UNIVERSITY OF WISCONSIN – LA CROSSE

Graduate Studies

IN VITRO REGULATION OF THE *seq* AND *sek* STAPHYLOCOCCAL ENTEROTOXIN GENES AMONG FOUR ALLELIC VARIANTS

A Chapter Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

Lindsay R. Sammons

College of Science and Health
Clinical Microbiology Concentration

December, 2010

IN VITRO REGULATION OF THE *seq* AND *sek* STAPHYLOCOCCAL ENTEROTOXIN GENES AMONG FOUR ALLELIC VARIANTS

By Lindsay R. Sammons

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology, Clinical Microbiology Concentration.

The candidate has completed the oral defense of the thesis.

William Schwan — 11-9-10

William Schwan, Ph.D. — Date
Thesis Committee Chairperson

Written thesis acceptable. No judgement on thesis oral defense as I was not invited to the thesis defense.

Sanjay K. Shukla — 9-27-10

Sanjay Shukla, Ph.D. — Date
Thesis Committee Member

Marc Rott — 9/10/10

Marc Rott, Ph.D — Date
Thesis Committee Member

Bernadette Taylor-Winfrey — 9/10/10

Bernadette Taylor-Winfrey, Ph.D. — Date
Thesis Committee Member

Thesis accepted

Vijendra K. Agarwal — 12/15/10

Vijendra K. Agarwal, Ph.D. — Date
Associate Vice Chancellor for Academic Affairs

ABSTRACT

Sammons, L. R. *In vitro* regulation of the *seq* and *sek* staphylococcal enterotoxin genes among four allelic variants. MS in Biology, Clinical Microbiology Concentration, December 2010, 91 pp. (W. Schwan)

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is considered to be more virulent than hospital-associated MRSA, presumably because it possesses additional, unidentified virulence factors. Two virulence proteins epidemiologically important to CA-MRSA are staphylococcal enterotoxins Seq and Sek. Four allelic variants of the *seq/sek* locus exist with type 4 being exclusive to the USA300 CA-MRSA strain type. Detection of *seq* and *sek* from a group of clinical methicillin-sensitive *S. aureus* (MSSA), clinical MRSA, and commensal nasal isolates revealed a high prevalence of these genes within CA-MRSA compared to other *S. aureus* cohorts. Furthermore, CA-MRSA displayed *seq/sek* allele types 3 and 4 exclusively. *In vitro* expression analysis of *seq* and *sek* among all four allele types was performed. Co-transcription of the enterotoxins was revealed for all allele types. Quantitative analysis showed very low overall expression of *seq* and *sek* during growth. The lowest expression occurred during the stationary phase for all allele types, while various strain- and allele-specific levels of absolute expression existed. When comparing all four allele types, the lowest *seq* and *sek* expression occurred in allele type 3, while the highest expression of *seq* occurred in allele type 4, suggesting a potential role in the virulence of the USA300 strain type.

ACKNOWLEDGEMENTS

It is a pleasure to thank those that made this thesis possible. Thank you to my advisor Dr. Sanjay Shukla for providing his time, resources, and direction on this project, without which this work would not have been possible. Thank you for imparting upon me your knowledge on this interesting and exciting field of research. Thank you also to my advisor, Dr. William Schwan, for his invaluable assistance. Thank you for your time and willingness to help me with many aspects of this research. You have taught me a great deal both in the laboratory and in the classroom.

Thank you to my other committee members, Drs. Marc Rott, and Bernadette Taylor, for your guidance, expertise, and encouragement. Each of you has been an inspiration to me. To other members of the Marshfield Clinic staff, Dr. Kurt Reed, Dr. Jennifer Meece, Mary Stemper, and Dr. Murray Brilliant, thank you for your generous support and mentorship.

To my fellow past and present researchers in the Emerging Infectious Disease Lab, you have been instrumental in this achievement. Thank you to Jennifer Kislow, Pravin Kaldhone, Ashley Pokallus, and Madhulatha Pantrangi for providing me with thoughtful assistance with my project and for moral support throughout this journey.

I would also like to thank Dr. Steve Waring for generously contributing his time to assist me with the statistical analysis of this project, and also Dr. Michael Hoffman for his help in assisting me through the requirements for completion of my master's degree.

I would especially like to thank my parents, family, friends, and Ben for their overwhelming support, love, and patience during the past three years. Thank you for providing me the strength and encouragement to accomplish this pursuit.

This work was supported in part by a grant from the Graduate Student Research, Service, and Educational Leadership Grant program at the University of Wisconsin – La Crosse and by an NIH grant

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Staphylococcus aureus Clinical Significance

Clinical Characteristics

Staphylococcus aureus is one of the most successful and versatile human pathogens (79). The species is characterized as Gram-positive cocci occurring in grape-like clusters with a golden-pigmented colony morphology (86). *S. aureus* is highly adaptable and capable of infecting nearly every organ in the body. In about one-third of healthy individuals, the bacterium can colonize skin or the anterior nares (70). When natural immune barriers are breached, *S. aureus* infections occur, ranging from minor skin and soft tissue infections to more severe and invasive disease, such as bacteremia, pneumonia, and endocarditis (2, 3, 67, 83).

Resistance to Antibiotics

The extensive use of penicillin in the 1940s quickly selected for *S. aureus* isolates that could produce the enzyme β-lactamase, converting penicillin into penicilloic acid (1). By 1960, most *S. aureus* isolates were resistant to penicillin (126). The introduction in 1959 of a semi-synthetic beta-lactam antibiotic, methicillin, gave rise to methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961 (107). Methicillin resistance is a result of the acquisition of a mobile genetic element, referred to as SCC *mec*, containing *mecA*, a gene encoding an altered penicillin-binding protein (PBP2a) (43). Since the development of MRSA in the 1960s, hospital-associated MRSA (HA-MRSA) infection

rates have continued to increase. In 1975, *S. aureus* infections due to MRSA in intensive care units were reported to be 2.5%. By 2004, MRSA rates among *S. aureus* infections had risen to 60% (22). In 2005, there were about 368,600 cases of MRSA infection in U.S. hospitals, which was a 10-fold increase from 1995, a three-fold increase from 2000, and 30% increase since 2004 (36, 69). In 2000, MRSA accounted for 3.95 per 1000 patient hospitalizations and in 2006 this was reported to be 34 per 1000 patient hospitalizations (62, 69). In a 10-year study in U.S. children's hospitals, MRSA incidence rose 10-fold from 2.0 cases per 1000 admissions in 1999 to 20.7 cases per 1000 admissions in 2008 (55).

Emergence of Community-Associated MRSA

Historically, MRSA has been predominately considered a nosocomial pathogen. Since the 1990s, the epidemiology of MRSA has continued to change. Newer strains of MRSA have spread rapidly through communities, causing infections in otherwise healthy individuals who do not have typical risk factors, such as recent hospitalization, dialysis, surgery, or antibiotic use (42). These community-associated MRSA (CA-MRSA) typically cause skin and soft tissue infections. However, they have recently been reported to cause diseases not commonly attributed to *S. aureus*, such as necrotizing fasciitis, purpura fulminans, and Waterhouse-Friderichsen syndrome (2, 67, 83). In recent years, several CA-MRSA outbreaks have occurred in populations characterized by close contact between individuals, a breakdown in natural immunity, or poor hygiene. These populations have included sports teams (20), prison inmates (21), high school wrestling teams (74), the military (17), and Native Americans (117).

Presently, two major clonal groups of CA-MRSA with clinical significance are found in the United States: USA400 and USA300. Both 'USA' strain types, like all of the eight initial MRSA strain types (USA100-USA800), have unique genetic backgrounds and were identified by the Centers for Disease Control (CDC) based on their genome macrorestriction patterns using pulsed-field gel electrophoresis (81). CA-MRSA also typically harbor SCC *mec* type IV and produce the cytolysin, Panton Valentine leukocidin (PVL).

The USA400 strain, often represented by the hypervirulent Midwestern strain, MW2, was first isolated in 1998 in North Dakota from a 16-month old girl with fatal septicemia (19). Beginning in the year 2000, an unrelated CA-MRSA group, USA300, emerged in western coastal states and later spread throughout the United States (65). The USA300 clone, often represented by genotype USA300-114 (e.g. strains LAC, FPR3757, etc.), has become the predominant strain, undergoing rapid dissemination in the community and causing unusually invasive diseases (30). It is still not understood what characteristics of these strains make them more invasive than HA-MRSA strains or the USA400 clone.

S. aureus Virulence Factors.

S. aureus is known to produce a multitude of virulence factors involved in pathogenesis, contributing to the wide variety of clinical manifestations. These factors can be cell-associated or secreted into the environment. Cell-associated proteins, which are mainly expressed during the exponential growth phase, allow for host tissue colonization. Specifically, cell-associated proteins are involved in encapsulation, biofilm formation, and adherence. Some examples include the biofilm-associated protein (Bap),

staphylococcal protein A (Spa), fibronectin-binding proteins A and B (FnbA and FnbB), clumping factors A and B (ClfA and ClfB), and collagen binding protein (Cna). Secreted virulence factors are generally expressed in the post-exponential growth phase and enable *S. aureus* to destroy host tissue and evade host defenses. These include exoproteins like coagulase (Coa), α-hemolysin (Hla), and staphylokinase (Sak), as well as exotoxins like toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (Seb) (3).

Genetic Variation in *S. aureus*

The development of antibiotic resistance and emergence of epidemic strains demonstrates the extraordinary ability of *S. aureus* to evolve (37). Both small- and large-scale genome variations can play important roles in this evolution.

Core *S. aureus* Genome

The core genome makes up around 75% of the total genome and is mostly conserved, consisting mainly of essential genes present in all strains of *S. aureus*. Genetic diversity of the core genome occurs due to small-scale variations, such as single nucleotide polymorphisms (SNPs) and changes in the number of small repeat sequences. Additionally, recombination events can lead to DNA loss and rearrangement of slightly larger regions. Although small-scale variations cause little genomic diversity, they can lead to important changes in gene expression and protein function, and ultimately to phenotypic differences among strains (76).

Accessory *S. aureus* Genome

The accessory genome, covering 25% of the genome, is highly variable and consists of non-essential genes, such as those involved in virulence and drug resistance. Genetic diversity of the accessory genome occurs at a larger scale and is mainly due to

horizontal gene transfer. In *S. aureus*, this is largely accomplished by transduction between isolates. During transduction, host DNA is accidently packaged into bacteriophage particles during replication and is then integrated into the genome of another bacterial cell during bacteriophage infection (116). When the new genes are successfully integrated into the chromosome, they may offer a selective advantage to the host that can often lead to the gene's rapid spread within a *S. aureus* population. Most of the accessory genome is horizontally transferable and these regions are known as mobile genetic elements (MGEs). The MGEs include prophages and *S. aureus* pathogenicity islands (SaPIs), which tend to harbor virulence genes. They also include staphylococcal cassette chromosomes (SCC), plasmids, and transposons, which transfer resistance genes. Because MGEs tend to contain genes involved in virulence and drug resistance, horizontal transfer of MGEs can play a critical role in the evolution of pathogenic *S. aureus* (76).

S. aureus Pathogenicity Islands

Staphylococcal pathogenicity islands are MGEs that are widespread among *S. aureus.* They are characterized by their high mobility and carriage of at least two or more staphylococcal superantigen genes and other virulence genes. To date, over 20 different SaPIs have been sequenced (90). They are inserted in a single orientation in one of six identified SaPI insertion sites in the *S. aureus* genome (119). Besides virulence genes, these 14-17 kbp SaPI elements tend to encode proteins involved in mobility, such as an integrase and terminase, and phage structure, such as capsid and tail proteins, but they lack the genes necessary for phage transfer. The prophages instead rely on the presence of a 'helper' phage for induction (77). The integrase, terminase, and virulence genes tend

to lie at the extreme end of the SaPI while the structural genes tend to exist centrally. Non-matching sequences that flank some of the virulence genes suggest that the genes have been inserted via non-homologous recombination (90). In many examples, the virulence genes are present exclusively on SaPIs. Toxic shock syndrome toxin 1 (TSST-1), the only superantigen to be associated with menstrual toxic shock syndrome (TSS), is not only found on the prototypical pathogenicity island SaPI1, but also on SaPI2 and SaPIbov (38, 77). Each SaPI tends to contain a specific set of virulence genes and competition between SaPIs at insertion sites may result in the inclusion or exclusion of certain virulence genes in the *S. aureus* genome, giving a *S. aureus* strain a particular virulence gene profile. The origin of SaPIs is poorly understood. However, there is evidence for recombination events that have led to the evolution of SaPIs. SaPI5 has been recently described in the USA300 clonal group with end regions that are highly similar to those of SaPI1, while the middle region has similarity with SaPImw2, a SaPI from the USA400 clonal group (30, 90).

Superantigens

Function of Superantigens

Superantigens (SAgs) are well characterized in *S. aureus* and *Streptococcus pyogenes*, encompassing a large family of exotoxins that are defined by their ability to induce a strong immune response within the host. Unlike conventional antigens, SAgs do not need to be processed by antigen-presenting cells (APCs) before being presented to T-cells. SAg binding sites lie outside the major histocompatibility complex class II (MHC II) peptide-binding groove, and therefore do not depend on T-cell antigenic specificity. During infection, the SAgs bind to the variable region of the α- or β-chain of the T-cell

receptor (Vα- or Vβ-TCR) and to the α- and/or β-chains of MHC II molecules on APCs. Cross-linking of the TCR with MHC II causes a large, polyclonal activation of T-cells leading to a massive production of cytokines, including interleukin-2 (IL-2), interferon gamma (IFN-γ), and tumor necrosis factor β (TNF-β). An enormous release of cytokines is thought to be a contributing factor in staphylococcal toxic shock syndrome (TSS). Symptoms of TSS include fever, hypotension, skin rash, and skin exfoliation (66). SAg production by *S. aureus* is probably not meant to induce systemic shock in the host, but more likely the role is to activate local T-cells to produce cytokines that suppress local inflammation during the early stages of infection (41). A study by Vojtov *et al*. showed no significant local inflammation in hairless mice injected with a *tst*$^{+}$ strain at four days compared to mice injected with an isogenic knockout strain, which produced an open abscess characteristic of subcutaneous *S. aureus* infection (129). SAgs can induce T-cell anergy and prevent the production of toxin-specific antibody (5, 100). These activities are proposed to confer immunological protection to the bacteria and prevent its clearance from the human host (134).

Staphylococcal Enterotoxins

The SAgs include staphylococcal enterotoxins (SEs), which are highly stable, heat- and enzyme-resistant exotoxins. To date, 21 different enterotoxins and related toxins have been described in *S. aureus* (95, 124) (FIG. 1). All of the enterotoxin genes are located on MGEs (9, 71, 92, 95, 134). The SEs, along with TSST-1, are included in SAg groups I-III and V based on their structural features and MHC-II binding abilities (80). Most SEs have emetic activity in addition to causing TSS symptoms and are thus

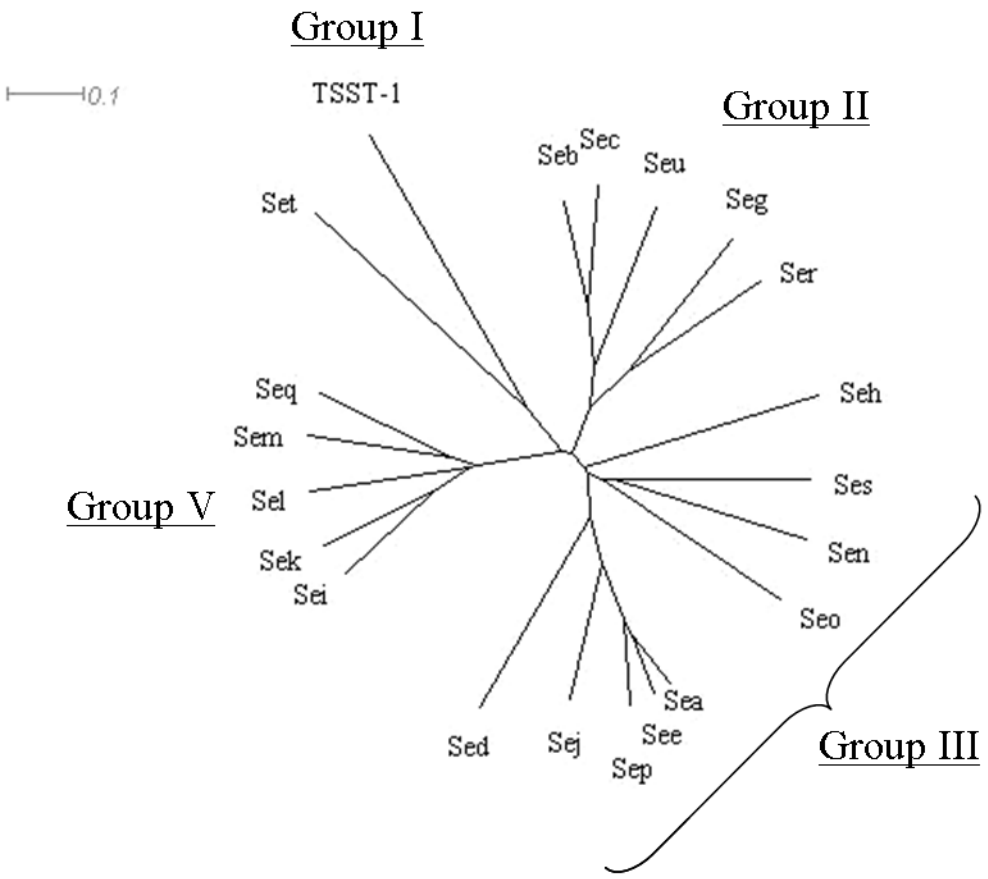


FIG. 1. A phylogenetic tree of SEs. Amino acid sequences of the mature toxins were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The alignment was performed with ClustalX software (version 2.0.12; University College Dublin [http://www.clustal.org]) and a tree was generated by means of the neighbor-joining method using SplitsTree4 software (version 4.11.3; Universität Tübingen [http://www-ab.informatik.uni-tuebingen.de/software/splitstree4]). The scale bar corresponds to 0.1 substitutions per site. The SEs are categorized into SAg groups based on structure and MHC-binding abilities.

known to be the cause of staphylococcal food poisoning. Symptoms of staphylococcal food poisoning include nausea, emesis, abdominal pain, and diarrhea 1-6 h after the ingestion of SE-contaminated food (127). Traditional enterotoxins include Sea, Seb, Sed, See, Seg and Sei. SEs with no proven emetic activity are referred to as SE-like and include Sek, Sel, and Seq, among others. TSST-1 is another SAg that is separated into its own group due to its low stability and high association with menstrual TSS (66).

Eighty percent of *S. aureus* human nasal isolates have been found to harbor at least one SE gene and most harbor many SE genes (75, 91, 110). One advantage for *S. aureus* producing multiple SEs is a greater ability to activate certain T-cell populations. Although there is some redundancy, each *S. aureus* SE is capable of activating between one and eight Vβ T-cell clones in humans. Also, each Vβ-TCR in the entire human panel is capable of stimulation by at least one known SE (125).

Production and Regulation of Staphylococcal SAgs

The amount of SAg protein production by *S. aureus* can vary depending on the SAg. Three SAgs; TSST-1, Seb, and Sec; are most often associated with TSS and when tested *in vitro* are produced in the highest concentrations (5-50 µg/ml) among all SAgs (8, 26, 108, 112, 114). Though data is limited, it is generally thought that most other SAgs are produced at much lower levels *in vitro* (92-94, 99). However, SAg amounts as low as 0.1 µg per human have been shown to cause TSS symptoms (46). Production of SAgs *in vivo* has been verified by the presence of TSS and related symptoms as well as through detection of toxins in patient serum samples (84). Additional evidence suggests that SAgs are produced in the absence of TSS and even during *S. aureus* colonization. Sea, Seb, Sec, and TSST-1 were detected in sera from sepsis patients not experiencing

septic shock (6). Also, the level of antibodies against SAgs increases in humans after the first three months of life (115).

Little is understood about the regulation of SAgs. The best known regulator is the accessory gene regulator (Agr), a component of a quorum-sensing system that is activated at high cell densities. Many genes encoding enterotoxin proteins, including Seb, Sec, Sed, TSST-1, are upregulated by Agr upon its activation during stationary growth phase (33, 60, 89). Other SAgs, like Sea, are constitutively expressed across the growth phases and are suggested to be regulated by processes that govern their surrounding phage-encoded factors (10, 27, 28, 121). The staphylococcal respiratory response A/B (SrrA-SrrB) system is considered to be a master switch repressor of exotoxins. This two-component regulatory system is activated under low-oxygen, low pH, and high glucose conditions. Upon signaling, the membrane-bound SrrB activates cytoplasmic SrrA to bind to the promoters of *spa*, *tst* and *agr*, causing repression, and to its own promoter, causing activation. Therefore, only *S. aureus* isolates containing truncated *srrA-srrB* genes are able to produce these exotoxins anaerobically (102, 103, 135). The staphylococcal accessory regulator (SarA), a DNA-binding protein, is an activator of virulence genes. In addition to being necessary for *agr* transcription, SarA independently activates TSST-1, Seb, Sec, and other exotoxin expression (23, 25, 54, 88, 104). Other regulators of enterotoxins may act through regulation of the *agr* locus. These include the two-component system, autolysis-related locus (ArlRS), which has been shown to down-regulate *hla*, *ssp* (serine protease), *spa*, and *agr* transcription while increasing *sarA* transcription (39, 40). The staphylococcal virulence regulator (SvrA) is a

membrane-associated protein that upregulates α-, β- , and δ-hemolysins and protein A and is required for *agr* transcription (44).

Seq and Sek Enterotoxins

Classification of Seq and Sek

The SE-like proteins Seq and Sek belong to the group V subfamily of staphylococcal SAgs (96, 97) (FIG.1). Like all SAgs, the biological activity of these enterotoxins includes superantigenicity, pyrogenicity, the capability to enhance endotoxin shock, and lethality (32, 111). The group V subfamily is defined by the lack of a cysteine loop structure hypothesized to be important for emetic activity. Additionally, this group has an extra 15-amino acid loop at the top of the β-grasp domain required for TCR binding (53, 66). Seq and Sek have been found to have unique profiles of Vβ-TCR specificity among the SEs with Seq capable of expanding Vβ-TCR subsets 2, 5.1, 5.2, 6.7 and 21.3 and Sek expanding subsets 1, 5.1, 5.2, and 6.7 (96, 97, 125).

Genomic Organization of *seq* and *sek*

The coding sequences of both the *seq* and *sek* genes are 729 nucleotides long and occur in tandem in the genome with *seq* located upstream of *sek* separated by 23 nucleotides. Nucleotide and amino acid sequence identity between *seq* and *sek* averages 61% and 55%, respectively. Clustering of enterotoxins in this fashion has been previously described for other SAgs, such as those found in the enterotoxin gene cluster (*egc*) and the *set* gene cluster (61, 133). Evolution of these clusters is likely the result of gene duplication and variation from an ancestral gene. This can result when recombination occurs between non-allelic regions by misalignment (73). As with all SAgs, *seq* and *sek* are located on MGEs. According to available genome sequencing data

from GenBank, four different MGEs (SaPI1, SaPI3, ΦSa3mw, and SaPI5) have been found to carry *seq* and *sek* and each has a distinct allelic variant of the enterotoxin locus (http://www.ncbi.nlm.nih.gov) (FIG. 2). The ΦSa3mw MGE has an identical *seq/sek* locus on ΦSa3ms found on a related strain (121). The MGEs have a low level of similarity and the similar regions that do exist suggest major recombination events have occurred (90, 121). Additionally, SaPIs 1, 3, and 5 all share the same integration site in the genome, while phage ΦSa3mw is found in an entirely different location in the genome, with a different integration site and a reverse orientation (90). In all MGEs, the *seq/sek* locus is located at one end of the MGE with the integrase located ~84-89 base pairs downstream of the locus in the same orientation and marking the end of the MGE. For the purposes of this study, the variant loci will be referred to as allele types 1-4 according to the order in which the MGE was first characterized: SaPI1, allele type 1; SaPI3, allele type 2; ΦSa3mw, allele type 3; SaPI5, allele type 4 (FIG. 2).

Allelic Variation of *seq* and *sek*

Variant alleles are common among SAgs and have been reported for other toxin genes, such as *sea*, *sec*, and *tst* (10, 56, 59). The entire *seq-sek* coding region, including the 23-bp intergenic sequence, is highly conserved among all the MGE variants, exhibiting at least 97% identity at the nucleotide level when comparing the most non-identical forms. Conversely, sequences of the upstream region of *seq* show little or no level of identity in all four variants (FIG. 2). Additional information on the four allelic variants of *seq* and *sek* from the GenBank database can be found in TABLE 1. It is important to note that *seq* from allele type 1 is annotated as being 771 nucleotides in length. The 42-bp extra sequence comes from a single base pair deletion in the upstream

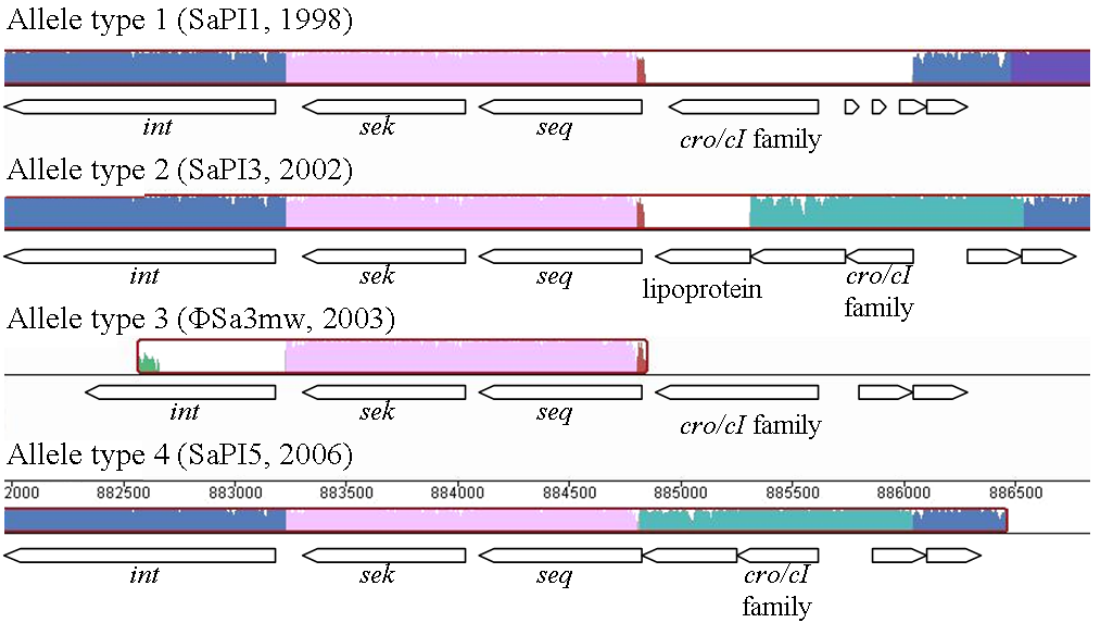


FIG. 2. Alignment of four *seq/sek* allele variants. Mauve genomic alignment software (http://gel.ahabs.wisc.edu/mauve) was used to construct an alignment of the four allelic variants of *seq* and *sek* and surrounding genes using available sequences in the GenBank database (version 2.3.0; Genome Evolution Laboratory, University of Wisconsin – Madison [http://www.ncbi.nlm.nih.gov/Genbank/index.html]). The alignment is anchored to the 882000-887000 bp region of the USA300-FPR3757 genome. Like colored regions indicate areas of high identity in the alignment whereas white or blank regions indicate no identity. Pointed bars indicate open reading frames and their orientation. *int*; integrase, *cro/cI* family; putative transcriptional regulator of the phage life cycle (refer to text).

TABLE 1. GenBank database[a] information for *seq* and *sek* from four reference *S. aureus* strains

Allele type	*S. aureus* strain (MGE[b])	Year sequenced	Genome orientation[c]	Gene annotation	Protein ID	Nucleotides	Amino acids	Genome region (bp)
1	RN4282 (SaPI1)	1998	Reverse	*seq* *sek*	AAL67620 AAC28968	771 729	256 242	~898931-899701 ~899725-900453
2	COL (SaPI3)	2002	Reverse	*sei (seq)* *sek*	AAW36440 AAW36439	729 729	242 242	906279-905551 904800-905528
3	MW2 (ΦSa3mw)	2003	Forward	*seg2 (seq)* *sek2 (sek)*	BAB95802 BAB95803	729 729	242 242	2086147-2086875 2086900-2087628
4	USA300-FPR3757 (SaPI5)	2006	Reverse	*seq* *sek*	ABD21542 ABD22279	729 729	242 242	884056-884784 883304-884032

[a] [http://www.ncbi.nlm.nih.gov/Genbank/index.html].
[b] MGE, mobile genetic element.
[c] Orientation defined relative to the origin of replication.

region compared to the identical sequence in allele type 2, which puts it in-frame with the rest of the coding region. However, due the high degree of identity among all *seq* variants and no occurrence of a similar sequence in any other SEs, the additional 42-bp sequence is likely not part of the coding region.

Evolution of *seq* and *sek*

The allelic variation and location of *seq* and *sek* on different mobile elements suggests the occurrence of recombination (134). The *seq/sek* locus located on the recently described SaPI5 (allele type 4) from the USA300 clone seems to be a hybrid of the same locus from earlier MGEs, SaPI3 (allele type 2) and ΦSa3mw (allele type 3). Some evidence shows SaPI recombination between COL and MW2, suggesting that the newest *seq/sek* variant could have also resulted from a recombination event (30). The recombined form of *seq* and *sek* could suggest slightly altered protein structure and function and, therefore, a potential change in virulence properties. Amino acid sequence differences between members of the pyrogenic family can result in markedly altered biological properties (133). Interestingly, because of the likely repositioning of the *seq/sek* locus during recombination, the upstream region of *seq* is almost entirely different for each allele type (FIG. 2). All four allele types have just 16 bases upstream of the start codon of *seq* that are similar. These unique upstream regions of *seq* suggest one of several possible mechanisms for variable expression among the allelic variants.

Seq and Sek Regulation

In previous studies, it has been shown that an MGE with a particular enterotoxin can be associated with a certain expression profile for that enterotoxin. Two different alleles of *sea* are located on two different phages, ΦFRI100 and ΦFRI281A. These *sea*

alleles had a minimal eightfold difference in mRNA production between them, which could not be solely explained by differences in the alleles themselves (10). Several studies have suggested mechanisms of regulation of the *seq/sek* locus.

Expression studies. Recently, the expression level of many SE genes, including *seq* and *sek*, was measured by qRT-PCR in several *S. aureus* food isolates during *in vitro*, planktonic growth (28). The study, by Derzelle *et al.*, indicated that most of the newly described SEs, including *seq* and *sek*, exhibited growth-independent expression patterns since the transcript levels remained unchanged throughout growth. However, strain-specific differences in the absolute levels of transcript existed. In contrast, the transcript levels of the well known SEs, like *seb* and *sec*, increased markedly during the transition from logarithmic to stationary growth phases. The researchers concluded that the new SE genes are likely not controlled by the Agr system, but could be regulated by factors that govern phage lysogeny, as most of the genes are phage encoded.

Sumby *et al*. showed by Northern blot hybridization and transcription start-site analyses that *seq* and *sek* expression in ΦSa3ms (allele type 3) is likely to be regulated from a common promoter of an upstream gene, *cI*, encoding a phage transcriptional repressor (121) (FIG. 1). Additionally, Yarwood *et al*. reported a similar level of gene expression between *seq, sek*, the downstream integrase, and three ORFs upstream of *seq* in allele type 2 using microarray analysis, suggesting control of *seq/sek* by an upstream encoded gene product. Interestingly, the first gene in this supposed operon also encodes a phage transcriptional regulator protein (134).

Co-transcribed genes are common in phage and phage-originated genomes due to coordinated temporal regulation at critical points within the phage life cycles (72). Since

the MGEs are likely of phage origin, *seq* and *sek* are likely co-transcribed and this is further supported by the fact that they have a shared orientation and close proximity. Other investigations have also supported *seq* and *sek* co-transcription. Microarray analysis has shown that under various experimental conditions, *seq* and *sek* expression levels in allele type 2 cluster together along with other surrounding genes (134). Northern blot analysis has detected both a common transcript for both genes in allele type 3 as well as a separate transcript for the *sek* gene (121). Predicted stem-loop structures just beyond the coding regions for both *seq* and *sek* in allele type 2 may serve as Rho-independent transcription terminators (134). These results suggest that the *seq/sek* locus may be regulated by multiple proteins and that the *sek* gene may possess its own specific promoter in addition to a shared promoter upstream of *seq*.

By examining the region upstream of the *seq* gene in all four allelic variants using GenBank annotations and predicted protein BLAST searches, the furthest upstream gene that exists in the same orientation as *seq* and *sek* was found to belong to the *cro*/*cI* family of phage transcriptional regulators (FIG. 2) (4). These phage transcriptional regulators, characterized in bacteriophage λ, comprise a binary switch that is able to exist stably in either of the two distinct, self-sustaining regulatory states (34). These two regulatory states are responsible for determining the lysogenic or lytic development of temperate phages which is dependent on many factors, including DNA damage and environmental conditions. The CI (Clear I) protein is expressed during phage lysogeny and blocks the transcription of the *cro* (Control of Repressor's Operator) gene, which is required for lytic development. Alternatively, Cro is expressed during the lytic development and prevents CI expression and, therefore, phage lysogeny (63, 82, 122). In accordance with

bacteriophage λ genome structure, putative *cro/cI* regulators indicated in FIG. 2 mostly resemble *cI* as indicated by the downstream *int* genes whose low and stable expression is controlled by CI and required for phage lysogeny. The *seq/sek* allele types 2 and 4 share a highly identical (99%) *cro/cI* regulator gene, however, a putative lipoprotein located just upstream of *seq* has been excised from allele type 4. This *cro/cI* regulator gene and the regulator genes in allele types 1 and 3 share no identity. Since regulation of *seq* has been linked in allele types 2 and 3 to the upstream promoter of the *cro/cI* regulator, a similar type of *seq* regulation may occur in the other allele types. On the other hand, these upstream regions share almost no identity, which may affect the downstream expression of *seq* and *sek*.

Despite variability between the MGEs, the *seq/sek* locus has remained highly conserved, suggesting some functional significance for these toxins during the disease process. In addition, the locus remains in the same relative location on the mobile elements with respect to non-homologous phage integrases and *cro/cI* regulator elements. One rationale for the common insertion location of *seq/sek* downstream of *cro/cI* regulator genes may be optimized gene expression. Phage lysogeny is maintained by constitutive expression of CI at low levels (72). If *seq/sek* is controlled by the promoter of the *cro/cI* regulators then this guarantees constitutive expression of the enterotoxins as well (121).

Protein studies. Several proteomic studies have shown that Seq and Sek are most highly secreted during the late-logarithmic/early-stationary phase of growth (15, 51, 99). In a global analysis of exoprotein production by automated direct infusion-tandem mass spectrometry (ADI-MS/MS), Seq and Sek protein production levels were different

between strains MW2 (allele type 3) and USA300-FPR3757 (allele type 4) (15). Sek production was detected only in USA300-PFR3757 during mid-logarithmic and stationary growth phase and never in MW2. Seq production was also detected in USA300-FPR3757 during both growth phases, while three-fold less Seq was detected during stationary phase growth only in MW2 (15). This data also supports the idea that these two enterotoxins may be under different regulatory control in different MGEs and/or genetic backgrounds.

To date, there has been no investigation to directly compare the expression levels of *seq* and *sek* among the different allelic variants. Also, no expression analysis of *seq* and *sek* has been done definitively for the newest allelic variant, allele type 4, or for allele type 1. It is possible that allele-specific expression of *seq* and *sek* contribute to patterns of virulence seen in *S. aureus* groups harboring those alleles. For example, since allele type 4 is exclusively found in the USA300 strain type, it is possible that allele-specific levels of expression of *seq* and *sek* contribute to the heightened virulence seen in this CA-MRSA group. The goal of this study was to both characterize and quantify the *seq* and *sek* transcripts *in vitro* for all four allelic variants. This work has demonstrated that the *seq* and *sek* genes are co-transcribed in all allele types and are expressed at very low levels *in vitro*. In addition, strain- and allele-specific levels of absolute expression were shown to exist, which may have a potential role in the virulence of certain *S. aureus* cohorts.

RESEARCH OBJECTIVES

The goal of this research was to test the hypothesis that *seq* and *sek* are differentially regulated in *S. aureus* isolates depending on their allele type. This hypothesis was based on the occurrence of these enterotoxins in four different MGE's and their suggested regulation by non-identical phage transcriptional regulators. It was also based on the evidence of different amounts of Seq and Sek production in two unrelated predominant CA-MRSA strains, USA300-FPR3757 (allele type 4) and MW2 (allele type 3) (15, 121, 134). The objectives for this study were to:

1. Determine if *seq* and *sek* are expressed and co-transcribed during three phases of growth in allele types 1-4 by reverse-transcription PCR using total RNA collected during mid-logarithmic, late-logarithmic and stationary growth phases.
2. Confirm the co-transcription of *seq* and *sek* and determine co-transcription of these genes with additional genes in all four allelic variants by Northern blot hybridization using total RNA collected from three phases of growth.
3. Measure the expression of *seq* and *sek* using real-time-reverse-transcriptase PCR in four reference and four clinical isolates of *S. aureus* grown in nutrient media.

RESEARCH DESIGN AND METHODS

Identification of *seq/sek* Allele Types

Bacterial Strains

Seventy-one *S. aureus* clinical and commensal isolates were selected for detection of *seq* and *sek* by polymerase chain reaction (PCR). These isolates were selected from a pool of 390 archived isolates collected from various institutions (patient samples from the Marshfield Clinic, Minnesota Department of Health, and Wisconsin State Lab of Hygiene; student samples University of Wisconsin – La Crosse, and) between 1990 and 2006 and represented five cohorts: commensal methicillin-sensitive *S. aureus* (cmMSSA), clinical MSSA (clMSSA), HA-MRSA, CA-MRSA, and Transitional-MRSA. The pool of isolates was used in a previous MRSA study investigating the molecular epidemiology of CA-MRSA (Shukla *et al*., submitted for publication). Clinical isolates, including all MRSA, were collected from clinical cases of skin and soft tissue infections, sepsis, and respiratory tract infections. Commensal isolates were obtained from the University of Wisconsin – La Crosse and were collected from the anterior nares of students without clinical syndromes. HA-MRSA isolates were defined by antibiotic resistance, presence of SCC *mec* types I, II, or III, and lack of the *pvl* genes. CA-MRSA was defined by PFGE patterns resembling clonal groups USA300 or USA400 and presence of SCC *mec* type IV and the *pvl* genes. Transitional-MRSA resembled CA-MRSA by their presence of SCC *mec* type IV; however, they lacked the *pvl* genes (11). *S. aureus* reference and clinical strains used to characterize the expression of the *seq/sek*

TABLE 2. *S. aureus* strains and characteristics for *seq/sek* allele expression studies

S. aureus strain	Properties[a]	*seq/sek* allelic type	Source or reference
Reference			
RN4282	clMSSA, SaPI1, *agr* type 1	1	Kreiswirth *et al.* (68)
COL	HA-MRSA, SaPI3, *agr* type 4	2	Gill *et al* . (47)
MW2	CA-MRSA, ΦSa3mw, *agr* type 3	3	CDC (19)
USA300-FPR3757	CA-MRSA, SaPI5, *agr* type 1	4	Diep *et al.* (30)
Clinical[b]			
UWL-96	cmMSSA, *agr* type 2	1	University of Wisconsin - La Crosse
5180	tMRSA, *agr* type 1	2	Marshfield Clinic
WSLH-6	CA-MRSA, *agr* type 3	3	Wisconsin State Lab of Hygiene
2004	CA-MRSA, *agr* type 1	4	Marshfield Clinic

[a] clMSSA, clinical methicillin-sensitive *S. aureus* ; HA-MRSA, hosptial-associated methicillin-resistant *S. aureus* ; CA-MRSA, community-associated methicillin-resistant *S. aureus* ; cmMSSA, commensal methicillin-sensistive *S. aureus* ; tMRSA, transitional methicillin-resistant *S. aureus.*

[b] Clinical strain information provided by Shukla *et al* ., submitted for publication.

alleles are listed in TABLE 2. The four clinical *S. aureus* isolates were selected based on results from PCR and DNA sequencing analysis. Each clinical strain carries a certain *seq/sek* allele that was confirmed to be identical to its reference strain.

PCR Detection of *seq* and *sek*

Genomic DNAs were extracted from *S. aureus* isolates using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and used as templates for PCR. Primers used for PCR amplification of *seq* and *sek* are listed in TABLE 3. Separate multiplexed reactions for *seq* (primers: Seq-CodR, Seq-PF-SaPI1, Seq-ProF-300, Seq-ProF-COL, Seq-ProF-MW2) and *sek* (primers: Sek-ProF, Sek-CodR-300COL, Sek-CodR-MW2, Sek-CR-SaPI1) were set up using HotStarTaq Master Mix (Qiagen, Valencia, CA) to detect all four *seq* and *sek* alleles. PCR amplification was carried out using a thermal cycler. The following parameters were used to amplify *seq*: 95ºC for 15 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension 72ºC for 1 min, followed by 72ºC for 7 min. For *sek*, the following parameters were used: 95ºC for 15 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s, and extension 72ºC for 1.5 min, followed by 72ºC for 7 min. All PCR products were separated and visualized by agarose gel electrophoresis to determine the presence of a PCR product of the correct size. Isolates that were negative for a specific gene following the multiplexed PCR were screened again using two primer sets (RTSeq-R-CUS/RTSek-300COL-F and Seg2F/Seg2R). These primers targeted the conserved coding regions of the *seq/sek* locus and served to identify any additional allele variants. PCR parameters for primer set RTSeq-R-CUS/RTSek-300COL-F followed conditions used for the *seq* multiplexed PCR. PCR parameters for primer set Seg2F/Seg2R was as follows: 95ºC for

TABLE 3. Oligonucleotide primers sets and hybridization probes used in this study

Primer or probe[a]	Sequence (5'-->3')	Length (bp)	PCR product (bp)	*seq/sek* allele type	Purpose or method[b]
seq					
seg2F[ch]	TCTCTGGGTCAATGGTAAGC	20	260	1-4	Confirmation of PCR negative isolates
seg2R[dh]	TTCCGGTGTAAAACAAATCG	20			
SeqB-F[c]	CACTGTTAGCTTGTTTTTCTTCACA	25	303	1-4	Northern/Southern DNA probe
SeqB-R[d]	TGCCAACGTAATTCCACCAT	20			
Seq-CodR[d]	TATCACCTTGAGCGCTGGCA	20	961	1-4	PCR detection and sequencing
Seq-PF-SaPI1[c]	CGTGTCCTAGCACATCTACGTC	22		1	
Seq-ProF-300[c]	TCCGAGTACCTACAACTGAGTGA	23	988	4	
Seq-ProF-COL[c]	AACGGTGATATCGATGATGCT	21	967	2	
Seq-ProF-MW2[c]	GATGATATCAAAGTCGCTTCGT	22	1087	3	
RTSeq-300COL-F[d]	TCAATCTCTTGAGCAGTTACCTCTT	25	136	1, 2, 4	RT-PCR (SEQ), qRT-PCR
RTSeq-CUS-R[cg]	GAATTACGTTGGCGAATCAA	20			
RTSeq-MW2-F[d]	TCAATCTCTTGAGCAGTTACTTCTTT	26	137	3	
RTSeq-MW2-R[c]	GGAATTACGTTGGCAAATCAA	21			
Pseq[e]	TGCTTACCATTGACCCAGAGATT	23		1-4	qRT-PCR

Primer or probe[a]	Sequence (5'-->3')	Length (bp)	PCR product (bp)	*seq/sek* allele type	Purpose or method[b]
sek					
SekB-F[c]	GGAATTGATAATCTCAGGAATTTTT	25	365	1-4	Northern/Southern DNA probe
SekB-R[d]	TCAATCTCTTGAGCGGTAACAA	22			
Sek-ProF[c]	TTTTACACCGGAACTGGTCA	20	1038	1-4	PCR detection and sequencing
Sek-CodR-300COL[d]	TTGGCAGTGAGAGGGTCTTT	20		2, 4	
Sek-CodR-MW2[d]	CAACGTGTTTTCATGCGTTC	20	966	3	
Sek-CR-SaPI1[d]	CAAACCACATTTTGCTTCTCC	21	966	1	
RTSek-300COL-F[dg]	TTTGATTTATGACCATATTCTTCTCC	26	116	1, 2, 4	RT-PCR (SEK), qRT-PCR
RTSek-CUS-R[c]	TTACCGCTCAAGAGATTGATGT	22			
RTSek-MW2-F[d]	TGATTTATGACCGTATTCTTCTCC	24	110	3	
RTSek-R[c]	TTGTTACCGCTCAAGAGATTG	21			
Psek[e]	TGCCGTTATGTCCATAAATGTTG	23		1-4	qRT-PCR
***seq-sek* (1)**					
Sek-ProF[c]	TTTTACACCGGAACTGGTCA	20	219	1-4	RT-PCR (1)
Seq-CodR[d]	TATCACCTTGAGCGCTGGCA	20			
***seq-sek* (2)**					
Sek-C[c]	GGTCAGGAATATGGATATCAGTCA	24	427	1-4	RT-PCR (2)
Seq-PC[d]	ACATTTGAAAATTGTAGTTGATTAGC	26			

Primer or probe[a]	Sequence (5'-->3')	Length (bp)	PCR product (bp)	*seq/sek* allele type	Purpose or method[b]
gmk					
GmkN-R[d]	CATTGAATTCTATTCTTCGCAAGTT	25	474	1-4	Northern/Southern DNA probe
RTGmk-F[c]	ACGAATATTTGAAGATCCAAGTACA	25	123	1-4	RT-PCR
RTGmk-R[d]	GCTTCAAACGCATCCCTAGT	20			
gyrB					
RTGyrB-F[cf]	CAAATGATCACAGCATTTGGTACAG	25	90	1-4	qRT-PCR
RTGyrB-R[df]	CGGCATCAGTCATAATGACGAT	22			
PgyrB[ef]	AATCGGTGGCGACTTTGATCTAGCGAAAG	29			

[a] Primers designed with Primer 3 software (version 0.4.0; Whitehead Institute for Biomedical Research [http://frodo.wi.mit.edu/primer3/]) unless noted otherwise.

[b] RT-PCR, reverse-transcriptase PCR; qRT-PCR, quantitative real-time PCR.

[c] Forward primer.

[d] Reverse primer.

[e] Taqman minor groove binding probe with 5' 6FAM (6-carboxyfluorescein) fluorescent dye and 3' non-fluorescent quencher.

[f] Primers and probes designed by Voyich *et al*. (131).

[g] Primers used to confirm PCR negative isolates, 975 bp product.

[h] Primers designed by Pokallus (101).

15 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension 72ºC for 45 s, followed by 72ºC for 7 min.

DNA Sequencing to Determine *seq/sek* Allele Type

Amplicons from PCR analyses were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) and used as a template for cycle sequencing. Cycle sequencing was performed on a thermal cycler using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and primers from the original PCR. Extension products were purified to remove unincorporated dye terminators using the DyeEx Spin kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Samples were then subjected to capillary electrophoresis using an ABI PRISM 3130xl Genetic Analyzer instrument (Applied Biosystems, Foster City, CA). Sequencing data was analyzed using Lasergene software (DNASTAR, Madison, WI). GenBank sequences from four sequencing projects, SaPI1, COL, MW2, and USA300-FPR3757 served as reference sequences for determining *seq/sek* allele type (TABLE 1).

Methods to Address Research Objective 1

Growth Curve Analysis

The growth curves of three *seq/sek* reference strains, COL, MW2, and USA300-FPR3757, were used to determine mid-logarithmic, late-logarithmic and stationary phases of growth. Strains were inoculated into 5 ml of tryptic soy broth (TSB, Becton, Dickinson, and Company, Franklin Lakes, NJ) and grown overnight in a shaking incubator. A 1:50 dilution was made into fresh TSB and shaken at 250 rpm at 37ºC. Aliquots were taken at the time of dilution and every 30 minutes during incubation for

absorbance readings at $A_{600.}$ The log of the average optical density for all aliquots from two separate cultures was plotted against time to determine the time points of the three growth phases.

Manipulation of RNA

RNA extraction. Total RNA was collected from *S. aureus* strains using combined methods from Theis *et al.* and Cheung *et al.* (24, 123). Briefly, cultures were grown to time points corresponding to mid-logarithmic, late-logarithmic and stationary phases. During each phase, a 1-4 ml aliquot of culture was removed and mixed with RNAprotect Bacterial Reagent (Qiagen, Valencia, CA) according to the manufacturer's instruction to stabilize RNA for accurate gene expression analysis. Cells were then washed and resuspended in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA). The suspension was transferred to 2.0-ml Lysing Matrix B tubes (MP Biomedicals, Solon, OH) and homogenized three times using a Mini-Beadbeater-1 (BioSpec Products, Bartlesville, OK) for 20 seconds each with incubation on ice between runs. Chloroform (200 μl) was then mixed with each sample and incubated at room temperature for 2 minutes. High-speed centrifugation (13,000 x *g*, 2 min) separated the phases for collection of the clear, upper, aqueous phase containing the RNA. Isopropyl alcohol (500 μl) was then gently added and the sample was applied to an RNeasy collection tube for RNA Cleanup using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA samples were eluted twice from the column using the same 50-μl diethylpyrocarbonate-treated (DEPC) water sample for maximum recovery and stored at -80ºC.

RNA quantification and determination of purity. RNA was quantified by measuring the absorbance at A_{260} of a 1:50 dilution of RNA sample in a SmartSpec Plus

Spectrophotometer (Bio-Rad, Hercules, CA). The A_{260}/A_{280} ratio was used to determine the purity of each extraction. RNA samples with ratios below 1.9 and above 2.1 were discarded and the extractions repeated. Each RNA sample was then treated with DNase to remove any residual DNA using the TURBO DNA-free Kit (Applied Biosystems/Ambion, Austin, TX). Integrity of the RNA was checked at the time of cDNA synthesis by electrophoresing 1 µl of each extraction on a 1.0% non-denaturing agarose gel. The presence of sharp 23S and 16S rRNA bands indicated intact RNA.

RT-PCR of *seq* and *sek*

The four reference strains were used for RT-PCR analysis (TABLE 2). RNAs prepared from mid-logarithmic, late-logarithmic, and stationary growth phases were used as templates for RT-PCR. RT-PCR was performed in two steps consisting of a reverse transcription step to synthesize complementary DNAs (cDNAs) from each RNA sample followed by a PCR step to amplify the cDNA template.

Reverse transcription. cDNA synthesis of total RNA was carried out with random primers on a thermal cycler using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Reactions were set up using DNAse-treated RNA at a final concentration of 100 ng/µl. Each reaction was done in duplicate with one reaction containing the reverse transcriptase enzyme and one without the enzyme to serve as a No-RT control (NRT). All cDNAs were stored at -20ºC until further use.

PCR to detect *seq* and *seq* cDNA. Synthesized cDNAs were used as templates for PCR to detect the individual *seq* and *sek* transcripts as well transcripts containing both genes. PCRs were performed with a thermal cycler using the HotStarTaq Master Mix

(Qiagen, Valencia, CA). All primers used for amplification are noted in TABLE 3. The quality of cDNA template was assessed by amplifying the housekeeping gene, *gmk* (primers: RTGmk-F/RTGmk-R), in both the RT and NRT samples. Any samples that produced an amplicon from the NRT reaction were presumed to have significant genomic DNA contamination and were excluded from the analysis. The *seq/sek* regions targeted for amplification included the coding region of each gene as well as the region spanning the *seq/sek* intergenic sequence. Due to the presence of polymorphic sites within the coding region of *seq* and *sek*, two sets of primers targeting each gene were used to cover all allelic types (primer sets for *seq*; RTSeq-300COL-F/RTSeq-CUS-R and RTSeq-MW2-F/RTSeq-MW2-R, primer sets for *sek*; RTSek-300COL-F/RTSek-CUS-R and RTSek-MW2-F/RTSek-R). These primers targeted the same region in all allelic types. In addition to gene-specific primer sets, two primer sets, 1 (Sek-ProF/Seq-CodR) and 2 (Sek-C, Seq-PC), were used to amplify across the intergenic region. PCR amplification was carried out under the following parameters: 95ºC for 15 min followed by 40 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, and extension 72ºC for 45 s, followed by 72ºC for 7 min. For the primer set 2 amplification, the cycle number for the PCR was 40 or more cycles to account for low abundance of the intact target. All PCR products were separated and visualized by agarose gel electrophoresis to determine the presence of a PCR product of the correct size. One PCR product from each primer set for strains RN4282 and USA300-FPR3757 was sequenced to determine amplification of the correct target.

Methods to Address Research Objective 2

Northern Blot Hybridization

Electrophoresis of RNAs. Northern blot hybridizations were carried out following combined methods provided in Sambrook and Russell and Streit *et al.* (106, 118). RNAs were extracted from USA300-FPR3757 cultures grown to stationary phase as previously described. If necessary, RNA samples were first concentrated by ethanol precipitation. To do this, two volumes of 100% ethanol and 1/10 volume 3 M sodium acetate in DEPC water were added to the RNA sample and stored at -20ºC overnight or at -80ºC for one to two hours. RNA was collected by centrifugation at maximum speed (13,000 *x g*) for 10 min in a microcentrifuge at 4ºC. The RNA pellet was washed with 70% ethanol in DEPC water, allowed to dry, and then resuspended in DEPC water. RNA denaturation reactions for electrophoresis were set up using 20-, 30-, and 40-µg aliquots of RNA mixed with 2X RNA Loading Dye Solution (Fermentas, Glen Burnie, MD). Five micrograms of RNA Millennium Markers (Applied Biosystems/Ambion, Austin, TX) was also prepared in the same manner to serve as an RNA ladder. All samples were heated at 70ºC for 10 minutes followed by chilling on ice. Samples were then loaded into a 1.5% agarose gel containing 2.2 M formaldehyde and 1X 3-(N-morpholino) propanesulfonic acid (MOPS) and placed in a horizontal electrophoresis box containing 1X MOPS electrophoresis buffer. Electrophoresis was carried out at 3 V/cm for 4-5 hours. The gel was photographed along with a fluorescent ruler using a UV transilluminator. The 16S and 23S rRNA bands served as a control to equalize the RNA in each lane.

Transfer of RNAs to membrane. RNA gels were rinsed with DEPC-treated water and soaked in 20X saline-sodium citrate (SSC) (3M NaCl, 0.3M Na-Citrate, DEPC water) on a rotary platform for 20 minutes. RNAs were then transferred to a positively charged Hybond-XL membrane (GE Healthcare, Waukesha, WI) via capillary transfer with 20X SSC transfer buffer overnight. Membranes were rinsed with 6X SSC and allowed to air dry. RNAs were cross-linked to the membrane using a Stratalinker 2400 instrument (Agilent Technologies, Santa Clara, CA) on the auto cross-link setting.

DNA probes. Three DNA probes were constructed by PCR to probe the RNA transcripts of *seq*, *sek*, and *gmk*. Primer sets used for PCR along with respective product sizes can be found in TABLE 3 and were as follows: *seq*; SeqB-F/SeqB-R, *sek*; SekB-F/SekB-R, *gmk*; RTGmk-F/GmkN-R. PCR amplification was carried out using a thermal cycler under the following parameters: 95ºC for 15 min followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, and extension 72ºC for 45 s, followed by 72ºC for 7 min. All PCR products were separated and visualized by agarose gel electrophoresis to determine the presence of a PCR product of the correct size. Products were then purified from the gel using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). DNA probes were labeled at the time of hybridization with [α-^{32}P] deoxyadenosine 5'-triphosphate (dATP) (3000 Ci/mmol) (PerkinElmer, Waltham, MA) using the Prime-IT II Random Primer Labeling Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instruction. Probes were filtered following labeling to remove unincorporated nucleotides using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instruction.

Hybridization and imaging. The membrane containing immobilized RNAs was divided to allow for multiple hybridizations using different probes. Prehybridization occurred at 50ºC for at least four hours in a hybridization oven using roller tubes containing prehybridization buffer {50% [vol/vol] formamide, 0.2% [wt/vol] sodium dodecyl sulfate (SDS), 5X SSC, 5X Denhardt's solution (0.5% [vol/vol] Ficoll, 0.5% [wt/vol] polyvinlypyrolidone, 0.5% [wt/vol] bovine serum albumin), 100 μg/ml salmon sperm DNA, and 50 mM sodium phosphate [pH 6.5]}. After prehybridization, DNA probes were denatured at 100ºC for five minutes followed by chilling on ice for two minutes and then added directly to the prehybridization buffer. Hybridization occurred overnight at 50ºC. Membranes were washed at 65ºC for 10 minutes each with Wash Solution I (2X saline-sodium phosphate-EDTA (SSPE) (3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA), 0.5% [wt/vol] SDS, DEPC water), followed by Wash Solution II (1X SSPE, 0.5% [wt/vol] SDS, DEPC water), and Wash Solution III (0.1X SSPE, 0.5% [wt/vol] SDS, DEPC water). Membranes were wrapped with Saran Wrap™ and exposed in a phosphorimaging cassette for five days. An image was obtained using a Molecular Dynamics Storm 860 (GE Healthcare, Waukesha, WI) scanning instrument according to the manufacturer's instruction. The scaled RNA ladder served as a reference for determining size of the transcripts.

Southern Blot to Determine DNA Probe Efficacy

Manipulation of DNAs. Genomic DNAs were extracted from a culture of USA300-FPR3757 grown overnight in a 37ºC shaking incubator using the MasterPure Gram Positive DNA Purification kit (Epicentre Biotechnologies, Madison, WI). The provided kit protocol was followed with the addition of a mechanical lysing step

following addition of the Proteinase K/Gram Positive Lysis Solution. Sterile 0.1 mm silica spheres (MP Biomedicals, Solon, OH) were added to the sample and vortexed vigorously for 30 s. Silica spheres were then pelleted by centrifugation at 10,000 *x g* for 2 min. The lysed sample was removed and placed in a new microcentrifuge tube and the kit protocol resumed. Approximately 15 µg of genomic DNAs were used for an overnight digestion with *Nsp* I (New England Biolabs, Ipswich, MA) enzyme according to the manufacturer's instruction. A small portion of the digestion was checked for completeness by agarose gel electrophoresis. Digested DNAs were then concentrated by ethanol precipitation as described in methods for electrophoresis of RNAs for Northern blot hybridization.

Electrophoresis of DNAs. The digested DNAs were divided evenly into three samples (~5 µg/well) for loading onto a 0.8% agarose gel. Prior to loading, samples were heated for 2-3 minutes at 56ºC to disrupt base pairing. A GeneRuler 1 kb DNA Ladder (Fermentas, Glen Burnie, MD) served as a scale for detecting hybridized DNAs of expected size. Electrophoresis was carried out overnight at <1 V/cm to allow for slow migration. The gel was then stained in a 0.5 µg/ml solution of ethidium bromide for 45 min and photographed with a fluorescent ruler using a UV transilluminator.

Transfer of DNAs to membrane. The DNAs were depurinated by soaking the gel for 15 min in 0.25 M HCl with constant gentle agitation on a rotary platform. The gel was then soaked in Denaturation Buffer (1.5M NaCl, 0.5M NaOH) for 20 min with constant agitation and repeated once more with fresh solution. The gel was then soaked twice in Neutralization Buffer (3M NaCl, 1M Tris-Cl [pH 8.0]) with constant agitation. Sterile, deionized water was used to rinse the gel between all steps. DNAs were then

transferred to a positively charged Hybond-XL membrane (GE Healthcare, Waukesha, WI) via capillary transfer and cross-linked to the membrane as described in methods for the transfer of RNAs to membrane.

DNA probes. The DNA probes for *seq*, *sek*, and *gmk* synthesized for Northern blot hybridization were also used for Southern blot hybridization and were labeled using the same methods as described. For Southern blot hybridization, probes remained unfiltered following labeling.

Hybridization and imaging. Hybridization and imaging steps followed those as described for Northern blot hybridization. To account for unfiltered probes, washing time was increased to 45 min for all wash solutions. An image was obtained after six days of exposure. The scaled DNA ladder served as a reference for determining size of detected DNAs.

Methods to Address Research Objective 3

Primers and Probes

Oligonucleotide primers and minor groove binding (MGB) Taqman probes (Applied Biosystems, Foster City, CA) used for detection of cDNA from the target genes, *seq* and *sek,* and reference gene, *gyrB,* are listed in TABLE 3. Primer sets were as follows: *seq*; RTSeq-300COL-F/RTSeq-CUS-R and RTSeq-MW2-F/RTSeq-MW2-R, *sek*; RTSek300COL-F/RTSek-CUS-R and RTSek-MW2-F/RTSek-R, *gyrB*; RTGyrB-F/RTGyrB-R. The housekeeping gene, *gyrB,* encodes DNA gyrase B subunits which are responsible for binding and hydrolyzing ATP, providing the energy requirement for DNA supercoiling (58, 85, 120). Selection of *gyrB* as a suitable reference gene was based on a previous study that indicated constitutive expression during *in vitro* growth in *S. aureus*

(48). Primers were designed for *seq* and *sek* to account for polymorphisms in the allelic variants in order to maximize PCR efficiency. As a result, allele type 3 had unique primer sets which prime to the same or very similar regions of the target as the other allele types.

qRT-PCR Conditions

Expression analysis was carried out using the LightCycler 480 instrument (Roche Applied Science, Indianapolis, IN). All reactions were set up using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction with the final reaction volume being 25 µl. Thermal cycling conditions included an AmpErase Uracil N-glycosylase activation step at 50ºC for 2 min followed by DNA polymerase activation at 95ºC for 10 minutes. The amplification steps consisted of 50 cycles with each including a denaturation step at 95ºC for 15 s and an annealing/extension step at 60ºC for 1 min. Fluorescence was measured after each annealing/extension step for each amplification cycle.

Determining PCR Efficiencies

Prior to relative quantification, PCR efficiencies were measured for each primer and probe set. Serial dilutions of prepared cDNA template spanning three orders of magnitude were used to generate standard curves using the Roche's LightCycler 480 Absolute Quantification software (Roche Applied Science, Indianapolis, IN). PCR efficiency values were automatically generated from the slope of the C_T value plotted against the log of concentration of the template ($E = 10^{(-1/slope)}$). Since the comparative cycle threshold method ($2^{-\Delta\Delta CT}$) of relative quantification relies on a PCR efficiency of 2, any primer/probe set with a calculated PCR efficiency less than 1.95 went through

efficiency correction during relative quantification analysis using the LightCycler software.

qRT-PCR Analysis

qRT-PCR experimental design. All eight reference and clinical strains were used for qRT-PCR analysis (TABLE 2). cDNAs prepared from mid-logarithmic, late-logarithmic, and stationary growth phases were used as templates for qRT-PCR analysis. A minimum of three cDNA preparations from two separate cultures was prepared at each growth phase for each strain. Each cDNA preparation was analyzed three times on the LightCycler instrument. For each LightCycler run, single-plexed reactions for *seq*, *sek*, and *gyrB* were set up simultaneously and in duplicate for each cDNA sample using 100 ng of template per well. Additionally, 100 ng of the corresponding NRT reaction for each cDNA sample was included in a *gyrB* reaction well. This control served to evaluate levels of genomic DNA contamination in the cDNA sample. cDNA samples that produced a cycle threshold (C_T) value of at least 10 cycles higher in the NRT reaction compared to the corresponding RT *gyrB* samples were presumed to have negligible amplification ($\leq 0.1\%$) due to genomic DNA template and were included in the analysis. Each run also contained a no-template control well for each *seq*, *sek*, and *gmk* master mix in which sterile water substituted cDNA template to monitor master mix contamination. The Newman strain, missing the *seq* and *sek* genes, was used to assess the specificity of the assay (7). Initial qRT-PCR products were checked by agarose gel electrophoresis to confirm their presence and correct size.

Relative quantification. LightCycler 480 Relative Quantification Software was used to quantify the expression of the target genes. Briefly, the software uses a

comparative cycle threshold method ($2^{-\Delta\Delta CT}$) to provide reliable expression data of target genes compared to the reference gene (16, 78, 98). PCR efficiency correction was used when necessary as previously mentioned. Expression ratios (target/reference) were automatically generated from average C_T values of duplicate reactions for each LightCycler run.

Statistical analysis. The results of *seq* and *sek* expression from qRT-PCR were analyzed by one-way ANOVA. Relative gene expression values were analyzed across growth phases to assess growth-dependent expression of *seq* and *sek* by both strain and allele type. Expression values of each gene were analyzed within allele type to assess degree of strain agreement in expression. Finally, *seq* and *sek* expression levels were compared within each growth phase to determine allele-specific rates of expression. P values of < 0.05 were considered significant.

RESULTS

Identification of *seq/sek* Allele Types

PCR Detection of *seq* and *sek* in Clinical Isolates

Multiplexed PCR analysis revealed 34 out of 71 (48%) clinical and commensal *S. aureus* isolates (representing all five cohorts) were positive for *seq* and *sek* (TABLE 4). Both genes were detected in all positive isolates. Seventy-four percent of the CA-MRSA strains were positive for *seq/sek*, while only 33% of clMSSA and HA-MRSA had the genes. The 37 isolates that were negative by the multiplexed PCRs were also negative using primer set RTSeq-R-CUS/RTSek-300COL-F. Two negative isolates, one clMSSA and one cmMSSA, appeared positive using the Seg2F/Seg2R primer set (data not shown), but were confirmed *seq/sek* negative by additional PCR analysis targeting the conserved areas of the gene locus.

All Four *seq/sek* Allele Types Identified

Data gathered from DNA sequencing included the entire ORF region for each gene as well as DNA sequence approximately 95 bp upstream and 50 bp downstream of the ORF region for *seq* and 75 bp upstream and 25 bp downstream for *sek*. The results of allele typing are shown in TABLE 5. All four known *seq/sek* allele types were represented among the positive isolates. Moreover, all sequences were identical to one of the four sequences available in Genbank for the reference strains with the exception of allele type 2. Nine out of 13 isolates carrying *seq/sek* allele type 2 contained a single

TABLE 4. Results of *seq/sek* PCR detection by *S. aureus* cohort

S. aureus cohort[a]	Isolates tested	Isolates positive[b]	% positive
cmMSSA	21	9	43
clMSSA	21	7	33
HA-MRSA	6	2	33
CA-MRSA	19	14	74
tMRSA	4	2	50
total	71	34	48

[a] cmMSSA, commensal methicillin-sensitive *S. aureus*; clMSSA, clinical methicillin-sensitive *S. aureus*; HA-MRSA, hospital-associated methicillin-resistant *S. aureus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; tMRSA, transitional methicillin-resistant *S. aureus.*

[b] The number of isolates testing postive for both *seq* and *sek.*

TABLE 5. Results of *seq/sek* sequencing by allele type

seq/sek allele type	Isolates positive	% total	*S. aureus* cohort[a]				
			cmMSSA	clMSSA	HA-MRSA	CA-MRSA	tMRSA
1	2	6	1	-	1	-	-
2	13[b]	38	7	3	1	-	2
3	15	44	1	4	-	10	-
4	4	12	-	-	-	4	-
total	34	100	9	7	2	14	2

[a] cmMSSA, commensal methicillin-sensitive *S. aureus*; clMSSA, clinical methicillin-sensitive *S. aureus*; HA-MRSA, hospital-associated methicillin-resistant *S. aureus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; tMRSA, transitional methicillin-resistant *S. aureus.*

[b] 9 of 13 isolates contained a single nucleotide polymorphism (T144A) in *sek*, including all isolates in clMSSA, 5 in cmMSSA, and 1 in tMRSA cohorts.

nucleotide polymorphism (T144A) in *sek*, causing a substitution of tyrosine by alanine in the β-grasp domain. Clinical isolate 5180 that was used for further *seq/sek* expression analysis for allele type 2 did not have this change. Allele type 3 was the most common type, representing 15 out of 34 (44%) *seq/sek* positive isolates and allele type 1 was least common, found in only 2 (6%) positive isolates. Both MSSA cohorts and HA-MRSA had allele types 1, 2, and 3 represented, but allele type 3 was more prevalent in the CA-MRSA cohort (10/15, 67%) than other cohorts (5/15, 33%). Allele type four was found exclusively in the CA-MRSA cohort.

Research Objective 1

Growth Curve Analysis of Different *S. aureus* Strains

To determine if there were any growth differences between the *S. aureus* strains, *S. aureus* reference strains COL, MW2, and USA300-FPR3757 were grown in TSB medium, plotting optical density against time (FIG. 3). Similar growth rates were observed for the three strains with an average generation time at mid-log phase ranging from 25 to 35 min. Time points were established for mid-logarithmic growth at 2 h, late logarithmic growth at 4.5 h, and stationary growth at 7 h.

RT-PCR Analyses of *seq* and *sek* Expression Under *In Vitro* Growth Conditions

Expression of *seq* and *sek* occurs *in vitro* throughout growth. cDNAs were prepared from total RNAs collected at three growth phases from the four reference strains: RN4282 (allele type 1), COL (allele type 2), MW2 (allele type 3) and USA300-FPR3757 (allele type 4). Detection of *seq* and *sek* cDNA was performed by PCR using primer sets targeting *seq*- and *sek*-specific regions and also the *seq/sek* intergenic region (FIG. 4A). All PCR products of amplification were visualized by agarose gel

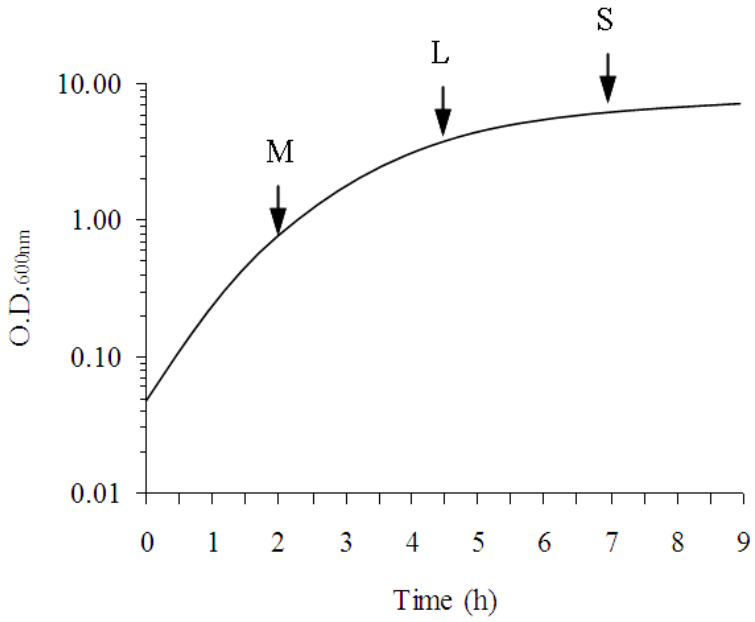
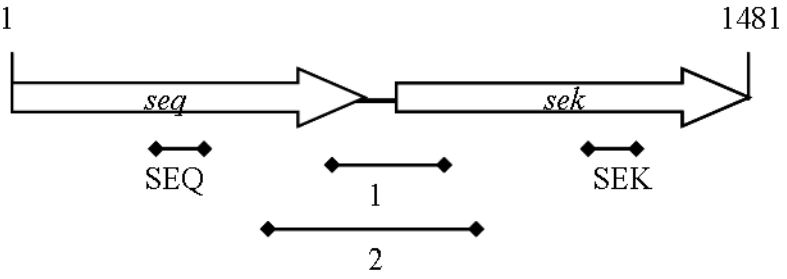
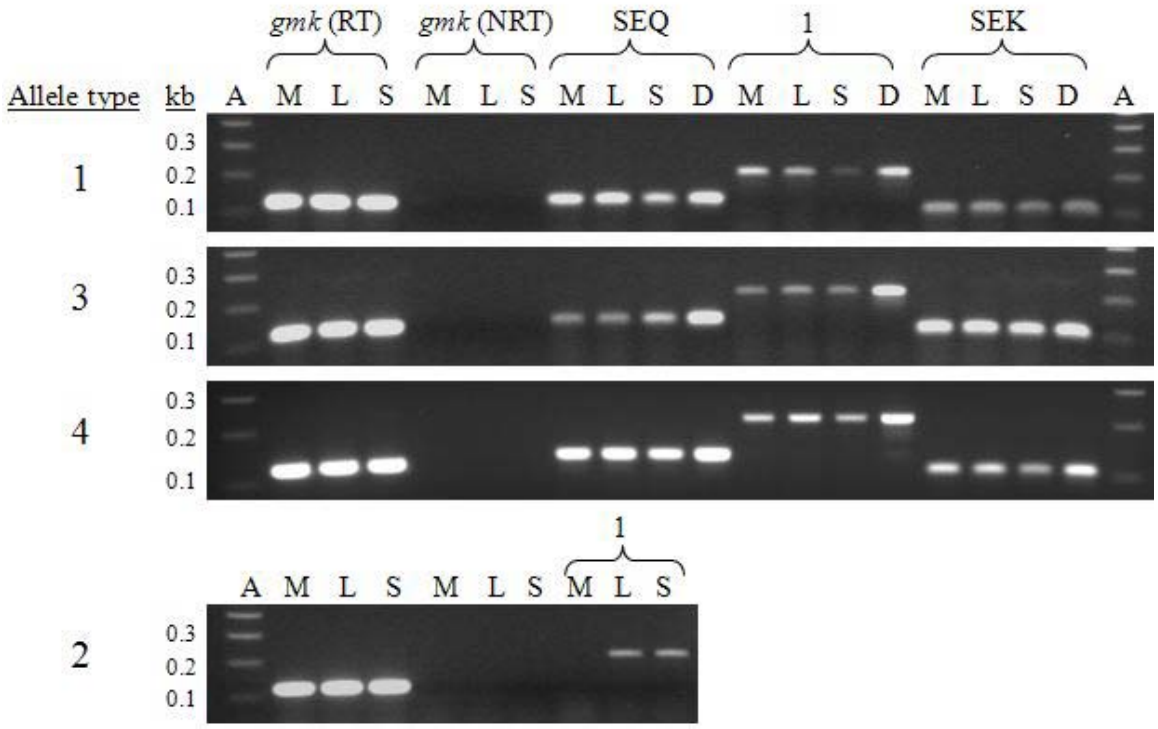


FIG. 3. Growth curve analysis of *S. aureus* reference strains in TSB media. The growth curve is based on the average optical density ($O.D._{600\ nm}$) of strains COL, MW2 and USA300-FPR3757. Time points were determined for growth at mid-logarithmic (M) phase at 2 h, late logarithmic (L) phase at 4.5 h and stationary (S) phase at 7 h.

A.


1
1481
seq
sek
SEQ
1
SEK
1
2

B.


gmk (RT)
gmk (NRT)
SEQ
1
SEK
Allele type
kb
A M L S M L S M L S D M L S D M L S D A
0.3
0.2
0.1
1
3
4
1
A M L S M L S M L S
2

C.

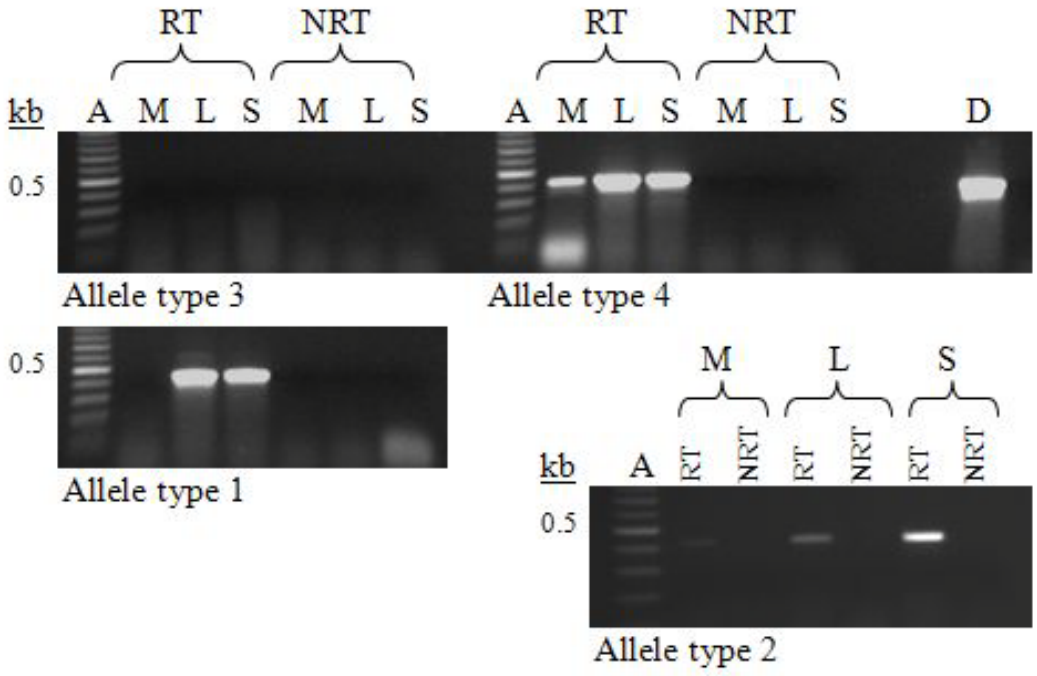


FIG. 4. Analysis of *seq* and *sek* transcripts by RT-PCR. A) Schematic map of the primer positions used for RT-PCR amplification of *seq* and *sek*. B) An RT-PCR analysis of cDNAs prepared from *S. aureus* reference strains for allele types 1-4 targeting *gmk, seq, sek* and intergenic region 1. C) RT-PCR results obtained using primer pairs for the intergenic region 2. Abbreviations are as follows: RT = reverse transcription positive control; NRT = reverse transcription negative control (no reverse transcriptase). Lanes A, 100-bp molecular size marker; lanes M, RT-PCR from extract at mid-logarithmic growth; lanes L, RT-PCR from extract at late logarithmic growth; lanes S, RT-PCR from extract at stationary growth; lanes D, PCR positive control (DNA as template). PCR product sizes are as follows: *gmk*, 123 bp; *seq*, 136 bp or 137 bp (allele type 3); *sek*, 116 bp or 110 bp (allele type 3); intergenic region 1, 219 bp; intergenic region 2, 427 bp.

electrophoresis (FIG. 4B and 4C). One PCR product from each primer set for strains RN4282 and USA300-FPR3757 was sequenced and determined to have amplified the correct target region (data not shown). FIG. 4B shows the RT-PCR results using the gene-specific primer sets and intergenic primer set 1. In all strains, amplification of *gmk* from RT samples confirmed the presence and quality of cDNA template, while no amplification of *gmk* from NRT samples confirmed the absence of contaminating genomic DNA. Amplification of gene specific regions occurred in all growth phases for allele types 1, 3, and 4. These results show that *seq* and *sek* is expressed in these allele types under the growth conditions tested. Amplification of gene specific regions occurred for allele type 2 but not without the presence of contaminating DNA (data not shown). However, further qRT-PCR analysis showed expression of *seq* and *sek* during all growth phases for allele type 2 (See RESULTS for Research Objective 3).

Transcripts containing both *seq* and *sek* occur in all allele types. Primer sets 1 and 2 were used to amplify across the *seq/sek* intergenic region (FIG. 4A). Using primer set 1, amplification of a 219-bp intergenic region was successful for all allele types and all growth phases except for the mid-logarithmic growth phase for allele type 2 (FIG. 4B). Amplification of a larger 427-bp intergenic region using primer set 2 was shown for allele type 1 during late-logarithmic and stationary growth phases and for allele types 2 and 4 at all growth phases (FIG. 4C). No amplification of allele type 3 cDNA occurred, however, amplification of allele type 3 genomic DNA was successful (data not shown). These combined results, taken together with the results from gene specific PCRs, indicate that the *seq* and *sek* genes are expressed in all allele types and are present on the same

mRNA transcript under all growth conditions tested, suggesting co-transcription of these genes in all allele types from a promoter region upstream of the *seq* gene.

Research Objective 2

Northern Blot Hybridization

***seq* and *sek* are expressed at very low levels *in vitro*.** Northern blot hybridization was performed to determine *seq* and *sek* transcript size and to characterize any additional co-transcribed genes. Total RNA was collected from USA300-FPR3757 cultures grown to stationary phase for use in detecting *seq* and *sek* transcripts. Amounts of total RNA that varied from 20 to 40 µg per lane were separated by electrophoresis on a denaturing gel followed by capillary transfer to a positively charged nylon membrane. The membrane was divided evenly and probed with [α-^{32}P] dATP-labeled DNA probes specific for the *seq*, *sek*, and *gmk* transcripts. In Northern blot hybridizations, *seq* and *sek* transcripts were not detected, whereas a *gmk* transcript of approximately 1.0 kb in size was detected (FIG. 5A). To ensure that the probes were hybridizing, Southern blot hybridizations were done on the corresponding genomic DNA from USA300-FPR3757 digested with *Nsp* I (New England Biolabs, Ipswich, MA). An expected 2.0-kb DNA fragment that represented the *seq* and *sek* genes hybridized with the appropriate probes and a 1.6-kb fragment containing *gmk* also hybridized using the *gmk* probe (FIG. 5B). Northern blot hybridizations were done using RNAs from other *S. aureus* reference strains and at different growth phases but no *seq* or *sek* transcripts were detected (data not shown). These results suggest the levels of *seq* and *sek* expression are too low to be detected by Northern blot hybridization as performed in this study.

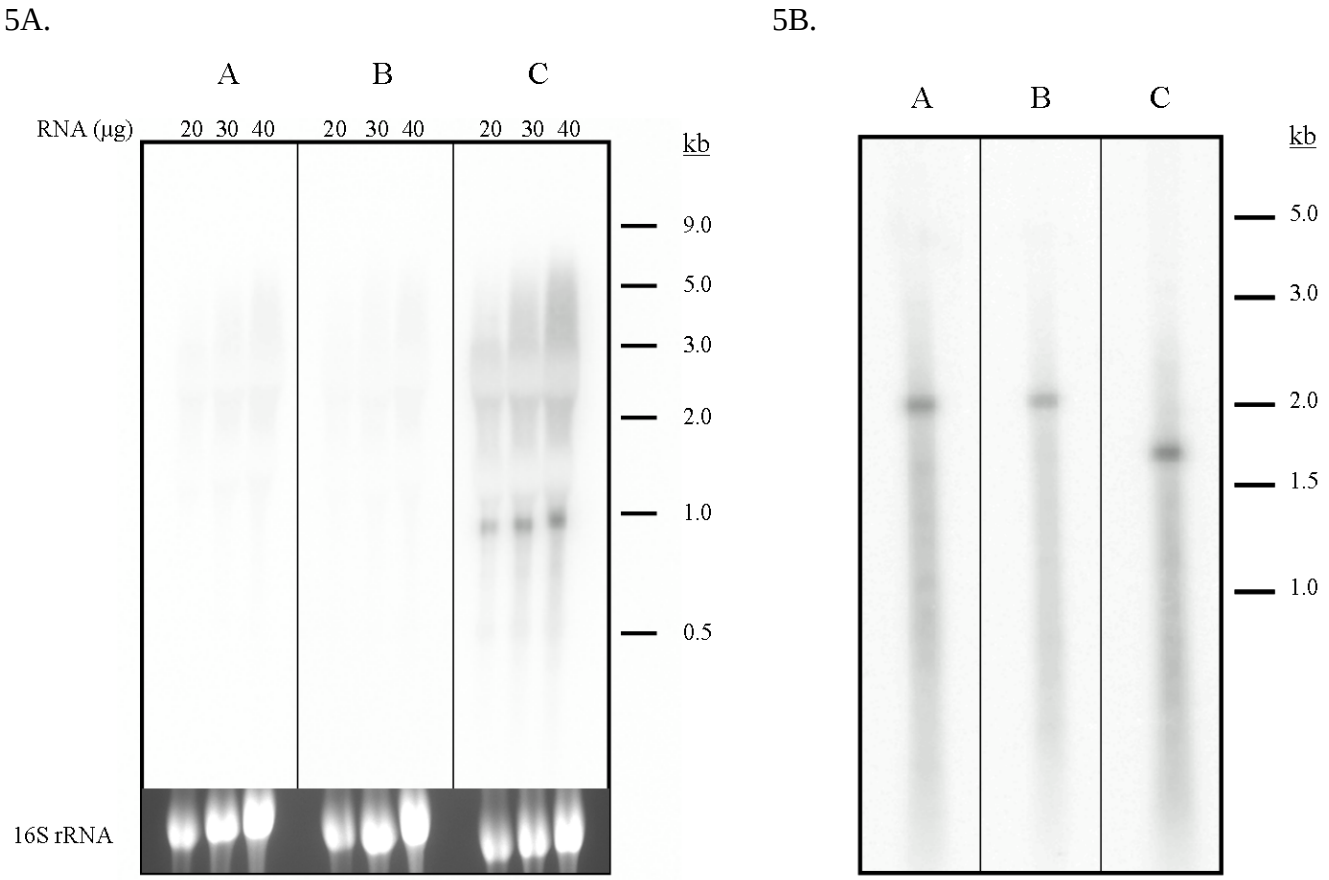


FIG. 5. Northern and Southern blot hybridizations probing for *seq* and *sek*. Nucleic acid for hybridizations was taken from reference strain USA300-FPR3757. Membranes were divided and probed with *seq* (A), *sek* (B), and *gmk* (C). 5A) Total RNA was extracted from culture grown to stationary growth phase and loaded in varying amounts. 16S rRNA from the EtBr gel acted as a control for RNA loading. 5B) Approximately 5 µg of DNA was digested with *Nsp* I (New England Biolabs, Ipswich, MA) and loaded for each lane.

Research Objective 3

PCR Efficiency and Primer and Probe Specificity for qRT-PCR Analyses

Quantitative expression analysis was performed to compare transcript levels during growth and between *seq/sek* allele types. The relative quantification of *seq* and *sek* was determined for four clinical and four reference *S. aureus* isolates during *in vitro* growth by qRT-PCR. PCR efficiencies for all primer/probe sets were determined to be proficient ($E \geq 1.95$) for relative quantification except for primer/probe set RTSek-300COL-F/RTSek-CUS-R/Psek, which had an efficiency of 1.94. All data collected using this primer/probe set underwent efficiency correction to account for suboptimal PCR efficiency during analysis with LightCycler 480 Relative Quantification Software. Initial products of qRT-PCR from all primer/probe sets were visualized by gel electrophoresis and determined to have the correct size and no non-specific amplification. As a negative control, the Newman strain, which has no *seq* and *sek* genes, was used. The qRT-PCR assays with the Newman strain were negative for both SE genes.

Suitability of the *gyrB* Reference Gene for qRT-PCR Analyses

Since an appropriate reference gene is necessary for accurate transcript measurement by qRT-PCR, the suitability of the *gyrB* gene was assessed using the collected data and available literature.

C_T values. A C_T value represents the PCR cycle at which amplification of the target is detectable above a defined threshold on the LightCycler instrument. The average raw C_T value for *seq* was 31.1 (range: 20.0-38.5), 25.1 for *sek* (range: 19.8-37.4), and 25.1 for *gyrB* (range: 17.7-29.4). Delta C_T values (target-reference) averaged 6.8 for *seq* (range: 1.16-13.13) and 5.0 for *sek* (range: 2.14-11.12). The *gyrB* gene was

expressed at an average 96-fold higher level than *seq* and at a 32-fold higher level than *sek.* This indicates that expression of *S. aureus seq* and *sek in vitro* is very low in relation to the housekeeping gene.

The basis for *gyrB* selection was limited. Selection of *gyrB* as a reference gene was based on a previous study in *S. aureus* that indicated constitutive expression of this gene during growth *in vitro* (48). Transcription of *gyrB* has also been used to normalize qRT-PCR data in a number of previous *S. aureus in vitro* gene expression studies (18, 45, 49-50, 64, 131). However, none of these studies indicated any validation of *gyrB* as a suitable reference gene. Furthermore, Goerke *et al*. determined constitutive expression of *gyrB* during *in vitro* growth by slot blot hybridization (48). However, this method has a low accuracy of quantification and fails to have the same high sensitivity as qRT-PCR (52).

Results of qRT-PCR Analyses for *seq* and *sek* During *In Vitro* Growth

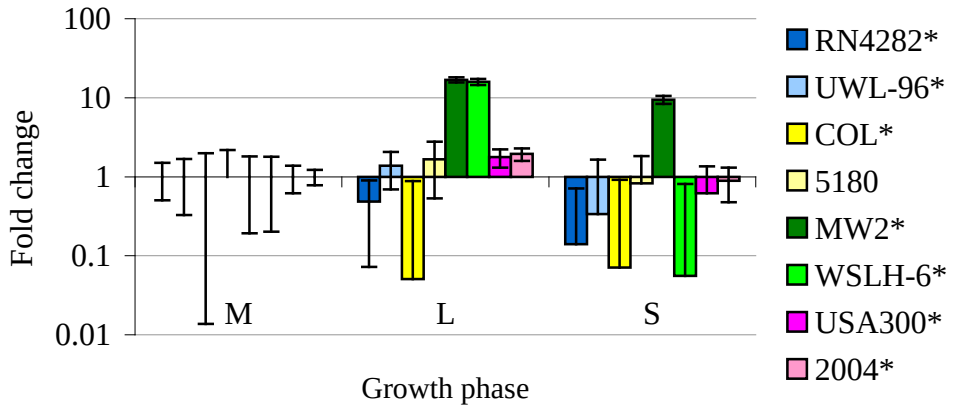
The relative quantification of *seq* and *sek* expression was determined by qRT-PCR for comparison during *in vitro* growth and between all four allele types. Expression of *seq* and *sek* was measured for all eight reference and clinical *S. aureus* strains from mid-logarithmic, early-logarithmic, and stationary growth phase cultures. The *gyrB* gene was used to normalize the data. Relative quantification was performed using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). Average expression data from at least nine separate LightCycler analyses on three cDNA preparations from two separate cultures was collected for each strain. Although expression of *seq* and *sek* transcripts were detectable for all strains during all growth phases, statistical analyses performed by one-

way ANOVA demonstrated that variance was at least 50% in over half (56%) of the data points and at least 75% in over a quarter (28%) of data points.

All *S. aureus* strains showed significant variation in *seq* expression during their growth except for strain 5180 (allele type 2) and *sek* expression showed significant variation during growth in all strains except for strains 5180 and USA300-FPR3757 (allele type 4) (FIG. 6). The expression patterns of both *seq* and *sek* were similar in each strain during growth. By comparing all strains, *seq* and *sek* expression was generally highest during late-logarithmic phase (*seq*: 1.4- to 16.9-fold increase; *sek*: 1.1- to 2.9-fold increase) except for strains RN4282 (allele type 1) and COL (allele type 2), which showed the highest expression during mid-logarithmic growth. Moreover, *seq* and *sek* expression was the lowest during stationary growth (*seq*: 1.1- to 17.86-fold decrease; *sek*: 1.4- to 6.8-fold decrease). This was not true for strains COL, which had the lowest expression of both genes during late-logarithmic growth, MW2 (allele type 3), which had lowest expression of *seq* during mid-logarithmic growth, and 2004 (allele type 4), which could not be determined. These results indicate expression of *seq* and *sek* may be growth dependent.

Allele variation in *seq* and *sek* expression during growth. Average relative expression data for each strain was combined into respective allele types and analyzed. The fold change in *seq* and *sek* expression with respect to mid-logarithmic phase for each allele is shown in FIGURE 7. All four alleles showed significant variation in *seq* and *sek* expression across growth, while allele type 2 did not show significant variation in expression of *sek* (FIG. 7B). In general, expression of *seq* and *sek* was lowest in

A.



B.

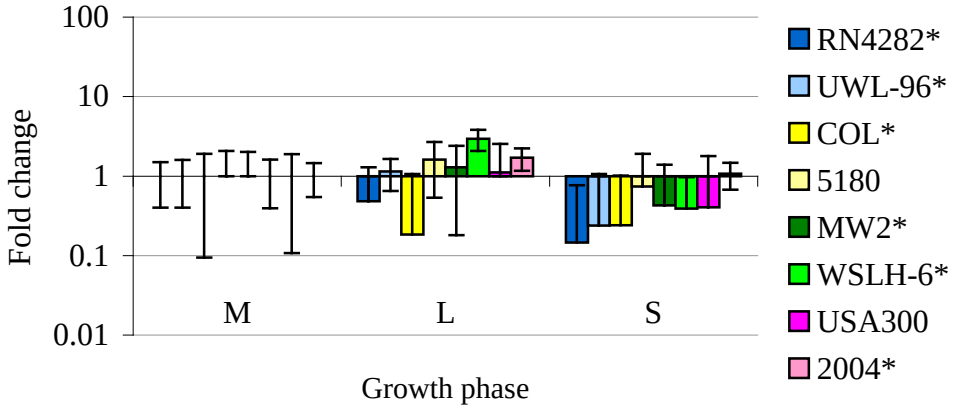
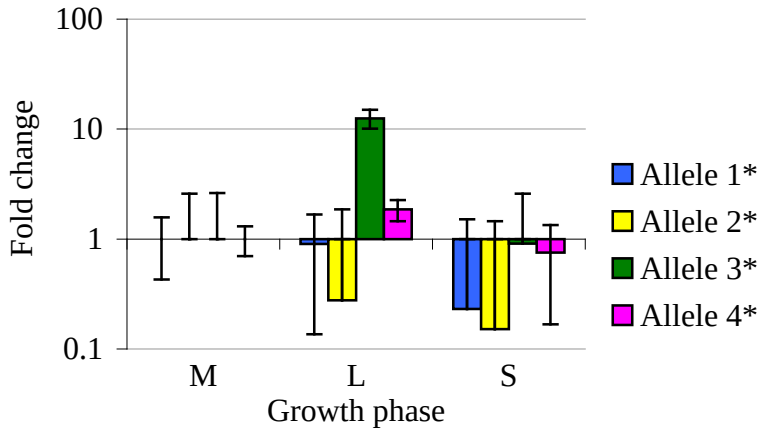


FIG. 6. Mean relative expression of A) *seq* and B) *sek* during growth in each strain by qRT-PCR. The results are based on cultures of 8 strains representing all four *seq/sek* allele types collected at three phases of growth: M, mid-logarithmic; L, late-logarithmic; and S, stationary. Mean values ± standard deviations represent samples from two independent cultures, each analyzed 3-6 times. Samples collected from the mid-logarithmic (M) growth phase were used as a calibrator for all subsequent samples. Strains labeled with an asterisk (*) indicate significant variation in gene expression during growth determined by ANOVA.

A.



B.

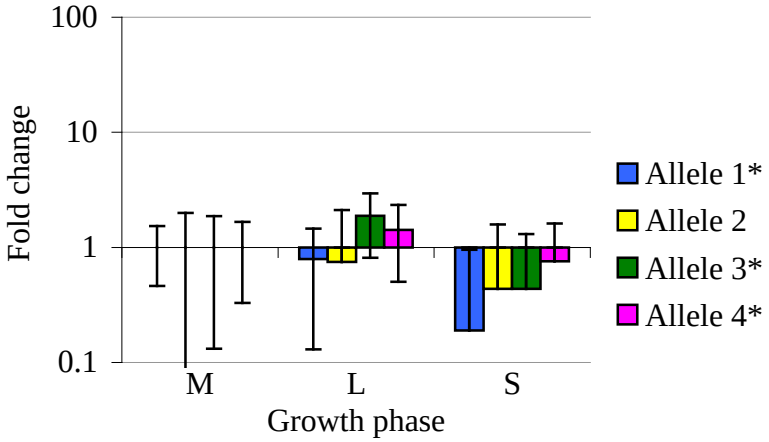


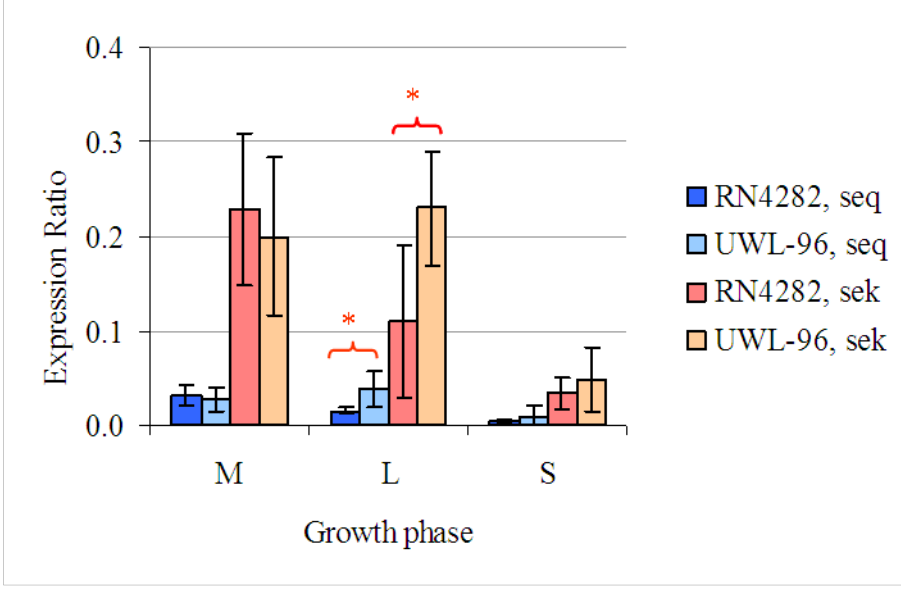
FIG. 7. Mean relative expression of A) *seq* and B) *sek* during growth in each allele by qRT-PCR. The results are based on cultures of 8 strains representing all four *seq/sek* allele types collected at three phases of growth: M, mid-logarithmic; L, late-logarithmic; and S, stationary. Mean values ± standard deviations represent the average expression of one reference and one clinical strain representing an allele type. Data for each strain represents samples from two independent cultures, each analyzed 3-6 times. Samples collected from the mid-logarithmic (M) growth phase were used as a calibrator for all subsequent samples. Allele types labeled with an asterisk (*) indicate significant variation in gene expression during growth determined by ANOVA.

stationary growth phase but higher in late-logarithmic phase for allele types 3 and 4 and mid-logarithmic phase for allele types 1 and 2.

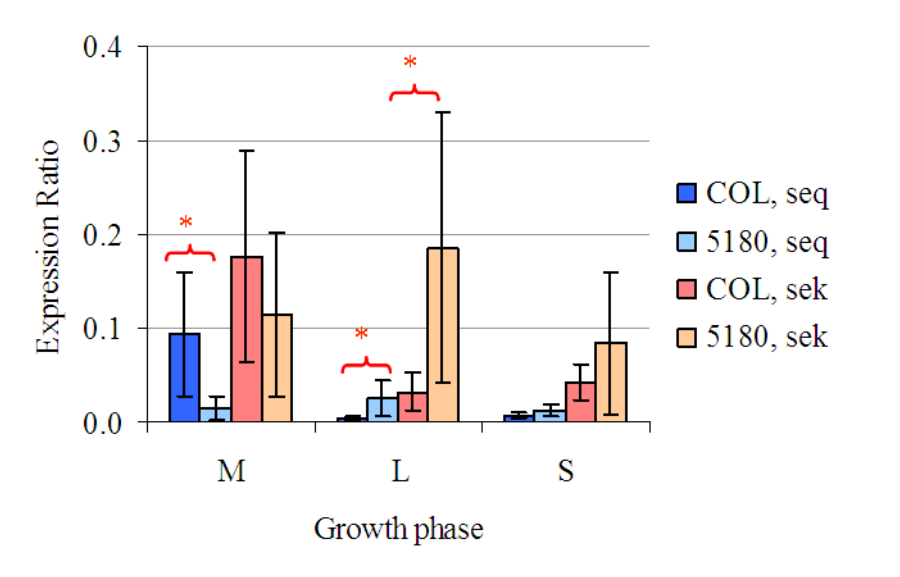
Expression levels of *seq* and *sek* compared within allele type. Expression levels of *seq* and *sek* were compared among strains of each allele type to determine the strain agreement in expression within the allele type. FIGURE 8 displays the raw expression ratios (target/reference) for *seq* and *sek* for the strains of each allele. For allele type 1, expression levels of each gene were similar, except during late-logarithmic phase, where strain UWL-96 showed significantly higher expression of both genes compared to RN4282 (FIG. 8A). For allele type 2, significant differences in expression were also seen during late-logarithmic phase with strain 5180 having higher expression of both genes compared to COL versus mid-logarithmic phase where *seq* expression was higher in COL (FIG. 8B). Allele type 3 *seq* expression levels were dissimilar across all growth phases, while *sek* levels were similar (FIG. 8C). Very similar levels of *sek* expression were observed for both allele type 4 strains, although strain 2004 had significantly higher expression than USA300-FPR3757 (FIG. 8D).

Comparison of *seq* to *sek* to assess co-transcription. Expression levels of *seq* to *sek* were also compared to look for any indication of co-transcription. Patterns of *seq* and *sek* expression during growth were similar for a specific strain or allele type (FIG. 7). For example, the progressive decrease in *seq* across growth in allele type 1 is mirrored by a similar *sek* pattern as well. Both *seq* and *sek* in allele types 3 and 4 are expressed at a higher level during late-logarithmic phase followed by a decrease in stationary phase. This suggests that both *seq* and *sek* are under the same regulatory control and further supports RT-PCR data showing co-transcription of the two genes.

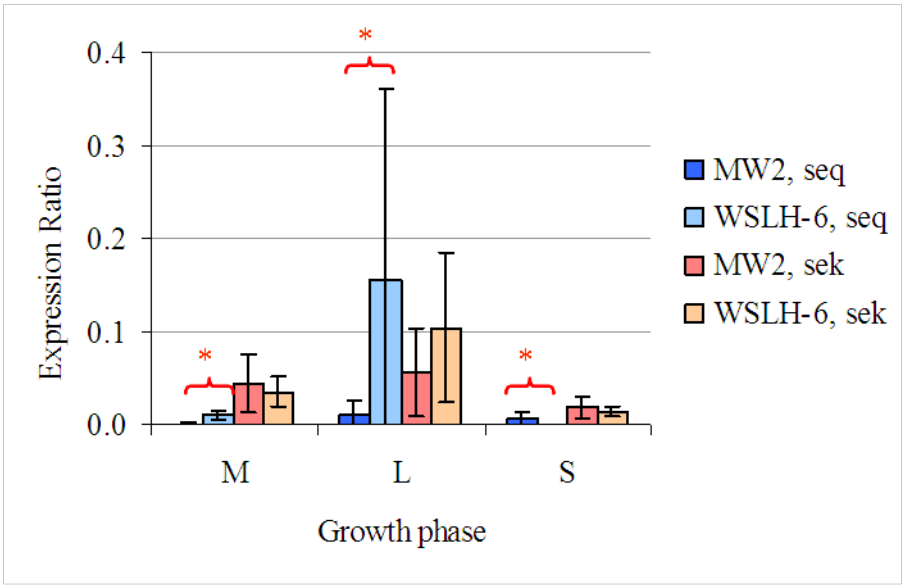
A.


0.4
0.3
0.2
0.1
0.0
Expression Ratio
*
*
M
L
S
Growth phase
RN4282, seq
UWL-96, seq
RN4282, sek
UWL-96, sek

B.


0.4
0.3
0.2
0.1
0.0
Expression Ratio
*
*
*
M
L
S
Growth phase
COL, seq
5180, seq
COL, sek
5180, sek

C.



D.

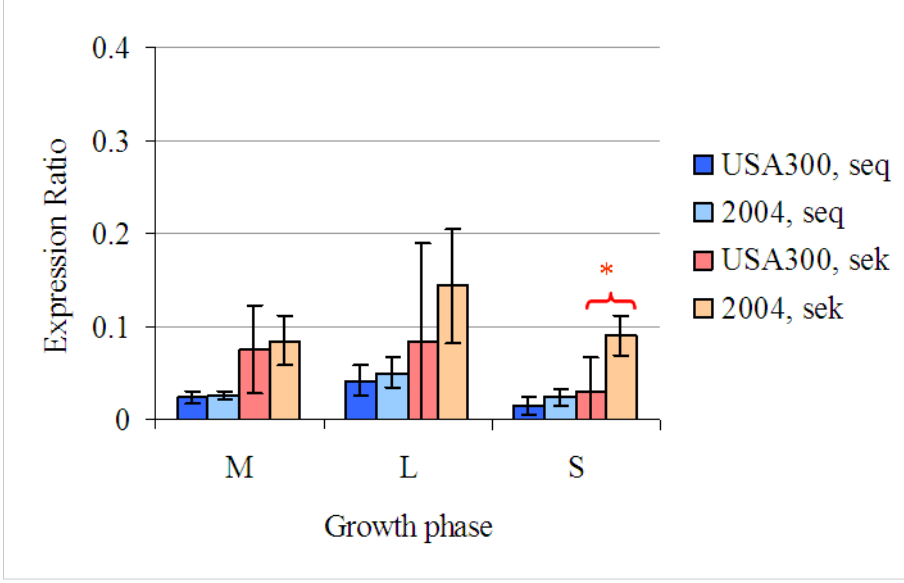
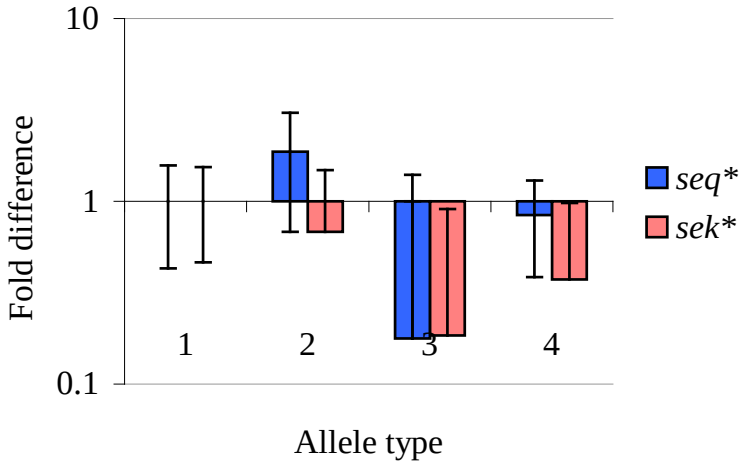


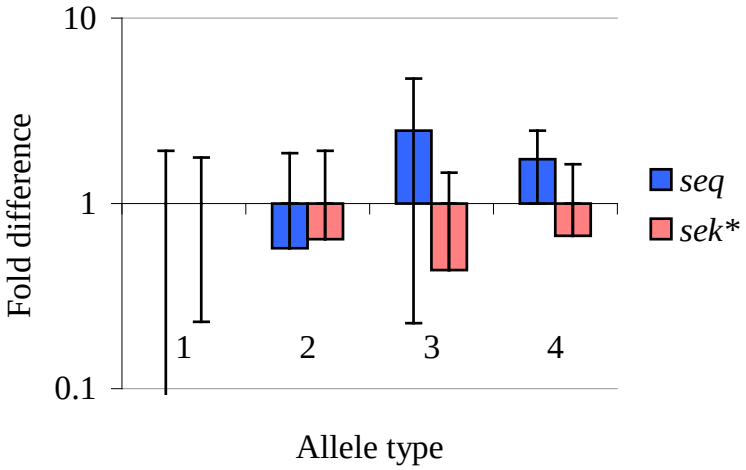
FIG. 8. Mean relative expression ratios for *seq* and *sek* for strains of allele types A) 1, B) 2, C) 3, and D) 4. The results are based on cultures of 8 strains representing the four allele types collected at three phases of growth: M, mid-logarithmic; L, late-logarithmic; and S, stationary. Mean values ± standard deviations represent samples from two independent cultures, each analyzed 3-6 times. Enterotoxin gene pairs labeled with an asterisk (*) indicate significant difference in gene expression determined by ANOVA.

Comparison of *seq* and *sek* expression in each growth phase. Expression of *seq* and *sek* was analyzed at each growth phase for all allele types. During mid-logarithmic and stationary growth phases, there were significant differences in *seq* and *sek* expression among the allele types (FIG. 9A and 9C), but only *sek* expression was significantly varied in the late-logarithmic stage of growth (FIG. 9B). In general, allele type 3 shows the lowest expression of *seq* and *sek* during the mid-logarithmic (*seq*: 4.7- to 10.5-fold lower; *sek*: 2.0- to 5.4-fold lower) and stationary (*seq*: 1.4- to 3.9-fold lower; *sek*: 2.3- to 3.5-fold lower) growth phases. Allele type 4 showed the highest expression of *seq* during stationary phase (2.2- to 3.9-fold higher). These results support allele specific expression, especially seen in allele types 3 and 4, and suggests the promoter strength of theses alleles may vary during *in vitro* growth.

A.


10
1
0.1
Fold difference
1
2
3
4
Allele type
seq*
sek*

B.


10
1
0.1
Fold difference
1
2
3
4
Allele type
seq
sek*

C.

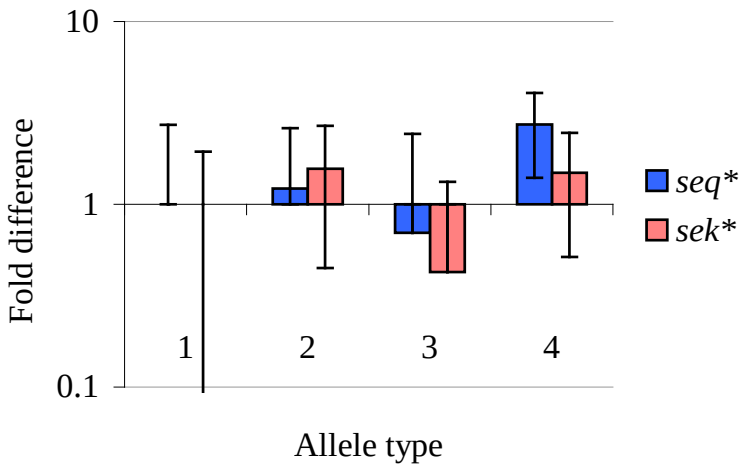


FIG. 9. Mean relative expression of *seq* and *sek* during A) mid-logarithmic, B) late-logarithmic, and C) stationary growth for each allele by qRT-PCR. The results are based on cultures of 8 strains representing all four *seq/sek* allele types. Mean values ± standard deviations represent the average expression of one reference and one clinical strain representing an allele type. Data for each strain represents samples from two independent cultures, each analyzed 3-6 times. Data collected from allele type 1 strains were used as a calibrator for all subsequent samples. Enterotoxin genes labeled with an asterisk (*) indicate significant differences in gene expression among allele types determined by ANOVA.

DISCUSSION

The success of *S. aureus* as a pathogen is in part due to its great ability to adapt through genetic diversity. The genotypically and phenotypically distinct strain types of CA-MRSA, USA400 and USA300, have recently emerged in community settings causing uncharacteristic disease in otherwise healthy individuals. In order to understand the success of CA-MRSA and other cohorts of *S. aureus*, it is important to elucidate which virulence factors may be contributing to pathogenesis. It is also important to understand how those virulence factors may have evolved to gain new roles in *S. aureus* disease. Enterotoxins Seq and Sek are known virulence factors of *S. aureus*, however, four allelic variants of this enterotoxin locus now exist due to recombination events between the MGEs on which they reside. The most recent variant, allele type 4, occurs exclusively in the particularly virulent USA300 strain type and may play a role in this strain's heightened virulence. A previous study has shown that Seq and Sek production is somewhat higher for the USA300 prototype strain compared to the USA400 prototype harboring allele type 3 (15). Transcriptional analysis has only been done for allele types 2 and 3 in separate studies and no direct comparison of regulation of the *seq* and *sek* genes among the allele variants has been performed (121, 134). This study is the first to show comparative, *in vitro* regulation of all four *seq/sek* allele variants, which may be correlated with disease severity in certain *S. aureus* cohorts.

A PCR analysis detected *seq* and *sek* genes in five cohorts of *S. aureus* isolates, including clinical MSSA and MRSA strains as well as commensal nasal isolates. The results also showed that the CA-MRSA group had the highest prevalence of *seq* and *sek* among other groups (74% positivity), reflecting a high degree of genetic similarity in this newest clonal expansion of *S. aureus* in comparison to the older, more genetically diverse groups of MSSA and HA-MRSA. Previous studies have shown a high prevalence of *seq* and *sek* genes in CA-MRSA (Shukla *et al*., submitted for publication, 87), which demonstrated the genetic homogeneity of CA-MRSA and identified a number of virulence genes that are more prevalent in this group compared to clinical or nasal carriage MSSA isolates, including *seq* and *sek*. Because the *seq* and *sek* genes were also found in commensal nasal isolates, this suggests that the presence of *seq* and *sek* is not always associated with *S. aureus* infection. Previous studies have also detected *seq* and *sek* in both clinical and nasal isolates (Shukla *et al*. submitted for publication, 57). The presence of SAgs in the *S. aureus* genome is more often associated with a certain clonal background than with clinical outcome. To verify SAg clonal presence in *S. aureus*, allele typing of the *seq/sek* loci revealed that CA-MRSA carried only allele types 3 and 4, reflecting the respective MGEs (ΦSa3mw/ΦSa3ms and SaPI5) in this group. Moreover, allele type 4 was found only in CA-MRSA, which is consistent with the previous finding that SaPI5 is exclusive to the USA300 strain type (30).

Both RT-PCR and qRT-PCR analyses of *seq* and *sek* from *in vitro* TSB cultures indicated that the *seq* and *sek* genes were expressed in all four allelic variants and during all tested phases of growth. Since qRT-PCR analyses showed allele type 2 expression of *seq* and *sek*, the negative results for allele type 2 gene specific RT-PCRs may be due to

low expression of the SE genes or low abundance of the intact templates. A previous study has also shown expression of *seq* and *sek* throughout *S. aureus* growth in brain heart infusion broth (BHI) and even in a 24-hour culture, however, allele types were not discerned. qRT-PCR analysis also determined that *seq* and *sek* expression was very low in TSB cultures. Based on ΔC_T values (target-reference), an average of 96-fold lower expression of *seq* and 32-fold lower expression of *sek* occurred compared to *gyrB*. Our data is consistent with previous studies that suggest SAgs; aside from TSST-1, Seb, and Sec; are produced in very low amounts (92-94, 99).

The role of SAgs during infection in the host is thought to be suppression of the local immune system. It is possible that low expression of these enterotoxins is ideal for the organism to establish infection. High amounts of enterotoxin could cause so much inflammation that the organism would quickly be eliminated by the host's immune system or the organism could quickly kill the host through TSS. Low levels of enterotoxin, on the other hand, could be just enough to suppress local immunity and establish infection without creating a large immune response within the host. However, more research is necessary to determine if these results from *in vitro* cultures are comparable to expression *in vivo*.

Both RT-PCR and qRT-PCR analyses of *seq* and *sek* from *in vitro* TSB cultures also indicated that the *seq* and *sek* genes were co-transcribed in all four allelic variants and during all tested phases of growth. Since all intergenic regions were amplified during RT-PCR analysis using at least one of the two primer sets targeting the intergenic region, negative results seen in RT-PCRs may be due to low expression of the SE genes, low abundance of an intact template, insufficient primer annealing or a combination of

these factors. The forward primer of primer set 2 contains one mismatch compared to allele type 3, which may have impaired primer annealing. qRT-PCR analyses revealed that *seq* and *sek* followed a similar pattern of expression during growth, indicating co-transcription, however, comparing absolute levels of *seq* to *sek* expression, revealed that *sek* almost always showed higher expression than *seq* (FIG. 8). This ranged from an average 2.9-fold difference in allele type 4 to a 17.0-fold difference in allele type 3. Since a ratio of expression closer to 1:1 would be expected for co-transcribed genes, this suggests that another factor is responsible for these observations. A likely possibility is that the rate of mRNA degradation may vary between *seq* and *sek*, with *seq* transcripts having a shorter mRNA half-life than *sek* transcripts. Another possibility is that *sek* may have its own specific promoter in addition to a common promoter, leading to amplification of *sek* expression. Yarwood *et al.* have predicted stem-loop structures just beyond the coding regions for both *seq* and *sek* in allele type 2, which may serve as Rho-independent transcription terminators. The presence of these terminators would suggest separate *seq* and *sek* transcripts and a need for a separate *sek* promoter (134).

The co-transcription of *seq* and *sek* is likely considering the close proximity of *seq* to *sek* and the fact that co-transcription tends to occur among phage genes due to coordinated temporal regulation. Transcription of the *seq* and *sek* loci may be controlled by a promoter from an upstream phage gene in all allelic variants. The transcriptional start-site for *seq* has previously been mapped to the upstream *cI* gene in allele type 3 and has been suggested for allele type 2 as well (121, 134). As mentioned earlier, the *cI* gene functions to maintain phage lysogeny by causing constitutive, low-level expression of the downstream integrase (*int*). Yarwood *et al*. was the first to suggest read through

transcription from the *cI* gene from their work in which they showed *seq, sek, int* and surrounding genes of allele type 2 having the same transcriptional profile by microarray (134). Since co-transcription occurs in all allele types, this research suggests the putative *cI* gene in each MGE may be responsible for transcription of this enterotoxin locus in all allele types. Despite several recombination events between different MGEs, the *seq/sek* locus remains intact and in the same relative location on the MGE so that it is controlled by non-identical phage transcriptional regulators, suggesting a selective advantage for *S. aureus* that retain the *seq/sek* locus in a certain location in the MGE.

Aside from transcriptional start-site analysis, another way to confirm that *seq* and *sek* are regulated by CI is to do phage induction experiments using a stress-inducing agent. Sumby *et al.* were able to determine increased expression of *seq* and *sek* upon addition of mitomycin C in cultures of both MSSA-476 (allele type 3) and an isogenic ΦSa3ms replication-deficient mutant.

The *seq* and *sek* transcripts of allele type 3 have previously been detected by Northern blot hybridization (121). Hybridizing species for *seq* and *sek* were weakly to moderately present in uninduced cultures while the addition of mitomycin C increased the level of hybridizing species of both enterotoxin genes. Although a Northern blot hybridization was done in this study, no *seq* or *sek* transcripts were detected. Since culture conditions and RNA extraction procedures were very similar between the two studies, the disparity in results may be attributable to strain variation in *seq* and *sek* expression or differences in the Northern blot protocol. Since expression of *seq* and *sek* were shown by RT-PCR, this indicated that the Northern blots were not sensitive enough to detect transcript due to very low expression of the enterotoxins.

Unfortunately, qRT-PCR analyses in this study were somewhat limited by the reference gene selected for the normalization of target gene expression. The accuracy of results obtained by qRT-PCR is largely dependent on accurate transcript normalization (52). Delta C_T values in this study (6.8 and 5.0 for *seq* and *sek*, respectively) indicate that expression of *gyrB* was too high to give reliably accurate expression measurements for the lowly expressed *seq* and *sek* targets. Optimal delta C_T values have an upper range of 2-3 (Roche Applied Science, personal communication). The large disparity in transcript abundance between the target and reference can put the measurements on different linear scales. For example, rare target transcripts may not be amplified when the RNA is partially degraded, whereas amplification of highly abundant reference transcripts still occurs, leading to false negative results (105). Further, a validation assay was not performed to assess the stability of *gyrB* for this particular study. It is, therefore, uncertain whether the variation in *gyrB* expression during growth was negligible compared to the variation in *seq* and *sek* expression during growth. The evaluation of numerous housekeeping genes as appropriate references has been performed in *Staphylococcus epidermidis* and *S. aureus* (35, 123, 128). However, these studies have not revealed an ideal reference gene that is not influenced by most metabolic conditions, growth phase, or experimental conditions, indicating the need for reference gene validation prior to experimentation (29).

Besides demonstrating that *seq* and *sek* were expressed at low levels, qRT-PCR analysis indicated that there may be growth-dependent regulation of *seq* and *sek* that was conserved despite the allele type. In general, expression of *seq* and *sek* was the lowest during the stationary growth phase and highest during mid-logarithmic and late-

logarithmic growth. However, this variation is rather insignificant compared to some enterotoxins, like *seh* and *sec,* which show an average 40-fold and 1,000-fold transcriptional increase, respectively, upon entry into the stationary phase (28). The growth-dependent regulation of *seq* and *sek* observed in this study is in contrast to a qRT-PCR study by Derzelle *et al*. that showed *S. aureus* food isolates grown in BHI broth had no significant difference in *seq* and *sek* expression during the various growth phases (28). However, no statistical analysis was performed in the prior study.

Based on data from this study, it is unlikely that the quorum sensing global regulator, Agr, has a role in the transcriptional differences we have noted. Induction of SE expression by Agr tends to occur during entry into stationary phase and then remain at high levels. However, we did not observe this transcriptional pattern for *seq* and *sek*. Potentially, other global regulators, such as SrrA-SrrB or SarA, may be controlling the expression of *seq* and *sek*.

Although *seq* and *sek* were expressed in all of the alleles, there were both strain-specific and allele-specific expression patterns. It has previously been acknowledged that *S. aureus* strains of undefined *seq/sek* allele type exhibited strain-specific differences in the absolute levels of *seq* and *sek* transcripts (28). Our study is the first to show that expression of *seq* and *sek* can vary depending on the allele type. This was most apparent in allele type 3, which showed the lowest expression of both *seq* and *sek*, especially during the mid-logarithmic and stationary growth phases. In addition, allele type 4 showed the highest expression of *seq* during the stationary growth phase. A previous study has also shown allele specific expression for an SE. Variant *sea* enterotoxin alleles located on two different phages had a minimal eight-fold difference in mRNA production

between them, which could not be explained by the differences in the alleles themselves (10).

The fact that allele type 4 shows the highest expression of *seq* may be interesting. These results agree with the study by Burlak *et al*. which showed an average 2.6-fold higher amount of Seq production in a TSB stationary phase culture of USA300-FPR3757 (allele type 4) compared to MW2 (allele type 3) (15). If this pattern of *in vitro* expression held true for *in vivo* or in *vivo*-like studies, there could be a possible role in clinical outcome. Allele type 4 occurs exclusively in the USA300 strain type. Presently, this USA300 CA-MRSA type has rapidly disseminated in the community, causing a wide spectrum of infections in healthy individuals, including invasive disease. Many studies have attempted to elucidate the virulence determinants of USA300, such as individual studies on PVL, α-toxin, and phenol-soluble modulators (PSMs) (12-14, 31, 130, 132). However, it is more likely that the collective action of many virulence factors may be involved. It is possible that higher expression of *seq* in this strain type may contribute to disease in healthy individuals. Though expression of this enterotoxin is still very low, slightly higher production may be just enough to give *S. aureus* an advantage when faced with a healthy immune system. More enterotoxin could lead to a superior ability to suppress local immune cells without causing a large immune response, allowing an infection to persist and progress. Further linking the enterotoxin expression to expression of other USA300 virulence factors could help explain why the USA300 strain is more successful in healthy individuals.

One limitation of this study is that *seq* and *sek* expression was measured *in vitro* and not *in vivo*. Virulence factor production is known to be regulated by the host niche

through nutrient availability and the presence of the innate and adaptive immune systems. For example, *S. aureus* has difficulty producing secreted virulence factors when present in the blood due to potential interactions of α- and β-globin chains in host blood with bacterial two-component regulatory systems (109, 113). Moreover, about 200 times more TSST-1 and Sec have been shown to be produced in biofilm culture versus planktonic culture, presumably due to quorum-sensing and other global transcription regulation (88, 113). It is possible that *seq* and *sek* are expressed at a much higher level *in vivo* (i.e. the human body). Therefore, it is necessary to assess the expression of these genes in an *in vivo*-like environment by altering growth conditions (for example, minimal nutrient media versus nutrient-rich TSB) to more resemble *in vivo* conditions, or by using biofilm cultures or animal infection models.

Aside from expression analyses of *seq* and *sek*, functional analysis would be informative since the allele variants differ slightly from each other. The predicted enterotoxin amino acids only differ by 0.8% to 3.7% among the four different *seq/sek* loci. However, slight amino acid sequence differences between members of the SAg family can result in markedly altered biological properties (133). Certain *seq/sek* allele types may have an enhanced ability to activate T-cells. Activation of a larger repertoire or a unique subset of T-cells, combined with additional activation by other SAgs, could give *S. aureus* an improved ability to suppress local immunity. It would be interesting to see if allele type 4 *S. aureus* exhibit unique T-cell activation that may have contributed to the success of the USA300 strain type.

REFERENCES

1. **Abraham, E. P., and E. Chain.** 1988. An enzyme from bacteria able to destroy penicillin. Rev. Infect. Dis. **10:**677-678.

2. **Adem, P. V., C. P. Montgomery, A. N. Husain, T. K. Koogler, V. Arangelovich, M. Humilier, S. Boyle-Vavra, and R. S. Daum.** 2005. *Staphylococcus aureus* sepsis and the Waterhouse-Friderichsen syndrome in children. N. Engl. J. Med. **353:**1245-1251.

3. **Ala'Aldeen, D., and K. Hiramatsu.** 2004. *Staphylococcus aureus*: molecular and clinical aspects. Horwood Publishing Limited, Chichester.

4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.

5. **Attinger, A., H. Acha-Orbea, and H. R. MacDonald.** 2000. Cutting edge: cell autonomous rather than environmental factors control bacterial superantigen-induced T cell anergy *in vivo*. J. Immunol. **165:**1171-1174.

6. **Azuma, K., K. Koike, T. Kobayashi, T. Mochizuki, K. Mashiko, and Y. Yamamoto.** 2004. Detection of circulating superantigens in an intensive care unit population. Int. J. Infect. Dis. **8:**292-298.

7. **Baba, T., T. Bae, O. Schneewind, F. Takeuchi, and K. Hiramatsu.** 2008. Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J. Bacteriol. **190:**300-310.

8. **Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis.** 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome *Staphylococcus aureus* isolates. Lancet **1:**1017-1021.

9. **Betley, M. J., and J. J. Mekalanos.** 1985. Staphylococcal enterotoxin A is encoded by phage. Science **229:**185-187.

10. **Borst, D. W., and M. J. Betley.** 1994. Phage-associated differences in staphylococcal enterotoxin A gene (*sea*) expression correlate with *sea* allele class. Infect. Immun. **62:**113-118.

11. **Brady, J. M., M. E. Stemper, A. Weigel, P. H. Chyou, K. D. Reed, and S. K. Shukla.** 2007. Sporadic "transitional" community-associated methicillin-resistant *Staphylococcus aureus* strains from health care facilities in the United States. J. Clin. Microbiol. **45:**2654-2661.

12. **Bubeck Wardenburg, J., T. Bae, M. Otto, F. R. Deleo, and O. Schneewind.** 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat. Med. **13:**1405-1406.

13. **Bubeck Wardenburg, J., A. M. Palazzolo-Ballance, M. Otto, O. Schneewind, and F. R. DeLeo.** 2008. Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant *Staphylococcus aureus* disease. J. Infect. Dis. **198:**1166-1170.

14. **Bubeck Wardenburg, J., and O. Schneewind.** 2008. Vaccine protection against *Staphylococcus aureus* pneumonia. J. Exp. Med. **205:**287-294.

15. **Burlak, C., C. H. Hammer, M. A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo.** 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. Cell. Microbiol. **9:**1172-1190.

16. **Butte, A. J., J. Ye, H. U. Haring, M. Stumvoll, M. F. White, and I. S. Kohane.** 2001. Determining significant fold differences in gene expression analysis. Pac. Symp. Biocomput**:**6-17.

17. **Campbell, K. M., A. F. Vaughn, K. L. Russell, B. Smith, D. L. Jimenez, C. P. Barrozo, J. R. Minarcik, N. F. Crum, and M. A. Ryan.** 2004. Risk factors for community-associated methicillin-resistant *Staphylococcus aureus* infections in an outbreak of disease among military trainees in San Diego, California, in 2002. J. Clin. Microbiol. **42:**4050-4053.

18. **Cassat, J., P. M. Dunman, E. Murphy, S. J. Projan, K. E. Beenken, K. J. Palm, S. J. Yang, K. C. Rice, K. W. Bayles, and M. S. Smeltzer.** 2006. Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology **152:**3075-3090.

19. **Centers for Disease Control and Prevention.** 1999. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus* infections—Minnesota and North Dakota, 1997–1999. Morb. Mortal. Wkly. Rep. **48** 707–710.

20. **Centers for Disease Control and Prevention.** 2003. Methicillin-resistant *Staphylococcus aureus* infections among competitive sports participants--Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000-2003. Morb. Mortal. Wkly. Rep. **52:**793-795.

21. **Centers for Disease Control and Prevention.** 2003. Methicillin-resistant *Staphylococcus aureus* infections in correctional facilities---Georgia, California, and Texas, 2001-2003. Morb. Mortal. Wkly. Rep. **52:**992-996.

22. **Centers for Disease Control and Prevention.** 2004. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. Am. J. Infect. Control **32:**470-485.

23. **Chan, P. F., and S. J. Foster.** 1998. Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. J. Bacteriol. **180:**6232-6241.

24. **Cheung, A. L., K. J. Eberhardt, and V. A. Fischetti.** 1994. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem. **222:**511-514.

25. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. J. Biol. Chem. **274:**37169-37176.

26. **Crass, B. A., and M. S. Bergdoll.** 1986. Involvement of staphylococcal enterotoxins in nonmenstrual toxic shock syndrome. J. Clin. Microbiol. **23:**1138-1139.

27. **Czop, J. K., and M. S. Bergdoll.** 1974. Staphylococcal enterotoxin synthesis during the exponential, transitional, and stationary growth phases. Infect. Immun. **9:**229-235.

28. **Derzelle, S., F. Dilasser, M. Duquenne, and V. Deperrois.** 2009. Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. Food Microbiol. **26:**896-904.

29. **Dheda, K., J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook, and A. Zumla.** 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques **37:**112-114, 116, 118-119.

30. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **367:**731-739.

31. **Diep, B. A., A. M. Palazzolo-Ballance, P. Tattevin, L. Basuino, K. R. Braughton, A. R. Whitney, L. Chen, B. N. Kreiswirth, M. Otto, F. R. DeLeo, and H. F. Chambers.** 2008. Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. PLoS One **3:**e3198.

32. **Dinges, M. M., P. M. Orwin, and P. M. Schlievert.** 2000. Exotoxins of *Staphylococcus aureus*. Clin. Microbiol. Rev. **13:**16-34

33. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. **183:**7341-7353.

34. **Eisen, H., P. Brachet, L. Pereira da Silva, and F. Jacob.** 1970. Regulation of repressor expression in lambda. Proc. Natl. Acad. Sci. U S A **66:**855-862.

35. **Eleaume, H., and S. Jabbouri.** 2004. Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during *in vitro* growth. J. Microbiol. Methods **59:**363-370.

36. **Elixhauser, A., and C. Steiner.** July 2007. Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) in U.S. hospitals, 1993-2005, HCUP Statistical Brief #35. Agency for Healthcare Research and Quality, Rockville, MD.

37. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. U S A **99:**7687-7692.

38. **Fitzgerald, J. R., S. R. Monday, T. J. Foster, G. A. Bohach, P. J. Hartigan, W. J. Meaney, and C. J. Smyth.** 2001. Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. J. Bacteriol. **183:**63-70.

39. **Fournier, B., and D. C. Hooper.** 2000. A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J. Bacteriol. **182:**3955-3964.

40. **Fournier, B., A. Klier, and G. Rapoport.** 2001. The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. **41:**247-261.

41. **Fraser, J. D., and T. Proft.** 2008. The bacterial superantigen and superantigen-like proteins. Immunol. Rev. **225:**226-243.

42. **Fridkin, S. K., J. C. Hageman, M. Morrison, L. T. Sanza, K. Como-Sabetti, J. A. Jernigan, K. Harriman, L. H. Harrison, R. Lynfield, and M. M. Farley.** 2005. Methicillin-resistant *Staphylococcus aureus* disease in three communities. N. Engl. J. Med. **352:**1436-1444.

43. **Fuda, C., M. Suvorov, S. B. Vakulenko, and S. Mobashery.** 2004. The basis for resistance to beta-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. J. Biol. Chem. **279:**40802-40806.

44. **Garvis, S., J. M. Mei, J. Ruiz-Albert, and D. W. Holden.** 2002. *Staphylococcus aureus svrA*: a gene required for virulence and expression of the *agr* locus. Microbiology **148:**3235-3243.

45. **Geiger, T., C. Goerke, M. Mainiero, D. Kraus, and C. Wolz.** 2008. The virulence regulator Sae of *Staphylococcus aureus*: promoter activities and response to phagocytosis-related signals. J. Bacteriol. **190:**3419-3428.

46. **Giantonio, B. J., R. K. Alpaugh, J. Schultz, C. McAleer, D. W. Newton, B. Shannon, Y. Guedez, M. Kotb, L. Vitek, R. Persson, P. O. Gunnarsson, T. Kalland, M. Dohlsten, B. Persson, and L. M. Weiner.** 1997. Superantigen-based immunotherapy: a phase I trial of PNU-214565, a monoclonal antibody-staphylococcal enterotoxin A recombinant fusion protein, in advanced pancreatic and colorectal cancer. J. Clin. Oncol. **15:**1994-2007.

47. **Gill, S. R., D. E. Fouts, G. L. Archer, E. F. Mongodin, R. T. Deboy, J. Ravel, I. T. Paulsen, J. F. Kolonay, L. Brinkac, M. Beanan, R. J. Dodson, S. C. Daugherty, R. Madupu, S. V. Angiuoli, A. S. Durkin, D. H. Haft, J. Vamathevan, H. Khouri, T. Utterback, C. Lee, G. Dimitrov, L. Jiang, H. Qin, J. Weidman, K. Tran, K. Kang, I. R. Hance, K. E. Nelson, and C. M. Fraser.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. **187:**2426-2438.

48. **Goerke, C., S. Campana, M. G. Bayer, G. Doring, K. Botzenhart, and C. Wolz.** 2000. Direct quantitative transcript analysis of the agr regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro. Infect. Immun. **68:**1304-1311.

49. **Goerke, C., U. Fluckiger, A. Steinhuber, V. Bisanzio, M. Ulrich, M. Bischoff, J. M. Patti, and C. Wolz.** 2005. Role of *Staphylococcus aureus* global regulators *sae* and sigmaB in virulence gene expression during device-related infection. Infect. Immun. **73:**3415-3421.

50. **Goerke, C., U. Fluckiger, A. Steinhuber, W. Zimmerli, and C. Wolz.** 2001. Impact of the regulatory loci *agr*, *sarA* and *sae* of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis. Mol. Microbiol. **40:**1439-1447.

51. **Grumann, D., S. S. Scharf, S. Holtfreter, C. Kohler, L. Steil, S. Engelmann, M. Hecker, U. Volker, and B. M. Broker.** 2008. Immune cell activation by enterotoxin gene cluster (egc)-encoded and non-egc superantigens from *Staphylococcus aureus*. J. Immunol. **181:**5054-5061.

52. **Guenin, S., M. Mauriat, J. Pelloux, O. Van Wuytswinkel, C. Bellini, and L. Gutierrez.** 2009. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J. Exp. Bot. **60:**487-493.

53. **Gunther, S., A. K. Varma, B. Moza, K. J. Kasper, A. W. Wyatt, P. Zhu, A. K. Rahman, Y. Li, R. A. Mariuzza, J. K. McCormick, and E. J. Sundberg.** 2007. A novel loop domain in superantigens extends their T cell receptor recognition site. J. Mol. Biol. **371:**210-221.

54. **Heinrichs, J. H., M. G. Bayer, and A. L. Cheung.** 1996. Characterization of the *sar* locus and its interaction with *agr* in *Staphylococcus aureus*. J. Bacteriol. **178:**418-423.

55. **Herigon, J. C., A. L. Hersh, J. S. Gerber, T. E. Zaoutis, and J. G. Newland.** 2010. Antibiotic management of *Staphylococcus aureus* infections in US children's hospitals, 1999-2008. Pediatrics **125:**e1294-1300.

56. **Ho, G., W. H. Campbell, M. S. Bergdoll, and E. Carlson.** 1989. Production of a toxic shock syndrome toxin variant by *Staphylococcus aureus* strains associated with sheep, goats, and cows. J. Clin. Microbiol. **27:**1946-1948.

57. **Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Broker.** 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45:**2669-2680.

58. **Hopewell, R., M. Oram, R. Briesewitz, and L. M. Fisher.** 1990. DNA cloning and organization of the *Staphylococcus aureus gyrA* and *gyrB* genes: close homology among gyrase proteins and implications for 4-quinolone action and resistance. J. Bacteriol. **172:**3481-3484.

59. **Hovde, C. J., S. P. Hackett, and G. A. Bohach.** 1990. Nucleotide sequence of the staphylococcal enterotoxin C3 gene: sequence comparison of all three type C staphylococcal enterotoxins. Mol. Gen. Genet. **220:**329-333.

60. **Jarraud, S., C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne, and F. Vandenesch.** 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect. Immun. **70:**631-641.

61. **Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina.** 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol. **166:**669-677.

62. **Jarvis, W. R., J. Schlosser, R. Y. Chinn, S. Tweeten, and M. Jackson.** 2007. National prevalence of methicillin-resistant *Staphylococcus aureus* in inpatients at US health care facilities, 2006. Am. J. Infect. Control **35:**631-637.

63. **Johnson, A., B. J. Meyer, and M. Ptashne.** 1978. Mechanism of action of the Cro protein of bacteriophage lambda. Proc. Natl. Acad. Sci. U S A **75:**1783-1787.

64. **Joost, I., D. Blass, M. Burian, C. Goerke, C. Wolz, L. von Muller, K. Becker, K. Preissner, M. Herrmann, and M. Bischoff.** 2009. Transcription analysis of the extracellular adherence protein from *Staphylococcus aureus* in authentic human infection and *in vitro*. J. Infect. Dis. **199:**1471-1478.

65. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA **298:**1763-1771.

66. **Kotb, M., and J. Fraser.** 2007. Superantigens: molecular basis for their role in human diseases. ASM Press, Washingtion, D.C.

67. **Kravitz, G. R., D. J. Dries, M. L. Peterson, and P. M. Schlievert.** 2005. Purpura fulminans due to *Staphylococcus aureus*. Clin. Infect. Dis. **40:**941-947.

68. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature **305:**709-712.

69. **Kuehnert, M. J., H. A. Hill, B. A. Kupronis, J. I. Tokars, S. L. Solomon, and D. B. Jernigan.** 2005. Methicillin-resistant-*Staphylococcus aureus* hospitalizations, United States. Emerg. Infect. Dis. **11:**868-872.

70. **Kuehnert, M. J., D. Kruszon-Moran, H. A. Hill, G. McQuillan, S. K. McAllister, G. Fosheim, L. K. McDougal, J. Chaitram, B. Jensen, S. K. Fridkin, G. Killgore, and F. C. Tenover.** 2006. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. J. Infect. Dis. **193:**172-179.

71. **Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet **357:**1225-1240.

72. **Lewin, B.** 2008. Genes IX, vol. 9. Jones and Bartlett Publishers, Boston, MA.

73. **Lewin, B.** 1990. Structural genes evolve in families. Oxford University Press, New York.

74. **Lindenmayer, J. M., S. Schoenfeld, R. O'Grady, and J. K. Carney.** 1998. Methicillin-resistant *Staphylococcus aureus* in a high school wrestling team and the surrounding community. Arch. Intern. Med. **158:**895-899.

75. **Lindsay, J. A., and M. T. Holden.** 2004. *Staphylococcus aureus*: superbug, super genome? Trends Microbiol. **12:**378-385.

76. **Lindsay, J. A., and M. T. Holden.** 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct. Integr. Genomics **6:**186-201.

77. **Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick.** 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. Mol. Microbiol. **29:**527-543.

78. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-428.

79. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:**520-532.

80. **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert.** 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu. Rev. Microbiol. **55:**77-104.

81. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 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.

82. **Meyer, B. J., R. Maurer, and M. Ptashne.** 1980. Gene regulation at the right operator (OR) of bacteriophage lambda. II. OR1, OR2, and OR3: their roles in mediating the effects of repressor and *cro*. J. Mol. Biol. **139:**163-194.

83. **Miller, L. G., F. Perdreau-Remington, G. Rieg, S. Mehdi, J. Perlroth, A. S. Bayer, A. W. Tang, T. O. Phung, and B. Spellberg.** 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N. Engl. J. Med. **352:**1445-1453.

84. **Miwa, K., M. Fukuyama, T. Kunitomo, and H. Igarashi.** 1994. Rapid assay for detection of toxic shock syndrome toxin 1 from human sera. J. Clin. Microbiol. **32:**539-542.

85. **Mizuuchi, K., M. H. O'Dea, and M. Gellert.** 1978. DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci. U S A **75:**5960-5963.

86. **Murray, P. R., Baron, E.J., Jorgensen J.H., et al.** 2007. Manual of clinical microbiology, 9th ed. ASM Press, Washington D.C.

87. **Naimi, T. S., K. H. LeDell, K. Como-Sabetti, S. M. Borchardt, D. J. Boxrud, J. Etienne, S. K. Johnson, F. Vandenesch, S. Fridkin, C. O'Boyle, R. N. Danila, and R. Lynfield.** 2003. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA **290:**2976-2984.

88. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. **48:**1429-1449.

89. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. **12:**3967-3975.

90. **Novick, R. P., and A. Subedi.** 2007. The SaPIs: mobile pathogenicity islands of *Staphylococcus*. Chem. Immunol. Allergy **93:**42-57.

91. **Omoe, K., D. L. Hu, H. Takahashi-Omoe, A. Nakane, and K. Shinagawa.** 2005. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. FEMS Microbiol. Lett. **246:**191-198.

92. **Omoe, K., K. Imanishi, D. L. Hu, H. Kato, Y. Fugane, Y. Abe, S. Hamaoka, Y. Watanabe, A. Nakane, T. Uchiyama, and K. Shinagawa.** 2005. Characterization of novel staphylococcal enterotoxin-like toxin type P. Infect. Immun. **73:**5540-5546.

93. **Omoe, K., K. Imanishi, D. L. Hu, H. Kato, H. Takahashi-Omoe, A. Nakane, T. Uchiyama, and K. Shinagawa.** 2004. Biological properties of staphylococcal enterotoxin-like toxin type R. Infect. Immun. **72:**3664-3667.

94. **Omoe, K., M. Ishikawa, Y. Shimoda, D. L. Hu, S. Ueda, and K. Shinagawa.** 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates Harboring *seg*, *seh*, or *sei* genes. J. Clin. Microbiol. **40:**857-862.

95. **Ono, H. K., K. Omoe, K. Imanishi, Y. Iwakabe, D. L. Hu, H. Kato, N. Saito, A. Nakane, T. Uchiyama, and K. Shinagawa.** 2008. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. Infect. Immun. **76:**4999-5005.

96. **Orwin, P. M., D. Y. Leung, H. L. Donahue, R. P. Novick, and P. M. Schlievert.** 2001. Biochemical and biological properties of staphylococcal enterotoxin K. Infect. Immun. **69:**360-366.

97. **Orwin, P. M., D. Y. Leung, T. J. Tripp, G. A. Bohach, C. A. Earhart, D. H. Ohlendorf, and P. M. Schlievert.** 2002. Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. Biochemistry **41:**14033-14040.

98. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**45.

99. **Pocsfalvi, G., G. Cacace, M. Cuccurullo, G. Serluca, A. Sorrentino, G. Schlosser, G. Blaiotta, and A. Malorni.** 2008. Proteomic analysis of exoproteins expressed by enterotoxigenic *Staphylococcus aureus* strains. Proteomics **8:**2462-2476.

100. **Poindexter, N. J., and P. M. Schlievert.** 1986. Suppression of immunoglobulin-secreting cells from human peripheral blood by toxic-shock-syndrome toxin-1. J. Infect. Dis. **153:**772-779.

101. **Pokallus, A. T.** 2008. Association of accessory gene regulator alleles with several virulence factor genes and *in vitro* expression of the *lpl10, sel2,* and *seg2* genes in *Staphylococcus aureus*. MS. University of Wisconsin - La Crosse, La Crosse.

102. **Pragman, A. A., L. Herron-Olson, L. C. Case, S. M. Vetter, E. E. Henke, V. Kapur, and P. M. Schlievert.** 2007. Sequence analysis of the *Staphylococcus aureus srrAB* loci reveals that truncation of *srrA* affects growth and virulence factor expression. J. Bacteriol. **189:**7515-7519.

103. **Pragman, A. A., J. M. Yarwood, T. J. Tripp, and P. M. Schlievert.** 2004. Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. J. Bacteriol. **186:**2430-2438.

104. **Rechtin, T. M., A. F. Gillaspy, M. A. Schumacher, R. G. Brennan, M. S. Smeltzer, and B. K. Hurlburt.** 1999. Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*. Mol. Microbiol. **33:**307-316.

105. **Roche Applied Science.** 2005. Selection of housekeeping genes: Technical Note. Roche Applied Science.

106. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

107. **Sampathkumar, P.** 2007. Methicillin-resistant *Staphylococcus aureus*: the latest health scare. Mayo Clinic Proceed. **82:**1463-1467.

108. **Schlievert, P. M.** 1986. Staphylococcal enterotoxin B and toxic-shock syndrome toxin-1 are significantly associated with non-menstrual TSS. Lancet **1:**1149-1150.

109. **Schlievert, P. M., L. C. Case, K. A. Nemeth, C. C. Davis, Y. Sun, W. Qin, F. Wang, A. J. Brosnahan, J. A. Mleziva, M. L. Peterson, and B. E. Jones.** 2007. Alpha and beta chains of hemoglobin inhibit production of *Staphylococcus aureus* exotoxins. Biochemistry **46:**14349-14358.

110. **Schlievert, P. M., L. C. Case, K. L. Strandberg, B. B. Abrams, and D. Y. Leung.** 2008. Superantigen profile of *Staphylococcus aureus* isolates from patients with steroid-resistant atopic dermatitis. Clin. Infect. Dis. **46:**1562-1567.

111. **Schlievert, P. M., L. M. Jablonski, M. Roggiani, I. Sadler, S. Callantine, D. T. Mitchell, D. H. Ohlendorf, and G. A. Bohach.** 2000. Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals. Infect. Immun. **68:**3630-3634.

112. **Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura.** 1981. Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. J. Infect. Dis. **143:**509-516.

113. **Schlievert, P. M., K. L. Strandberg, Y. C. Lin, M. L. Peterson, and D. Y. Leung.** 2010. Secreted virulence factor comparison between methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, and its relevance to atopic dermatitis. J. Allergy Clin. Immunol. **125:**39-49.

114. **Schlievert, P. M., T. J. Tripp, and M. L. Peterson.** 2004. Reemergence of staphylococcal toxic shock syndrome in Minneapolis-St. Paul, Minnesota, during the 2000-2003 surveillance period. J. Clin. Microbiol. **42:**2875-2876.

115. **Schroder, E., G. Kunstmann, H. Hasbach, and G. Pulverer.** 1988. Prevalence of serum antibodies to toxic-shock-syndrome-toxin-1 and to staphylococcal enterotoxins A, B and C in West-Germany. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **270:**110-114.

116. **Sorensen, S. J., M. Bailey, L. H. Hansen, N. Kroer, and S. Wuertz.** 2005. Studying plasmid horizontal transfer *in situ*: a critical review. Nat. Rev. Microbiol. **3:**700-710.

117. **Stemper, M. E., S. K. Shukla, and K. D. Reed.** 2004. Emergence and spread of community-associated methicillin-resistant *Staphylococcus aureus* in rural Wisconsin, 1989 to 1999. J. Clin. Microbiol. **42:**5673-5680.

118. **Streit, S., C. W. Michalski, M. Erkan, J. Kleeff, and H. Friess.** 2009. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat. Protoc. **4:**37-43.

119. **Subedi, A., C. Ubeda, R. P. Adhikari, J. R. Penades, and R. P. Novick.** 2007. Sequence analysis reveals genetic exchanges and intraspecific spread of SaPI2, a pathogenicity island involved in menstrual toxic shock. Microbiology **153:**3235-3245.

120. **Sugino, A., N. P. Higgins, P. O. Brown, C. L. Peebles, and N. R. Cozzarelli.** 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. U S A **75:**4838-4842.

121. **Sumby, P., and M. K. Waldor.** 2003. Transcription of the toxin genes present within the staphylococcal phage phiSa3ms is intimately linked with the phage's life cycle. J. Bacteriol. **185:**6841-6851.

122. **Takeda, Y.** 1979. Specific repression of *in vitro* transcription by the Cro repressor of bacteriophage lambda. J. Mol. Biol. **127:**177-189.

123. **Theis, T., R. A. Skurray, and M. H. Brown.** 2007. Identification of suitable internal controls to study expression of a *Staphylococcus aureus* multidrug resistance system by quantitative real-time PCR. J. Microbiol. Methods **70:**355-362.

124. **Thomas, D., S. Chou, O. Dauwalder, and G. Lina.** 2007. Diversity in *Staphylococcus aureus* enterotoxins. Chem. Immunol. Allergy **93:**24-41.

125. **Thomas, D., O. Dauwalder, V. Brun, C. Badiou, T. Ferry, J. Etienne, F. Vandenesch, and G. Lina.** 2009. *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. Infect. Immun. **77:**2043-2050.

126. **Thornsberry, C.** 1995. Trends in antimicrobial resistance among today's bacterial pathogens. Pharmacotherapy **15:**3S-8S.

127. **Tranter, H. S.** 1990. Foodborne staphylococcal illness. Lancet **336:**1044-1046.

128. **Vandecasteele, S. J., W. E. Peetermans, R. Merckx, and J. Van Eldere.** 2001. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. J. Bacteriol. **183:**7094-7101.

129. **Vojtov, N., H. F. Ross, and R. P. Novick.** 2002. Global repression of exotoxin synthesis by staphylococcal superantigens. Proc. Natl. Acad. Sci. U S A **99:**10102-10107.

130. **Voyich, J. M., M. Otto, B. Mathema, K. R. Braughton, A. R. Whitney, D. Welty, R. D. Long, D. W. Dorward, D. J. Gardner, G. Lina, B. N. Kreiswirth, and F. R. DeLeo.** 2006. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J. Infect. Dis. **194:**1761-1770.

131. **Voyich, J. M., C. Vuong, M. DeWald, T. K. Nygaard, S. Kocianova, S. Griffith, J. Jones, C. Iverson, D. E. Sturdevant, K. R. Braughton, A. R. Whitney, M. Otto, and F. R. DeLeo.** 2009. The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. J. Infect. Dis. **199:**1698-1706.

132. **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. **13:**1510-1514.

133. **Williams, R. J., J. M. Ward, B. Henderson, S. Poole, B. P. O'Hara, M. Wilson, and S. P. Nair.** 2000. Identification of a novel gene cluster encoding staphylococcal exotoxin-like proteins: characterization of the prototypic gene and its protein product, SET1. Infect. Immun. **68:**4407-4415.

134. **Yarwood, J. M., J. K. McCormick, M. L. Paustian, P. M. Orwin, V. Kapur, and P. M. Schlievert.** 2002. Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. Implications for the evolution of staphylococcal pathogenicity islands. J. Biol. Chem. **277:**13138-13147.

135. **Yarwood, J. M., J. K. McCormick, and P. M. Schlievert.** 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.