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A Tn*917* transposon mutant of *Staphylococcus aureus* S6C was isolated and analyzed due to its deficiency in hemolysin and lipase activities. The transposon insertion did not occur in any of the known genetic regulators, which suggested the insertion occurred in a novel regulator of at least, hemolysin and lipase activities. One end of the region where the insertion occurred was isolated, sequenced, and compared with known DNA databases. Sequence comparisons revealed the insertion occurred in one of six rRNA DNA operons, which was confirmed by Southern analysis. Transduction of the transposon insertion back into the parental strain did not result in a mutant phenotype thereby indicating that the transposon insertion into a rRNA DNA operon was not responsible for the observed mutant phenotype. Further analysis of the parent strain, *S. aureus* S6C, revealed a population of four relatively stable variants differing in their hemolysin and catalase activities. These data suggest that the Tn*917* mutant was one of these four S6C variants.

ANALYSIS OF A Tn*917* TRANSPOSON MUTANT AND PRELIMINARY CHARACTERIZATION OF NONHEMOLYTIC, CATALASE-DEFICIENT VARIANTS OF *Staphylococcus aureus.*

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THESIS

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INTRODUCTION

Staphylococcus aureus is a gram-positive nonsporeforming member of the Micrococcaceae family which produces catalase, ferments mannitol, and can grow in the presence of ten percent sodium chloride (Kloos and Schleifer, 1984). *S. aureus* is a leading cause of nosocomial infections and is capable of causing a wide variety of diseases from simple skin infections to more life threatening diseases such as endocarditis (Waldvogel, 1995). In addition, this organism has the propensity to acquire resistances to most antibiotics (for review see Lyon and Skurray, 1987). Reports have shown that as many as 45% to 90% of health care workers are carriers of *S. aureus* (Casewell and Hill, 1986; Waldvogel, 1995). Furthermore, a report stated that 2.5% of hospital workers carried methicillin-resistant *S. aureus* (Reboli, 1990). Most methicillin-resistant strains of *S. aureus* are resistant to multiple antibiotics and pose a severe health hazard particularly for hospitalized persons (Boyce, 1997). These reasons mentioned above demonstrate the importance of *S. aureus* as a pathogen. Most likely the main reason *S.*

aureus is such a capable pathogen derives from the production of numerous proteins and toxins. This study was initially directed toward the identification of genetic systems which regulate the production of these proteins and toxins.

Diseases caused by *S. aureus* range from toxic food poisoning, furuncles, impetigo, and other skin infections to more life threatening diseases, such as, toxic shock syndrome, pneumonia, osteomyelitis, endocarditis, and meningitis (Waldvogel, 1995). Infections caused by *S. aureus* usually begin with a characteristic local abscess lesion. This lesion is the result of a breach in the skin and mucous membrane barriers, followed by penetration of *S. aureus* into the underlying tissue. If the organism is not contained within the lesion it can gain access to the lymphatic system and blood stream. This staphylococcal septicemia can then lead to more serious metastatic infections such as endocarditis, pneumonia, or osteomyelitis (Waldvogel, 1995). *S. aureus* is also a leading cause of osteomyelitis and endocarditis (Gentry, 1997; Ing et. al, 1997). In a survey of 103 adult osteomyelitis patients, *S. aureus* was the etiological agent in 50 to 70 percent of these cases (Cierny, 1990).

Likewise, Benn and colleagues determined that *S. aureus* was isolated most often from patients suffering from endocarditis (Benn *et. al.*, 1997). Furthermore, in a study of 300 patients with endocarditis, *S. aureus* was the etiological agent with the highest rate of endocarditis complications, which was defined as any clinically unfavorable event occurring during treatment (Mansur *et. al.*, 1992). Staphylococcal skin lesions and wound infections are best treated by debridement or with antiseptic solutions at the site of infection while staphylococcal bacteremia, osteomyelitis, endocarditis, and toxic shock syndrome are best treated with oral or intravenous antibiotics (Waldvogel, 1995).

Most strains of *S. aureus* isolated from nosocomial infections are resistant to a wide variety of antibiotics (Waldvogel, 1995). Vancomycin has been used for over 30 years to treat serious multiple antibiotic resistant clinical isolates presumably due to the bacterium's inability to acquire or become intrinsically resistant to the antibiotic (Kloos, 1997; Paulsen *et. al.*, 1997). However, a documented case last year described a *S. aureus* strain resistant to intermediate levels (8 microgram/ml) of vancomycin (Hiramatsu, 1997). This strain was isolated

from a 4 month old male infant recovering from heart surgery in Japan (Hiramatsu, 1997). Since then, two cases in the United States have been reported in which strains of *S. aureus* were found to also have reduced susceptibility to vancomycin (MIC = 8 microgram/ml) (MMWR, 1997). One strain was isolated in Michigan from a peritonitis patient, while another was isolated in New Jersey from a patient with staphylococcal bacteremia (MMWR, 1997).

S. aureus has been studied for over 100 years, and since then researchers have isolated and characterized over thirty extracellular and cell wall-associated proteins (for review see Iandolo, 1990). Most of these proteins are recognized as being involved in some aspect of disease (Iandolo, 1990). For example, toxic shock syndrome toxin-1 is the only element needed to cause symptoms of toxic shock syndrome (Todd, 1978). Other proteins enable the bacterium to colonize or evade host defense mechanisms. Protein A assists *S. aureus* in evading the host defense mechanisms by binding to the Fc portion of IgG while fibronectin and collagen binding proteins facilitate adherence to their respective extracellular matrix proteins, fibronectin and collagen (Sulica *et. al.*, 1979; Kuusela, 1978; Fröman *et. al.*, 1987; Patti *et. al.*, 1992). Undoubtedly,

staphylococcal disease involves the concerted action of multiple proteins and toxins, therefore researchers have directed their efforts to understanding the genetic regulators which control the expression of these factors.

There are two well characterized genetic systems which regulate the production of extracellular and cell wall-associated proteins in *S. aureus;* the accessory gene regulator (*agr*) (Recsei *et. al.,* 1986; Morfeldt *et. al.,* 1988; Peng *et. al.,* 1988) and the staphylococcal accessory regulator (*sar*) (Cheung *et. al.,* 1992; Cheung and Projan, 1994; Bayer *et. al.,* 1996). These two systems are involved with temporal regulation of a number of extracellular and cell wall-associated proteins (Recsei *et. al.,* 1986; Morfeldt *et. al.,* 1988; Peng *et. al.,* 1988). Temporal regulation describes the synthesis of surface proteins during the exponential phase of growth and the synthesis of toxins and exoenzymes during the stationary phase of growth (Iandolo *et. al.,* 1997). It is hypothesized that the exponential phase of growth corresponds to initial infection and multiplication of the organism in the host, and the stationary phase is similar to the stage of infection where the organism is crowded and releases its toxins and exoproteins in order to escape the

localized infection and colonize elsewhere (Projan and Novick, 1997). The *agr* locus demonstrates temporal regulation by up-regulating, in vitro, the expression of extracellular proteins such as alpha-toxin, beta-toxin, delta-toxin, protease, and nuclease, while the cell wall-associated proteins, fibronectin binding protein and protein A are down-regulated (Saravia-Otten *et. al.*, 1997; Recsei *et. al.*, 1986). The *agr* locus is transcribed as two divergent transcripts, RNAII and RNAIII, from their respective promoters, P2 and P3 (Peng *et. al.*, 1988; Janzon *et. al.*, 1989; Kornblum *et. al.*, 1990). The RNAII message contains four open reading frames; *agr*A - D, all of which have been shown to be involved in the autoregulation of *agr* (Novick *et. al.*, 1993). Although the RNAIII transcript contains the gene for delta-toxin, it is actually the transcript that is the effector molecule responsible for regulating the production of staphylococcal proteins and toxins (Kornblum *et. al.*, 1990; Janzon and Arvidson, 1990). RNAIII has been shown to regulate protein production at the transcriptional and translational levels (Novick *et. al.*, 1993).

The *sar* locus is similar in its regulation to *agr* in that it down-regulates the production of protein A and up-

regulating alpha and beta-toxin. However, instead of down-regulating the production of fibronectin-binding protein, *sar* up-regulates it (Cheung *et. al.*, 1994; Heinrichs *et. al.*, 1996; Cheung *et. al.*, 1992). In addition, several laboratories showed that a translated product from the *sar* locus, SarA, is a DNA binding protein and interacts with the *agr* locus and is necessary for complete *agr* expression (Cheung and Projan, 1994; Morfeldt *et. al.*, 1996; Chien and Cheung, 1998). This demonstrates a multi-regulator involvement for staphylococcal toxins and proteins.

The clinical importance of the two regulatory loci, *agr* and *sar*, has been demonstrated using animal models for endocarditis (Cheung *et. al.*, 1994), osteomyelitis (Abdelnour *et. al.*, 1993), arthritis (Abdelnour *et. al.*, 1993), and endophthalmitis (Booth *et. al.*, 1995). In these models *agr* or *sar* mutants were shown to be less virulent than the corresponding wild-type strains. In addition, experiments using a rabbit model of endocarditis demonstrated that an *agr/sar* double mutant was less virulent than either of the single mutants (Cheung *et. al.*, 1994).

Until recently, it was believed that a third distinct regulatory locus exists, encoding the extracellular protein

regulator (*xpr*), which was thought to be involved with exoprotein regulation in *S. aureus* (Smeltzer *et. al.*, 1992; Smeltzer *et. al.*, 1993). It was hypothesized that the *xpr* locus regulated the expression of alpha-toxin, delta-toxin, staphylococcal enterotoxin B, protease, and nuclease (Smeltzer *et. al.*, 1993). However, recent studies have demonstrated that the exoprotein deficient phenotype, observed in this *xpr* mutant, was the result of a point mutation in the *agr* locus (McNamara and Iandolo, 1998).

Since *S. aureus* produces a large number of extracellular and cell wall-associated proteins, it seems logical to believe that these proteins are regulated by many systems in a coordinate fashion. This could be in response to external and internal signals that result in a specific response to the environment. Evidence in support of a multiple-regulator involvement is provided by gel mobility shift assays involving the *sar* and *agr* loci (Hendrichs *et. al.*, 1996). Hendrichs and colleagues demonstrated that cellular extracts from an *sar* mutant would not retard the migration of the P2 promoter within the *agr* locus (Hendrichs *et. al.*, 1996). However, cellular extracts taken from the parent strain and an *sar* mutant complemented with a plasmid containing the *sar* locus would

retard the migration of the *agr* P2 promoter region (Hendrichs *et. al.*, 1996). Also in this study, Northern analysis revealed that the *sar* mutant reduced the levels of the *agr* transcripts, a plasmid containing the *sar* locus complemented this mutation (Hendrichs *et. al.*, 1996). More recently, SarA, a protein translated by the *sar* gene, was also shown to be a DNA-binding protein that binds between the *agr* promoters, P2 and P3, and activates *agr* transcription (Chien and Cheung, 1998). These data demonstrate how the *sar* locus regulates the *agr* locus. Because it has been shown that these two systems work together to regulate certain toxins and proteins, it is conceivable that additional genetic systems may be involved.

In an effort to find additional extracellular and cell wall-associated regulatory loci, a *S. aureus* S6C Tn*917* transposon mutant library was generated. *S. aureus* S6C was chosen because it is a hyperproducer of staphylococcal enterotoxin B, lipase, alpha-toxin, and beta-toxin (Gaskill and Khan, 1984; Smeltzer *et. al.*, 1993; Hart *et. al.*, 1993). Transposon mutants of *S. aureus* S6C were plated on sheep blood agar and colonies deficient in hemolysis activity were transferred to tributyrin plates and tested

for lipase activity. A *S. aureus* S6C Tn*917* mutant was isolated which lacked both hemolysin and lipase activity. Lipase and hemolysin activities were chosen because the hemolysin genes are located on the *Sma*I B and F fragments of the staphylococcal chromosome, while the lipase gene is located on the *Sma*I E fragment (Iandolo *et. al.*, 1997). Therefore, it was hypothesized that a mutant deficient in both hemolysin and lipase activities contained the transposon in a genetic region responsible for regulating these activities, and possibly other virulence factors.

The goals of this project were to determine whether or not the Tn*917* insertion occurred in the two known regulatory loci (*agr* or *sar*), use transduction to determine if the transposon insertion is the reason for the observed mutant phenotype, and clone a restriction fragment containing the locus where the insertion occurred. Data will be presented which suggests the transposon is most likely not the cause of the observed mutant phenotype and that the transposon did not interrupt any staphylococcal exoprotein regulatory loci.

When it became evident that the mutation was independent of the loss of hemolysin and lipase activities, the nature of the phenotype was investigated by examining

the parent strain, S6C. These studies revealed four phenotypic variants differing in hemolysis and catalase activity; wildtype hemolytic, catalase positive (NTH48); hemolytic, catalase negative (NTH50); nonhemolytic, catalase positive (NTH51); and nonhemolytic, catalase negative (NTH49). Because catalase is used to distinguish staphylococci from streptococci and thought to be involved in staphylococcal disease, I felt an investigation into the nature of these variants was warranted (Mandell, 1975).

Catalase is an enzyme produced in most aerobic bacteria and catalyzes the cleavage of hydrogen peroxide to water and oxygen (Schonbaum, 1976). Hydrogen peroxide is an oxidant that is lethal to staphylococcal cells (Lever and Sutton, 1996). One role of catalase is to degrade the toxic hydrogen peroxide molecule that is produced during aerobic growth conditions. This harmful byproduct must be dealt with and the catalase enzyme is the method most bacterial species employ. In *S. aureus*, catalase is also thought to promote evasion of host defense mechanisms (Mandell, 1975). This is hypothesized because, after the skin and mucous membranes of the host are breached, the next line of defense against staphylococcal cells are the phagocytes, especially polymorphonuclear leukocytes (PMN)

(Verhoef, 1997). After the bacterial cell is opsonized by antibodies and complement, the PMN engulfs the bacterial cell which triggers a cascade of events orchestrated to destroy the phagocytosed bacterium (Verhoef, 1997). One event, called the respiratory burst, begins by reducing O_2 to $\bullet O_2^-$ which dismutates to H_2O_2 (Verhoef, 1997). The H_2O_2 molecule can harm the engulfed bacterium in two ways; one, it is a powerful oxidant to biological materials and can react with the bacterial cell itself, and two, it can react with other molecules to give rise to more potent microbicidal agents (Verhoef, 1997). Therefore, by catalase deactivating H_2O_2, *S. aureus* could possibly survive within the phagocyte. Rogers and colleagues provided evidence that survival of staphylococcal cells within leukocytes may contribute to pathogenicity (Rogers *et. al.*, 1952). They incubated pathogenic and nonpathogenic strains of staphylococci with human leukocytes and monitored the survival of the bacteria (Rogers *et. al.*, 1952). The results revealed that pathogenic staphylococci survived much better than the nonpathogenic (Rogers *et. al.*, 1952). In support of the hypothesis that catalase can contribute to virulence, Mandell reported that low catalase producing staphylococci were less virulent in mice than high catalase

producing staphylococci (Mandell, 1975). Mandell generated a low-catalase producing mutant strain by incubating *S. aureus* Wood 46 with a subinhibitory amount of rifampicin. The virulence of the mutant was then compared to the parent strain Wood 46, a high-catalase producing strain. The comparison was done by mouse lethality experiments in which the high-catalase producing strain killed a greater percentage of mice than the low-catalase producing strain (Mandell, 1975). In addition, when exogenous catalase was added with the low-catalase producing strain, the results from the mouse lethality experiments were similar to the high-catalase producing strain (Mandell, 1975). However, rifampicin, the mutagen used in this experiment, is not specific in its mutagenesis and may have affected more than just the catalase system. Hence, it cannot be concluded that catalase assists *S. aureus* in virulence. This demonstrates the need for a study to prove or disprove the importance of catalase in staphylococcal virulence.

Because of what has been mentioned above about the role catalase may play in staphylococcal virulence, efforts were shifted to substantiate the hypothesis that catalase is a *S. aureus* virulence factor. Since the catalase gene of *S. aureus* has not been cloned, efforts of this study

were shifted to attempt to clone the catalase gene from *S. aureus*.

This thesis is presented in two parts; characterization of a *S. aureus* transposon mutant deficient in lipase and hemolysin activities and the attempt to clone the catalase gene from *S. aureus*. Because the transposon mutant was deficient in both hemolysin and lipase activities, it was hypothesized that the transposon inserted into a genetic locus responsible for the regulation of staphylococcal toxins and proteins. In this thesis it was clearly demonstrated that the transposon insertion site did not occur in either of the known regulators (*agr* and *sar*), the transposon was moved back into the parent strain by transduction, and the region containing the insertion site was successfully cloned. However, it was demonstrated that the transposon insertion was not responsible for the hemolysin and lipase deficient phenotype. Sequence analysis of the insertion revealed that the transposon inserted into one of six ribosomal RNA operons found in *S. aureus* (Wada *et. al.*, 1993). Since the transductants did not exhibit the same loss of hemolysin and lipase activity as the Tn*917* mutant, it was concluded that the transposon insertion is independent of the

observed phenotype. The isolation of hemolysin and catalase deficient variants from the parent strain, S6C, provides a source for the Tn*917* mutant's loss of hemolysin and lipase activity.

In order to further understand this catalase deficient phenomenon, frequencies of the four variants within *S. aureus* S6C (hemolytic, catalase positive; hemolytic, catalase negative; nonhemolytic, catalase positive; and nonhemolytic, catalase negative) were determined. In addition, the stability of each *S. aureus* S6C variation was tested and several clinical isolates were also examined for catalase and hemolytic deficiencies. There were no catalase deficiencies found in the *S. aureus* clinical isolates, however only a limited number of isolates were tested. To determine if this catalase variation is found within staphylococcal clinical isolates, more strains need to be tested.

Cloning the catalase gene was conceived because it was noted that the catalase gene from *S. aureus* had not been cloned and that catalase may play a role in staphylococcal virulence (Mandell, 1975). In order to demonstrate that catalase is a virulence factor in *S. aureus*, the first step is to clone the catalase gene. Until the catalase gene is

cloned, experiments cannot be conducted which isolate the importance of catalase in staphylococcal virulence. Regretfully, this thesis does not present data about successfully cloning the catalase gene from *S. aureus*. Polymerase chain reaction using degenerate primers designed from the catalase genes of *B. subtilis* and *E. coli* was chosen to isolate the staphylococcal catalase gene. The amplified fragments discussed in this thesis prove not be fragments of the *S. aureus* catalase gene.

Strain or plasmid	Source	Comments
Bacterial strains		
S. aureus		
S6C		Wild-type (hemolysis +, lipase +)
8325		Contains phi 11
Tn*917* mutant	This thesis	S6C with Tn*917* insertion (hemolysis +, lipase +)
NTH48	This thesis	Hemoloytic, catalase positive variant (S6C)
NTH49	This thesis	Nonhemolytic, catalase negative variant (S6C)
NTH50	This thesis	Hemolytic, catalase negative (S6C)
NTH51	This thesis	Nonhemolytic, catalase positive (S6C)
E. coli		
XL1-Blue	Stratagene	Highly transformable *E. coli* strain
Nova Blue	Novagen	Highly transformable *E. coli* strain
Plasmids		
pRCH	This thesis	Contains left-end of Tn*917* and the 1.5-kb *Hin*dIII chromosomal DNA from the *Tn917* mutant of *S. aureus* S6C
pRCP	This thesis	Contains left-end of Tn*917* and the 3.0-kb *PstI* chromosomal DNA from the *Tn917* mutant of *S. aureus* S6C
pTVT	John Iandolo, UOHSC	Contains Tn*917*
pT7 Blue (*agr*12)	Mark Smeltzer, UAMS	Contains the *agr* locus
pT7 + sar	Mark Smeltzer, UAMS	Contains the *sar* locus
pCR2.1	Invitrogen	TA cloning vector

Table 1. List of bacterial strains and plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage

S. aureus S6C is the parent strain used in the transposon mutagenesis experiments, which is cured of all plasmids and is a hyperproducer of enterotoxin B (Gaskill and Khan, 1988), lipase (Smeltzer *et. al.*, 1992), and several other extracellular proteins (Hart *et. al.*, 1993). Dr. Mark E. Hart generated the Tn917 mutant while a postdoctoral fellow at Kansas State University, Manhattan. The mutant was generated by transforming *S. aureus* S6C with the plasmid pTVT, (kindly provided by John J. Iandolo, University of Oklahoma Health Science Center, Oklahoma City) and incubating at 37°C. The plasmid, pTVT, contains the transposon, Tn*917*, which when introduced into staphylococcal cells transposes into the staphylococcal chromosome. Tn*917* contains a gram-negative origin of replication and an ampicillin resistance gene for replication and selection within gram-negative organisms. pTVT also contains a gram-positive temperature sensitive replicon which prohibits replication of the plasmid at the nonpermissive temperature of 43°C. Therefore the plasmid

is lost during cell division and the transposon within the chromosome is replicated along with the cell. The Tn*917* mutant was plated on 5% sheep blood (Remel, Lenexa, Kansas) and tributyrin (Eastman Kodak Co., Rochester, N.Y.) agar plates (Smeltzer *et. al.*, 1992) and found to be deficient in hemolysis and lipase activities. The mutant was maintained on tryptic soy agar (TSA; Difco Laboratories Detroit, Mich) plates containing 10 µg/ml of erythromycin (Sigma Chemical Co., St Louis, Mo.) and 10 µg/ml of tetracycline (Sigma Chemical Co.).

The plasmid pT7Blue(*agr*12) (Novagen, Madison, Wis) contains a PCR generated 1.75 kb fragment which was used to probe the entire *agr* locus (Kornblum *et. al.*, 1990). The plasmid pT7+*sar* (kindly provided by Mark S. Smeltzer, University of Arkansas for Medical Sciences, Little Rock) contains a 317 bp *sar* fragment, which was used as a probe to determine if the *sar* locus is intact in the mutant. Bacteriophage phi 11 was propagated from *S. aureus* 8325 and used to transduce the transposon mutation from the Tn*917* mutant into the parent strain S6C.

Culture maintenance

All strains were obtained and streaked onto TSA plates with appropriate antibiotics (if needed) and incubated

overnight at 37°C. Six milliliters of brain heart infusion broth (BHI; Difco Laboratories) containing 10% (v/v) glycerol (Fisher Scientific, Pittsburgh, Pa) were pipetted onto the plate and cells were scraped off the agar with an inoculating loop and dispensed into three sterile cryovial tubes. These were labeled and stored at -85°C. To obtain working cultures, ice scrapings from frozen -85°C cultures were streaked onto TSA plates with appropriate antibiotics. These plates were routinely stored at 4°C and wrapped in parafilm (to prevent drying) for up to two weeks.

Starter cultures

Twenty milliliters of sterile tryptic soy broth (TSB; Difco Laboratories) were pipetted into a sterile screw-capped 50-ml Erlenmeyer flask (flask to volume ratio of 2.5). The appropriate antibiotic was added if needed and bacteria were inoculated into the flask using a loopful of growth from the first quadrant of a less than two week old streak plate. Cultures were incubated at 37°C with rotary aeration (180 rpm) for 15-18 hours using a Model G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). These cultures were used to inoculate other batches of media for larger volumes of cultures.

Isolation of staphylococcal chromosomal DNA

The staphylococcal chromosomal DNA isolation procedure was based on those of Dyer *et. al.*, 1983. Briefly, a 20 ml starter culture of the desired *S. aureus* strain was prepared and poured into a 25 ml, screw-capped Corex centrifuge tube (Corning Inc., Corning, N.Y.). The cells were centrifuged at 8 - 10,000 x *g* using a model J-2K preparative centrifuge (Beckman Instruments, Inc., Fullerton, Calif) and JA-20 rotor (Beckman Instruments) at 4°C for 15 min, washed twice with 20 ml of TES (100 mM Tris, 78 mM disodium ethylenediaminetetracetic acid, tetrasodium salt [EDTA] and NaCl 150 mM, [pH = 7.5]), and then suspended in 5 ml of high salt TES (TES plus 2.5 M NaCl). Twenty-five microliters of a 10 mg/ml stock solution of recombinant lysostaphin (AMBI, Inc., Tarrytown, N.Y.) were added to the suspended cells and incubated for 30 minutes. Protoplast formation was checked by adding 40 µl of 2% sodium dodecyl sulfate (SDS) (Fisher Scientific) to 40 µl of the suspension. The protoplasts were incubated at 60°C for 15 minutes after which 0.5 ml of 20% sodium-N-lauroyl-sarcosinate (Sarkosyl) was added. Guanidine hydrochloride (Fisher Scientific) was added to yield a final concentration of 7.0 M, and incubated at 65°C while

rocking intermittently for 60 minutes. The suspension was pipetted using the mouth end of a 10-ml, glass pipette into centrifuge tubes containing 2 ml of 2.85 M cesium chloride (CsCl; Fisher Scientific) layered on top of 2 ml of 5.7 M CsCl. Preparations were centrifuged for 24 hours at 12-16,000 x g at 20°C using the SW-40Ti rotor (Beckman Instruments) and a model XL-70 Ultracentrifuge (Beckman Instruments). Using an 18-gauge needle, a hole was punctured in the bottom of the tube. The viscous fraction was collected in a 15-ml polypropylene conical centrifuge tube. The preparation was diluted by adding two volumes of deionized glass-distilled water, before pouring the entire preparation into a 25-ml, glass graduated cylinder containing approximately 25 ml of ice-cold, 100% ethanol. Chromosomal DNA was allowed to float to the surface of the ethanol and using a sealed Pasteur pipette, the DNA was collected and placed in a 1.5 ml sterile microcentrifuge tube containing 250-500 µl of TE (10mM Tris, 1mM EDTA [pH 8.0]).

Large scale isolation of staphylococcal plasmid DNA

A 20 ml starter culture of the desired *S. aureus* strain was grown for 15-18 hours and four, one ml portions were each used to inoculate four, 500-ml Erlenmeyer flasks

each containing 200 ml of TSB and the appropriate antibiotic if needed. The flasks were incubated at 37°C with rotary aeration (180 rpm) for 15-18 hours. Each 200-ml culture was transferred to a 250-ml polypropylene centrifuge bottle (Nalgene Co., Rochester, N.Y.) and centrifuged at 8-10,000 x *g* for 15 minutes at 4°C using the Beckman model JA14 rotor. The cells were washed twice with 200 ml of TES and each pellet was suspended in 9 ml of high-salt TES. Suspensions were combined as two, 18 ml suspensions in two, 125-ml sterile screw-capped flasks. Ninety microliters of a 10 mg/ml stock solution of recombinant lysostaphin (AMBI) was added to each suspension and incubated at 37°C for 30-60 minutes. Then 18 ml of 2% SDS was added to each suspension, gently mixed, and incubated on ice for at least 2 hours. Each lysate was transferred to a SW-28 centrifuge tube and centrifuged at 50,000 x *g* for 1 - 2 hours at 4°C using the SW-28Ti rotor (Beckman Instruments) and the model XL-70 ultracentrifuge (Beckman Instruments). The supernatants were poured into 50-ml graduated cylinders, and the DNA was precipitated by adding 0.1 volumes of 3.0 M sodium acetate and 0.6 volumes of isopropyl alcohol, and allowed to incubate at room temperature for 30 minutes. Each mixture was poured into a

25-ml, glass Corex centrifuge tube (Corning Inc.) and centrifuged at 12,000 x *g* for 30 minutes at room temperature. The supernatants were decanted and the pellets were allowed to air dry. The pellets were then suspended in 10 ml of TE (10mM Tris, 1mM EDTA [pH 8.0]) containing RNase A (20 µg/ml) (Sigma Chemical Co.) and incubated at room temperature for 30 minutes. Ten grams of cesium chloride and 0.8 ml of 10 mg/ml ethidium bromide (Fisher Chemical Co.) were added to each suspension. The suspensions were transferred to heat sealing centrifuge tubes, sealed, and loaded into the NVT65 rotor (Beckman Instruments) and centrifuged 402,000 x g 6-8 hours at 20°C using the model XL-70 ultracentrifuge (Beckman Instruments). The centrifugation produced two bands, the lower is the desired plasmid DNA. An 18-gauge needle was used to puncture a hole in the bottom of the tube and the plasmid DNA was collected in a 15-ml polypropylene conical centrifuge tube. The ethidium bromide was extracted from the DNA using several volumes of water-saturated N-butanol until the aqueous phase was colorless. Each aqueous phase was transferred to a 25-ml, Corex centrifuge tube (Corning Inc.) and the DNA was precipitated using 3 volumes of water and 2 volumes of room temperature isopropyl alcohol. The

sample was then centrifuged at 12,000 x *g* at room temperature for 20 minutes. The supernatant was poured off and the pellet suspended in TE (pH 8.0).

Large Scale isolation of *E. coli* plasmid DNA

Two, one-liter flasks containing sterile TSB were each inoculated with 13 ml of an overnight (15-18 h) *E. coli* starter culture. The two flasks were incubated for 15-18 hours at 37°C with rotary aeration (180 rpm) using an environmental incubator shaker. The cultures were poured into two, 250-ml polypropylene centrifuge bottles (Nalgene Co.), and centrifuged at 8-10,000 x *g* for 15-20 minutes using the Beckman JA 14 rotor in the model J-2K preparative centrifuge. The cells were washed with 100 ml of sterile, ice-cold deionized, glass-distilled water. The pellet was then suspended in 18 ml of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA [pH 8.0]) prior to the addition of 0.36 ml of a freshly prepared 50 mg/ml lysozyme (Sigma Chemical Co.) solution. This incubated at room temperature for 5 minutes, then 40 ml of Solution II [0.2 N NaOH (freshly diluted from a 10 N stock), 1% SDS] was added to the suspension. The bottle was inverted gently several times to mix the contents and stored on ice for 10 minutes, followed by the addition of 20 ml of ice-cold Solution III

(5 M potassium acetate). The contents were shaken until there was no longer two distinguishable liquid phases, then stored on ice for 10 minutes. The preparation was centrifuged and the supernatant was carefully poured into a 100-ml, glass graduated cylinder followed by the addition of 0.6 volumes of room temperature isopropyl alcohol. The sample preparation was mixed and dispensed into 30-ml, Corex centrifuge tubes (Corning, Inc.) and allowed to stand for 10 minutes at room temperature. The tubes were then centrifuged at 12,000 x *g* for 15-20 minutes at 4°C. The supernatant was poured off and the pellets were allowed to air dry. The pellet was then rinsed with 70% isopropyl alcohol and again allowed to air dry. Once dry, the pellet was suspended in 10 ml of TE (pH 8.0) prior to the addition of 20 µl of 10 mg/ml of RNase A (Sigma Chemical Co.). This incubated at room temperature for 30 minutes, then 0.8 ml of ethidium bromide (Sigma Chemical Co.) (10 mg/ml) and 10 grams CsCl was added. The preparations were centrifuged and plasmid bands procured as previously described.

Southern Analysis

Purification and labeling of fragments

Plasmids containing various DNA fragments used as probes were digested with the appropriate restriction endonucleases and the fragments were separated by electrophoresis through 0.8% low melting temperature agarose (FMC BioProducts, Rockland, Maine). The desired fragment was purified using the BioRad Prep-A-Gene DNA Purification System (BioRad Laboratories, Hercules, Calif.)

Purified fragments were labeled by random priming using digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, Ind.) and Klenow fragment (Promega Corp., Madison, Wis.)

Transferring of DNA and processing of membrane

Chromosomal DNA digested with the appropriate restriction endonucleases were electrophoresed through a 0.8% agarose (FMC BioProducts) gel. The DNA was depurinated by incubating the gel at room temperature in 0.25 N HCl for 15 minutes with agitation using a Belco orbital shaker (Belco Glass, Inc., Vineland, N.J.). The HCl was poured off and the gel was rocked gently in 0.5 N NaOH- 0.6 M NaCl for 30 minutes at room temperature. This solution was poured off and the gel was rocked gently again

for 30 minutes at room temperature in a solution of 1.5 M NaCl-0.5 M Tris-HCl, pH 7.5. The DNA was then transferred by passive diffusion onto neutral nylon (MagnaGraph; Micron Separation Inc., Westborough, Mass.) using 10X SSC (1.5 M sodium chloride, 1.5 M sodium citrate). The membranes were lifted off the gel and placed onto a glass plate and the DNA was UV crosslinked to the membrane using a BioRad Genelinker chamber using the Southern damp membrane program at 15 mJoules (BioRad, Laboratories). The DNA was denatured in 0.4 N NaOH for 30-60 seconds, followed by neutralization in 0.2 M Tris-HCl, pH 7.5, 2X SSC. The membranes were then transferred to 1.5" x 7" Magnabase (BioComp Instruments Inc., Fredericton, N.B., Canada) roller bottles followed by the addition of approximately 20 ml of a preheated (65°C) solution containing 1% SDS, 1 M NaCl, 10 % dextran sulfate (Fisher Scientific). The membrane was prehybridized by adding 0.2 ml denatured salmon sperm DNA (Sigma Chemical Co.) (10 mg/ml suspension sonicated to make DNA fragments) and incubated at 65°C for 1-2 hours in a Hybaid hybridization oven (National Labnet Co., Woodridge, N.J.) with rotisserie mixing. After prehybridization, 10 µl of the probe suspended in 100 µl of deionized glass-distilled water is denatured and added to

the appropriate membrane and allowed to incubate overnight at 65°C with rotisserie mixing. The hybridization solution was poured off and the membrane was washed twice with 50 ml of 2X SSC at room temperature for five minutes each with constant rotisserie agitation using a rotating magnetic plate (Navigator; BioComp Instruments, Inc.). The membrane was then washed twice with 50 ml of 2X SSC and 1.0% SDS for 30 minutes each at 65°C with constant rotisserie agitation in the hybridization oven. The membrane was washed twice with 50 ml of 0.1X SSC at room temperature for 30 minutes each with constant agitation. The membrane was then washed twice with 50 ml each of blocking buffer (0.2% sodium caseinate, 1X PBS [0.29 M Na_2HPO_4, 0.085 M $NaH_2PO_4 \cdot H_2O$, 0.34 M NaCl], 0.1% Tween-20) at room temperature for five minutes each with constant agitation, and then incubated at room temperature with constant agitation for 30 minutes with 50 ml of blocking buffer. The blocking buffer was poured off and the membrane was incubated with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) diluted 1:10,000 in 25 ml of Conjugate Buffer (0.2% sodium caseinate, 1X PBS), with constant agitation for 30 minutes. The membrane was washed once with blocking buffer, four times with wash

buffer (1X PBS, 0.3% Tween 20), and twice with assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$,) all at room temperature with constant agitation for five minutes each. The membrane was then transferred to a four-millimeter thick plastic bag (Consolidated Plastics Co., Inc., Twinsburg, OH) and sealed using electric impulse sealer (TEW Electric Heating Co.,). One milliliter of assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$) containing 5.5 µl of disodium 4-chloro-3-(methoxyspiro {1,2- dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.^{3,7}] decan}-4-yl) phenyl phosphate (CDP-*Star;* Boehringer Mannheim Corp.) substrate was added. The substrate was worked into the blot, and the bag was opened to allow the excess substrate to be removed from the bag. The bag was sealed again, and exposed to X-ray film (Fuji Medical Systems, Stamford, CT) for various times to achieve the apporpriate exposure. The film was developed using a Kodak M35A X-Omat precessor (Eastman Kodak Co.).

Transformation

Electroporation

Electrocompetent *E. coli* cells were prepared by first inoculating five, one-liter flasks each containing 200 ml of sterile TSB with 2 ml (1/100) of an overnight (15-18 h)

starter culture. Flasks were incubated at 37°C with rotary aeration (180 rpm). The optical density at 550 nm was taken every hour until the density of 0.5 - 1.0 was obtained. The flasks were chilled on ice for 15-30 minutes then transferred to four, 250-ml sterile centrifuge bottles (Nalgene, Co.). The cells were pelleted by centrifuging at 8-10,000 x *g* for 15 minutes, washed with 250 ml of sterile, ice-cold, deionized glass-distilled water and finally suspended in 125 ml of sterile, ice-cold, deionized glass-distilled water. Cell suspensions were combined as two, 250 ml portions and centrifuged again. Cell pellets were suspended in 10 ml of 10% glycerol and transferred to two, 25-ml, Corex centrifuge tubes (Corning Inc.) and centrifuged at 8-10,000 x *g* at 4°C for 15-20 minutes as previously discussed. Each cell pellet was suspended in one ml 10% glycerol, combined, and dispensed into sterile 0.6 microcentrifuge tubes as 200-µl aliquots. Cells were stored at -85°C until needed.

Electrocompetent *E. coli* XL1- Blue (Stratagene) or *E. coli* Nova Blue (Novagen) cells were thawed on ice and transformed by mixing 1-2 µl of DNA with 40-µl of cells. The cell/DNA mixtures were transferred to an electroporation cuvette (0.2-cm electrode gap; BioRad

Laboratories) and electroporated using a BioRad Gene Pulser II Apparatus (BioRad Laboratories). Settings were 25 microfaradays, 2.5 kilovolts, and 200 ohms which should yield a maximum time constant of five milliseconds. After electroporation, cells were immediately recovered in 1 ml of SOC [2% Bacto tryptone (Difco Laboratories), 0.5% Bacto yeast extract (Difco Laboratories), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose], and transferred to a sterile 12 x 75 mm Falcon tube (Fisher Scientific). Cells were incubated for 1 hour at 37°C with rotary aeration (225 rpm) before 100-µl portions were plated on TSA plates containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

Cloning the Tn*917* transposon insertion site

*Hin*dIII or *Pst*I restriction endonuclease digestions were set up with 10 µg of *S. aureus* Tn*917* mutant chromosomal DNA. Chromosomal fragments were resolved by electrophoresis through 0.8% low melting temperature agarose (FMC BioProducts). *Hin*dIII fragments of 3.0 - 5.0 kb and *Pst*I fragments of 4.5 - 6.5 kb were excised from the gel and purified separately using the BioRad Purification kit (BioRad Laboratories). The range of purified fragments was verified by again running a small portion of the

preparation through a 0.8% agarose gel. The purified fragments were ligated by incubating overnight at 14°C using five µl of purified restriction fragments, two µl ligation buffer (10X), one µl T4 DNA ligase (Promega), and 12-µl deionized, glass-distilled water. Two microliters of the ligated mixture were electroporated into electrocompetent *E. coli* XL1- Blue cells (Stratagene) as described previously. The transformants were plated onto TSA plates containing 100 µg/ml ampicillin (Sigma Chemical Co.).

Transduction

Isolation of phi 11

The bacteriophage phi 11 was isolated by growing an overnight (15-18 h) starter culture of *S. aureus* 8325, which contains the prophage phi 11, then transferring the culture to a 25-ml, Corex centrifuge tube (Corning Inc.) and centrifuging for 15-20 minutes at 8-10,000 x *g* at 4°C. The phage-containing supernatant was filter sterilized through a 0.2 µm filter membrane using a 20-ml syringe, and collected into a sterile, 15-ml polypropylene conical centrifuge tube.

Propagation of phage

Propagation was performed using the above isolated phi 11 preparation. Five tubes containing tryptic soy soft agar (TSB + 0.5% agar, 4 ml/ tube in 16 x 125 mm tubes) equilibrated to 45°C were each inoculated with 20-µl of an overnight starter culture of the *S. aureus* strain to be infected. Portions (10, 50, 100, or 200 µl) of the crude phage suspension were added to each tube and gently mixed by vortexing. Each suspension was poured on top of freshly solidified TSA plates containing 0.005 M $CaCl_2$. The soft agar was allowed to solidify, then the plates were incubated overnight at 37°C in an upright position. The plate containing the largest number of plaques which was not totally cleared by the phage was chosen for harvesting. To harvest the phage, four ml of sterile TSB were added to the plate and a glass rod was used to loosen the soft agar from the agar underneath. The soft agar and TSB were drawn up using a 10-ml, sterile glass pipette and expelled back into the plate. This was repeated several times until the soft agar was homogenized. The homogenized suspension was transferred to a 15-ml, glass Corex centrifuge tube (Corning Inc.) and centrifuged using the JA-20 (Beckman Instruments) rotor at 2740 x g at room temperature for 20

minutes. The supernatant was filter sterilized through a 0.2-µm filter membrane using a 5-ml syringe into a 15-ml, conical polypropylene tube. The phage preparation was then tested for sterility by spreading 0.2 ml of the phage onto a predried, brain heart infusion agar (BHIA; Difco Laboratories) plate containing 0.5 g/liter of sodium citrate, and incubated overnight at 37°C.

Phage were also propagated using the procedure described by Novick *et. al.* (Novick, 1991). Ten milliliters of sterile TSB in a 50-ml flask were inoculated with an overnight (15-18 h) starter culture of the appropriate *S. aureus* strain to an OD_{550} of approximately 0.05. The culture was incubated at 37°C with rotary aeration (180 rpm) until an OD_{550} of 1.0 was achieved. To a sterile 50-ml flask, 1.25 ml of the culture was added to 1.25 ml phage buffer (0.001 M $MgSO_4$, 0.004 M $CaCl_2$, 0.05 M Tris, pH 7.8, 5.9 g/liter NaCl) and 2.25 ml of the isolated phage (9.7×10^7 plaque-forming units/ ml). The suspension incubated overnight at 30°C with rotary aeration (180 rpm). The infective lysate was transferred to a 1.5-ml microcentrifuge tube (Fisher Scientific) and centrifuged at room temperature for 10 minutes at 16,250 x g using a microcentrifuge (IEC, Needham heights, Mass.). This was

then filter sterilized and tested for sterility as described previously in the first propagation method.

Determining the titer of phage preparations

In order to determine the titer of the phage preparation, portions were serially-diluted ten-fold with sterile TSB. A 100-µl aliquot of each dilution was transfered to a tube containing soft agar followed by the addition of 20-µl of an overnight culture of *S. aureus* 8325-4. *S. aureus* 8325-4 was chosen because ϕ11 originated in this strain. The tubes were gently vortexed and the suspensions poured onto a freshly solidified TSA plates containing 0.005 M $CaCl_2$. The plates were incubated overnight at 37°C, and plaques were counted in order to determine the plaque forming units per ml (pfu/ml).

Transduction

The transduction was performed on the hemolytic, catalase positive variant of *S. aureus* S6C (NTH48) using a phage lysate harvested from the mutant strain. A 20 ml starter culture of *S. aureus* S6C-HC was grown overnight in a 50-ml flask and diluted to approximately 10^8 cells per ml. Then, 0.2 ml of 0.05 M $CaCl_2$ was added to the flask. Two, one ml aliquots of this suspension were pipetted into two

separate sterile 50-ml flasks; one, a transduction flask and the other a control flask. One milliliter of ϕ11, propagated from the Tn*917* mutant (described previously), with a concentration of approximately 10^8 pfu/ml, and 2 ml TSB containing 0.005 M $CaCl_2$ were pipetted into the transduction flask. Then 3 ml of TSB containing 0.005 M $CaCl_2$ was pipetted into the control flask. This was incubated for 20 minutes at 35°C while shaking vigorously at 225 rpm. Two milliliters of ice-cold, 0.02 M sodium citrate was added to the flask prior to transfering the mixtures to two, separate 25-ml Corex centrifuge tubes and centrifuged at 2750 x g for 15 minutes with the JA-20 rotor at 4°C using the Beckman model J-2K centrifuge. The supernatants were decanted and each pellet suspended in 1 ml ice-cold, 0.02 M sodium citrate. One hundred microliters of the suspended pellets were plated onto TSA plates containing 15 µg/ml erythromycin, 10 µg/ml tetracycline, and 500 mg/liter sodium citrate. The plates were incubated at 37°C until transductants appeared.

tetracycline, and 500 mg/liter sodium citrate. The plates were incubated at 37°C until transductants appeared.

Comparison of sequence from Tn*917* insertion clones

The clones which contain the left end of the transposon, Tn*917*, and the adjacent mutant chromosomal DNA were sequenced in an effort to ascribe an identity. A primer (5′ CAC AAT AGA GAG ATG TCA CCG 3′)was designed from the left-ended sequence of Tn*917* (Shaw *et. al.*, 1985) such that the adjacent mutant chromosomal DNA was sequenced. The sequence was generated by Alan Geis at Kansas State University using automated sequencing. All six of the open reading frames in the sequence was then compared to known gene sequences using the BLAST sequence similarity search database maintained by the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nig.gov.).

Analysis of hemolysis and catalase activity

The catalase and hemolytic activities of *S. aureus* S6C, the four S6C variants, and *S. aureus* clinical isolates were tested by first growing the desired organism in a 20 ml TSB starter culture. The cells were then transfered to a 50-ml, screw-capped conical tube, sonicated at 50% power for 10 seconds using a Fisher model F50 Sonic Dismembrator

(Fisher Scientific), and serially diluted ten-fold using sterile deionized glass-distilled water. The diluted cells were plated in duplicate using 200-µl of the 10^{-7} dilution tube, 100-µl of the 10^{-6} dilution tube, and 50-µl of the 10^{-6} dilution tube onto TSA plates containing 5% sheep blood and incubated overnight at 37°C. Plating the cultures this way provided a range of dilutions in which two plates would have a sufficient number of colonies to test. The colonies were then analyzed for hemolysis of sheep blood by determining if there was a clearing of blood cells around individual colonies. The colonies were also tested for catalase activity by dropping 3% hydrogen peroxide on the individual colonies. The colonies were positive for catalase activity if effervescence was detected.

Cloning the catalase gene from *Staphylococcus aureus*

Primer design

Forward and reverse degenerate primers, CatF2 and CatB3, respectively were constructed using the deduced amino acid sequence of the catalase genes (*katE)* from *E. coli* (von Ossowski *et. al.,* 1991) and *Bacillus subtilis* (Engelmann *et. al.,* 1995) with a bias for codons used in *S. aureus*.. The two catalase gene sequences were compared and the forward primer (CatF2) was constructed using the *E.*

coli sequence from 1187 to 1208 bp and the *B. subtilis* sequence from 781 to 1002 bp. The reverse primer (CatB3) was constructed using the homology between the *E. coli* sequence from 2035 to 2059 bp and *B. subtilis* sequence from 1629 to 1653 bp. The two oligonucleotides were generated by Integrated DNA Technologies, Inc., Coralville, Iowa as follows: CatF2-- 5' CCG GA(A/G) CG(C/T) (A/G)TT GT(G/T) CAT GC(A/G) 3' and CatB3-- 5' GAA (A/C)A(A/G) (A/C)CG (C/T)CC CTG CAA (C/T)AG CGG 3'.

Amplification by polymerase chain reaction

Amplification of possible catalase fragments was performed with the forward and reverse primers, CatF2 and CatB3 using the Opti-Prime PCR Optimization Kit (Stratagene) which gives 12 different pH, magnesium chloride, and potassium chloride conditions to optimally amplify PCR fragments. The instructions were followed exactly for buffer determination by mixing 400-µl of sterile deionized glass-distilled water, 12.5-µl Master Mix 50X buffer, 6.25-µl 10X dNTP (5mM of each NTP), 7.9-µl CatF2 (40 nmoles/ml), 7.0-µl CatB3 (40 nmoles/ml), 125-µl *S. aureus* 8325-4 chromosmal DNA, and 6-µl Taq polymerase (Promega Biotech) at 5 U/µl. Five microliters of buffers #1-12 (12 different conditions with respect to pH, magnesium chloride, and potassium chloride) were pipetted

into 12 separate 0.6 ml microcentrifuge tubes and 45-µl of the above mixture was pipetted into each of the 12 microcentrifuge tubes. The tubes were mixed gently and placed a model PTC-100-60 programmable thermal cycler (MJ Research, Inc., Watertown, Mass.). The polymerase chain reaction amplification was done using the following touchdown program: Step 1- 94°C for 1 minutes, Step 2- 92_ for 30 seconds, Step 3- 45°C for 40 seconds (minus 0.5°C per cycle), Step 4- Goto step 2, 19 times, Step 5- 92°C for 30 seconds, Step 6- 35°C for 40 seconds (plus 1 second per cycle), Step 7- Goto step 5, 19 times, Step 8- 72°C for 7 minutes. After the amplification was performed, the PCR mixtures were electrophoresed through 0.8% LE agarose gels to determine size and appearance of products.

PCR products generated using CatF2 and CatB3 primers

After amplification of DNA, the PCR products were ligated into pCR2.1 (Invitrogen Corp., Carlsbad, Calif.) as described by the manufacturer. Electrocompetent NovaBlue cells (Novagen) were mixed with 1-2 µl of the above ligation mixture, incubated on ice for approximately 1 minute, and electroporated as previously described. Cells were then plated on TSA plus 100 µg/ml ampicillin plates. These plates were incubated at 37°C until transformants appeared and transferred to fresh TSA plus 100 µg/ml

ampicillin plates. Plasmid DNA was isolated from the transformants using the BioRad Purification kit (BioRad Laboratories). Plasmids isolated and pCR2.1 without an insert were electrophoresed through 0.8% agarose (FMC Bioproducts). Sizes of plasmids isolated from tranformants were compared to the size of pCR2.1 to determine if the transformant contained an insert. Plasmid DNA containing an insert was then digested with *Eco*RV, which excises inserts from pCR2.1, and electrophoresed through a 0.8% agarose as previously described. This will determine the size of the insert within pCR2.1.

RESULTS

This research was initially directed at characterizing the insertion site of a transposon mutant strain of *S. aureus* S6C. This Tn*917* mutant lacked both hemolysin and lipase activities which suggested an insertion in a regulatory element needed for these activities. After careful evaluation, it was discovered that the transposon insertion was independent of the observed mutant phenotype. Once it was determined that Tn*917* was not the reason for the loss of hemolysin and lipase activity, the nature of this phenotype was investigated. When overnight broth cultures of *S. aureus* were diluted and plated on sheep blood agar, several of the isolated colonies were nonhemolytic. Upon further examination of *S. aureus* S6C, it was also discovered that a number of the hemolytic and nonhemolytic colonies were also deficient in catalase activity. In all, four variants of *S. aureus* S6C were procured; hemolytic, catalase positive (NTH48); hemolytic, catalase negative (NTH50); nonhemolytic, catalase positive (NTH51); and nonhemolytic, catalase negative (NTH49). Therefore, the results section of this thesis is divided

into two parts; characterization of the *S. aureus* Tn*917* mutant deficient in hemolysin and lipase activities and analysis of the catalase and hemolysin variation in *S. aureus* S6C.

Part I. Characterization of a *S. aureus* Tn*917* mutant deficient in hemolysin and lipase activity

Characterizing the transposon insertion site. Chromosomal DNA isolated from *S. aureus* S6C and its corresponding Tn*917* mutant was digested with either *Hin*dIII or *Pst*I. *Hin*dIII and *Pst*I were chosen because they cleave within the transposon approximately 2.8-kb from the left-end (Figure 1). This left-end contains a gram negative ampicillin resistance marker and origin of replication both of which were utilized later on in this study to clone a portion of the chromosomal region where the transposon insertion occurred (Figure 1). The *Hin*dIII and *Pst*I chromosomal digestions of parent and mutant strains were analyzed by Southern analysis using a 2.8-kb *Eco*RI fragment isolated from the left-end of the transposon as a probe. Results from these experiments revealed one hybridizing band from

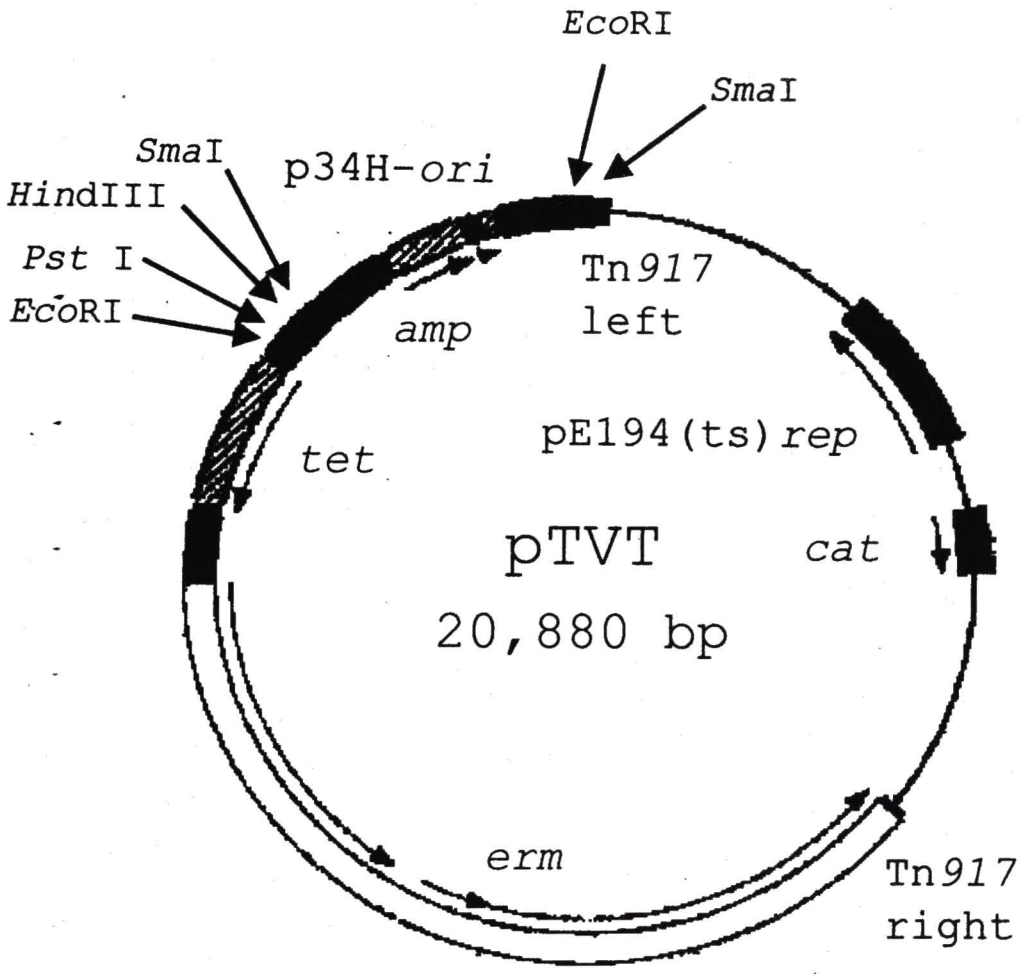

Figure 1. Plasmid pTVT with key endonuclease restriction sites and functions utilized in generating transposon mutations and subsequent insertion site cloning. Resistance genes for tetracycline (tet), ampicillin (amp), erythromycin (erm), and chloramphenicol (cat) as well as the gram-positive, temperature-sensitive origin of replication (pE194[ts]rep) and the gram-negative origin of replication (p34H-*ori*) are denoted.

each digestion for the mutant, while DNA from the parent strain did not produce any detectable hybridizing bands (Figure 2). The band observed from the *Hin*dIII digested DNA from the mutant strain was approximately 4.3-kb while the *Pst*I digested DNA yielded a band of approximately 5.8-kb (Figure 2). These data indicate the transposon was not present in the parent strain and the insertion occurred only once in the chromosome of the mutant. In addition, the results from these experiments estimated the relative size of the staphylococcal chromosomal fragment that extends from the left-end of the transposon to the respective *Hin*dIII or *Pst*I restriction site in the chromosome (Figures 1 and 2). The estimated size of the *Hin*dIII and *Pst*I staphylococcal chromosomal fragments was 1.5-kb and 3.0-kb, respectively (Figures 1 and 2).

Southern analysis was used to determine if the Tn*917* mutant contained the transposon in either of the known regulatory loci, *agr* or *sar*. Chromosomal DNA isolated from *S. aureus* S6C and the Tn*917* mutant strains was digested with *Eco*RV and probed with a PCR-generated, 1.75-kb *agr* fragment (Figure 3). *Eco*RV-digested DNA of wild-type *S. aureus*

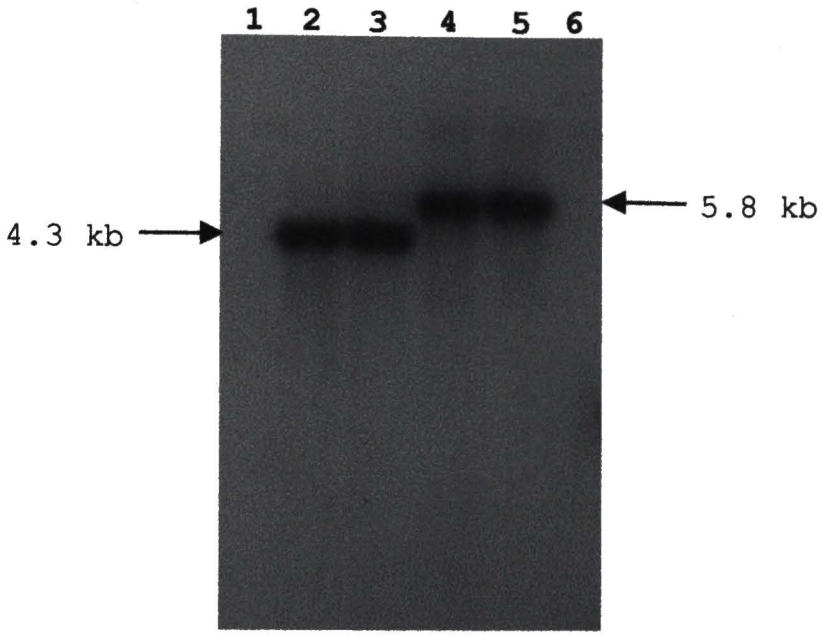

Figure 2. Southern analysis of *S. aureus* S6C and its corresponding Tn*917* mutant strain for the presence of Tn*917*. Lanes 1 and 6 are S6C digested with *Hin*dIII and *Pst*I respectively, lanes 2 and 3 are the Tn*917* mutant strain digested with *Hin*dIII, and lanes 4 and 5 are the Tn*917* mutant strain digested with *Pst*I. Digested DNAs were probed with the 2.8 kb *Eco*RI fragment of the left end of Tn*917* (see Figure 1).

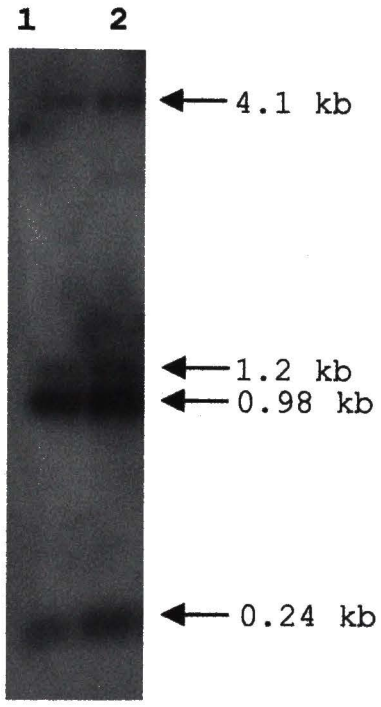

Figure 3. Southern analysis of *S. aureus* S6C (lane 1) and its corresponding Tn*917* mutant strain (lane 2) for the presence of an intact *agr* region. Chromosomal DNA was digested with *Eco*RV and probed with a PCR-generated, 1.75 kb *agr* fragment.

strains generates four characteristically hybridizing *agr* bands of 4.1, 1.2, 0.98 and 0.24-kb (Kornblum *et. al.*, 1990). The probe hybridized to 4.1, 1.2, 0.98 and 0.24-kb *Eco*RV fragments for both the Tn*917* mutant and *S. aureus* S6C DNA (Figure 3). Because this banding pattern is indicative of an intact wild-type *agr* locus, the Tn*917* transposon did not occur in the *agr* locus (Kornblum *et. al.*, 1990).

In addition, chromosomal DNA isolated from the parent and mutant strain and digested with *Bam*HI and *Acc*I was probed with a 317-bp PCR internal fragment of *sar* (Figure 4). If the *sar* locus is intact, a single 2.5-kb fragment should be observed (Cheung and Projan, 1994). The 2.5-kb fragment was observed for both the wildtype *S. aureus* S6C and the Tn*917* mutant strains. Based on these data, the Tn*917* transposon did not insert in either of the known regulators of extracellular and cell wall-associated protein expression, thus suggesting that the insertion occurred in a unique locus responsible for expression of hemolysin and lipase.

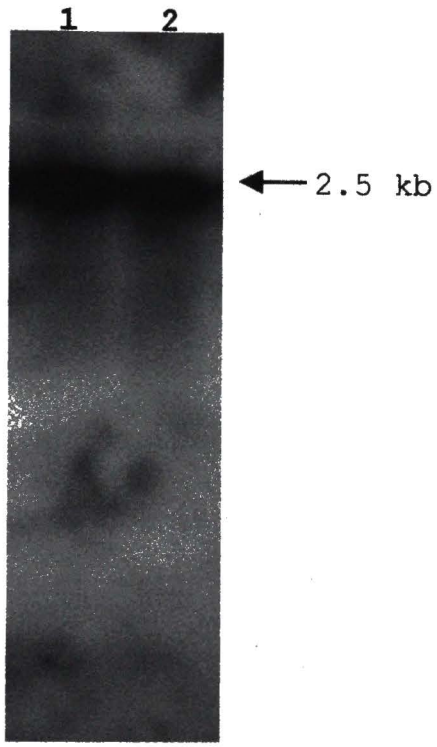

Figure 4. Southern analysis of *S. aureus* S6C (lane 1) and its corresponding Tn*917* mutant (lane 2) for the presence of an intact *sar* region. Chromosomal DNA was digested with *Bam*HI and *Acc*I and probed with a PCR-generated, 317 bp internal *sar* fragment.

Isolation and identification of the Tn*917* insertion site. Chromosomal DNA isolated from the mutant was digested using either *Hin*dIII or *Pst*I. Chromosomal digests were resolved through a 1.0 %, low temperature agarose gel and *Hin*dIII and *Pst*I fragments corresponding to 4.3-kb and 5.8-kb, respectively were excised and purified away from the agarose matrix. Purified fragments were ligated using T4 DNA ligase and used to transform *E. coli* by electroporation. Colonies exhibiting resistance to ampicillin were analyzed for the presence of plasmids that would correspond to the circularized left-end Tn*917* region and either the *Hin*dIII or *Pst*I chromosomal region where the transposon insertion occurred. Plasmids isolated from these ampicillin resistant colonies were digested with *Sma*I and separated on 0.8% agarose gel (Figure 5). *Sma*I was used to excise the insertion site chromosomal DNA because there are two *Sma*I cut sites located within the left-end of Tn*917;* one at the distal end of the left end of Tn*917* and the other just inside *Hin*dIII and *Pst*I cut sites in Tn*917* (Figures 1 and 6). Two positive clones, pRCP-1 and pRCP-2, generated from the *Pst*I ligation mixture, demonstrated *Sma*I fragment sizes of 2.8-, 2.4-, and 0.6-kb (Figure 5).

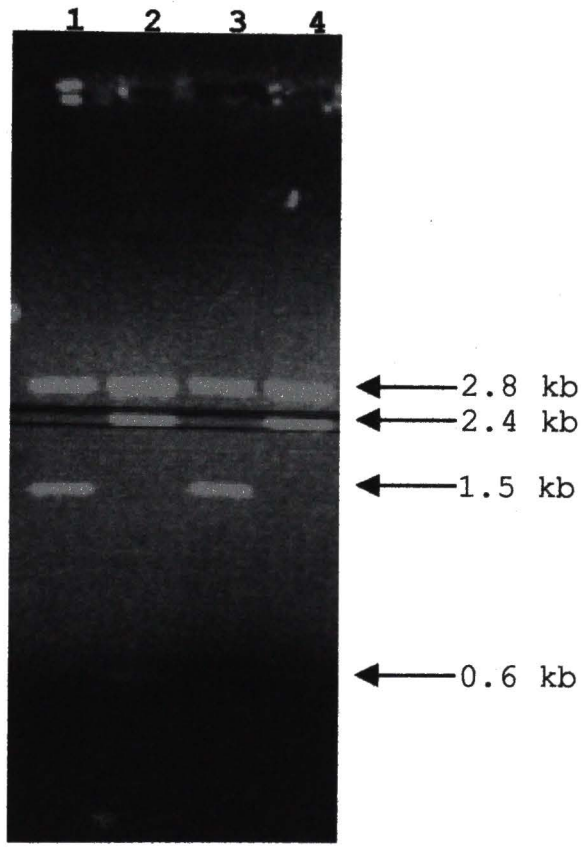

Figure 5. *Sma*I restriction endonuclease map of Tn*917* insertion clones pRCH-1 (lane 1), pRCP-1 (lane 2), pRCH-2 (lane 3), and pRCP-2 (lane 4). Plasmids were isolated, digested with *Sma*I, and resolved by electrophoresis through 0.8 % agarose and visualized by ethidium bromide staining.

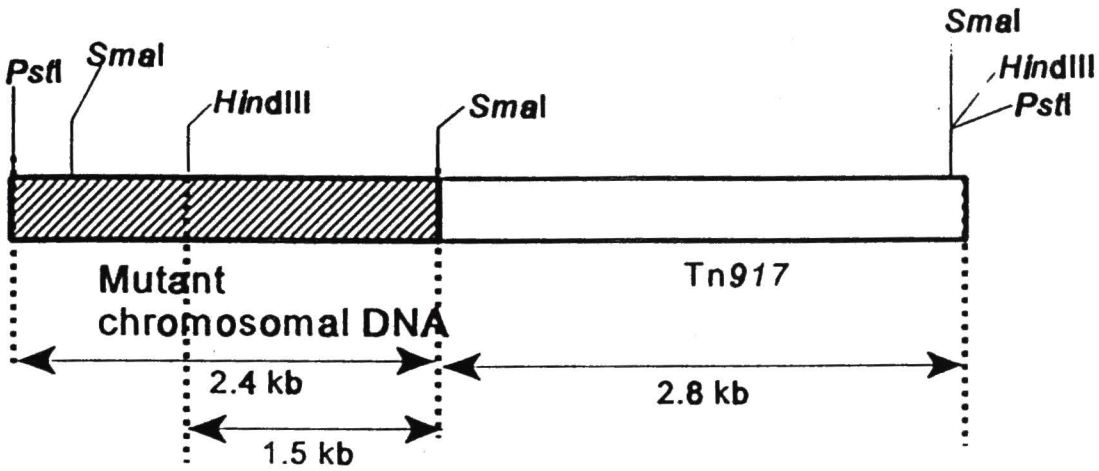

Figure 6. Schematic of the left-end of Tn*917* with the adjacent mutant chromsomal DNA.

Clones isolated from the *Hin*dIII ligation mixture, pRCH-1 and pRCH-2, demonstrated fragments of 2.8- and 1.5-kb (Figure 5). The 2.8-kb fragment observed in all clones represents the *Sma*I fragment located within the left end of *Tn917* and verifies the presence of the transposon insertion within these clones (Figures 1 and 6). The presence of the transposon insertion is further verified because the sum of the *Sma*I fragments in pRCP and pRCH is 5.8- and 4.3-kb respectively, which is equal to the *Pst*I and *Hin*dIII restriction fragments identified to contain the transposon insertion by Southern analysis (Figures 2 and 5). Since there are three fragments generated from the pRCP *Sma*I digestions, it was also concluded that the *Pst*I chromosomal fragment contained one of the approximately seventeen mapped *Sma*I chromosomal restriction sites known to exist in *S. aureus* (Iandolo *et. al.*, 1997).

In order to clone the Tn*917* insertion site, chromosomal DNA from the parent and mutant strains was digested with either *Hin*dIII or *Pst*I and probed with the *Sma*I chromosomal fragment isolated from either pRCH-1 or pRCP-1, respectively. It was anticipated that the respective probes would hybridize to either a single *Pst*I

or *Hin*dIII fragment where the transposon inserted. Surprisingly, chromosomal DNA from the parent strain digested with *Pst*I had hybridizing bands that corresponded to fragment sizes of 6, 7, 9, 10, and one greater than 13 kb, while chromosomal DNA from the mutant strain exhibited the same banding pattern as the parent minus the greater than 13-kb hybridizing band (Figure 7). Likewise, DNA from the parent strain digested with *Hin*dIII demonstrated hybridizing bands of approximately 3, 4, 4.9, 7, 10, and one greater than 13 kb, while the mutant strain exhibited the same banding pattern minus the 7-kb band (Figure 8). These data were surprising because a single restriction fragment was predicted, identifying the region containing the transposon insertion. Because of this the chromosomal region of the transposon insertion site was sequenced.

Using the known sequence of Tn*917*, a primer was designed, and the chromosomal DNA region within pRCH and pRCP was sequenced. A Blast search of the sequenced chromosomal DNA region identified the transposon insertion site to have a high degree of similarity to the staphylococcal ribosomal RNA DNA sequences (Figure 9).

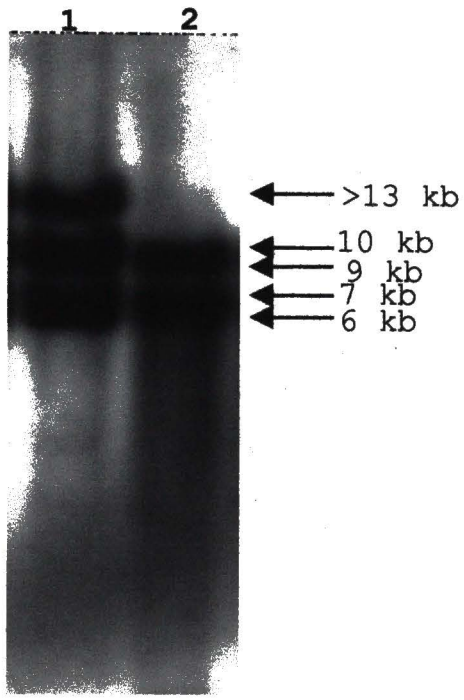

Figure 7. Southern analysis of *S. aureus* S6C (lane 1) and its corresponding Tn*917* mutant (lane 2) for the presence of a *Pst*I fragment containing the Tn*917* insertion site. Chromosomal DNAs were digested with *Pst*I and probed with the *Sma*I chromosomal fragment of pRCP-1.

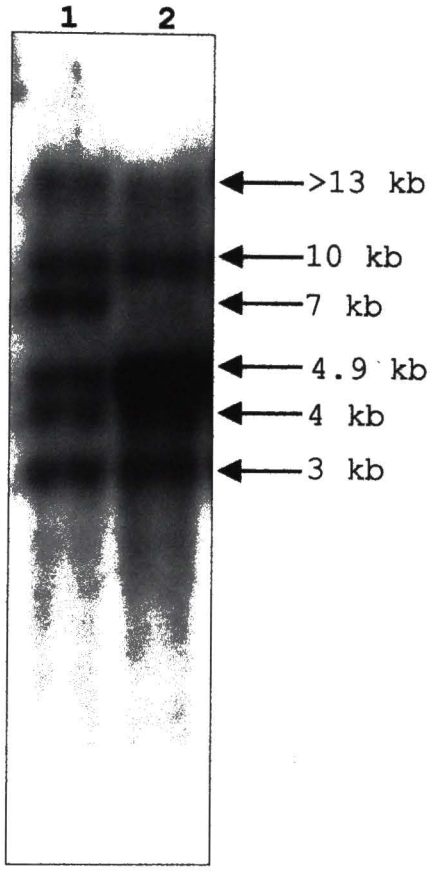

Figure 8. Southern analysis of *S. aureus* S6C (lane 1) and its corresponding Tn*917* mutant(lane 2) for the presence of a *Hin*dIII fragment containing the Tn*917* insertion site. Chromosomal DNAs were digested with *Hin*dIII and probed with the *Sma*I chromosomal fragment of pRCH-1.

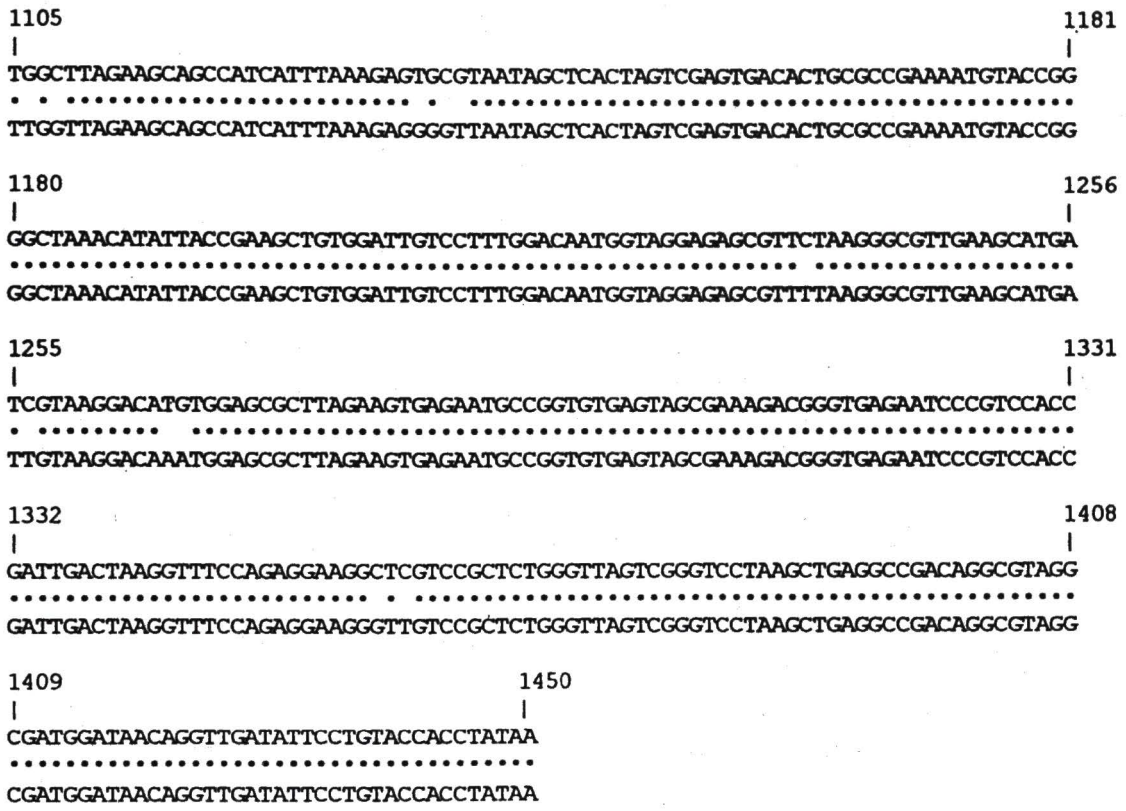

Figure 9. Comparison of the 23s *S. aureus* rRNA sequence (top) with the transposon insertion sequence (bottom).

Upon investigation of the literature, it was found that *S. aureus* chromosomal DNA digested with either *Hin*dIII or *Pst*I and probed with a 23S rRNA probe revealed hybridizing *Hin*dIII bands of 5.5, 6, 8, 9.2, and one greater than 13 kb and *Pst*I bands of 3.1, 4, 4.7, 7, 9, and one greater than 13 kb (Wada *et. al.*, 1993). The banding pattern described by Wada *et. al.*(1993), is similar to the banding pattern in the Southern analysis above using the transposon insertion site as a probe. Based on these data, I concluded Tn*917* inserted into one of the six rRNA DNA operons.

Phenotypic analysis of transductants containing the Tn*917* mutation. Given the above data which suggests the transposon inserted into one of six rRNA DNA operons it is unlikely that the mutation is responsible for the observed phenotype of the Tn*917* mutant. However, if the transposon and the region of insertion can be moved back into the parent strain with the subsequent loss of hemolysin and lipase activities, then in all probability the transposon insertion into one of the rRNA operons accounted for the observed phenotype. Using the bacteriophage phi 11, a transducing lysate of the Tn*917* mutant was generated and

used to move the transposon back into the parent strain, S6C. Forty transductants exhibiting resistance to erythromycin and tetracycline were recovered from the transduction. All forty transductants were hemolytic on 5% sheep blood agar and exhibited lipase activity on tributyrin agar. Two representative transductants were used to verify, by Southern analysis, the presence of the transposon. The 2.8-kb Tn*917* *Eco*RI fragment was used again as a probe against chromosomal DNA isolated from the transductants and digested with *Hin*dIII or *Pst*I (Figure 10). The probe hybridized to 4.3-kb *Hin*dIII and 5.8-kb *Pst*I fragments as expected. These data indicate that the chromosomal region containing the transposon was successfully moved from the Tn*917* mutant into the parent strain by transduction. The mutant phenotype (loss of hemolysin and lipase activities) was not observed in any of the transductants, indicating that the loss of hemolysin and lipase activity was not due to the insertion of Tn*917* into one of six rRNA DNA operons of *S. aureus* S6C.

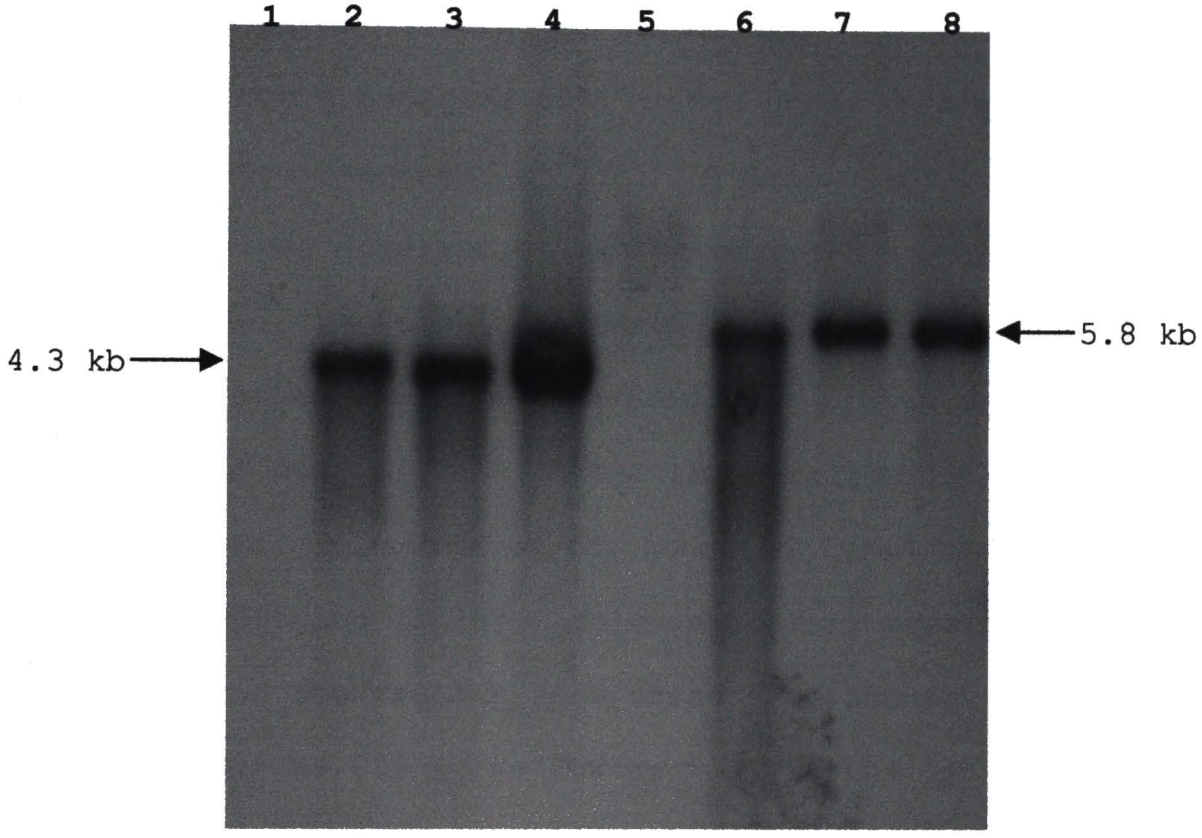

Figure 10. Southern analysis of *S. aureus* S6C (lanes 1 and 5) its corresponding Tn*917* mutant (lanes 2 and 6), and two representative transductants (lanes 3,4,7, and 8). Chromosomal DNAs were digested with either *Hin*dIII (lanes 1-4) or *Pst*I (lanes 5-8) and probed with the 2.8 kb, *Eco*RI fragment of Tn*917*.

Part II. Characterization of the *S. aureus* S6C catalase and hemolysin variants

Analyzing the frequency of hemolysin deficient colonies within the parent strain, *S. aureus* S6C. *S. aureus* S6C was streaked from a negative 85°C frozen stock culture onto TSA and incubated overnight at 37°C. A loopful of bacteria from the first quadrant of growth was used to inoculate 20 mls of TSB. The inoculated broth was incubated overnight at 37°C, serially diluted ten-fold, and plated onto 5% sheep blood agar plates. After an overnight incubation at 37°C, plates were observed for zones of lysed red blood cells surrounding isolated colonies. This experiment was repeated and the results from these experiments indicated that as much as 11% to as few as 1.3% of the isolated colonies were deficient in hemolytic activity, which was defined as hemolytic zones of clearing significantly smaller than that of the wildtype (Table 2). The wildtype *S. aureus* S6C hemolytic colonies demonstrated hemolytic zones of clearing with a radius of approximately 3mm. To ensure that these nonhemolytic colonies were *S. aureus*, each was Gram stained, tested for their ability to grow in

Experiment	Hemolytic	Nonhemolytic
One	98.7.0% (148/150)	1.3% (2/150)
Two	89.0% (89/100)	11.0% (11/100)

Table 2. The percentage of hemolytic and catalase variation within a stock culture of *S. aureus* S6C. Of the 150 colonies examined in experiment 1, 34 were deficient in catalase activity and of the 100 colonies examined in experiment 2, 17 were deficient in catalase activity.

the presence of 7.5% sodium chloride, ferment mannitol, and produce coagulase and catalase. These nonhemolytic colonies were all identified as gram-positive cocci in grapelike clusters that grew in the presence of 7.5% sodium chloride, fermented mannitol, and produced coagulase however, one of the colonies did not demonstrate catalase activity when 3% hydrogen peroxide was applied. Surprisingly when the hemolytic colonies were tested for catalase activity approximately 20% of these colonies did not effervesce when 3% hydrogen peroxide was applied (Table 2). Hence, four phenotypic variants were isolated and procured; wild-type hemolytic, catalase positive (NTH48); hemolytic, catalase negative (NTH50); nonhemolytic, catalase positive (NTH51); and nonhemolytic, catalase negative (NTH49). To verify that these colonies were *S. aureus,* plate cultures were submitted to the Microbiology Laboratory at Osteopathic Medical Center of Texas for identification. Each variant was used to inoculate a Positive Combination Type 6 Panel (Dade International Inc., West Sacramento, California). Panels were incubated for 16-24 hours at 35°C before being read by a Microscan Walkaway automated instrument (Dade International, Inc.). All four variants exhibited an identical biochemical (18

separate tests) and antimicrobial susceptibility (18 different antibiotics) pattern identifying each variant as *S. aureus* with a 99.9% probability.

Determining the stability of catalase and hemolytic activities in the four *S. aureus* S6C variants. In these experiments, each of the *S. aureus* S6C variants (NTH 48-51) was used to inoculate a flask containing 20-ml TSB. The cultures were incubated overnight (15-18 hrs) at 37°C and used to inoculate a second set of flasks at a 1/1000 dilution. In addition, a portion of each overnight culture was serially diluted 10-fold and plated on 5% sheep blood agar. Each variant was inoculated in this manner for a total of five consecutive overnight transfers. Each day a portion was plated as described and the hemolytic and catalase activities were determined. It is normally inappropriate to determine catalase activity on blood agar plates, due to the catalase activity found in red blood cells. However, *S. aureus* S6C produces such a strong and rapid effervescence when tested with 3% hydrogen peroxide, I determined that the catalase positive and catalase negative colonies of *S. aureus* S6C could be easily distinguished on blood agar plates.

The inoculation of the first 20-ml flask of TSB was performed in one of two ways. In one set of experiments, a loopful of bacteria from the first quadrant of growth was used to inoculate the broth culture and in the second set of experiments an isolated colony with the appropriate variant phenotype was used to inoculate the broth culture. Using an isolated colony to begin the test is more appropriate for determining the stability of the variants because when inoculation is performed with a loopful of bacteria, most likely all variant phenotypes are represented in the inoculum. However, the experiment using a loopful of bacteria revealed some useful data (Tables 3-6).

In most cases, results using a loopful of bacteria for inoculation reveal data very similar to that obtained when an isolated colony was used for inoculation. For example, only one hemolytic colony was isolated from the nonhemolytic catalase positive variant when either an isolated colony or a loopful of bacteria was used (Tables 4 and 8). However, the hemolytic, catalase positive and hemolytic, catalase negative variants generated high percentages of nonhemolytic colonies when using a loopful of bacteria for inoculation. The hemolytic, catalase

Table 3. Nonhemolytic colonies isolated from NTH48 and NTH50.

	Number of nonhemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	21/144 (14.6)	15/134 (11.2)	29/157 (18.5)	52/157 (33.1)	144/259 (55.6)
Hemolytic, catalase negative (NTH50)	5/122 (4.1)	35/149 (23.5)	45/119 (37.8)	48/ 98 (49.0)	97/145 (66.9)

Table 4. Hemolytic colonies isolated from NTH51 and NTH49.

	Number of hemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Nonhemolytic, catalase positive (NTH51)	0/218 (0)	0/309 (0)	0/526 (0)	1/359 (0.3)	0/741 (0)
Nonhemolytic, catalase negative (NTH49)	0/276 (0)	0/282 (0)	3/497 (0.6)	0/648 (0)	1/265 (0)

Tables 3 and 4. 20 mls of TSB was inoculated with a loopful of bacteria of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for hemolytic activity.

Table 5. Catalase negative colonies isolated from NTH48 and NTH51.

	Number of catalase negative colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	0/144 (0)	0/134 (0)	0/157 (0)	0/157 (0)	1/259 (0.4)
Nonhemolytic, catalase positive (NTH51)	0/218 (0)	0/309 (0)	0/526 (0)	0/359 (0)	0/741 (0)

Table 6. Catalase positive colonies isolated NTH49 and NTH50.

	Number of catalase positive colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase negative (NTH50)	0/122 (0)	0/149 (0)	0/119 (0)	0/98 (0)	0/145 (0)
Nonhemolytic, catalase negative (NTH49)	0/276 (0)	0/282 (0)	1/497 (0.2)	0/648 (0)	0/265 (0)

Tables 5 and 6. 20 mls of TSB was inoculated with a loopful of bacteria of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for catalase activity.

positive variant generated nonhemolytic colony percentages of 14.6, 11.2, 18.5, 33.1, and 55.6 over a 5-day period, respectively, while the hemolytic, catalase negative variant generated nonhemolytic colony percentages of 4.1, 23.5, 37.8, 49.0, and 66.9 over a 5-day period, respectively (Table 3). These data indicate that the hemolytic variants will generate more nonhemolytic colonies over a 5-day period when the initial inoculation begins with a loopful of bacteria versus an isolated colony. These data suggest that the nonhemolytic colonies are already present in the frozen *S. aureus* S6C stock culture used to isolate these four variants.

Results from stability studies using an isolated colony revealed the variants are very stable (Tables 7-10). This experiment was repeated for a second time to ensure the validity of the results (Tables 11-14). In the first experiment the hemolytic variants proved to be somewhat stable, with the greatest percentage of nonhemolytic colonies isolated, within a given day, being 1.3% (Table 7). Also the nonhemolytic variants proved to be very stable with only one hemolytic colony isolated out of a total of 3885 colonies tested within a five day period (Table 8). The catalase positive variants revealed some

Table 7. Nonhemolytic colonies isolated from NTH48 and NTH50.

	Number of nonhemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	1/324 (0.3)	0/476 (0)	0/127 (0)	0/218 (0)	0/144 (0)
Hemolytic, catalase negative (NTH50)	0/154 (0)	0/131 (0)	0/207 (0)	3/284 (1.1)	3/225 (1.3)

Table 8. Hemolytic colonies isolated from NTH49 and NTH51.

	Number of hemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Nonhemolytic, catalase positive (NTH51)	0/864 (0)	0/624 (0)	1/941 (0.1)	0/1019 (0)	0/437 (0)
Nonhemolytic, catalase negative (NTH49)	0/694 (0)	0/348 (0)	0/658 (0)	0/593 (0)	0/590 (0)

Tables 7 and 8. 20 mls of TSB was inoculated with a single isolated colony of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for hemolysin activity.

Table 9. Catalase negative colonies isolated from NTH48 and NTH51.

	Number of catalase negative colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	0/324 (0)	0/476 (0)	7/127 (5.5)	24/218 (11.0)	6/144 (4.2)
Nonhemolytic, catalase positive (NTH51)	0/864 (0)	0/908 (0)	0/1401 (0)	0/1238 (0)	0/437 (0)

Table 10. Catalase positive colonies isolated from NTH50 and NTH49.

	Number of catalase positive colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase negative (NTH50)	0/154 (0)	0/131 (0)	0/207 (0)	0/284 (0)	0/225 (0)
Nonhemolytic, catalase negative (NTH49)	0/694 (0)	0/348 (0)	0/658 (0)	0/593 (0)	0/590 (0)

Tables 9 and 10. 20 mls of TSB was inoculated with a single isolated colony of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for catalase activity.

Table 11. Nonhemolytic colonies isolated from NTH48 and NTH50.

	Number of nonhemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	0/225 (0)	0/129 (0)	0/169 (0)	0/118 (0)	0/112 (0)
Hemolytic, catalase negative (NTH50)	0/296 (0)	0/161 (0)	0/132 (0)	0/468 (0)	0/208 (0)

Table 12. Hemolytic colonies isolated from NTH51 and NTH49.

	Number of hemolytic colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Nonhemolytic, catalase positive (NTH51)	0/268 (0)	1/843 (0.1)	0/574 (0)	0/418 (0)	0/586 (0)
Nonhemolytic, catalase negative (NTH49)	0/774 (0)	0/617 (0)	0/586 (0)	0/589 (0)	0/611 (0)

Tables 11 and 12. 20 mls of TSB was inoculated with a single isolated colony of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for hemolytic activity.

Table 13. Catalase negative colonies isolated from NTH48 and NTH51.

	Number of catalase negative colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase positive (NTH48)	0/225 (0)	0/129 (0)	0/169 (0)	0/118 (0)	0/112 (0)
Nonhemolytic, catalase positive (NTH51)	0/268 (0)	0/843 (0)	0/574 (0)	0/418 (0)	0/586 (0)

Table 14. Catalase positive colonies isolated from NTH50 and NTH49.

	Number of catalase positive colonies/ total number (%)				
Variant	Day 1	Day 2	Day 3	Day 4	Day 5
Hemolytic, catalase negative (NTH50)	0/296 (0)	0/161 (0)	0/132 (0)	0/468 (0)	0/208 (0)
Nonhemolytic, catalase negative (NTH49)	0/774 (0)	0/617 (0)	0/586 (0)	0/589 (0)	0/611 (0)

Tables 13 and 14. 20 mls of TSB was inoculated with a single isolated colony of the respective variant, incubated overnight at 37°C, serially diluted, and plated onto sheep blood agar plates. The plates were incubated overnight at 37°C and colonies were analyzed for catalase activity.

higher percentages of colonies isolated that were catalase negative (5.5%, 11.0%, and 4.2%) which could indicate that the catalase activity has a greater propensity to mutate to catalase negative than the hemolytic activity (Table 9). The catalase negative variant proved to be very stable with no colonies demonstrating catalase activity over a 5-day period (Table 10). The second time this experiment was repeated for each variant over a period of 5-days, all of the variants were very stable (Tables 11-14). In fact, only one colony had a different phenotype than the variant being tested. This particular hemolytic colony was isolated from the nonhemolytic, catalase positive variant (Tables 11-14). These data indicate that most of these variants are stable over a 5-day period, except for the wild-type hemolytic, catalase positive that generated some catalase negative colonies over the 5-day period.

Hemolytic and catalase variation within *S. aureus* clinical isolates. Since the variation was noticed in a laboratory strain of *S. aureus*, namely S6C, I decided to test several *S. aureus* strains isolated from clinical settings. This analysis will reveal if this phenomenon occurs in disease-causing *S. aureus* strains that have not been cultured and

transferred as extensively as laboratory strains. Since catalase and hemolytic activities may prove to be more prevalent in a particular staphylococcal disease, five *S. aureus* isolates from different patients with various types of staphylococcal diseases were analyzed for catalase and hemolysin deficient colonies. These strains were isolated from patients suffering from staphylococcal septicemia, respiratory infection, tissue infection, and wound abscesses. Isolates were streaked onto a TSA plates from -85°C frozen stock cultures and incubated overnight at 37°C. A loopful of bacteria was used to inoculate 20 mls of TSB which was incubated overnight at 37°C. These were then serially diluted 10-fold and plated onto 5% sheep blood agar plates and approximately 200 colonies from each of the five isolates were tested. Only one isolate, which was isolated from the respiratory sputum of a patient, was found to have any variation (Table 15). Of the 239 colonies tested of this isolate only 2 were found to be nonhemolytic (Table 15). Although the number of clinical isolates examined was small, it appears that variation in hemolysin and catalase activities is a rare event and may indicate a phenomenon unique to *S. aureus* S6C.

Clinical isolate	Nonhemolytic colonies/ Total tested	Catalase negative colonies/ Total tested
Wound Tissue infection	0/ 530	0/ 166
Respiratory Sputum	2/ 239	0/ 247
Wound Abcess	0/ 222	0/ 222
Blood, Venous	0/ 530	0/ 166
Wound Tissue infection	0/ 293	0/ 293

Table 15. Analysis of *Staphylococcus aureus* clinical isolates. A loopful of bacteria from the clinical isolate was used to inoculate 20 mls of TSB which was incubated overnight at 37°C, serially diluted ten-fold and plated onto 5% sheep blood agar plates. The plates were incubated overnight at 37°C and examined for hemolytic and catalase activity.

Attempting to clone the *S. aureus* catalase gene. Since it has been hypothesized that catalase is an important staphylococcal virulence factor (Mandell, 1975) and the *S. aureus* catalase gene has not been cloned, I decided to clone the catalase gene from *S. aureus*. Catalase may be an important staphylococcal virulence factor (Mandell, 1975) because hydrogen peroxide is an oxidant used to destroy phagocytosed bacterial cells, and if *S. aureus* can defend itself by inactivating hydrogen peroxide it has a better chance of surviving the oxidative burst within the phagocyte and thus surviving in the host. Using the known catalase amino acid sequences (*katE*) from *E. coli* and *B. subtilis* (Ossowski *et. al,* 1991; Engelmann *et. al.,*1995) a search for regions of homology was performed. Regions identified were used to generate a set of oligonucleotide primers with a *S. aureus* codon bias. Both *E. coli* and *B. subtilis* are known to contain two or more peroxidases that exhibit activity for hydrogen peroxide however, I chose to use the hydrogen peroxide-specific peroxidase known as catalase which is encoded in both genera by the *katE* gene (Ossowski *et. al,* 1991; Engelmann *et. al.,* 1995). A PCR was designed in which the denaturing temperature was 92°C, the annealing temperature was between 35-45°C, and the

extension temperature was 72°C for seven minutes for a total of 19 cycles. From this reaction, two fragments of 850-bp and 710-bp were amplified from chromosomal DNA isolated from *S. aureus* 8325-4, the laboratory strain of *S. aureus* used to generate the chromosomal map of *S. aureus* (Iandolo *et. al.*, 1997). Based on the known sequence of the *katE* genes of *B. subtilis* and *E. coli*, these primers should amplify catalase fragments from *E. coli* and *B. subtilis* chromosomal DNA of approximately 870-bp. Since the 850 and 750-bp fragments generated from *S. aureus* 8325-4 chromosomal DNA were very similar in size to the predicted catalase fragments from *B. subtilis* and *E. coli*, I believed these two fragments might contain a portion of the *S. aureus* catalase gene. Each of the two PCR products (850 and 710-bp) were gel purified and cloned. To verify the fragment inserted into the vector, plasmids were isolated and digested with *Eco*RI and analyzed by gel electrophoresis. Plasmids were isolated from clones containing the 850- and 710-bp fragments and the fragments were sequenced. A Blast search of the sequences generated from the 850- and 710-bp PCR fragments revealed that the sequences had very little similarity with any other known gene sequences. It was anticipated that one of these PCR

fragments would have similarity to a catalase gene sequence of another species, which would indicate that the amplified fragment might be a portion of the *S. aureus* catalase gene. However, since the 850- and 710-bp PCR fragments shared very little similarity with any other gene sequences, I determined that the 850- and 710-bp PCR products do not contain a portion of the *S. aureus* catalase gene.

In order to identify restriction fragments which contain the catalase gene in *S. aureus*, the degenerate PCR primers designed from the *katE* gene sequences of *E. coli* and *B. subtilis* were used to amplify catalase gene fragments from *E. coli* and *B. subtilis* chromosomal DNA. The PCR products from these reactions were the anticipated size for fragments amplified from the *katE* gene of *E. coli* and *B. subtilis*. The two fragments were labeled and used as probes against staphylococcal chromosomal DNA isolated from *S. aureus* 8325-4 and S6C digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I. In order to make the experimental parameters less stringent, the Southern analysis procedure was modified in these two experiments by lowering the hybridization temperature to 45°C. It was anticipated that these probes would identify chromosomal fragments containing the *S. aureus* catalase gene. However, the

results revealed no hybridizing bands for any staphylococcal chromosomal DNA, therefore no chromosomal restriction fragments containing the *S. aureus* catalase gene were identified (data not shown).

Using the catalase forward and reverse degenerate PCR primers as probes in a Southern analysis, I attempted to identify *S. aureus* restriction fragments which contain the catalase gene. Chromosomal DNA isolated from *S. aureus* 8325-4 was digested with various restriction endonucleases (*Apa*I, *Ava*I, *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Sal*I, *Sca*I, *Sph*I, *Xba*I, and *Xho*I). The results using the catalase PCR primers did not produce any restriction fragments that were suspected to be catalase gene fragments (data not shown). It was anticipated that the catalase PCR primers would identify restriction fragments containing the *S. aureus* catalase gene which could then be cloned.

DISCUSSION

S. aureus is a human and animal pathogen capable of causing a wide variety of diseases. One reason *S. aureus* is such a capable pathogen is its ability to produce over thirty extracellular and cell wall-associated proteins, most of which are recognized as being involved with some aspect of disease (Iandolo, 1990). Two distinct genetic loci have been shown to regulate the production of extracellular and cell wall-associated proteins in *S. aureus;* the accessory gene regulator (*agr*) (Recsei *et. al.*, 1986; Morfeldt *et. al.*, 1988; Peng *et. al.*, 1988) and the staphylococcal accessory regulator (*sar*) (Cheung *et. al.*, 1992; Cheung and Projan, 1994; Bayer *et. al.*, 1996). *S. aureus agr* and *sar* mutants used in animal models for staphylococcal disease have been useful tools in demonstrating the clinical importance of *agr* and *sar* (Abdelnour *et. al.*, 1993; Cheung *et. al.*, 1994; Booth *et. al.*, 1995). Additional data indicate that the *agr* and *sar* loci interact and that the *sar* locus may regulate the *agr* locus (Hendrichs *et. al.*, 1996; Chien and Cheung, 1998). Since these two systems work in a coordinate fashion to

regulate staphylococcal proteins, it is possible that additional genetic systems may be involved with the regulation of staphylococcal extracellular and cell wall-associated proteins. The initial goal of this thesis was to characterize a Tn*917* transposon mutant deficient in hemolysin and lipase activity. Since the hemolysin and lipase genes are located on different *Sma*I fragments of the *S. aureus* chromosome, it was anticipated that the transposon inserted into a novel genetic region responsible for the regulation of these exoproteins and other extracellular and cell wall-associated proteins of *S. aureus*.

To establish the role of this locus as a regulator I set out to characterize the region where the Tn*917* insertion occurred. This was done by first determining if the transposon inserted into either of the known regulators, *agr* or *sar*, isolating chromosomal restriction fragments containing the transposon insertion site, and determining whether or not the mutation generated by the transposon was the reason for the loss of hemolysin and lipase activity.

The transposon, Tn*917*, did not insert into either *agr* or *sar* in the Tn*917* mutant. Southern analysis was performed on chromosomal DNA isolated from the parent and Tn*917* mutant strains using a probe specific for the staphylococcal regulatory loci, *agr* and *sar*. These experiments demonstrated restriction banding patterns characteristic of intact *agr* and *sar* loci (Kornblum *et. al.*, 1990 and Cheung and Projan, 1994) indicating that the transposon did not insert in either of the known regulators but possibly inserted into a novel genetic locus responsible for the regulation of hemolysin and lipase activities.

The Tn*917* mutant did not contain the transposon in a novel regulatory locus; it inserted into one of six staphylococcal rRNA DNA operons. The transposon insertion clones generated, pRCH1 and pRCP1, which contain the left-end of Tn*917* and a portion of the transposon insertion site were sequenced. The sequence was compared to known gene sequences, using a Blast search, and the results revealed that the insertion site sequence had a high degree of similarity to staphylococcal rRNA DNA sequences. In addition, the restriction endonuclease profiles of *S. aureus* chromosomal DNA probed with the Tn*917* insertion site

were similar to the profiles observed for staphylococcal chromosomal DNA using a rRNA probe (Wada, 1995). Since it is unlikely that a transposon insertion in one of six rRNA DNA operons would account for the loss of hemolysin and lipase activity, I decided to move the mutation back into the parent strain using the staphylococcal bacteriophage ϕ11. If the mutant phenotype co-transduced with Tn*917* then the mutation in the rRNA DNA operon would most likely represent a novel means of regulating hemolysin and lipase activity. However, transduction experiments yielded approximately forty transductants resistant to erythromycin (encoded on the transposon) yet exhibited wild-type phenotypes for hemolysin and lipase. This demonstrated that in all probability the transposon insertion for the loss of hemolysin and lipase activities in the Tn*917* mutant.

In an effort to determine the reason for the *S. aureus* Tn*917* mutant's loss of hemolysin and lipase activity the parent strain, *S. aureus* S6C, was examined. Stock culture collections in the laboratory are maintained as frozen cell suspensions stored at 85°C. Frozen cells are routinely scraped with a sterile inoculating loop and streaked for isolation on TSA plates. No colonial variants were observed for *S. aureus* S6C when streaked for isolation in

this manner. However, when *S. aureus* was streaked for isolation on sheep blood agar plates several isolated colonies exhibited a nonhemolytic phenotype. Upon further analysis, four *S. aureus* S6C variants were isolated; hemolytic, catalase positive (wild-type); hemolytic, catalase negative; nonhemolytic, catalase positive; and nonhemolytic, catalase negative. At this time it is not known why these variants appear however, the occurrence of a nonhemolytic variant of S6C suggests a possible source for the Tn*917* mutant that exhibited a loss of hemolysin and lipase activity.

The extracellular protein regulator (*xpr*) locus, first described by Smeltzer *et. al.*, (1992, 1993) was thought to be a staphylococcal global regulatory locus. This *xpr* locus was identified by isolating a Tn*551* transposon mutant of *S. aureus* S6C with reduced levels of hemolysin and lipase activity as well as other extracellular proteins (Smeltzer *et. al.*, 1993). The transposon insertion site was localized to the 670-kb *Sma*I fragment of S6C generally known as the A fragment (Smeltzer *et. al.*, 1992) and designated as Ω1058. Because the *xpr* mutant demonstrated decreased amounts of lipase, alpha-toxin, delta-toxin, staphylococcal enterotoxin B (SEB), protease, and nuclease

(Smeltzer *et. al.*, 1992; Smeltzer *et. al.*, 1993) it was believed that the Tn*551* insertion was the reason for the reduced levels of extracellular proteins. Previous attempts to complement the mutation using DNA from the *xpr* locus were unsuccessful (McNamara and Iandolo, 1998). However complementation using DNA from the *agr* locus was successful (McNamara and Iandolo, 1998). Because complementation was successful with DNA from the *agr* locus, McNamara and Iandolo (1998) sequenced the *agr* locus in the *xpr* mutant. These data revealed a frameshift mutation within the *agr* locus (McNamara and Iandolo, 1998). Also, transduction of the Tn*551* insertion from the *xpr* mutant into the parent strain, S6C, yielded approximately 50% of the transductants with the same loss of extracellular protein activity (McNamara and Iandolo, 1998). If the transposon insertion were truly the reason for the *xpr* mutant's loss of exoprotein activity, then 100% of the transductants would demonstrate a loss of exoprotein activity. Based on these data, McNamara and Iandolo (1998) concluded that the loss of extracellular protein activity in the *xpr* mutant is not the result of a Tn*551* insertion into the region known as *xpr* but due to a frameshift mutation within *agr* locus.

The Tn*917* mutant generated in our laboratory could be the result of a mutation within the *agr* locus similar to what was discovered in the *xpr* mutant for several reasons. The Tn*917* and *xpr* mutants were both deficient in hemolysin and lipase activities (Smeltzer *et. al.*, 1993). In addition, the *S. aureus* S6C strain which I isolated the four hemolytic and catalase variants and the S6C strain used to generate the Tn*917* mutant is from the same lineage as the -85°C frozen stock used to generate the *xpr* mutant. Therefore, it is possible that some of the nonhemolytic variants isolated in this thesis are the result of a mutation within the *agr* locus.

From the transductants generated by moving the *xpr* mutation into the parent strain *S. aureus* S6C only 50% were exoprotein deficient. McNamara and Iandolo (1998) decided to determine if this was specific to S6C by transducing the *xpr* mutation into two other *S. aureus* strains; Wood 46 and 8325-4. None of the transductants generated from the other strains produced exoprotein deficient colonies (McNamara and Iandolo, 1998). Since 50% of the transductants generated with S6C are exoprotein deficient and no exoprotein deficient transductants were generated from Wood 46 and 8325-5, McNamara and Iandolo (1998) concluded that

the transduction event induces a mutation in the *agr* locus and that it only happens in the S6C background. Data in this thesis indicate that these exoprotein deficient mutants can be isolated from the *S. aureus* S6C stock culture and is independent of a transduction event.

In summation, the Tn*917* mutant contains a transposon insertion in one of six staphylococcal rRNA DNA operons. After careful evaluation it is believed that the insertion is not responsible for the Tn*917* mutant's phenotype (loss of hemolysin and lipase activities). Given the study of McNamara and Iandolo (1998) it is believed that the loss of hemolysin and lipase activities in the Tn*917* mutant might be the result of a spontaneous mutation within the *agr* region. Data presented in this thesis and McNamara and Iandolo (1998) indicate that this mutation occurs in the S6C background and can be isolated from frozen stock cultures of *S. aureus* S6C.

In the process of isolating the nonhemolytic *S. aureus* S6C variant we isolated additional variants deficient in catalase activity. Four variants were isolated based on their hemolytic and catalase activities; hemolytic, catalase positive (NTH48); hemolytic, catalase negative (NTH50); nonhemolytic, catalase positive (NTH51); and

nonhemolytic, catalase negative (NTH49). Stability studies using isolated colonies of each variant revealed very little variation with respect to catalase and hemolysin activity thus it appears that the four *S. aureus* S6C variants are stable. Since the Tn*917* mutant and the four hemolytic and catalase variants were isolated from *S. aureus* S6C and the Tn*917* mutant demonstrates a nonhemolytic, catalase positive phenotype, and the transposon insertion is independent of the mutant's phenotype, it most likely represents the nonhemolytic, catalase positive variant found in the stock culture of *S. aureus* S6C. Since it is possible that the Tn*917* mutant contains a mutation within the *agr* locus, the nonhemolytic catalase positive *S. aureus* S6C variant could have the same mutation within its *agr* region. This is supported by Northern analysis using an RNAIII probe against the four *S. aureus* S6C variants. RNAIII is one of the two transcripts transcribed by the *agr* locus (Peng *et. al.*, 1988; Janzon *et. al.*, 1989; Kornblum *et. al.*, 1990). The RNAIII transcript is the regulatory element in the *agr* system that regulates the expression of staphylococcal extracellular and cell wall-associated proteins (Kornblum *et. al.*, 1990; Janzon and Arvidson, 1990). Results from Northern analysis

of the four S6C variants revealed very low levels of RNAIII expressed by the nonhemolytic, catalase positive variant whereas wild-type levels were observed for the other three variants (Hart, unpublished data). These data indicate that the *agr* system is inoperative in the nonhemolytic catalase positive variant thus, explaining the observed phenotype for this variant. However, the reason that the catalase negative variants were isolated from *S. aureus* S6C is unknown at present.

It has been hypothesized that catalase plays a role in staphylococcal virulence (Mandell, 1975; Verhoef, 1997; Kanafani and Martin, 1984) therefore I decided to investigate the nature of the catalase deficiency observed for two of the four variants of *S. aureus* S6C. Since the catalase gene of *S. aureus* has not been cloned, I decided to begin by first cloning the gene or genes responsible for catalase activity in *S. aureus*. Several approaches were used in an attempt to clone the catalase gene. These approaches included; using degenerate primers designed from *E. coli* and *B. subtilis* catalase gene sequences to amplify, by PCR, a catalase fragment, and using the degenerate primers to identify, by Southern analysis, a *S. aureus* restriction fragment containing the catalase gene.

However, these attempts were unsuccessful. Interestingly when PCR was used, a fragment of the expected size was amplified and isolated, however the sequence of the fragment did not match any known gene sequences. The techniques used were based on the hypothesis that the catalase gene sequence of *S. aureus* is similar to other catalase gene sequences, therefore these data suggest that the *S. aureus* catalase gene sequence might be distinct from other catalases. Studies are currently underway by other investigators in the laboratory to isolated and clone the catalase gene of *S. aureus* using alternative methods.

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