need for *in vivo* analysis of virulence factor regulation. FEMS Immunol Med Microbiol 42: 147-154.
79. Yarwood JM, Schlievert PM (2000) Oxygen and carbon dioxide regulation of toxic shock syndrome toxin 1 production by *Staphylococcus aureus* MN8. J Clin Microbiol 38: 1797-1803.
80. Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, et al. (1986) Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol Gen Genet 202: 58-61.
81. Qazi SN, Counil E, Morrissey J, Rees CE, Cockayne A, et al. (2001) agr expression precedes escape of internalized *Staphylococcus aureus* from the host endosome. Infect Immun 69: 7074-7082.
82. Rogasch K, Ruhmling V, Pane-Farre J, Hoper D, Weinberg C, et al. (2006) Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. J Bacteriol 188: 7742-7758.
83. Fournier B, Klier A, Rapoport G (2001) The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol Microbiol 41: 247-261.
84. Yarwood JM, McCormick JK, Schlievert PM (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J Bacteriol 183: 1113-1123.
85. Chen LF, Chopra T, Kaye KS (2009) Pathogens resistant to antibacterial agents. Infect Dis Clin North Am 23: 817-845, vii.
86. Nailor MD, Sobel JD (2009) Antibiotics for gram-positive bacterial infections: vancomycin, teicoplanin, quinupristin/dalfopristin, oxazolidinones, daptomycin, dalbavancin, and telavancin. Infect Dis Clin North Am 23: 965-982, ix.
87. Schlievert PM, Kelly JA (1984) Clindamycin-induced suppression of toxic-shock syndrome--associated exotoxin production. J Infect Dis 149: 471.
88. Ohlsen K, Ziebuhr W, Koller KP, Hell W, Wichelhaus TA, et al. (1998) Effects of subinhibitory concentrations of antibiotics on alpha-toxin (hla) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob Agents Chemother 42: 2817-2823.
89. Stevens DL, Wallace RJ, Hamilton SM, Bryant AE (2006) Successful treatment of staphylococcal toxic shock syndrome with linezolid: a case report and *in vitro*

evaluation of the production of toxic shock syndrome toxin type 1 in the presence of antibiotics. Clin Infect Dis 42: 729-730.
90. Hoyt JC, Robbins RA (2001) Macrolide antibiotics and pulmonary inflammation. FEMS Microbiol Lett 205: 1-7.
91. Rempe S, Hayden JM, Robbins RA, Hoyt JC (2007) Tetracyclines and pulmonary inflammation. Endocr Metab Immune Disord Drug Targets 7: 232-236.
92. Krakauer T, Buckley M (2003) Doxycycline is anti-inflammatory and inhibits staphylococcal exotoxin-induced cytokines and chemokines. Antimicrob Agents Chemother 47: 3630-3633.
93. Norrby-Teglund A, Kaul R, Low DE, McGeer A, Newton DW, et al. (1996) Plasma from patients with severe invasive group A streptococcal infections treated with normal polyspecific IgG inhibits streptococcal superantigen-induced T cell proliferation and cytokine production. J Immunol 156: 3057-3064.
94. Darenberg J, Ihendyane N, Sjolin J, Aufwerber E, Haidl S, et al. (2003) Intravenous immunoglobulin G therapy in streptococcal toxic shock syndrome: a European randomized, double-blind, placebo-controlled trial. Clin Infect Dis 37: 333-340.
95. Barry W, Hudgins L, Donta ST, Pesanti EL (1992) Intravenous immunoglobulin therapy for toxic shock syndrome. JAMA 267: 3315-3316.
96. Kaul R, McGeer A, Norrby-Teglund A, Kotb M, Schwartz B, et al. (1999) Intravenous immunoglobulin therapy for streptococcal toxic shock syndrome--a comparative observational study. The Canadian Streptococcal Study Group. Clin Infect Dis 28: 800-807.
97. Laupland KB, Kirkpatrick AW, Delaney A (2007) Polyclonal intravenous immunoglobulin for the treatment of severe sepsis and septic shock in critically ill adults: a systematic review and meta-analysis. Crit Care Med 35: 2686-2692.
98. Hartung HP, Mouthon L, Ahmed R, Jordan S, Laupland KB, et al. (2009) Clinical applications of intravenous immunoglobulins (IVIg)--beyond immunodeficiencies and neurology. Clin Exp Immunol 158 Suppl 1: 23-33.
99. Ruzin A, Novick RP (2000) Equivalence of lauric acid and glycerol monolaurate as inhibitors of signal transduction in *Staphylococcus aureus*. J Bacteriol 182: 2668-2671.
100. Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP (1994) Glycerol monolaurate inhibits the production of beta-lactamase, toxic shock toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J Bacteriol 176: 4204-4209.
101. Schlievert PM, Deringer JR, Kim MH, Projan SJ, Novick RP (1992) Effect of glycerol monolaurate on bacterial growth and toxin production. Antimicrob Agents Chemother 36: 626-631.
102. Peterson ML, Schlievert PM (2006) Glycerol monolaurate inhibits the effects of Gram-positive select agents on eukaryotic cells. Biochemistry 45: 2387-2397.
103. Schlievert PM, Strandberg KL, Brosnahan AJ, Peterson ML, Pambuccian SE, et al. (2008) Glycerol monolaurate does not alter rhesus macaque (*Macaca mulatta*) vaginal lactobacilli and is safe for chronic use. Antimicrob Agents Chemother 52: 4448-4454.

104. McNamara PJ, Syverson RE, Milligan-Myhre K, Frolova O, Schroeder S, et al. (2009) Surfactants, aromatic and isoprenoid compounds, and fatty acid biosynthesis inhibitors suppress *Staphylococcus aureus* production of toxic shock syndrome toxin 1. Antimicrob Agents Chemother 53: 1898-1906.
105. Brissette JL, Cabacungan EA, Pieringer RA (1986) Studies on the antibacterial activity of dodecylglycerol. Its limited metabolism and inhibition of glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans BHT*. J Biol Chem 261: 6338-6345.
106. Buonpane RA, Churchill HR, Moza B, Sundberg EJ, Peterson ML, et al. (2007) Neutralization of staphylococcal enterotoxin B by soluble, high-affinity receptor antagonists. Nat Med 13: 725-729.
107. Schaffer AC, Lee JC (2009) Staphylococcal vaccines and immunotherapies. Infect Dis Clin North Am 23: 153-171.
108. Tuchscherr LP, Buzzola FR, Alvarez LP, Lee JC, Sordelli DO (2008) Antibodies to capsular polysaccharide and clumping factor A prevent mastitis and the emergence of unencapsulated and small-colony variants of *Staphylococcus aureus* in mice. Infect Immun 76: 5738-5744.
109. Nanra JS, Timofeyeva Y, Buitrago SM, Sellman BR, Dilts DA, et al. (2009) Heterogeneous *in vivo* expression of clumping factor A and capsular polysaccharide by *Staphylococcus aureus*: implications for vaccine design. Vaccine 27: 3276-3280.
110. Kuehnert MJ, Hill HA, Kupronis BA, Tokars JI, Solomon SL, et al. (2005) Methicillin-resistant-*Staphylococcus aureus* hospitalizations, United States. Emerg Infect Dis 11: 868-872.
111. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, et al. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis 5: 751-762.
112. Tenover FC, McAllister S, Fosheim G, McDougal LK, Carey RB, et al. (2008) Characterization of *Staphylococcus aureus* isolates from nasal cultures collected from individuals in the United States in 2001 to 2004. J Clin Microbiol 46: 2837-2841.
113. Wertheim HF, van Leeuwen WB, Snijders S, Vos MC, Voss A, et al. (2005) Associations between *Staphylococcus aureus* Genotype, Infection, and In-Hospital Mortality: A Nested Case-Control Study. J Infect Dis 192: 1196-1200.
114. Peacock SJ, Moore CE, Justice A, Kantzanou M, Story L, et al. (2002) Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect Immun 70: 4987-4996.
115. Bhakdi S, Tranum-Jensen J (1991) Alpha-toxin of *Staphylococcus aureus*. Microbiol Rev 55: 733-751.
116. Hecker M, Engelmann S, Cordwell SJ (2003) Proteomics of *Staphylococcus aureus*--current state and future challenges. J Chromatogr B Analyt Technol Biomed Life Sci 787: 179-195.
117. Burlak C, Hammer CH, Robinson MA, Whitney AR, McGavin MJ, et al. (2007)

Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced *in vitro* and during infection. Cell Microbiol 9: 1172-1190.
118. MacDonald KL, Osterholm MT, Hedberg CW, Schrock CG, Peterson GF, et al. (1987) Toxic shock syndrome. A newly recognized complication of influenza and influenzalike illness. JAMA 257: 1053-1058.
119. Schlievert PM, Shands KN, Dan BB, Schmid GP, Nishimura RD (1981) Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. J Infect Dis 143: 509-516.
120. Squier CA, Mantz MJ, Schlievert PM, Davis CC (2008) Porcine vagina *ex vivo* as a model for studying permeability and pathogenesis in mucosa. J Pharm Sci 97: 9-21.
121. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
122. Fichorova RN, Rheinwald JG, Anderson DJ (1997) Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 57: 847-855.
123. Murray DL, Earhart CA, Mitchell DT, Ohlendorf DH, Novick RP, et al. (1996) Localization of biologically important regions on toxic shock syndrome toxin 1. Infect Immun 64: 371-374.
124. Mollby R, Wadstrom T (1971) Separation of Gamma Hemolysin from *Staphylococcus aureus* Smith 5R. Infect Immun 3: 633-635.
125. Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339: 520-532.
126. Belin D (2003) Why are suppressors of amber mutations so frequent among Escherichia coli K12 strains? A plausible explanation for a long-lasting puzzle. Genetics 165: 455-456.
127. Montgomery CP, Boyle-Vavra S, Adem PV, Lee JC, Husain AN, et al. (2008) Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. J Infect Dis 198: 561-570.
128. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, et al. (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103: 8487-8492.
129. Kalia A, Bessen DE (2004) Natural selection and evolution of streptococcal virulence genes involved in tissue-specific adaptations. J Bacteriol 186: 110-121.
130. Josefsson E, Kubica M, Mydel P, Potempa J, Tarkowski A (2008) *In vivo* sortase A and clumping factor A mRNA expression during *Staphylococcus aureus* infection. Microb Pathog 44: 103-110.
131. Diep BA, Otto M (2008) The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol 16: 361-369.
132. Dajcs JJ, Thibodeaux BA, Girgis DO, O'Callaghan RJ (2002) Corneal virulence of *Staphylococcus aureus* in an experimental model of keratitis. DNA Cell Biol 21:

375-382.
133. Imamura T, Tanase S, Szmyd G, Kozik A, Travis J, et al. (2005) Induction of vascular leakage through release of bradykinin and a novel kinin by cysteine proteinases from *Staphylococcus aureus*. J Exp Med 201: 1669-1676.
134. Gordon RJ, Lowy FD (2008) Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis 46 Suppl 5: S350-359.
135. Chuang YY, Huang YC, Lin TY (2005) Toxic shock syndrome in children: epidemiology, pathogenesis, and management. Paediatr Drugs 7: 11-25.
136. Jarman A, Duggal A, Korus L, Tredget EE (2007) Toxic shock syndrome in an adult burn patient. Burns 33: 1051-1053.
137. Trop M, Zobel G, Roedl S, Grubbauer HM, Feierl G (2004) Toxic shock syndrome in a scald burn child treated with an occlusive wound dressing. Burns 30: 176-180.
138. Schlievert PM, Case LC, Strandberg KL, Galask RP, Pack MC, et al. (2007) Vaginal *Staphylococcus aureus* toxin production is reduced by glycerol monolaurate in tampons. Obstet Gynecol 109: 1S-127S.
139. Li Q, Estes JD, Schlievert PM, Duan L, Brosnahan AJ, et al. (2009) Glycerol monolaurate prevents mucosal SIV transmission. Nature 458: 1034-1038.
140. Strandberg KL, Peterson ML, Lin YC, Pack MC, Chase DJ, et al. (2010) Glycerol monolaurate inhibits *Candida* and *Gardnerella vaginalis in vitro* and *in vivo* but not *Lactobacillus*. Antimicrob Agents Chemother 54: 597-601.
141. Ved HS, Gustow E, Mahadevan V, Pieringer RA (1984) Dodecylglycerol. A new type of antibacterial agent which stimulates autolysin activity in *Streptococcus faecium* ATCC 9790. J Biol Chem 259: 8115-8121.
142. Ved HS, Gustow E, Pieringer RA (1984) The involvement of the proteinase of *Streptococcus faecium* ATCC 9790 in the stimulation of its autolysin activity by dodecylglycerol. J Biol Chem 259: 8122-8124.
143. Ved HS, Gustow E, Pieringer RA (1990) Synergism between penicillin G and the antimicrobial ether lipid, rac-1-dodecylglycerol, acting below its critical micelle concentration. Lipids 25: 119-121.
144. Haynes MP, Buckley HR, Higgins ML, Pieringer RA (1994) Synergism between the antifungal agents amphotericin B and alkyl glycerol ethers. Antimicrob Agents Chemother 38: 1523-1529.
145. Pankey GA, Sabath LD (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clinical infectious diseases 38: 864-870.
146. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
147. Schlievert PM (1988) Immunochemical assays for toxic shock syndrome toxin-1. Methods Enzymol 165: 339-344.
148. Scott DF, Kling JM, Kirkland JJ, Best GK (1983) Characterization of *Staphylococcus aureus* isolates from patients with toxic shock syndrome, using polyethylene infection chambers in rabbits. Infect Immun 39: 383-387.
149. Schlievert PM (2007) Chitosan malate inhibits growth and exotoxin production of

toxic shock syndrome-inducing *Staphylococcus aureus* strains and group A streptococci. Antimicrob Agents Chemother 51: 3056-3062.
150. Yarwood JM, McCormick JK, Paustian ML, Kapur V, Schlievert PM (2002) Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. J Bacteriol 184: 1095-1101.
151. Kitahara T, Koyama N, Matsuda J, Aoyama Y, Hirakata Y, et al. (2004) Antimicrobial activity of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus*. Biol Pharm Bull 27: 1321-1326.
152. Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP (1972) Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2: 23-28.
153. Holland KT, Taylor D, Farrell AM (1994) The effect of glycerol monolaurate on growth of, and production of toxic shock syndrome toxin-1 and lipase by, *Staphylococcus aureus*. J Antimicrob Chemother 33: 41-55.
154. Preuss HG, Echard B, Enig M, Brook I, Elliott TB (2005) Minimum inhibitory concentrations of herbal essential oils and monolaurin for gram-positive and gram-negative bacteria. Mol Cell Biochem 272: 29-34.
155. Kelsey JA, Bayles KW, Shafii B, McGuire MA (2006) Fatty acids and monoacylglycerols inhibit growth of *Staphylococcus aureus*. Lipids 41: 951-961.
156. Kenny JG, Ward D, Josefsson E, Jonsson IM, Hinds J, et al. (2009) The *Staphylococcus aureus* response to unsaturated long chain free fatty acids: survival mechanisms and virulence implications. PLoS One 4: e4344.
157. Johnson W, Jr. (2004) Final report of the amended safety assessment of Glyceryl Laurate, Glyceryl Laurate SE, Glyceryl Laurate/Oleate, Glyceryl Adipate, Glyceryl Alginate, Glyceryl Arachidate, Glyceryl Arachidonate, Glyceryl Behenate, Glyceryl Caprate, Glyceryl Caprylate, Glyceryl Caprylate/Caprate, Glyceryl Citrate/Lactate/Linoleate/Oleate, Glyceryl Cocoate, Glyceryl Collagenate, Glyceryl Erucate, Glyceryl Hydrogenated Rosinate, Glyceryl Hydrogenated Soyate, Glyceryl Hydroxystearate, Glyceryl Isopalmitate, Glyceryl Isostearate, Glyceryl Isostearate/Myristate, Glyceryl Isostearates, Glyceryl Lanolate, Glyceryl Linoleate, Glyceryl Linolenate, Glyceryl Montanate, Glyceryl Myristate, Glyceryl Isotridecanoate/Stearate/Adipate, Glyceryl Oleate SE, Glyceryl Oleate/Elaidate, Glyceryl Palmitate, Glyceryl Palmitate/Stearate, Glyceryl Palmitoleate, Glyceryl Pentadecanoate, Glyceryl Polyacrylate, Glyceryl Rosinate, Glyceryl Sesquioleate, Glyceryl/Sorbitol Oleate/Hydroxystearate, Glyceryl Stearate/Acetate, Glyceryl Stearate/Maleate, Glyceryl Tallowate, Glyceryl Thiopropionate, and Glyceryl Undecylenate. Int J Toxicol 23 Suppl 2: 55-94.
158. Weber N (1985) Metabolism of orally administered rac-1-O-[1'-14C]dodecylglycerol and nutritional effects of dietary rac-1-O-dodecylglycerol in mice. J Lipid Res 26: 1412-1420.
159. Schlievert PM, Blomster DA (1983) Production of staphylococcal pyrogenic exotoxin type C: influence of physical and chemical factors. J Infect Dis 147: 236-242.
160. Hill DR, Brunner ME, Schmitz DC, Davis CC, Flood JA, et al. (2005) *In vivo*

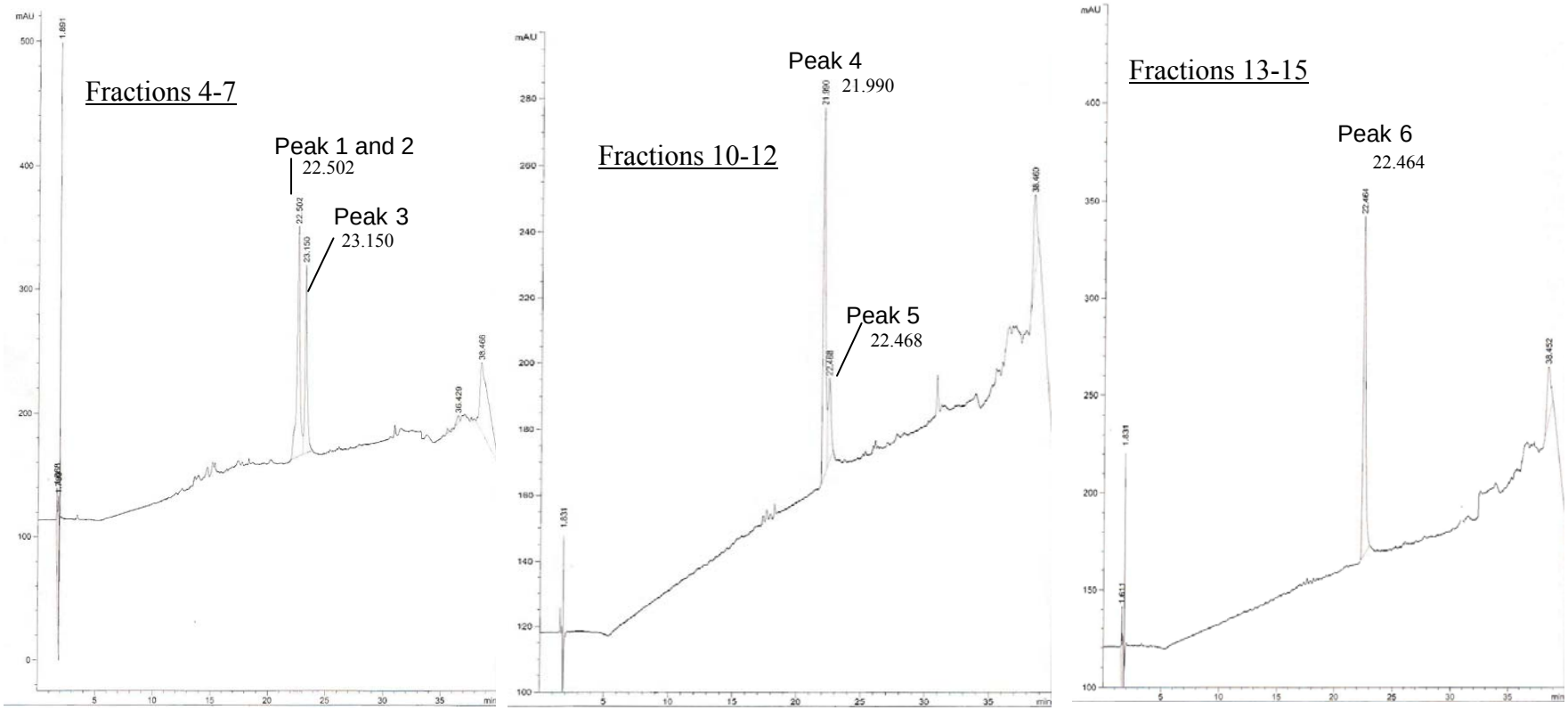
assessment of human vaginal oxygen and carbon dioxide levels during and post menses. J Appl Physiol 99: 1582-1591.
161. Todd JK, Todd BH, Franco-Buff A, Smith CM, Lawellin DW (1987) Influence of focal growth conditions on the pathogenesis of toxic shock syndrome. J Infect Dis 155: 673-681.
162. Smagur J, Guzik K, Bzowska M, Kuzak M, Zarebski M, et al. (2009) Staphylococcal cysteine protease staphopain B (SspB) induces rapid engulfment of human neutrophils and monocytes by macrophages. Biol Chem 390: 361-371.
163. Jones RC, Deck J, Edmondson RD, Hart ME (2008) Relative quantitative comparisons of the extracellular protein profiles of *Staphylococcus aureus* UAMS-1 and its sarA, agr, and sarA agr regulatory mutants using one-dimensional polyacrylamide gel electrophoresis and nanocapillary liquid chromatography coupled with tandem mass spectrometry. J Bacteriol 190: 5265-5278.
164. Kurek M, Pryjma K, Bartkowski S, Heczko PB (1977) Anti-staphylococcal gamma hemolysin antibodies in rabbits with staphylococcal osteomyelitis. Med Microbiol Immunol 163: 61-65.
165. Yang ES, Tan J, Eells S, Rieg G, Tagudar G, et al. (2009) Body site colonization in patients with community-associated methicillin-resistant *Staphylococcus aureus* and other types of S. aureus skin infections. Clin Microbiol Infect. [Epub]
166. Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, et al. (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A 101: 9786-9791.
167. Strandberg KL, Peterson ML, Schaefers MM, Case LC, Pack MC, et al. (2009) Reduction in *Staphylococcus aureus* growth and exotoxin production and in vaginal interleukin 8 levels due to glycerol monolaurate in tampons. Clin Infect Dis 49: 1711-1717.
168. Bubeck Wardenburg J, Williams WA, Missiakas D (2006) Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. Proc Natl Acad Sci U S A 103: 13831-13836.
169. LeClaire RD, Hunt RE, Bavari S (2002) Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. Infect Immun 70: 2278-2281.
170. Clyne M, De Azavedo J, Carlson E, Arbuthnott J (1988) Production of gamma-hemolysin and lack of production of alpha-hemolysin by *Staphylococcus aureus* strains associated with toxic shock syndrome. J Clin Microbiol 26: 535-539.
171. Isak G, Ryden-Aulin M (2009) Hypomodification of the wobble base in tRNAGlu, tRNALys, and tRNAGln suppresses the temperature-sensitive phenotype caused by mutant release factor 1. J Bacteriol 191: 1604-1609.
172. Kiktev D, Moskalenko S, Murina O, Baudin-Baillieu A, Rousset JP, et al. (2009) The paradox of viable sup45 STOP mutations: a necessary equilibrium between translational readthrough, activity and stability of the protein. Mol Genet

Genomics 282: 83-96.
173. Nilsson M, Ryden-Aulin M (2003) Glutamine is incorporated at the nonsense codons UAG and UAA in a suppressor-free *Escherichia coli* strain. Biochim Biophys Acta 1627: 1-6.
174. van der Poll T, Opal SM (2009) Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 374: 1543-1556.
175. Watt JP, Wolfson LJ, O'Brien KL, Henkle E, Deloria-Knoll M, et al. (2009) Burden of disease caused by *Haemophilus influenzae* type b in children younger than 5 years: global estimates. Lancet 374: 903-911.

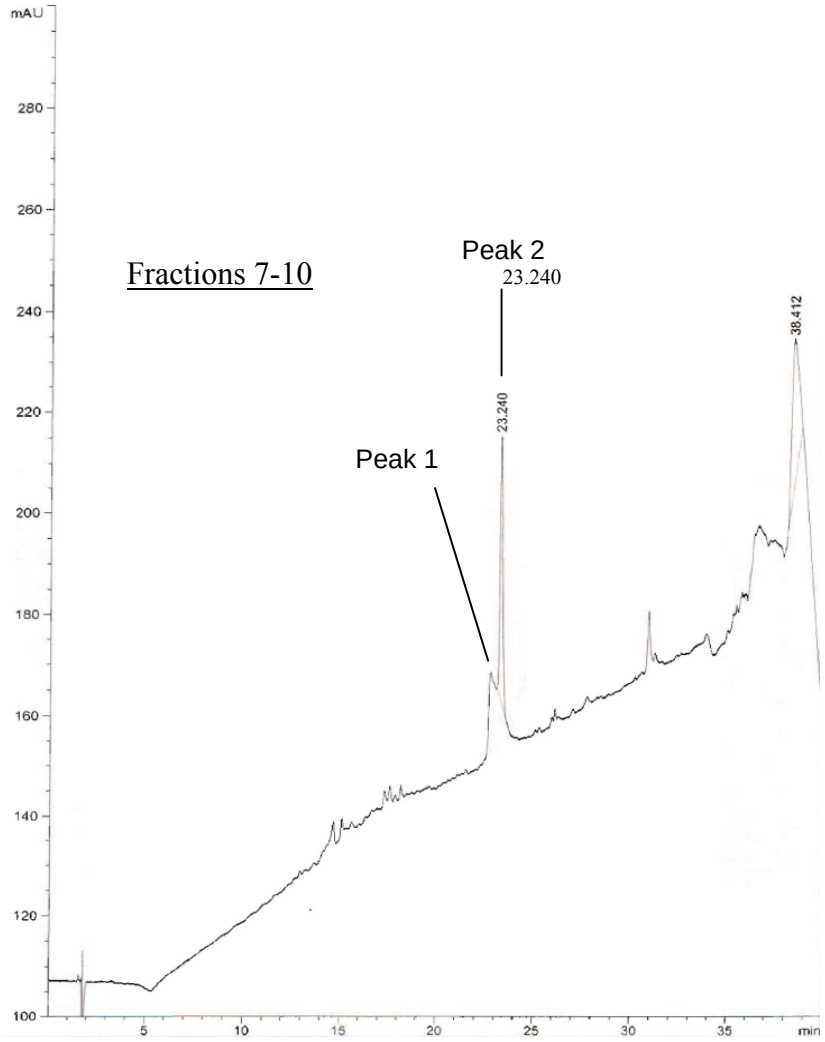
Appendices

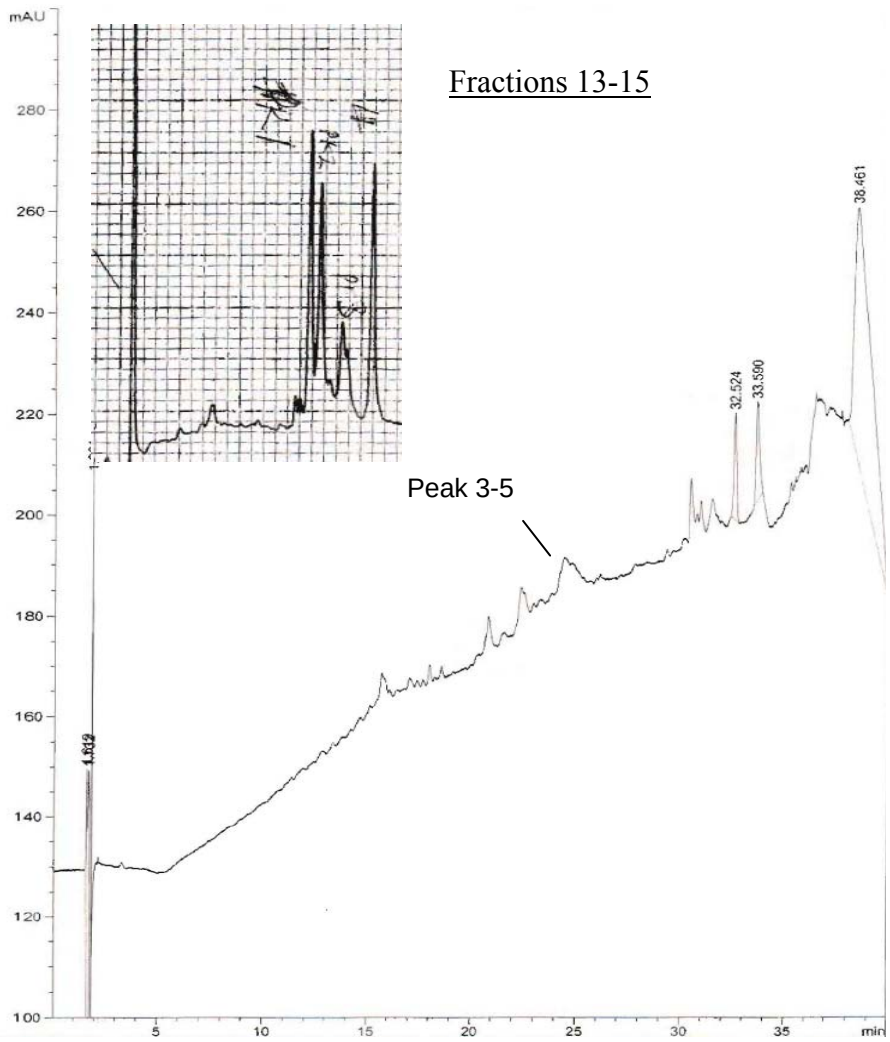
A1: rHPLC chromatograms of MNPE and CDC587 protein fractions

(A) Chromatograms of MNPE fractions



(B) Chromatograms of CDC587 fractions





A2: CDC587 α-toxin sequences identified by mass spectrometry

gi|2914574 Chain G, Alpha-Hemolysin From *Staphylococcus aureus* (61% coverage)

ADSDINIKTGTTDIGSNTTVKTGDLVTYDKENGMHKKVFYSFIDDKNHNKKLLVIRTK**GTIAGQYRVYSEE**

GANKSGLAWPSAFKVQ**L**QLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGK**IGGLIGANVSI**

the amino acid substituted by a stop codon

GHTLKYVQPDFKTILESPTDKKVGWKVIFNNMVNQNWGPYDR**DSWNPVYGNQLFMKTRNGSMKAADNFLDP**

NKASSLLSSGFSPDFATVITMDRKASK**QQTNIDVIYERVRDDYGLHWTSTNWK**GTNTKDKWTTDRSS

ER**YKIDWEKEEMTN**

Note: Peptides that were positively identified by mass spectrometry were bolded and underlined.

A3: Overview of data collections during the rabbit Wiffle ball experiment

Rabbit	Treatment	Temp	Diarrhea; weight changes	Wiffle ball fluids		
				Bacterial counts	TSST-1	TNF-α
Day 0	x	x	x	x		x
Day 1		x	x	x		x
Day 2	x	x	x	x	x	x
Day 3		x	x	x		x
Day 4	x	x	x	x		x
Day 5		x	x	x		x
Day 6	x	x	x	x		x
Day 7		x	x	x	x	x

x indicates that the data was collected on that day.