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Transposon mutagenesis (Tn*551*) was used to generate *agr*-suppressor mutations in the *agr*-null *Staphylococcus aureus* strain RN6911 (Δ*agr*::*tmn*). Fifty-four suppressor mutants displaying changes in hemolysin, protease, and lipase activities were isolated, and only twenty-six mutants contained Tn*551* within their chromosomes. Transposon insertion sites for seven mutants were determined by sequencing amplicons generated by arbitrary-PCR. One of the insertion sites was within the *s*erine *p*rotease-*l*ike *F* (*splF*) gene. Alpha-toxin message levels for the *splF* mutant were similar to RN6911. However, alpha-toxin activity in spent media isolated from the *splF* mutant was increased ten-fold as compared to RN6911. Transduction of the *splF*::Tn*551* mutation back into the parental strain verified the link between the phenotype and the mutation. Whole cell lysates from *Escherichia coli* cells containing a plasmid copy of *splF* displayed protease activity on casein. These data suggest that SplF may be post-translationally modifying alpha-toxin through proteolysis.

EFFECTS OF SERINE PROTEASE-LIKE F (SPLF)

ON ALPHA-TOXIN EXPRESSION

IN *Staphylococcus aureus.*

Mark E. Pulse, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Microbiology and Immunology

Dean, Graduate School of Biomedical Sciences

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THESIS

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Mark Pulse, B.S.

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CHAPTER I

INTRODUCTION

Background

Staphylococcus aureus belongs to the *Micrococcaceae* family and is one species of staphylococci that produces coagulase (67). This major human pathogen is a hardy organism that is able to survive various environmental stresses, which include extreme changes in dry and osmotic/ionic conditions (76). The hardiness of *S. aureus* makes it very difficult to eliminate this bacterium from the human environment, and, as a result, humans are frequently colonized without any signs of disease. Additionally, the percentage of healthy carriers dramatically increases among health care professionals, which is a significant reason why *S. aureus* is major cause of nosocomial infections worldwide (16). The prevalence of drug resistance among clinical isolates has made treating individuals with *S. aureus* infections very difficult at times (17). This situation has reached a new level of severity with the isolation of a vancomycin-resistant *S. aureus*, VRSA, strain in 2002 (18). Vancomycin has been the last remaining antibiotic to which *S. aureus* is uniformly susceptible. Therefore, successfully treating infections caused by drug resistant strains will be difficult to accomplish with the emergence of VRSA. The seriousness of this situation demonstrates the need to understand the virulence

mechanisms of *S. aureus* in order to design new and successful therapies for and against infection.

The virulence of *Staphylococcus aureus* is linked to its ability to produce a diverse array of proteins (i.e. virulence factors), many of which have been identified with particular disease processes (35). Based on their postulated roles in *S. aureus* pathogenicity, virulence factors are grouped into one of the four following catergories: factors that aid in attachment, factors that help evade the host immune system, factors with cytolytic activities, or factors that act as immunomodulators (63). These categories suggest that virulence factor expression is regulated by *S. aureus* in response to the different microenvironments of the host. It is thought that this pathogen could utilize its network of regulatory genes to coordinate the expression of those factors over the course of an infection (5, 21, 29, 39, 48, 51, 68, 72, 79).

The genetics of virulence factor regulation in *S. aureus* is largely understood through two well-characterized global virulence regulatory loci (21, 39), *agr* (*a*ccessory *g*ene *r*egulator) and *sar* (*s*taphylococcal *a*ccessory *r*egulator). The *agr* locus is known to regulate the expression of numerous extracellular proteins in *S. aureus* (64). This operon is comprised of two divergent transcripts, RNAII and RNAIII, which are initiated from promoters P2 and P3, respectively (39; Figure 1). The RNAII transcript contains four open reading frames that sequentially encode for proteins AgrB, AgrD, AgrC, and AgrA (56). AgrC and AgrA are thought to act as the sensor and activator of a two-component signal transduction system (39, 43, 56), while AgrD and AgrB are involved in the synthesis and secretion of an octapeptide that serves as a quorum-sensing molecule (56).

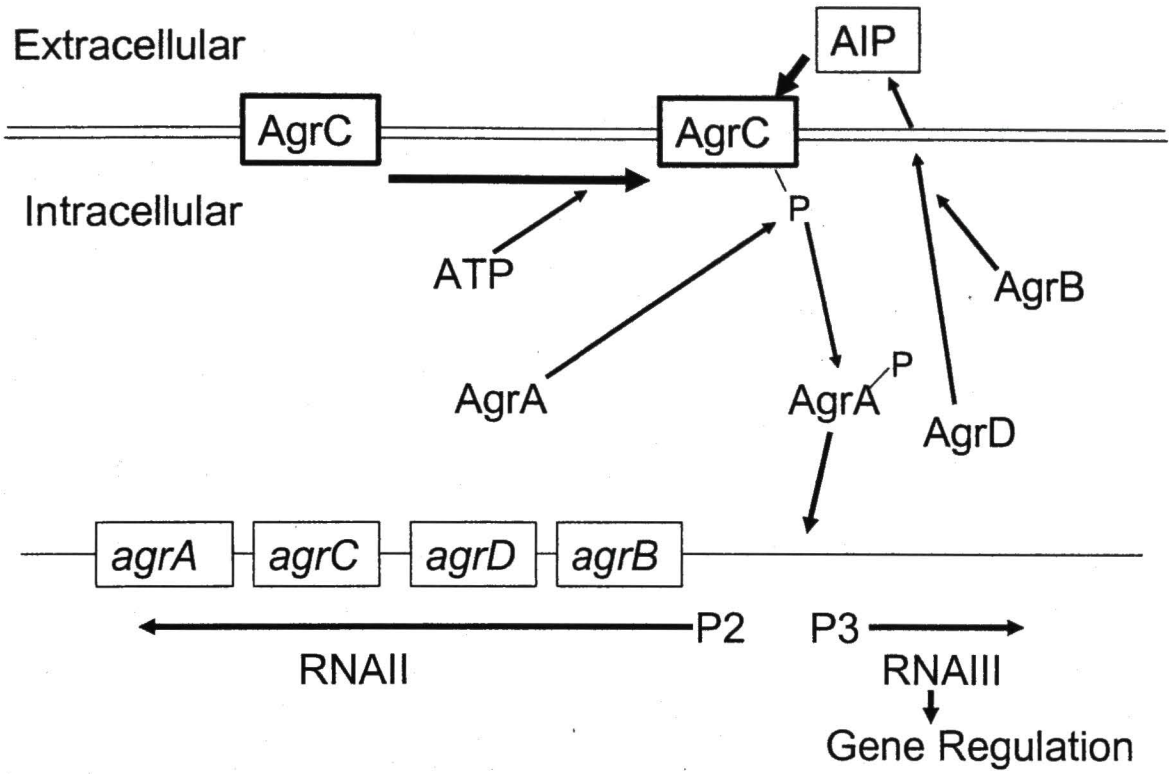


FIGURE 1. Model of *agr* regulatory locus in *S. aureus* (39). Two divergent transcripts are expressed, RNA II and RNAIII, from separate promoters. Products encoded by RNAII make up the quorum sensing component of *agr*. RNAIII is the effector molecule that regulates the expression of many genes in *S. aureus*. AIP is the Auto-Inducing Peptide synthesized by AgrD and AgrB.

Theoretically, the transmembrane sensor component, AgrC, becomes activated by phosphorylation through interactions with the octapeptide (56). The activated AgrC then interacts with the cytosolic activator component, AgrA, resulting in its phosphorylation and eventual activation (56). Subsequent transcriptional enhancement of the *agr* operon occurs through a proposed interaction of activated AgrA and another molecule, SarA, with the *agr* promoter region (21, 24, 54; Figure 2). The other transcript of the *agr* operon, RNAIII, is the major effector molecule of this regulatory locus, which interacts with targeted genes to modulate their transcription and possibly their translation as well (57). As the message levels of the RNAIII transcript increase, the expression of several secreted virulence factors are up-regulated in conjunction with a down-regulation of many cell-surface associated factors (54, 57, 64). This phenotype is observed when in vitro growth reaches the post-exponential phase, where cell density is high and overall nutrient availability is limited (64). With this in mind, a simple correlation can be made between the virulence factors produced during different phases of in vitro growth and their proposed roles throughout the course of an infection (63). Virulence factor expression by *S. aureus* appears to be influenced by the *agr* locus. However, the significance of *agr* in determining virulence factor expression may be dependent on its interactions with other regulatory loci like *sar* (5).

Cheung *et al.* (1992) described a *S. aureus* transposon mutation that phenotypically had reduced production of extracellular and cell-surface associated virulence factors. An *agr*-independent virulence regulatory locus, *sar*, was identified by cloning and sequencing the chromosomal region where transposition had occurred (21). This locus

generates three overlapping transcripts, each containing an open reading frame that encodes for the effector molecule of the *sar* locus, SarA (21, 47; Figure 2). Transcription initiation can occur at one of three distinct promoters located within this regulatory locus (7, 21, 47). The *sarA* P1 and P2 promoters, identified as being highly active during exponential phase, are SigA dependent, which is the primary sigma factor required for the transcription of several housekeeping genes in *S. aureus* (21, 47). The P3 promoter, known to be active primarily during the post-exponential phase, is SigB dependent, which is a stress factor that is responsive to various environmental pressures encountered by *S. aureus* (26, 41, 42). Protein-DNA binding analysis revealed that SarA binds to an area within the P1-P2 promoter region of the *agr* operon (22, 23). As a result of this interaction, transcription of the *agr* operon is enhanced, thereby increasing the amount and consequently the effects of RNAIII within the cell. In addition to influencing virulence factor expression by interacting with the *agr* locus, *sar* has been shown to independently alter the expression of virulence factors in *S. aureus* (11, 44; Figure 2). For example, SarA represses the expression of extracellular proteases regardless of cellular RNAIII levels (44). The potency of this repression is evident by the fact that *sarA* mutants overly express extracellular proteases during in vitro growth. Therefore, the *sar* locus, like the *agr* locus, appears to influence virulence expression in *S. aureus*. However, the expression of many virulence factors appears to be dependent on other regulatory elements outside of or in addition to *agr* and *sar* (5).

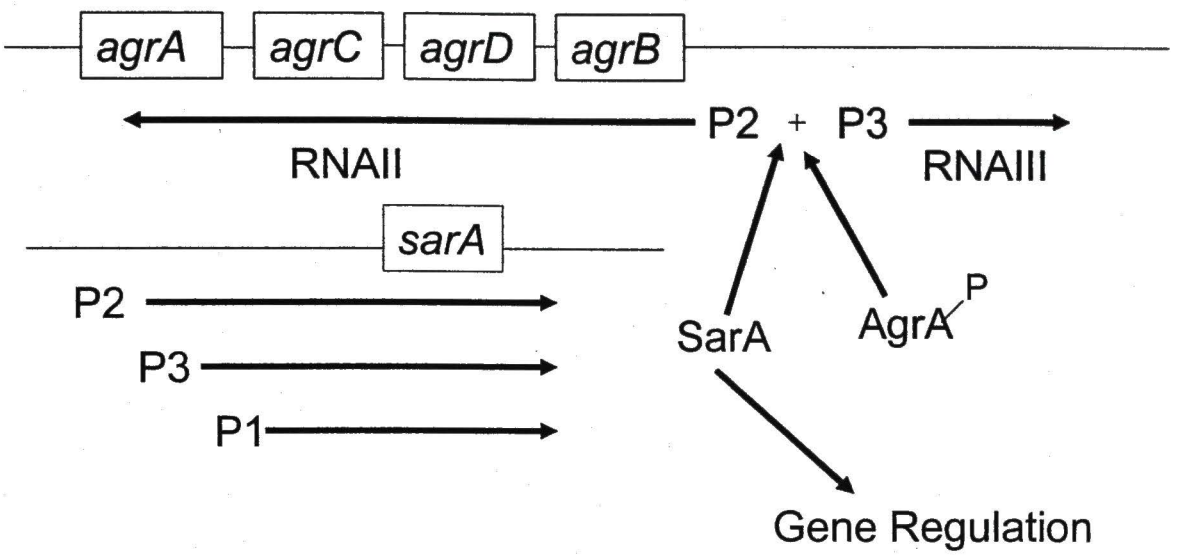


FIGURE 2. Model of *agr* and *sar* regulatory loci in *S. aureus* (21, 39). Three overlapping transcripts are expressed from the *sar* locus. Each of these transcripts contains an open-reading-frame for the effector molecule of *sar*, SarA. The interaction of SarA with active AgrA at the *agr* promoter region results in enhanced (+) transcription of RNAII and RNAIII. Besides influencing gene expression through its interactions with *agr*, SarA can independently regulate the expression of genes in *S. aureus*.

Current evidence suggests that virulence factor expression is not solely directed by the *agr* and *sar* loci (20, 22, 78). In fact genetic studies of the interaction between SarA and the *agr* locus have shown that when a *sarA* mutant is complemented in *trans*, only partial restoration of the wild type message levels for RNAIII occurred (22). This suggests that additional, unidentified factors are involved in the expression of RNAIII, and subsequently, the activation and repression of various virulence factors in *S. aureus*. Another study revealed that mutations in both the *sar* and *agr* loci reduced *S. aureus* virulence in an animal model; however, those mutants were not rendered completely avirulent (20). Additionally, the development of staphylococcal toxic shock syndrome (TSS) in a subcutaneous rabbit model was not dependent on the activation of *agr* (78). These studies suggest that other regulatory elements produced by *S. aureus* are influencing virulence factor expression within in vivo and probably in vitro environments.

Other regulators determining virulence factor expression have been identified within *S. aureus* (5, 29, 30, 48, 51, 68, 72, 79). However, several of these regulators have unresolved questions concerning their expression and/or their influence on both virulence factors and other regulatory elements, which suggests that additional regulatory loci remain unidentified within *S. aureus*. In an effort to identified additional regulators of virulence factor expression, transposon mutagenesis was used to generate *agr*-suppressor mutants in the *agr*-null background of *S. aureus* RN6911. The in vitro phenotype for this strain is low hemolysin and protease activities combined with a high lipase activity. Changes to this phenotype as a result of transposition would suggest that a mutation

might have occurred within a regulatory gene. As a result of this investigation, a novel interaction between alpha-toxin (hemolysin) and a serine protease was uncovered.

CHAPTER II

SUPPRESSOR-MUTANT LIBRARY

Introduction

The importance of *agr* and *sar* in determining virulence factor expression in *Staphylococcus aureus* is clear (5). However, their overall impact on virulence remains unclear (20, 78). In addition to *agr* and *sar*, other potential regulators have been identified within the genome of *S. aureus* (5, 29, 30, 48, 51, 68, 72, 79). A study conducted by Cheung *et al.* (1995) involved isolating a *S. aureus* transposon mutant that phenotypically had both an increase in alpha-toxin levels and a decrease in protein A levels. The site of disruption was also found to be distinct from *agr* and *sar*. Several two-component systems have been identified as regulators of virulence expression within *S. aureus* (29, 30, 79). The *sae* locus, for *S. aureus* exoprotein expression, is a regulon that encodes a putative two-component signal transduction system, which stimulates the production of alpha-toxin, beta-hemolysin, and coagulase independent of both *agr* and *sar* (30). Another two-component regulatory system, *srrAB* (for staphylococcal respiratory response), was identified by Yarwood *et al.* (2001), which has the ability to regulate the production of both extracellular and cell-surface associated virulence factors in response to environmental oxygen levels. The two-component system of ArlS-ArlR

interacts with components of *agr* and *sar* to regulate the expression of protein A, alpha-toxin, and V8 protease within *S. aureus* (29).

Several SarA homologues have been identified within *S. aureus* (5, 48, 51, 68, 72). Tegmark *et al.* (2000) identified a SarA homologue, SarH1 or SarS, that acts downstream of *sar* and *agr* to activate the transcription of *spa*, which encodes for protein A. Additionally, this same group identified two more SarA homologues, known as SarU and SarV, by searching for percent identities within *S. aureus* databases maintained at The Institute for Genomic Research (http://www.tigr.org) and the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html) (5). The regulatory locus *rot*, for repressor of toxins, was identified by McNamara *et al.* (2000) as a repressor for alpha-toxin expression within *S. aureus*. Rot, another SarA homologue, was shown to have a global regulatory effect on the expression of several *S. aureus* virulence factors (66). Another SarA homologue, SarR, was shown to repress SarA expression during the post-exponential phase of in vitro growth (48). Moreover, Schmidt *et al.* (2001) identified a homologue of SarA, SarT, which was shown to be a repressor of alpha-toxin synthesis, and that SarA repressed *sarT* expression. Therefore, many of the SarA homologues appear to effect virulence gene expression within *S. aureus* by cross-talking with one another.

The combined effects of the newly defined regulators, along with the influences of *agr* and *sar*, suggests that a complex regulatory network controls virulence gene expression in *S. aureus*. Many questions regarding the expression and/or influence on both virulence factors and other regulatory elements remain unanswered for all of the

recently defined regulators (5, 29, 30, 48, 51, 68, 72, 79). This incomplete picture suggests that additional regulatory loci remain unidentified within *S. aureus*. The following study describes an effort to identify additional *S. aureus* virulence factor regulators by using transposon mutagenesis to generate *agr* suppressor-mutations in an *agr*-null or –mutant strain. Out of 4×10^4 erythromycin resistant colonies screened for phenotypic variation from the enzymatic-deficient phenotype of RN6911, only fifty-four suppressor mutant colonies were isolated with detectable changes in hemolysin, protease, and lipase activities. Southern analysis revealed that only twenty-six suppressor mutants contain the transposon within their chromosomes. Transpositional insertion sites were identified for seven of the mutants by sequencing arbitrary-PCR products generated from chromosomal DNA. Sequence analysis for one of the seven revealed that the transposon, Tn*551*, disrupted the open reading frame of the *s*erine *p*rotease-*l*ike *F* (*splF*) gene. This finding resulted in additional work being done with the *splF* mutation.

Materials and Methods

Transposon mutagenesis and phenotypic screening. A list of the strains and plasmids used in this study are found in table 1. The following summary of transposon mutagenesis in *S. aureus* was adapted and modified from a protocol obtained from Laura (Hruska) McDowell at Microcide Pharmaceuticals, Inc. The temperature sensitive plasmid pI258*repA36* (kindly provided by Laura Hruska-McDowell), which harbors the staphylococcal transposon Tn*551*, was transformed into electrocompetent *S. aureus* RN6911 (Δ*agr*::*tmn*) by electroporation as recommended by the manufacturer (Bio-Rad

TABLE 1. Strains and plasmids used in suppressor library.

Strain or plasmid	Relevant characteristic(s)	Origin or reference
S. aureus		
RN6390	8325-4; Prototypic strain	M.S. Smeltzer, University of Arkansas for Med. Sci.
RN6911	8325-4 Δ*agr*::*tmn*	M.S. Smeltzer, University of Arkansas for Med. Sci.
Plasmids		
pI258*repA36*	Temperature sensitive vector; contains Tn*551*	Laura McDowell, Microcide Pharmaceuticals, Inc.

Laboratories, Richmond, Ca.). A negative control was subjected to the same conditions except that no plasmid was added to electrocompetent cells. After pulsing (2.5 kV, 100 ohms, 25 μF) the cells, they were suspended in 1 ml of nutrient rich broth (SMMP) and incubated with high rotary aeration (200 rpm) for one hour at 37°C. Volumes of 0.1 ml were spread on tryptic soy agar (TSA; Difco Laboratories, Detroit Mich.) containing penicillin G (15 μg/ml; Sigma Chemical Co., St. Louis, Mo.). After incubating overnight (18 h) at 30°C, penicillin G-resistant colonies were streaked to additional TSA-penicillin G media and incubated overnight (18 h) at 30°C. Plate growth was transferred and suspended in 1 ml of tryptic soy broth (TSB; Difco Laboratories). Those suspensions were serially diluted ten-fold in 1 ml of TSB, and 0.1 ml of each dilution was spread on TSA containing both 2% (v/v) defibrinated rabbit erythrocytes (Remel, Inc., Lenexa, Ks.) and erythromycin (10 μg/ml; Sigma Chemical Co.). A total of two sets with ten plates each were completed. One set was incubated overnight at the permissive temperature of 30°C, while the other set was incubated at the non-permissive temperature of 43°C to promote the movement of the transposon into chromosomal DNA. Colony forming units (cfu) were determined from statistically countable plates (30-300 colonies/plate). Transposition frequencies were calculated, and the dilution yielding approximately 300 cfu/plate after incubation at 43°C was determined. The original suspension was diluted in the volume of TSB that yielded the desired 300 cfu/plate, and the total volume of that dilution was spread in 0.1-ml volumes onto at least twenty individual TSA plates containing 2% rabbit erythrocytes and erythromycin (10 μg/ml). After overnight (24 h) incubation at 43°C, erythromycin resistant (Em^r) colonies were screened for the lysis of

rabbit erythrocytes, which is indicated by clear zones surrounding bacterial growth. Hemolytic positive colonies were numbered and streaked to the same antibiotic medium for additional growth and verification of hemolysin activity. Those colonies were additionally streaked to TSA containing 5% (w/v) skim milk (Difco) and to TSA containing penicillin G (15 μg/ml). Plates were incubated at 37°C for 18 hours. Penicillin G sensitive (Pc^s) colonies suggested that transposition had occurred and that the plasmid had been lost. Pc^s-Em^r colonies displaying hemolysin and/or protease activities were spread on TSA containing erythromycin (10 μg/ml) and incubated overnight (18 h) at 37°C. The plate growth was harvested into brain-heart infusion broth (BHI; Difco) containing 25% glycerol, and then stored at -85°C for additional studies.

Preparation of spent media. Cultures for all *S. aureus* strains (RN6911 and RN6390) and transposon mutants were incubated for 18 hours with rotary aeration (180 rpm) at 37°C. Cultures (10 ml) were standardized using optical density (absorbance) at 550 nm. Standardization was done in TSB to a total volume of 5 ml. The standardized cultures were poured into sterile, 25-ml glass tubes (Corex), and centrifuged (4°C) for 15 minutes at 8,000 × *g* (J-21C Centrifuge and JA-20 rotor; Beckman Coulter, Inc., Fullerton, CA). Cell-free spent media were syringe-filtered through 0.22 μm filters into sterile, 1.6-microcentrifuge tubes (Fisher Scientific, Fisher Scientific, Fairlawn, N.J.) placed in ice. Spent media were stored at -20°C in 1 ml aliquots.

Hemolysis Assay. The microtiter hemolysis assay used in this study was adapted from previously described protocols (59, 74). Thawed spent media were serially diluted two-fold in 1 × phosphate buffer saline (PBS) to a total volume of 225 μl. A 10% (v/v) rabbit

erythrocyte suspension, washed in 1 × PBS, was added (25 µl) to each 0.6 microcentrifuge (Fisher) tube containing dilutions of the spent media. The reaction mixtures were incubated at 37°C for 25 minutes. Each 0.6 tube was cold-centrifuged (4°C) for 10 minutes at 1000 × *g* (854 rotor; Centra MP4R, IEC, Needham Heights, Mass.) to pellet cellular debris in the reaction mixture. Debris-free supernatant was loaded (180 µl) into individual wells of a 96-well plate. These plates were read at 490 nm in a microplate reader, and units of activity were assigned as the reciprocal of the lowest dilution yielding 50% lysis. The 50% lysis points were calculated by interpolating data from standard curves generated from 0.2 ml suspensions containing a volume percentage (0-100% v/v) of a 1% hemoglobin suspension and a 1% erythrocyte suspension. The 1% hemoglobin suspension was obtained by adding 1ml of a 10% erythrocyte suspension to 8 ml of sterile distilled water, shaking the suspension to promote cellular lysis, and adding 1ml of (10 ×) phosphate buffered saline. The 0.6 microcentrifuge tubes containing the standards were centrifuged (4°C) for 10 minutes at 1000 × *g* (854 rotor; Centra MP4R, IEC), and 180 µl was pipetted into individual wells on a 96-well plate. Standard curves were generated with GraphPad Prism software (GraphPad Software, Inc., San Diego, Ca.), and 50% lysis points were determined from those values. Hemolytic assays were repeated in duplicate for each spent media.

Protease activity. Agar plugs were punched out of TSA containing 5% (w/v) skim milk (Difco). Wells were loaded (30 µl) with standardized spent media from the transposon mutants and strains used in this study. The plates were incubated at 37°C for 24 hours. Protease activity was determined by measuring the diameters of cleared zones minus the

diameter of the punch (5 mm). This protease activity assay was repeated in triplicate for each mutant and strain, and a protease activity range was determined from the measured diameters.

Lipase activity. To verify differences in lipase activity, standardized spent media from the strains and mutants were added (30 µl) to wells punched in 0.5% (v/v) tributyrin (Sigma)-agarose (1.0%; BioWhittaker Molecular Applications, Rockland, Maine) plates. Tributyrin was emulsified in 25 mM (w/v) $CaCl_2$ with a tip sonicator for 10 minutes at 50% output. These plates were incubated for 24 hours at room temperature. Lipase activity was determined by measuring the diameters of cleared zones minus the diameter of the punch (5 mm). This lipase activity assay was repeated in triplicate for each mutant and strain, and a lipase activity range was determined from the measured diameters.

CTAB (CetylTrimethylAmmonium Bromide) extraction of chromosomal DNA. The following is a description of the CTAB method that was used to isolate and purify chromosomal DNA from the transposon mutants, RN6911, and RN6390. A total of 1.5 ml of each overnight culture was pelleted by centrifugation for 10 minutes at 8,000 × *g* (854 rotor; Centra MP4R, IEC). After suspension in TE (Tris-EDTA, pH 8.0), lysostaphin (final concentration 5 µg/ml; Applen and Barrett LTD., England) and RNase A (5µg/ml; Fisher) were added to the suspension, followed by an incubation at 37°C for 1 hour. 10% SDS (Fisher), Proteinase K (10 µg/ml; Fisher), and RNase A (5 µg/ml) were added to the reaction, which was incubated at 37°C for 1 hour. Molecular grade NaCl (5 M; Fisher) was added to the reaction tube, followed by vigorously shaking the tube for 15 seconds. CTAB (2 ×; Aldrich Chemical Company, Milwaukee, Wi.) was added to the

tube and shaken for an additional 15 seconds. Each tube was then incubated for 20 minutes at 65°C, followed by addition of TE (pH 8.0; 1:1 volume ratio) and centrifugation at 16,000 × *g* for 5 minutes (854 rotor; Centra MP4R, IEC). The aqueous phase was pipetted to a sterile, 1.5 microcentrifuge tube (Fisher). Phenol:chloroform (1:1; Fisher) were added to the phase at a volume ratio of 1:1. The mixture was shaken and centrifuged as before. Phenol:chloroform were again added to isolated aqueous phase and centrifuged again. This process was consecutively repeated until a clear interface was obtained. The aqueous phase was washed twice in excess chloroform (2 ×'s the aqueous phase volume), shaken, and centrifuged as before. Room temperature isopropanol was added to the aqueous phase to precipitate the DNA out of solution. After centrifugation, the pellet was washed in 70% ethanol, re-pelleted, and allowed to dry at room temperature for at least 5 hours. Each pellet was suspended in either 50 µl of TE buffer or nuclease-free deionized, distilled water.

Restriction digestion and gel electrophoresis. Freshly isolated chromosomal DNA was diluted 1:100 in sterile deionized, distilled water and added (5 µl) to a sterile, 0.6 microcentrifuge tube (Fisher). *Eco*RI and H buffer (10 ×) were added to a total 50 µl reaction volume as recommended by the manufacturer (Promega Corp., Madison, Wi.). Each tube was incubated for 1 hour at 37°C. The entire restriction volume (50 µl) was combined with 6 × tracking dye and loaded onto a 0.8% LE agarose gel (BioWhittaker Molecular Applications). The DNA was resolved for 1.5 hours at 65 V/cm^2. Gels were stained in ethidium bromide (5 µg/ml) for 20 minutes and destained in deionized, distilled water for 20 minutes with gentle agitation. Gels were UV illuminated, and

images were captured using Alpha Imaging Software (Alpha Imaging Technologies, Inc., Lowell, Ma.).

Southern analysis. The following is a summary of the Southern analysis method adapted and modified from protocols originally described by the membrane (MagnaGraph; Micron Separations, Inc., Westborough, Mass.) and the chemiluminescence manufacturers (Roche Molecular Biochemicals, Indianapolis, Ind.). After the DNA fragments were passively transferred in 10 × SSC (sodium chloride-sodium citrate) to a neutral nylon membrane (Micron Separations) from the 0.8% LE agarose gel, they were UV fixed (UV Chamber) and completely denatured in an alkaline solution (5 mM NaOH). The membrane was hybridized overnight (16-24 h) at 65°C (Hybaid Oven; Thermo Hybaid, Ashford, Middlesex, UK) to a Tn*551* specific probe randomly labeled with digoxigenin-11-UTP (Roche Molecular Biochemicals) and the Klenow fragment of DNA polymerase (Promega). Membranes were subsequently washed in SSC solutions with varying concentrations (0.1 × to 2 ×) at both room temperature and 65°C. The digoxigenin-labeled Tn*551* probe hybridized to membrane-fixed DNA was detected with an alkaline phosphate-conjugated anti-digoxygenin $F(ab')_2$ antibody fragments (Roche Molecular Biochemicals). After subsequent 5 minute washes with blocking, washing, and assay buffers, each membrane was placed into seal-a-meal bags. Bands of hybridization were detected by autoradiography once the chemiluminescent substrate CDP-*Star* (Roche Molecular Biochemicals) was added. Molecular weights (kb) of the bands were estimated by comparing the ethidium bromide stained gel picture to the film.

Arbitrary PCR and sequence identification. The DNA sequence flanking Tn*551* was determined using an arbitrary PCR method (14). In this method, flanking chromosomal DNA is enriched through two subsequent rounds of PCR amplification using primers specific for one end of Tn*551* and primers to random sequence, which can anneal to chromosomal DNA adjacent to the transposon. Arbitrary PCR was performed in 50 µl volumes containing 200 nM of each primer and a *Taq/Pyroccoccus species* GB-D DNA polymerase mixture from the Elongase Enzyme Mix kit (Invitrogen Corp., Carlsbad, Ca.). In the first round (30 cycles), a primer 260 nucleotides from the left end of Tn*551* (Tn1, 5'-GGGTTAACCATACGCAAGACCAAT-3') and a primer with 3' random sequence (ARB1, 5'-GGCCACGCGTCGACTAGTACNNN-NNNNNNNGATAT-3') were used to amplify random PCR fragments from transposon mutants under low stringent conditions (annealing temperature 45°C). The second round (35 cycles) utilized the PCR products from the first round as the DNA template for amplification under higher stringency conditions (annealing temperature 53°C). A primer approximately 70 nucleotides from the left end of Tn*551* (INSI, 5'-GAGAGATGTCACCGTCAAGTT-AAATCTA-3') and a primer specific to the 5' tag of ARB1 (ARB2, 5'-GGCCACGCGT-CGACTAGTAC-3') were used to amplify specific fragments in the second round of arbitrary PCR. PCR products from the second round were resolved in a 1.0% GTG agarose gel (BioWhittaker Molecular Applications), and detectable bands after ethidium bromide staining were gel purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, Ca.). Arbitrary-PCR products were sequenced with the INSI primer at the University of Arkansas for Medical Sciences

DNA Sequencing Core Facility (Little Rock, Ark.) using a DNA sequencer (Perkin-Elmer Biosystems, Foster City, Ca.). The unknown chromosomal sequences flanking the transposon's sequence were BLASTN searched for identity at *S. aureus* DNA databases maintained at The Institute for Genomic Research (http://www.tigr.org) and the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html). DNAMAN software (Lynnon Corp., Quebec, Canada) was used to generate a conceptual translation of putative open reading frames (orfs) disrupted by transposition. The amino acid sequences for disrupted orfs were BLASTP searched for similarity at the NCBI database (http://www.ncbi.nlm.nih.gov/).

Results

Transposon mutagenesis and phenotypic analyses. A total of fifty-four *agr*-suppressor mutants positive for increased hemolysin activities were isolated out of approximately 4 $\times 10^4$ transposon mutant colonies. Transposition frequency was estimated to be 0.001 with all of the transposon mutation experiments. The fifty-four *agr*-suppressor mutants were Em^r-Pc^s and assigned a colony number (Table 2).

Units of activity for spent media hemolysing rabbit erythrocytes ranged from two to sixty-four for the fifty-four suppressor-mutants, which was well above the hemolytic ability of RN6911 (≤ 0.5). Lipase activity was as high or higher for the spent media of RN6911 than it was for several of the suppressor mutants. Those with corresponding lipase activities to RN6911 displayed little to no protease activities. Several of the

TABLE 2. Phenotypic summary of *S. aureus* strains and suppressor mutants

Strain or Mutant #[a]	Hemolytic Titer[b]	[c]Zones on 5% Skim Milk	[c]Zones on 0.5% Tributyrin
S. aureus RN6390	125±4.3	+	+++
S. aureus RN6911	<0.5	-	+++
17	7.5±0.8	++	+
20	16.8±2.0	-	+++
22	16.2±1.8	-	+++
24	8.4±0.8	-	+++
38	17.2±2.0	-	+++
54	17.0±1.2	+	++
61	4.8 ± 0.4	+	++
64	7.5±0.8	-	+++
82	14±2.0	-	+++
108	5.3±1.0	+	++
109	15.2±1.6	+	++
110	4.8 ± 0.4	-	+++
111	8.4±1.0	+	++
155	17.2±2.0	++	++
171	16.3±2.1	++	+
175	15.5±1.0	++	+
201	3.0±0.1	-	+++
233	7.5±0.8	-	+++
238	4.5±0.6	+	++
248	14±2.0	-	+++
291	15.5±1.2	-	+++
294	7.5±0.8	-	+++
350	16.3±2.1	+	++
363	16.2±1.8	-	+++
371	17.2±2.0	-	+++
568	8.4±0.8	-	+++
21	16.8±2.0	++	++
23	16.2±1.8	++	+
25	15.9±1.8	+++	++
26	16.8±2.0	++	+
27	16±2.0	+++	+
28	3.8±0.5	++	+

Table 2 continued			
29	7.8±0.8	+	+
30	7.5±0.8	++	+
31	17.2±2.0	+	++
32	15.2±1.0	++	+
33	14±2.0	++	-
34	16.3±2.1	+++	+
35	16.3±2.1	+	++
36	15.2±1.0	++	+++
37	17.0±1.2	+	++
42	63.4±4.2	+++	-
43	7.5±0.8	+++	+
49	2.5±0.1	-	+++
50	15.0±1.0	++	++
51	8.4±1.0	++	++
52	15.2±1.4	+	++
53	7.5±0.8	++	-
65	15.5±1.0	++	+
83	8.3±1.0	+	++
84	2.3±0.5	++	++
100	8.4±1.1	+	++
120	16.3±2.1	++	+
121	16±1.4	++	+

[a] Mutant strains positively identified with intact Tn*551*by Southern analysis are bolded. Strains lacking intact Tn*551* are not bolded.

[b] Units of activity are expressed as the reciprocals of the highest dilutions of the original culture supernatants yielding a minimum of 50% hemolysis of rabbit erythrocytes.

[c]Assayed by measuring the zones of hydrolysis on 5% skim milk and 0.5% tributyrin agar plates. -, no activity; +, weak; ++ moderate; +++, strong.

mutants were highly protease positive as compared to the *agr* wild-type background of RN6390 (Table 2).

Southern analysis. Southern analysis for Tn*551* revealed that only twenty-six *agr*- suppressor mutants contained an intact transposon within their chromosomes (Table 2; Fig. 3). Figure 3 is an example of the results with Southern analyses. In this image, only one positive band of hybridization was detected in lane six containing chromosomal DNA from mutant colony 110. The other lanes, besides the lane with the positive control (lane 1), contained chromosomal DNA from other mutants that apparently did not contain an intact transposon. These results were verified in two separate experiments that utilized chromosomal DNA isolated from different cultures of each mutant. Those that did not contain the transposon within their chromosomes were erythromycin resistant (encoded by Tn*551*) and exhibited some of the highest units of activity for hemolysis (Table 2).

Arbitrary PCR and sequence analysis. The sequences of seven arbitrary PCR products were analyzed for identities within the *S. aureus* chromosomal sequences maintained at The Institute for Genomic Research (http://www.tigr.org) and the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html). These products contained chromosomal DNA sequence flanked by sequences for primer ARB-2 and Tn*551* (Fig. 4). The identities for the seven chromosomal sequences were determined to be as follows: mutant #17, conserved hypothetical protein at the chromosomal position of 1226.49 to 1229.091 kb; mutant #110, serine protease-like F at the chromosomal position of 1842.685 to 1842.826 kb; mutant #171 cytochrome C oxidase assembly protein at the chromosomal position of

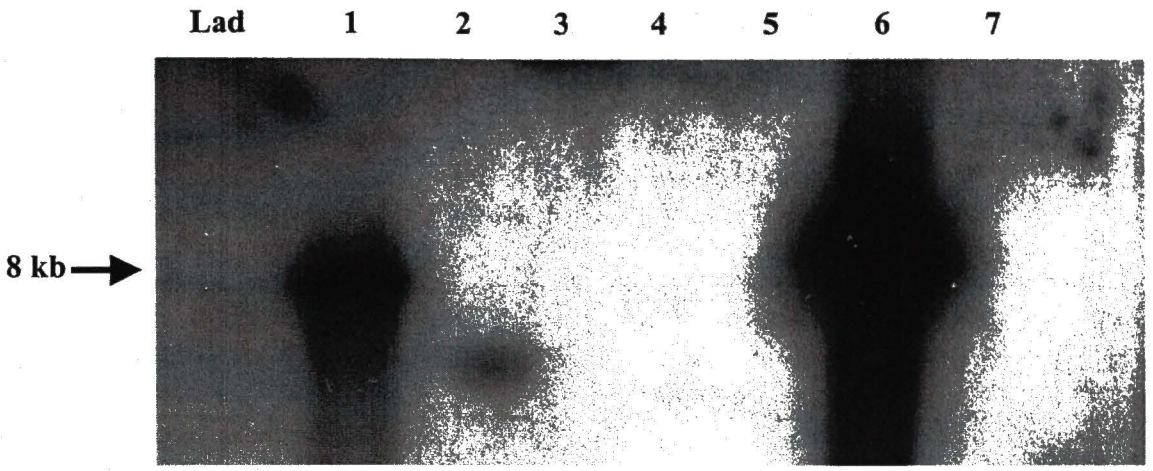


FIGURE 1. Southern analysis of *S. aureus* chromosomal DNA hybridized to Tn*551* specific probe. Lane 1, 171; Lane 2, 51; Lane 3, 52; Lane 4, 53; Lane 5, 65; Lane 6, 110; Lane 7, 121. Approximate molecular weights of *Eco*RI chromosomal fragments containing Tn*551*. Hybridization was detected by autoradiography utilizing chemiluminescence chemistry.

5'-GAAATTTTTAAATCTATTTCATTATCGATACAAATTTCTCGTAGGCG
CTCGGGACCCCCATTAGGATTTGGATAACCAATGACTGATATAGGTTC
ATTTTCTTTAGCTTCTGATGCTATATTAAATTTACTAGTGAAATCTTTG
AATTTTCTACCTTTTGGTTGTGTTGATTTTTCTTCAACTTGTACAACCG
CAATATCCCCCCCCCCCCGTACTAGTCGACGCGTGGCC-3'

FIGURE 2. Sequence of arbitrary PCR product generated from transposon mutant 110. Chromosomal sequence is underlined. Upstream sequence Tn*551*; downstream ARB-1 primer sequence.

1096 to 1097 kb; mutant #291, non-encoding sequence at the chromosomal position of 1941 to 1942 kb; mutant #350, conserved hypothetical protein at the chromosomal position of 1744.143 to 1745.084 kb; mutant #371, dihydropteroate synthase chain A synthetase at the chromosomal position of 545.390 to 546.193 kb; mutant #568, tryptophan synthase β chain protein at the chromosomal position of 768.430 to 769.205 kb (Table 3). The chromosomal kb positions are based on the sequence data for *S. aureus* 8325-4 maintained at the University of Oklahoma's Advanced Center for Genome Technology.

Discussion

This study describes the results from generating an *agr*-suppressor mutation in an *agr*-null strain (RN6911) of *S. aureus*. Suspected mutants were screened for variations from the enzymatic deficient phenotype of RN6911 by assaying for hemolysin, protease, and lipase activities. A total of fifty-four suppressor mutants were identified with various enzymatic phenotypes. Out of those fifty-four mutants, only twenty-six positively contained an intact transposon within their chromosomes as verified by Southern analysis. Transposition sites for seven of the twenty-six were determined from sequences of arbitrary PCR products generated from their chromosomal DNA.

The effects of *agr* on extracellular virulence factor expression in *S. aureus* have been well documented (57, 64). As *S. aureus* cultures reach the late-exponential phase of in vitro growth, the *agr* phenotype occurs, which is characterized by an increase in secreted virulence factors and a decrease in cell-surface associated virulence factors (57, 64). The prototypic lab strain, RN6390, has been used extensively in mutational studies to

TABLE 3. Insertional sites of Tn*551*.

Colony Number	Mutation Location	Gene Function
17	Conserved Hypothetical Protein	Unknown
110	Serine protease-like F (SplF)	Serine Protease
171	Cytochrome C Oxidase Assembly Protein	Assembly of Cytochrome C Oxidase
291	Non-encoding region of chromosome	Unknown
350	Conserved Hypothetical Protein	Unknown
371	Dihydropteroate Synthase Chain A Synthetase	Synthesis of Dihydropteroate Synthase Chain A
568	Tryptophan Synthase β Chain	Component of Typtophan Synthase

investigate *S. aureus* pathogenecity (64). As a result, several isogenic mutants of RN6390 have been generated that are useful in studying virulence factor expression. Strain RN6911 is an *agr* mutant with an in vitro phenotype opposite of RN6390 (64). Transposable elements have been used in RN6911 to generate mutations that have lead to the identification of other regulatory loci in *S. aureus* (21, 51). Tn*551* is a staphylococcal transposon used to generate mutations within the genomes of staphylococci (77). Using Tn*551* in this study resulted in genetic anomalies occurring within RN6911 that exhibited both antibiotic and enzymatic phenotypes associated with transposition. Additional studies using transposon mutagenesis with similar results have not been evident. Therefore, it is difficult to ascertain why such anomalies would occur.

The twenty-six mutants that did contain intact transposons in their chromosomes exhibited altered phenotypes compared to RN6911. Mutants exhibiting high protease levels have reduced lipase activities, while other mutants had high lipase activities with low or null protease levels. These results suggest that protease levels determine lipase activities in spent media of *S. aureus*. Kawano et al. (2001) demonstrated that extracellular proteases produced by *S. aureus* proteolytically degrade lipase proteins in spent media (38). Therefore, the lipase and protease activity phenotypes of RN6390, RN6911 and several transposon mutants suggest that the level of proteolysis corresponding to protease expression determines total lipase activity. This type of activity is intriguing given the fact that one of the identified sites of disruption was in a serine protease gene (65; *splF*, for *s*erine *p*rotease-*l*ike *F*). This mutant (# 110) exhibited a hemolytic titer of about five (Table 1), which suggests that the product of this gene may

be regulating hemolysin activity in *S. aureus*. The effects of this mutation were further investigated in additional studies.

The possible effects on virulence expression were not as evident for the other identified sites of disruption. The conserved hypothetical proteins did not show extensive similarities to other regulatory elements found in *S. aureus* or in other bacterial species. However, expression of hypothetical proteins has been observed during infections by in vivo expression technology (IVET; 45). Therefore, it is possible that these proteins are affecting virulence factor expression in *S. aureus* by an unknown function. Other sites of disruption include a cytochrome C oxidase assembly protein, a tryptophan synthase β chain protein, and dihydropteroate synthase chain A synthetase. These are all essential genes and are not considered virulence factors or regulators of virulence in *S. aureus* (63). It is possible that mutations within these genes induced a stress-response that altered the phenotype of RN6911. For example, tryptophan analogues have been shown to influence the synthesis of beta-hemolysin in *S. aureus* (34). However, the effects of these essential genes on *S. aureus* virulence expression remain unclear.

This study revealed a possible link between hemolysin activity and protease production. More specifically, a possible link between alpha-toxin activity and the product of the *splF* gene may have been discovered. This is based on the fact that rabbit erythrocytes are very sensitive to alpha-toxin activity (9). The link will be further investigated to establish the type of interaction that may be occurring between those two virulence factors.

CHAPTER III

EFFECTS OF *splF*::Tn*551* ON ALPHA-TOXIN ACTIVITY

Introduction

S. aureus has the ability to produce more than 40 different extracellular toxins, enzymes and cell surface-associated proteins (5). This armament of diverse virulence factors is believed to contribute to the overall pathogenicity of this bacterium, which probably accounts for the range of disease states associated with *S. aureus* infections (35, 46). *S. aureus* virulence factors can be classified according to their activities into one of the four following categories: those that aid in attachment, those that help evade the host immune system, those that promote tissue damage and penetration, and those that are immunomodulatory (5, 63). A specific group of toxins that are classified as tissue damaging are the hemolysins (5, 63). These cytotoxins attack target cells by disrupting their membrane permeability through pore formation, bilayer insertion and translocation, or lipase activity (2, 62). If the host cell cannot recover from leakage caused by the action of these toxins, then cellular lysis will occur. The four currently identified hemolysins produced by *S. aureus* are alpha-toxin (hemolysin), beta-toxin (hemolysin), gamma-hemolysin, and delta-hemolysin (5).

Alpha-toxin is one of the most understood virulence factors produced by *S. aureus*, and does not belong to either of the two traditional protein subdivisions of being

soluble or membrane bound (10, 32, 36, 52). Instead, alpha-toxin produced by a bacterial cell is secreted into the extracellular environment and must travel to the membrane of a target cell (60). Initially, alpha-toxin is secreted as a hydrophilic monomer, with an approximate weight of 34 kDa (32). The monomers bind to unidentified site(s) on the target cell's membrane and begin to oligomerize. Apparently, oligomerization triggers membrane insertion of a hydrophobic domain by each monomer subunit, forming a transmembrane channel (52). Once the channel is formed, there is a rapid efflux of K^+ and influx of Na^+, Ca^+ and other small molecules. The osmotic pressure increases, and the cell eventually lyses (10, 73). Cellular type and organism source determines the sensitivity of cells to alpha-toxin lysis (8, 9, 10, 71, 75). For example, rabbit erythrocytes are 1000-fold more sensitive to alpha-toxin lysis than are human erythrocytes, and fibroblasts are less sensitive than erythrocytes. Additionally, many cell types are sensitive to alpha-toxin, which is thought to be significant in promoting disease associated with *S. aureus* infections (10, 40, 75). Tissue damage caused by alpha-toxin has indeed been linked to various diseases ranging from superficial skin lesions to more severe ailments (13, 33, 55, 58). Within various animal models, the alpha-toxin negative strain is always less virulent than its alpha-toxin producing isogenic parent, thereby demonstrating its importance in *S. aureus* infections and related diseases (13, 15, 55).

The multiple proteases produced by *S. aureus* are another group of virulence factors that contribute to the overall pathogenicity of this bacterium (3, 28). The four major extracellular proteases produced by *S. aureus* include the V8 protease (SspA), aureolysin (Aur), staphopain (Ssp) and a second cysteine protease (SspB) (5). Recently,

an additional serine protease operon known as *spl*, for *s*erine *p*rotease-*l*ike, was identified in *S. aureus* (65). The 5.5 kb polycistronic message of *spl* contains open-reading frames for six separate proteins designated as SplA, SplB, SplC, SplD, SplE, and SplF (65). All six protein sequences have similar identities to that of the V8 protease (SspA) and the exfoliative toxins A (ETA) and B (ETB) (65). More significantly, the catalytic triad conserved within the three known serine proteases of *S. aureus*, SspA, ETA, and ETB, is also conserved within the amino acid sequence of each Spl protein (12, 25, 65). Conservation of this sequence suggests that all six proteins are proteolytically active, which has already been confirmed for SplB and SplC (65). However, a significant effect on virulence was not observed after rats were intraperitoneally injected with an *spl* mutant strain (65). Therefore, how the *spl* operon contributes to *S. aureus* virulence remains undetermined.

The designation of specific virulence factor roles for extracellular proteases has not been apparent within the pathogenicity of *S. aureus*. Postulated roles have included destruction of tissue protein, protection from antimicrobial peptides like defensins, and cleavage of antibodies within the host (3, 31, 69). Evidence has also supported the idea that proteases may be modifying the expression of other virulence factors produced by *S. aureus* (37, 38, 44, 49). Additionally, protease production could mediate the transition from one cellular phenotype (adhesive) to another phenotype (non-adhesive) (37, 38, 49). With the exception of aureolysin, the expression level of extracellular proteases is highest during the stationary phase of in vitro growth (4, 5, 65). The levels of bacterial surface proteins are reduced in this growth phase, resulting in predominately a non-adhesive

phenotype (1, 5). Transitioning from an adhesive phenotype to a non-adhesive phenotype could be largely attributed to the degradation of surface adhesins by numerous proteases existing within the extracellular medium. This cell density-dependent expression of extracellular proteases by *S. aureus* is thought to be largely modulated through the actions of two staphylococcal global regulatory loci known as *agr*, for *a*ccessory *g*ene *r*egulator, and *sar*, for *s*taphylococcal *a*ccessory *r*egulator (21, 53, 61, 64). The effector molecule of *sar*, SarA, is a potent repressor to protease expression within *S. aureus*; while the expression of several proteases appears to be induced by RNAIII, the effector molecule of *agr* (5, 19). A proposed model for protease regulation in *S. aureus* suggests that RNAIII acts as an anti-repressor to SarA (5). In this model, protease expression levels are low during the early exponential growth phase, which is a result of high SarA levels as compared to the levels of RNAIII. Once a culture of *S. aureus* reaches the late-exponential to early-stationary growth phase, RNAIII levels increase and protease expression is induced due to RNAIII out-titrating SarA within the cell. Thus, cellular transitions from one phenotype to another could result from extracellular proteases extensively degrading other self-proteins produced by *S. aureus*. If this hypothesis is true, then the concerted effects of all the *S. aureus* proteases play a major role in the virulence states of this organism.

Secreted virulence factors like alpha-toxin and lipase have been shown to be sensitive to proteolysis by *S. aureus* extracellular proteases (38, 44). In the *sarA* mutant strain PC1839, Lindsay et al. (1999) demonstrated that the high protease levels produced by this strain led to the cleavage of alpha-toxin in vitro. This type of modification may be

necessary to regulate the effectiveness of alpha-toxin in *S. aureus* virulence, which is exemplified by the fact that strains overly expressing alpha-toxin demonstrated attenuated virulence in a rabbit model of experimental endocarditis (6). Furthermore, proteolytically nicked alpha-toxin is still hemolytically active, but appears to be unable to permeabilize nucleated cells (60). Taken all together, this data suggests that alpha-toxin activity, along with the activities of other virulence factors, may depend upon the extracellular proteases produced by *S. aureus*. This current study describes the results of an effort to identify additional staphylococcal factors involved in regulating the hemolytic abilities of *S. aureus*. A *s*erine *p*rotease-*l*ike *F* (*splF*) mutant strain, with an increased ability to lyse rabbit erythrocytes, was identified initially as a suppressor mutation generated by transposon mutagenesis in *S. aureus* RN6911 (Δ*agr*::*tmn*). The sensitivity of rabbit erythrocytes to alpha-toxin, along with no obvious differences in alpha-toxin message levels existing between the *splF* mutant and RN6911, suggests that the SplF protein may be proteolytically modifying alpha-toxin protein during in vitro growth. The possibility of this interaction adds to the already complex regulatory network influencing alpha-toxin expression in *S. aureus* (5).

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were routinely grown overnight (15 to 18 h) in tryptic soy broth (TSB; Difco Laboratories, Detroit Mich.) at 37°C with rotary aeration (180 rpm) or on TSA plates (TSB containing 1.5% agar). *Escherichia coli* strains were grown routinely at 37°C with rotary aeration (225 rpm) in Luria-Bertani (LB) broth or on

TABLE 1. Strains and plasmids used in *splF*::Tn*551* study.

Strain or plasmid	Relevant characteristic(s)	Origin or reference
S. aureus		
RN6390	Prototypic strain	M.S. Smeltzer, University of Arkansas for Med. Sci.
RN6911	RN6390 (Δ*agr*::*tmn*)	M.S. Smeltzer, University of Arkansas for Med. Sci.
RN6911 (*splF*::Tn*551*)	*splF* transposon mutant	This study
T-RN6911 (*splF*::Tn*551*)	Transductant (*splF*::Tn*551*) of RN6911	This study
RN6390 (*splF*::Tn*551*)	Transductant (*splF*::Tn*551*) of RN6390	This study
RN4220	Nitrosoguanidine-induced restriction mutant	J.J. Iandolo, University of Oklahoma Health Sci.Ctr.
E. coli		
HB101	F⁻ *leuB6 supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 galK2 lacY1 rpsL20 xyl-5*	Laboratory stock
JM109	F′ *traD36 proAB lacI*^{q}*ZΔM15 recA1 endA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44*	Promega
Plasmids		
pCL15	Expression shuttle vector	C.Y. Lee, University of Kansas Med. Ctr.
pGEM-T Easy	Cloning vector	Promega
pI258*repA36*	Temperature sensitive vector contains Tn*551*	Laura McDowell, Microcide Pharm., Inc.
pME1	pGEM-T Easy *splF*	This study
pME2	pCL15*splF*	This study

LB-agar plates (1.5% agar). Antibiotic-resistant strains of *S. aureus* were selected with and maintained on media containing erythromycin, tetracycline, or chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) at 5 μg/ml, or penicillin G at 15 μg/ml (Sigma). Antibiotic-resistant strains of *E. coli* were selected with and grown in media supplemented with carbenicillin (Sigma) at 100 μg/ml.

Overnight (15-18 h) cultures of RN6911 (Δ*agr*::*tmn*), RN6911 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), RN6390 (wild-type), and RN6390 (*splF*::Tn*551*) were used to inoculate 50-ml flasks containing 40 ml of TSB to an initial optical density of approximately 0.05 at 550 nm. The inoculated medium for each culture was dispensed as 20 ml portions into 2, 50-ml flasks and incubated at 37°C with rotary aeration (180 rpm). A growth curve for each strain was generated spectrophotometrically by monitoring optical density at 550 nm.

Transposon (Tn*551*) mutagenesis and phenotypic screening. Transposon mutagenesis was performed in strain RN6911 containing the plasmid pI258*repA36* (49; kindly provided by Laura McDowell, Microcide Pharmaceuticals, Inc.). Plasmid pI258*repA36* is a temperature sensitive plasmid that is defective for replication at 43°C. This plasmid carries a penicillin G resistance marker and Tn*551*, which is a staphylococcal transposon that encodes erythromycin resistance (Em^r). To obtain a pool of cells for transposon mutagenesis, RN6911 containing pI258*repA36* was streaked on TSA supplemented with penicillin G and grown overnight (15-18 h) at a permissive temperature of 30°C. Volumes (0.1 ml) of 10-fold, serial diluted cells suspended in TSB were spread on TSA containing erythromycin and 2% rabbit blood (Remel, Inc., Lenexa, Ks.). After

overnight incubation at the non-permissive temperature of 43°C, hemolytic colonies were procured for further study.

Assay for alpha-toxin activity. Standardized spent media harvested from 18-hour cultures of RN6911 (Δ*agr*::*tmn*), RN6911 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), RN6390 (wild-type), and RN6390 (*splF*::Tn*551*) were used to determine alpha-toxin activities. After filter sterilization through 0.22-μm filters (Fisher Scientific, Fairlawn, N.J.), portions (0.02 ml) of the spent media were added to wells in TSA containing 5% rabbit blood, and the plates were incubated at 37°C overnight (18-24 h). Diameters of cleared zones were determined and compared between strains. Additionally, alpha-toxin activities of spent media were also assayed with a microtiter method adapted from previously described protocols (59, 74). Briefly, standardized spent media that had been filter sterilized were serially diluted, two-fold in phosphate-buffered saline, and each dilution was incubated for 25 minutes at 37°C with 1% rabbit erythrocytes (Remel). Unlysed blood cells were pelleted by centrifugation (4°C at 1,000 × *g*) for 10 minutes, and 180 μl of the cell-free supernatant was added to individual wells of a 96-well microtiter plate. Plates were read at 490 nm in a microtiter plate reader, and units of activity are expressed as the reciprocal of the dilution giving 50% lysis. The 50% lysis points were calculated by interpolating data from standard curves generated from 0.2 ml suspensions containing a volume percentage (0-100% v/v) of a 1% hemoglobin suspension and a 1% erythrocyte suspension. The 1% hemoglobin suspension was obtained by adding 1ml of a 10% erythrocyte suspension to 8 ml of

sterile distilled water, shaking the suspension to promote cellular lysis, and adding 1ml of (10 ×) phosphate buffered saline.

Arbitrary PCR amplification and DNA sequence analysis. Chromosomal DNA was isolated from *S. aureus* RN6911 (*splF*::Tn*551*) using the method of Dyer and Iandolo (27). The DNA sequence flanking Tn*551* was determined using an arbitrary PCR method (14). In this method, flanking chromosomal DNA is enriched through two subsequent rounds of PCR amplification using primers specific for one end of Tn*551* and primers to random sequence, which can anneal to chromosomal DNA adjacent to the transposon. Arbitrary PCR was performed in 50 µl volumes containing 200 nM of each primer and a *Taq/Pyroccoccus species* GB-D DNA polymerase mixture from the Elongase Enzyme Mix kit (Invitrogen Corp., Carlsbad, Ca.). In the first round (30 cycles), a primer 260 nucleotides from the left end of Tn*551* (Tn1, 5'-GGGTTAACCATACGCAAGACC-AAT-3') and a primer with 3' random sequence (ARB1, 5'-GGCCACGCGTCGACTA-GTACNNNNNNNNNGATAT-3') were used to amplify random PCR fragments from RN6911 (*splF*::Tn*551*) under low stringent conditions (annealing temperature 45°C). The second round (35 cycles) utilized the PCR products from the first round as the DNA template for amplification under higher stringency conditions (annealing temperature 53°C). A primer approximately 70 nucleotides from the left end of Tn*551* (INSI, 5'-GAGAGATG-TCACCGTCAAGTTAAATCTA-3') and a primer specific to the 5' tag of ARB1 (ARB2, 5'-GGCCACGCGTCGACTAGTAC-3') were used to amplify specific fragments in the second round of arbitrary PCR. PCR products from the second round were resolved in a 1.0% GTG agarose gel (BioWhittaker Molecular Applications,

specific fragment of approximately 250-bp was gel purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, Ca.). This PCR fragment was sequenced with the INSI primer at the University of Arkansas for Medical Sciences DNA Sequencing Core Facility (Little Rock, Ark.) using a DNA sequencer (Perkin-Elmer Biosystems, Foster City, Ca.). The unknown sequence (153 nt) was BLASTN searched for identity at *S. aureus* DNA databases maintained at The Institute for Genomic Research (http://www.tigr.org) and the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html). DNAMAN software (Lynnon Corp., Quebec, Canada) was used to generate a conceptual translation of a putative open reading frame (orf) disrupted by transposition. The amino acid sequence of that orf was BLASTP searched for similarity at the NCBI database (http://www.ncbi.nlm.nih.gov/).

PCR amplification and cloning of *splF*. Oligonucleotide primers (SPLF-1, 5'-TCATCT-CTGTGATTCCATTTATTCGAAA-3'; and SPLF-2, 5'-TTTGTCAGGAT-TGGGTGAATGT-CTAA-3') were designed using sequence data from The Institute for Genomic Research and the University of Oklahoma's Advanced Center for Genome Technology and used to amplify a 847-bp contiguous region from *S. aureus* RN6390 by PCR. The PCR product was ligated into pGEM-T Easy vector (Promega Corp., Madison, Wi.) and transformed into *E. coli* JM109. Transformants were selected as recommended by the manufacturer (Promega). Plasmid DNA was isolated from an antibiotic-resistant transformant using a plasmid miniprep kit (Bio-Rad Laboratories, Richmond, Ca.) and restriction digested with *Eco*RI (Promega) to verify the presence of an approximately

900-bp insert. The plasmid, designated as pME1, containing the desired contiguous *splF* chromosomal region was sequenced at the University of Arkansas for Medical Sciences DNA Sequencing Core Facility (Little Rock, Ark.).

The 900-bp insert containing *splF* was subcloned into the expression shuttle vector pCL15 (kindly provided by Chia Lee at the University of Kansas Medical Center), which contains an inducible *lac* promoter. This construct, designated as pME2, was transformed in succession through electrocompetent *E. coli* HB101, *S. aureus* RN4220, and *S. aureus* RN6911 (*splF*::Tn*551*), and plasmid DNA was isolated from each antibiotic-resistant transformant with a plasmid midiprep kit (Qiagen). The midiprep procedure was modified for *S. aureus* by incubating the cells with 50 μg/ml of lysostaphin (Applen and Barrett LTD., England) for 30 minutes at 37°C. Correct orientation of the insert in relation to the *lac* promoter of pCL15 was verified by restriction analysis with *Eco*RI and *Xmn*I (Sigma). *S. aureus* RN6911 (*splF*::Tn*551*) transformed with pME2 was procured for additional studies.

Transduction of *splF*::Tn*551* into RN6390 and RN6911. Transduction of *splF*::Tn*551* from RN6911 (*splF*::Tn*551*) to RN6390 and RN6911 was mediated by the bacteriophage ϕ11 as described by McNamara et al. (50). Briefly, a phage lysate from infected RN6911 (*splF*::Tn*551*) grown in soft agar (TSB, 0.5% agar) overlay on TSA containing $CaCl_2$ (5 mM) was filter-sterilized through 0.22-μm syringe filters (Fisher Scientific). Plaque assays on RN6390 and RN6911 determined the titer for the phage preparation, which was used to perform transduction with multiplicities of infection of 1.0 and 0.1. The phage lysate was added to TSB containing $CaCl_2$ (5 mM) and $\geq 10^9$ colony forming units (cfu) of

RN6390 or RN6911 per ml. After a 5-minute incubation at room temperature, transduction was performed at 37°C with rotary aeration (250 rpm) for 20 minutes. Ice-cold sodium citrate (20 mM) was added to chelate the $CaCl_2$ and stop transduction. Cells were pelleted by centrifugation, suspended in ice-cold sodium citrate (20 mM), and plated onto TSA plates containing sodium citrate and erythromycin. Plates were incubated at 37°C for 18 to 24 hours. Erythromycin-resistant transductants were subcultured to selective media, analyzed for their hemolytic abilities, and subjected to Southern analysis. Transductants containing the *splF*::Tn*551* were procured for further analysis.

Southern analysis of transposition site. Chromosomal DNA isolated from *S. aureus* strains RN6911 (Δ*agr*::*tmn*), RN6911 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), RN6390 (wild-type), and RN6390 (*splF*::Tn*551*) was digested with *Eco*RI and electrophoretically resolved through a 0.8% LE agarose gel (BioWhittaker Molecular Applications). Resolved DNA was passively transferred to a neutral nylon membrane (MagnaGraph; Micron Separations, Inc., Westborough, Mass.), UV-fixed, and hybridized overnight (18 to 24 h) at 65°C to a DNA probe specific for Tn*551*. After autoradiography (Fujifilm Medical Systems USA, Inc., Stamford, Con.), alkali-labile Tn*551* specific probes were removed from membrane-fixed DNA upon exposure to an alkaline probe-stripping solution (0.2 M NaOH, 0.1% SDS). DNA on the membrane was then hybridized to a DNA probe specific for *splF* under the same conditions described above. The *splF* specific probe was generated from the *Eco*RI fragment of plasmid pME1, and the Tn*551* specific probe from the 1.2-kb *Hind*III fragment of plasmid pTVT

(kindly provided by Laura McDowell, Microcide Pharmaceuticals, Inc.). Each probe was randomly labeled with digoxigenin-11-UTP (Roche Molecular Biochemicals, Indianapolis, Ind.) and the Klenow fragment of DNA polymerase (Promega). Probes hybridized to specific sequences were detected by autoradiography with alkaline phosphate-conjugated anti-digoxygenin $F(ab')_2$ antibody fragments (Roche Molecular Biochemicals) and the chemiluminescent substrate CDP-*Star* (Roche Molecular Biochemicals).

RNA isolation and northern analysis. Overnight (18 h) cultures of RN6911 (Δ*agr*::*tmn*), RN6911 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), RN6390 (wild-type), and RN6390 (*splF*::Tn*551*) was added (10 ml) to 10 ml of ice-cold ethanol/acetone (1:1). Total cellular RNA was isolated from the ethanol/acetone suspensions as described by Hart et al. (34). Briefly, cells were pelleted by centrifugation (11,500 × *g*), washed and suspended in HSTES (2.5 M NaCl, 78 mM DiNaEDTA, 100 mM Tris [pH 7.5]). Suspended cells were incubated for 30 minutes at 37°C with lysostaphin (50 μg/ml), and RNAzol B (Tel-Test, Inc., Friendswood, Tx.) was added to promote lysis of protoplasts. Chloroform was added (0.6 ml), and the mixture was shaken and incubated on ice for 15 minutes. The mixture was centrifuged (4°C at 11,500 × *g*) to facilitate phase separation, and 1.2 ml of the aqueous phase was combined with ice-cold isopropanol and incubated overnight (24 h) at -20°C. Precipitated RNA was pelleted by centrifugation (4°C at 15,000 × *g*) for 30 minutes, washed twice in 70% ethanol, and dried for 40 minutes at room temperature. Dried RNA pellets were suspended in DEPC (diethylpyrocarbonate; Sigma) -treated water, and stored at -85°C.

RNA sample concentrations were determined specrophotometrically at 260 nm, and RNA purity was assessed as absorbance (A_{260}/A_{280}) ratios of 1.9 to 2.0. Samples were diluted to 1 μg/ml in DEPC-treated water, and the comparative intensities of rRNA bands were visualized by gel electrophoresis. The 1 μg/ml samples were serial diluted, and RNAs were denatured in glyoxal (Eastman Kodak Co., Rochester, N.Y.) and dimethyl sulfoxide (Fisher Scientific) at 50°C for 1 hour. Denatured samples were electrophoresed through a 1.4% GTG agarose gel (BioWhittaker Molecular Applications). RNA was passively transferred to a neutral nylon membrane (MagnaGraph), UV fixed, and hybridized overnight (18-24 h at 65°C) with a DNA probe specific for the alpha-toxin transcript. After autoradiography (Fujifilm Medical Systems USA, Inc., Stamford, Con.), alkali-labile alpha-toxin specific probes were removed from membrane-fixed RNA upon exposure to an alkaline probe-stripping solution (0.2 M NaOH, 0.1% SDS). RNA on the membrane was then hybridized to a DNA probe specific for 16Srna under the same conditions described above. This served as a load control for the comparison of band intensities between different films. Hybridized probes were detected by autoradiography as described for Southern analyses.

Preparation of cell lysates and zymographic analysis. The expression of *splF* in *E. coli* HB101 containing pME2 was induced with IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM; Fisher Scientific) 1 hour post-inoculation. Cells were harvested after 5 hours of incubation. To serve as a control, IPTG was not added to a second culture of HB101 cells containing pME2.

Whole-cell lysates were prepared from the two cultures using the procedure of Blevins et al. (11). Briefly, cells were harvested by centrifugation (12,000 × *g* for 10 minutes at 4°; J-21C Centrifuge and JA-20 rotor; Beckman Coulter, Inc., Fullerton, CA), washed in an equal volume of TEG buffer (25 mM Tris, 25 mM EGTA [pH 8.0]), and suspended in 0.4 ml of TEG buffer. The cellular suspensions were added to 2.0-ml Fast Prep Blue tubes (Bio 101, Vista, Cal.) containing acid-washed, RNase-free 0.1-mm silica beads. Each tube was placed into a high-speed reciprocator (Bio 101) and agitated at 6 m/s for 40 seconds. The tubes were placed on ice for 15 minutes and centrifuged (16,170 × *g*; 854 rotor; Centra MP4R, IEC, Needham Heights, Mass.) for 10 minutes at 4°C. Cellular debris free supernatants were recovered from the tubes and stored at -20°C until needed. Protein concentrations of the lysates were determined using the BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL.).

Protease activity of SplF was determined by utilizing essentially the zymographic method described by Arvidson et al. (4). A 2.5-µl volume of each sample was combined with 2.5 µl of 2× SDS sample buffer (without DTT or β-mercaptoethanol) and denatured for 15 minutes at room temperature. Purified *S. aureus* V8 protease (5 µg/µl; US Biological, Swapscott, Mass.) was used as a positive control, and 2.5 µg of this protease was denatured under the same conditions as the samples. The samples and control were loaded onto a precast, 12% polyacrylamide gel containing 0.1% casein (Bio-Rad Laboratories), and electrophoresed for 1 hour and 15 minutes at 120 V. Proteins were renatured for 30 minutes in 2.5% (v/v) Triton X-100 with gentle agitation at room temperature. The gel was incubated in development buffer (50 mM Tris, 200 mM NaCl,

5 mM $CaCl_2$, 0.02% Brij-35 [pH 7.5]) overnight (12 h) at 37°C, and stained with 0.5% Coomassie blue. Destaining with a 40% methanol-10% acetic acid solution resulted in clear zones of hydrolysis against a blue background.

Complementation of *splF*::Tn*551*. An overnight culture of *S. aureus* RN6911 (*splF*::Tn*551*, pME2) was used to inoculate a 50-ml flask containing 20 ml of TSB supplemented with chloramphenicol (15 μg/ml; Sigma). The initial optical density of the inoculum was determined to be approximately 0.05 at 550 nm. Expression of *splF* was induced with the addition of IPTG (0.5 mM; Fisher Scientific) 1 hour post-inoculation. The spent media was harvested at 12 hours by centrifugation (8,000 × *g*) for 15 minutes at 4°C. Filter sterilized (0.22 μm; Fisher Scientific) spent media was analyzed for hemolytic activity and stored at -20°C for further use.

Results

Transposon mutagenesis and analysis of *agr*-null suppressor mutants. The *S. aureus* *agr*-null strain, RN6911, was subjected to mutagenesis with the staphylococcal transposon Tn*551*. A total of fifty-four hemolytic positive colonies were isolated out of approximately 4×10^4 erythromycin-resistant colonies. The alpha-toxin activity of each hemolytic positive mutant was quantitatively determined by a microtiter assay of supernatants recovered from 18-hour cultures. Units of activity ranged from two to sixty-four for the fifty-four suppressor-mutants.

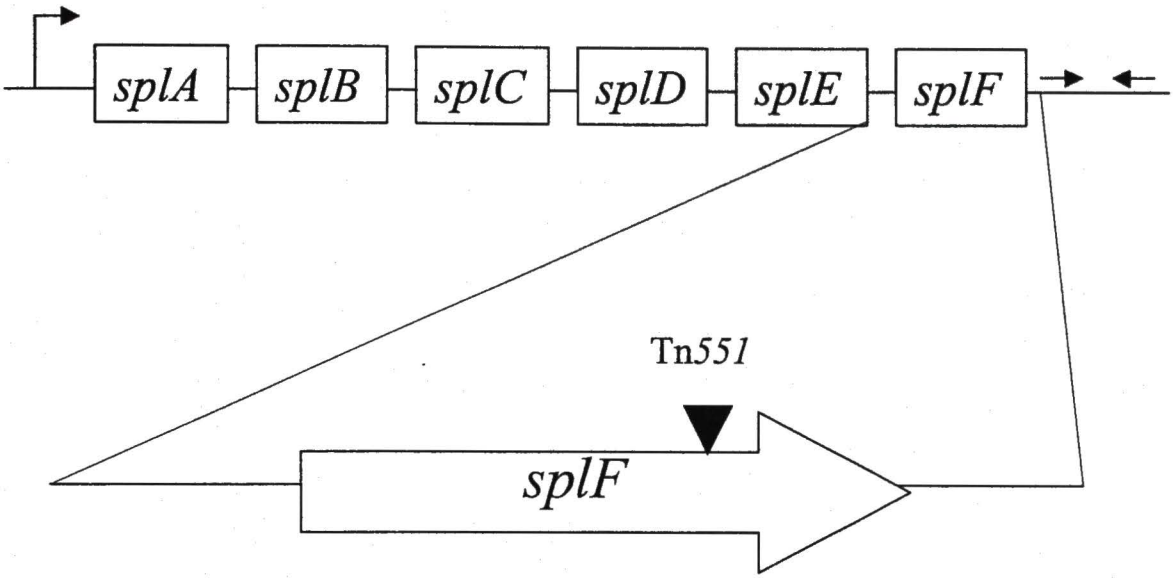
Southern analysis for Tn*551* revealed that only twenty-six of the fifty-four mutants contained an intact transposon within their chromosomes. Chromosomal transposition

sites were determined for seven of the twenty-six mutants by BLASTN searching sequence data obtained from arbitrary-PCR products against the maintained databases at the Institute for Genomic Research (http://www.tigr.org) and the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html). Genomic identification of PCR sequences revealed that transposition had occurred in various structural genes within six suppressor-mutants. However, one PCR sequence with a length of approximately 250 bp contained chromosomal sequence specific for the *s*erine *l*ike-*p*rotease *F* gene, *splF* (Fig. 1A and B).

Verification and initial characterization of the *splF*::Tn*551* mutation. The *splF* open reading frame (ORF) has an estimated length of 718 nt, and encodes for a protein with an approximate molecular weight of 21.9 kDa (65). Tn*551* inserted itself between nucleotides 447 (adenine) and 448 (cytosine) of *splF*, disrupting the peptide link between amino acids 149 (glutamine) and 150 (proline) located within the 203 amino acid polypeptide (Fig. 1A and B). A PCR product with a length of 847 bp was amplified from the chromosome of *S. aureus* RN6390 using primers SPLF-1 and SPLF-2. The 847-bp fragment cloned into pGEM-T Easy (Promega), designated pME1, was sequenced and determined to contain a non-mutated *splF* allele within its sequence.

Southern analysis with the *splF* specific probe detected a 5 kb increase in the molecular size of chromosomal *Eco*RI fragments containing *splF*::Tn*551* (Fig. 2B). This shift in size was evident for strains RN6390 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), and RN6911 (*splF*::Tn*551*), which corresponded to the approximate size of hybridization bands detected with the Tn*551* specific probe (Fig. 2A). These results

A



B

5'-GAAATTTTTAAATCTATTTCATTATCGATACAAATTTCTCGTAGG
CGCTCGGGACCCC**CATTAGGATTTGGATAACCAATGACTGATAT**
AGGTTCATTTTCTTTAGCTTCTGATGCTATATTAAATTTACTAG
TGAAATCTTTGAATTTTCTACCTTTTGGTTGTGTTGATTTTTCT
TCAACTTGTACAACCGCAATATCCCCCCCCCCCCGTACTAGTCGA
CGCGTGGCC-3'

FIGURE 1. (A) Organization of the *spl* operon as described previously (65). The transcriptional start site and the Rho-independent terminator are represently by the bent arrow and converging arrows respectively. ORF's are indicated by boxes, and the directionality of *splF* by the block arrow. The site of Tn*551* insertion is also indicated. (B) Sequence of arbitrary-PCR product. Chromosomal sequence containing *splF* is bolded.

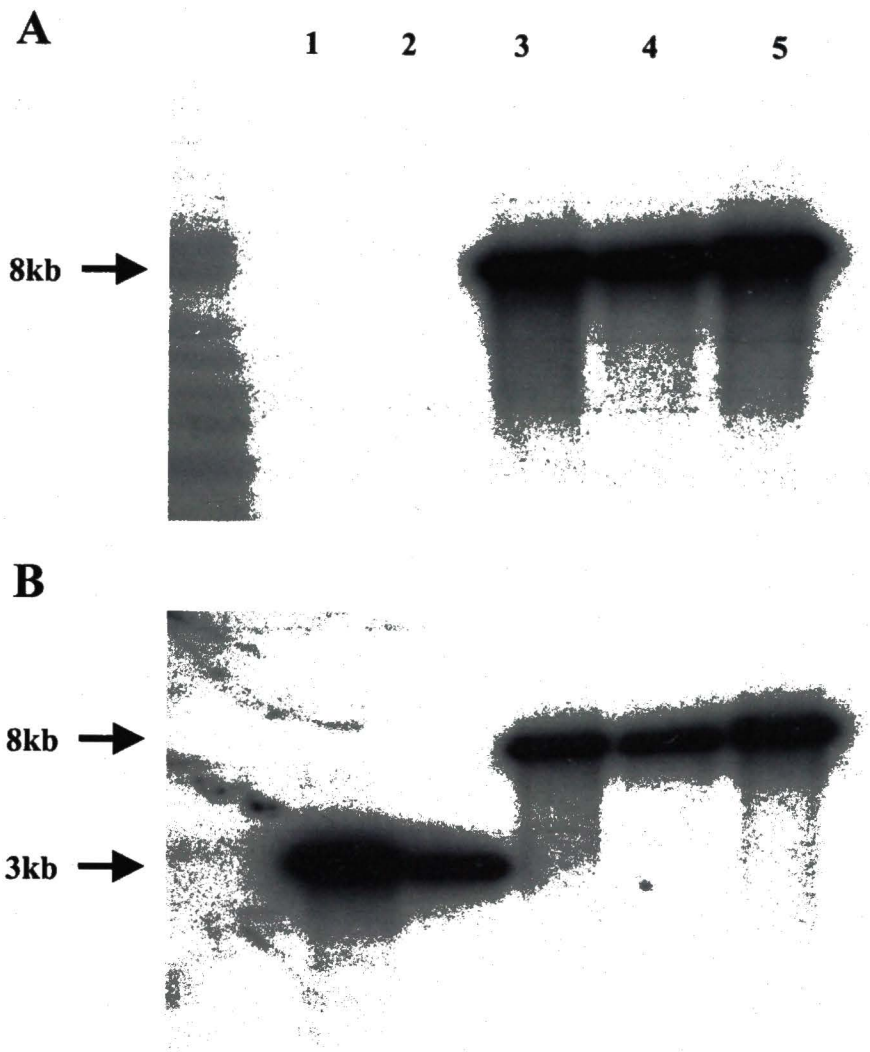


FIGURE 2. Southern analysis of *S. aureus* chromosomal DNA hybridized to specific probes for Tn*551* (A) and *splF* (B). Lane 1, RN6390; Lane 2, RN6911; Lane 3, transduced RN6390 (*splF*::Tn*551*); Lane 4, transduced RN6911 (*splF*::Tn*551*); Lane 5, RN6911 (*splF*::Tn*551*). Arrows indicate approximate molecular weights of *Eco*RI chromosomal fragments containing Tn*551* and *splF*. Hybridization was detected by autoradiography utilizing chemiluminescence chemistry.

verify the presence of Tn*551* within *splF*. To support these results, PCR product was generated from the transposon-chromosomal junction of RN6911 (*splF*::Tn*551*) with primers SPLF-1 and INSI. The primer INSI anneals to Tn*551* sequence approximately 70 bases from the left-end of the transposon. The estimated molecular size of 170 bp for the PCR product was confirmed by gel electrophoresis (data not shown).

The *splF* mutation in *S. aureus* RN6911 (*splF*::Tn*551*) generated a hemolytic titer approximately ten times that of RN6911 (Table 2). To verify the link between transposon mutagenesis and increased alpha-toxin activity, the *splF*::Tn*551* mutation was transduced back into the *agr*-null background of RN6911. Hemolytic titer for the RN6911 (*splF*::Tn*551*) transductant approximately corresponded to that determined for RN6911 (*splF*::Tn*551*), which verifies the suppressor-mutation effect of *splF*::Tn*551*on alpha-toxin activity (Table 2). To further verify the effects of the *splF* mutation on alpha-toxin activity, the 900-bp *Eco*RI fragment of pME1 containing the *splF* ORF was subcloned into the expression shuttle vector pCL15. This construct, pME2, was propagated first in *E. coli* HB101, then in the nitrosoguanidine-induced restriction mutant strain, RN4220, before being moved into RN6911 (*splF*::Tn*551*). After exposure to IPTG (isopropyl-β-D-thiogalactopyranoside) for 11 hours, the spent medium from RN6911 (*splF*::Tn*551*, pME2) was analyzed for hemolytic activity. The hemolytic titer for the *splF* complemented strain, where complementation was induced in *trans*, corresponded to the titer for RN6911 (Table 2). Furthermore, the diameters of cleared zones were smaller for RN6911 (*splF*::Tn*551*, pME2) than they were for RN6911 (*splF*::Tn*551*). These results suggest that the product of *splF* may influence alpha-toxin

TABLE 2. Alpha-hemolysin activities of *S. aureus* strains and *splF*::Tn*551* mutants.

Strain	Activity measured by:	
	Clearing of blood agar[a]	Hemolytic titer[b]
RN6911	0	<0.5
RN6911 (*splF*::Tn*551*)	3.8 ± 0.1	4.8 ± 0.4
Transduced RN6911 (*splF*::Tn*551*)	3.4 ± 0.4	5.1 ± 0.3
RN6390	7 ± 0.9	125 ± 4.3
Transduced RN6390 (*splF*::Tn*551*)	6.9 ± 0.8	123.4 ± 5.1
RN6911 (*splF*::Tn*551*, pME2)	0.6 ± 0.02	<0.5

[a]Assayed by measuring the diameter of a cleared zone in mm minus the diameter of the punch (5 mm). Represents the mean ± standard error of three independent assays.

[b]Units of activity or hemolytic units are expressed as the reciprocal of the interpolated dilution value yielding 50% lysis. Represents the mean ± standard error of two independent assays.

expression within *S. aureus*. To determine how significant those effects are, *splF*::Tn*551* was transduced from RN6911 (*splF*::Tn*551*) into the wild-type background of RN6390 by ϕ11. The hemolytic titer was as equally high for transduced RN6390 (*splF*::Tn*551*) as they were for RN6390 (Table 2). Additionally, no large difference in cleared zone diameters was observed between the *splF*::Tn*551* transductant and its parent strain. The *agr* wild-type background evidently produces factors that compensate for the effects of the *splF* mutation on alpha-toxin expression within *S. aureus*.

To determine if the *splF* product affects alpha-toxin expression at the transcriptional level, northern analysis for the alpha-toxin transcript (*hla*) was done with total cellular RNA isolated from 18-hour cultures of RN6911, RN6911 (*splF*::Tn*551*), transduced RN6911 (*splF*::Tn*551*), RN6390, and RN6390 (*splF*::Tn*551*) (Fig. 3 and 4). The two-fold serially diluted RNA samples for RN6911 and RN6911 (*splF*::Tn*551*) revealed no quantifiable difference in alpha-toxin message levels (Fig. 3A and 3B). Additionally, no quantifiable difference in alpha-toxin transcipt levels was observed for RNA samples isolated from RN6390 and RN6390 (*splF*::Tn*551*) (Fig. 4). These two samples were four-fold serially diluted to obtain reduced band intensities on the image. The results of the northern analyses imply that the *splF* product does not affect alpha-toxin expression at the transcriptional level.

Zymographic analysis of SplF. To determine if *splF* encodes protease activity, *splF* expression from pME2 was induced by adding IPTG (0.5 mM) to a culture of *E. coli* HB101 transformed with pME2. Additionally, a second culture with the same transformed *E. coli* strain did not have IPTG added to it. The whole cell lysate of each

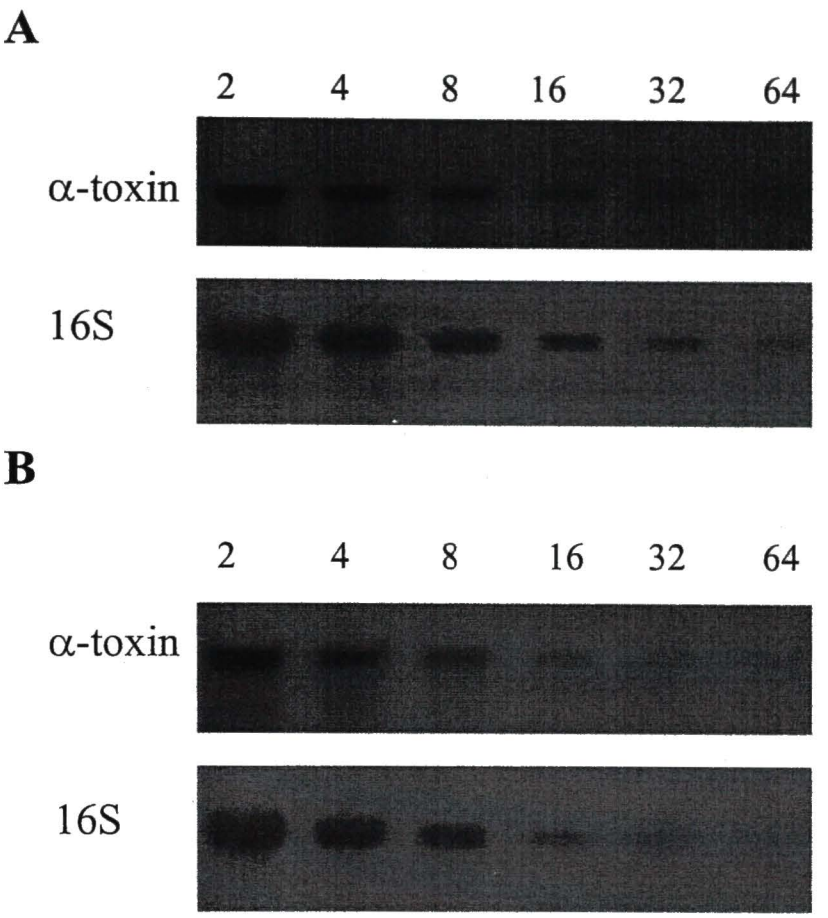


FIGURE 3. Northern analysis of alpha-toxin and 16S messages in total RNA isolated from post-exponential (18h) cultures of *S. aureus* RN6911 (*splF*::Tn*551*) (A) and RN6911 (B). Numbers at the top of each figure indicate the reciprocal of two-fold serially diluted RNA (1μg/ml) samples. Hybridization to alpha-toxin and 16S transcripts was detected by autoradiography utilizing chemiluminescence.

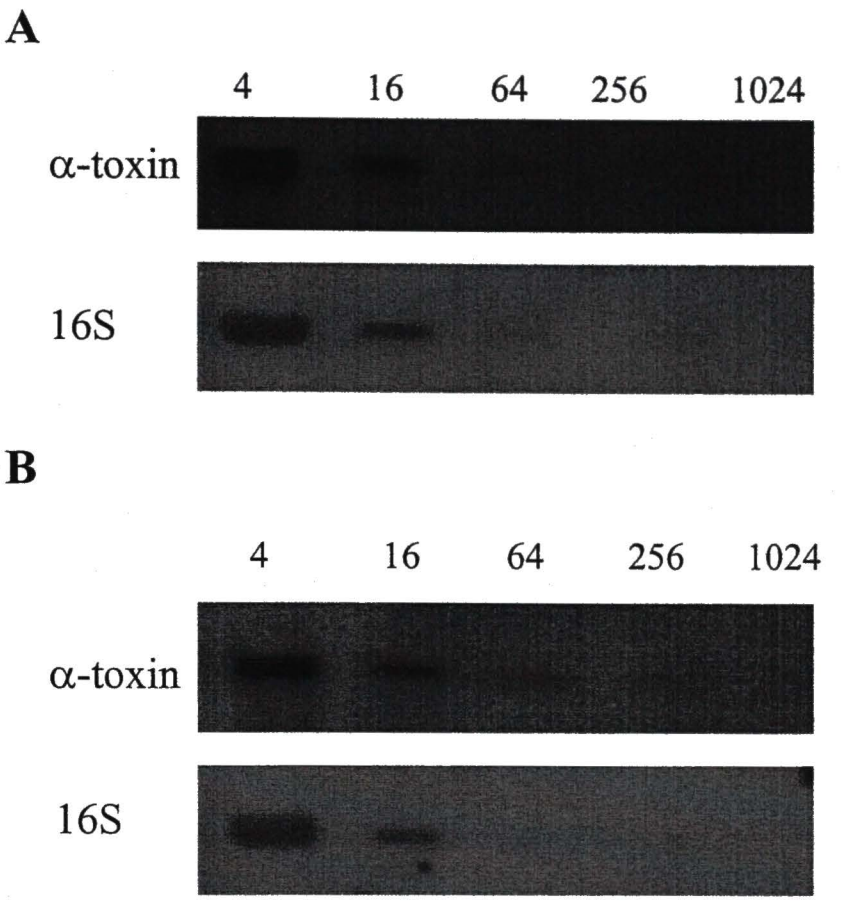


FIGURE 4. Northern analysis of alpha-toxin and 16S messages in total RNA isolated from post-exponential (18h) cultures of *S. aureus* RN6390 (*splF*::Tn*551*) (A) and RN6390 (B). Numbers at the top of each figure indicate the reciprocal of four-fold serially diluted RNA (1μg/ml) samples. Hybridization to alpha-toxin and 16S transcripts was detected by autoradiography utilizing chemiluminescence.

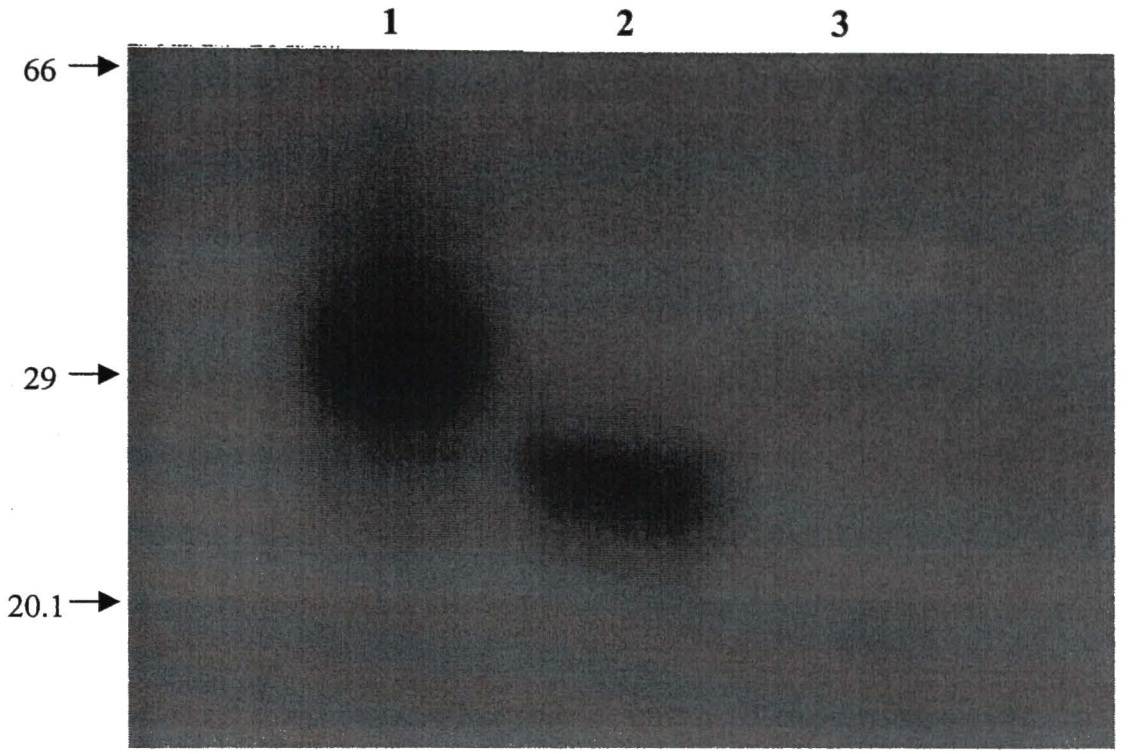


FIGURE 5. Zymographic analysis of SplF induced from an expression vector in *E. coli* HB101. Whole cell lysates were analyzed on a precast 12% polyacrylamide gel containing 0.1% casein (Bio-Rad Laboratories). Lane 1, 2.5 μg of purified V8 protease (US Biological); Lane 2, lysate from IPTG (0.5 mM)-induced HB101 cells; Lane 3, Lysate from non-induced HB101 cells. Molecular size markers (kDa) are those of the Kaleidoscope Molecular Standards (Bio-Rad Laboratories). Image presented is a negative of the original.

culture was zymographically analyzed for protease activity on a 12% polyacrylamide gel containing 0.1% casein (Bio-Rad Laboratories). Cleared zones of casein hydrolysis were evident in the lanes containing purified V8 protease (Fig. 5, lane 1) and the lysate of transformed *E. coli* HB101 cells grown in the presence of IPTG (Fig. 5, lane 2). The estimated migration position of SplF (~22 kDa) corresponds to the zone of hydrolysis located in lane 2 of figure 5. Therefore, the product of *splF*, SplF, is proteolytically active and may post-translationally modify alpha-toxin protein.

Discussion

The *spl* operon was previously identified and described as a genetic locus in *S. aureus* that encodes for six serine protease-like proteins from a 5.5-kb polycistronic transcript (65). In vitro expression of the *spl* operon is highest during the transition from late-exponential to early-stationary growth phase and is influenced by *agr* but not *sar* (65). Sequences of all six *spl*- proteins revealed that each one contained putative 35- or 36-amino-acid signal peptides for Sec-dependent secretion (65). Additionally, catalytic amino acid triads associated with enzymes in the trypsin-like serine protease family were identified in all six sequences (12, 65). Rats intraperitoneally injected with an *spl* isogenic mutant, where the entire *spl*-operon was deleted, showed no significant differences in lethality as compared to those infected with RN6390. Therefore, how the *spl*- proteases contribute to *S. aureus* virulence remains unclear.

This study describes the isolation and characterization of an *agr*-suppressor mutant of *S. aureus* RN6911 (Δ*agr*::*tmn*) that contains a transpositionally disrupted *s*erine *p*rotease-*l*ike *F* gene (*splF*). Identification of this disruption was determined from the sequence of

a 250-bp arbitrary PCR product generated from RN6911 (*splF*::Tn*551*) (Fig. 1). The microtiter method for assaying alpha-toxin activity revealed a ten-fold increase in activity for spent media isolated from an 18-hour culture of RN6911 (*splF*::Tn*551*) as compared to that of RN6911 (Table 2). Differences in growth curves or alpha-toxin message levels were not noticeably significant for RN6911 (*splF*::Tn*551*) as compared to RN6911 (Fig. 3; data not shown for growth curves). Additionally, zymographic analysis of whole cell lysates from IPTG-induced *E. coli* HB101 cells carrying pME2 revealed that the *splF* product possesses protease activity (Fig. 5). These data suggest that SplF may be post-translationally modifying alpha-toxin protein.

Lindsay et al. (1999) attributed the low alpha-toxin activity of the *S. aureus sarA* mutant strain PC1839 to the high protease levels it produced after 12 hours of growth. A major breakdown product of alpha-toxin was detected in spent media from PC1839 with an anti-alpha-toxin antibody used in western analysis. Additionally, high levels of V8 serine protease were detected in spent media from PC1839 by western analysis utilizing an anti-V8 serine protease antibody. This group concluded that serine proteases appear to be proteolytically cleaving alpha-toxin protein in spent media of *S. aureus* (44). Another study revealed extensive loss of exoproteins in spent media from *S. aureus* NCTC8325 harvested at late-stationary growth phase (38). Approximately two to three hundred spots were detected on two-dimensional gels containing spent media harvested at the early-stationary growth phase. The majority of these spots were absent on gels containing spent media harvested at late-stationary growth. N-terminal sequencing revealed that lipase, triacylglycerol lipase, and two other exoproteins were detected as multiple spots

on the gel containing the spent media harvested at early-stationary growth. It was determined that each of those exoproteins was being cleaved by proteases present within the spent media of *S. aureus* NCTC8325. These two studies support the idea that proteases are able to cleave other exoproteins produced by *S. aureus* during in vitro growth.

The significance of SplF in determining total alpha-toxin activity in *S. aureus* appears to be related to the presence and activity of *agr* within a cell. Spent media from RN6390 and the *splF*::Tn*551* transductant of RN6390 had similar hemolytic activities after 18 hours of in vitro growth (Table 2). This suggests that other factors generated in an *agr* background during in vitro growth overrides the effects of the *splF*::Tn*551* mutation on alpha-toxin activity. It is known that RNAIII is necessary for the transcription and presumably the translation of alpha-toxin within *S. aureus* (5). However, the maximal expression of the *spl* operon also requires the presence of *agr* within a cell (65). If this is the case, then alpha-toxin and SplF, as well as the other *spl*- proteases, are maximally expressed at the same time (late-exponential phase to stationary phase) during in vitro growth. Proteolysis of alpha-toxin is probably occurring, but its effect on hemolysis appears to be insignificant at this stage of growth. However, cleaved alpha-toxin has been shown to retain hemolytic activity (60). It was also revealed that pores created by cleaved alpha-toxin in erythrocytes are considerably smaller than those generated by its native form, and that the nicked form could not permeabilize nucleated cells like monocytes (60). Therefore, the importance of regulating alpha-toxin activity by proteolysis during in vitro growth may not be as evident as it would be within the in vivo

environment of the host. The importance of this type of regulation during infection is exemplified in a study demonstrating that *S. aureus* strains overproducing alpha-toxin had reduced virulence in an experimental endocarditis model (6). It appears that proteolysis may serve as a safeguard to the effects of overly expressing alpha-toxin during an infection.

It is evident that *S. aureus* retains tight control over alpha-toxin expression, and SplF appears to play a role in regulating its activity. The significance of SplF in determining alpha-toxin activity may not be readily evident in certain situations, but in others it may be essential. Further investigations with SplF will need to be conducted in order to fully understand its contributions to *S. aureus* pathogenecity. The findings of this study contribute to the growing amount of evidence suggesting that extracellular proteases produced by *S. aureus* are significant in determining the overall virulence of this organism.

CONCLUSIONS

It is apparent that a complex network of regulatory elements controls the expression of virulence factors in *S. aureus* (5). Extensive interactions appear to exist between many of the defined regulators, including *agr* and *sar*. The exact mechanisms of interaction remain unknown for most of the regulatory elements, and models for virulence regulation based on these interactions are purely speculative. Nevertheless, such models can explain in vitro phenotypes for many regulator mutants. In one proposed model, SarA and its homologues are assumed to act as repressors, while RNAIII acts as an anti-repressor to the Sar repressors (5). As the cell density increases during infection processes or during in vitro growth, RNAIII levels increase and bind up all of the Sar repressors in the cell. This de-repression results in the increased expression of secreted virulence factors such as alpha-toxin and extracellular proteases. As protease levels increase, the stability and activity of other virulence factors are affected.

Several virulence factors have been shown to be sensitive to protease cleavage during in vitro growth (37, 38, 44, 49). Protease cleavage of cell-surface associated virulence factors appears to mediate a quick transition from an adhesive phenotype to an invasive phenotype (37, 49). This type of phenotypic transition could be essential in spreading infection to other sites within the host by in enabling the escape of cells from localized infections, like abscesses. Additionally, cytotoxin production is thought to be necessary to escape local infections by damaging the surrounding host tissues and cells (73).

Alpha-toxin may be one of those toxins mediating this escape. Apparently alpha-toxin production is tightly regulated at multiple levels within *S. aureus* (29, 30, 51, 64, 68, 72). This regulation may include post-translationally modifying the activity of alpha-toxin by proteolysis (44). More specifically, proteases like SplF may modify alpha-toxin activity by selectively nicking this virulence factor post-translationally. Nicked alpha-toxin can still bind to the surface of target cells in vitro, but lack the ability to lyse nucleated cells (60). Therefore, proteolytically nicked alpha-toxin could theoretically modulate the effectiveness of full-length alpha-toxin by reducing the number of available binding sites on the surface of a target cell. If strains overly producing alpha-toxin were significantly less virulent than those expressing normal levels of this hemolysin (6), then it is reasonable to think that protease activity may determine the effective threshold for alpha-toxin activity during an infection. Furthermore, protease activity may also be involved in determining the effectiveness of other virulence factors produced by *S. aureus*.

It is obvious that SplF effects alpha-toxin activity during in vitro growth. The significance of this interaction on *S. aureus* virulence remains to be determined. However, in theory this interaction may help determine the overall outcome of a *S. aureus* infection. It would be interesting to see if the other Spl proteins have the ability to modify the expression of other virulence factors as well. The similarities in sequence between those proteins would suggest that this type of post-translational modification might be occurring (65). Taken all together, a complex regulatory network orchestrates virulence factor expression in *S. aureus*, and proteases appear to play an important role in

this complex process. The redundancy of this regulation may be essential for the survival of *S. aureus* under diverse environmental conditions encountered in the infected host.

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