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# Identification and Characterization of Helper Phage Gene Products Involved in Mobilization of Staphylococcal Pathogenicity Island SaPI1

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# IDENTIFICATION AND CHARACTERIZATION OF HELPER PHAGE GENE PRODUCTS INVOLVED IN MOBILIZATION OF STAPHYLOCOCCAL PATHOGENICITY ISLAND SAPI1

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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# Dedication

I dedicate this to my family and friends, most notably my husband Robert. Their unwavering love, support, and encouragement as I pursued my studies helped sustain me and give me strength right to the end.

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# List of abbreviations

| | |
|---|---|
| A | adenine |
| AA | amino acid |
| att | attachment site |
| BHI | brain heart infusion |
| bp | base pair |
| BSA | bovine serum albumin |
| C | cytosine |
| CCD | charge-coupled device |
| CDD | Conserved Domain Database |
| CFU | colony forming units |
| cm | centimeter |
| COG | Clusters of Orthologous Groups |
| CsCl | cesium chloride |
| CSPD | Disodium-3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.13,7]decan)-4-yl)phenyl phosphate |
| CY-GL | casamino acids yeast extract glycerophosphate |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| DIG-11-dUTP | Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dTTP | deoxythymidine triphosphate |
| dUTP | deoxyuridine triphosphate |
| EDTA | ethylenediamine tetraacetic acid |
| *E. coli* | *Escherichia coli* |
| g | gram |
| G | guanine |
| gp | gene product |
| Jcm | Joules per centimeter |
| kb | kilobase pairs |
| L | liter |

| | |
|---|---|
| LB | Luria-Bertani |
| M | molar |
| ml | milliliter |
| mM | millimole |
| MOI | multiplicity of infection |
| mol | mole |
| msec | millisecond |
| MWCO | molecular weight cut-off |
| n | nano |
| NCBI | National Center for Biotechnology Information |
| NCTC | National Collection of Type Cultures |
| $^{o}C$ | degree centigrade |
| OD | optical density |
| ORF | open reading frame |
| p | pico |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PFU | plaque forming units |
| RDF | recombination directionality factor |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| *S. aureus* | *Staphylococcus aureus* |
| SaPI | *Staphylococcus aureus* pathogenicity island |
| SaPI1 | *Staphylococcus aureus* pathogenicity island one |
| SDS | sodium dodecyl sulfate |
| *sir* | SaPI1 Interference Resistant |
| SSC | saline sodium citrate buffer |
| TAE | Tris acetate EDTA |
| TE | Tris EDTA |
| TEM | transmission electron microscopy |
| Tris | Tris Hydroxymethylaminoethane |
| TSA | Tryptic soy broth |
| TU | transducing units |
| U | uracil |
| UV | ultraviolet |
| vol/vol | volume per volume |
| wt/vol | weight per volume |

| | |
|---|---|
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactoside |
| μ | micro |
| ρ | rho |

# Abstract

Identification and Characterization of Helper Phage Gene Products Involved in Mobilization of Staphylococcal Pathogenicity Island SaPI1

By Sandra McKenzie Tallent, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

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Staphylococcal pathogenicity island SaPI1 is excised from genomic DNA and extrachromosomal copies are amplified during the vegetative growth of staphylococcal phage 80α. The amplified genetic element is subsequently encapsidated and transduced at very high frequency. Previous

studies have demonstrated that the transducing particles have virions with tails that appear identical to those of helper phage 80α but have smaller capsids, commensurate with the smaller genome of the SaPI (Lindsay *et al.*, 1998). The morphology of the transducing particles, coupled with the observation that the genomic sequence of SaPI1 (GenBank U93688) does not reveal any obvious phage structural proteins, has led to the hypothesis that SaPI1 is encapsidated in a virion comprised of 80α structural proteins. Analysis of SaPI1 transducing particles supports this hypothesis. Further investigation of 80α genes involved in SaPI1 mobilization was accomplished by selection of phage mutants resistant to SaPI1 interference. Two classes of SaPI1 interference resistant (*sir*) mutants were obtained, and point mutations were identified in two adjacent genes. In order to confirm the roles of these genes, an in-frame deletion of each candidate gene was constructed in an 80α prophage. All mutant phage and deletion constructs were evaluated for phage replication, SaPI1 replication, SaPI1 transduction and SaPI1 interference. One gene (ORF21) was required for 80α growth and replication, but was not required for SaPI1 growth or replication. The second gene (ORF22) was not essential for phage replication, but was required for SaPI1 replication and high frequency transduction. The product of this gene was subsequently shown to be required for SaPI1 excision.

# Chapter 1 Introduction

General characteristics of *S. aureus.* *S. aureus* has emerged as an important pathogen in both hospital and community settings, resulting in increased morbidity, hospital stay and financial burden (Rubin *et al.*, 1999) (Shorr *et al.*, 2006). Approximately two million people annually are reported to have a hospital acquired staphylococcal infection (Rubin, 1999). In 2000, 54% of *S. aureus* strains causing hospital acquired infections in the critical care setting were resistant to methicillin, a considerable increase compared to the previous 5 year period (CDC report, 2000). Methicillin resistant *S. aureus* (MRSA), once associated with hospital acquired infections, has recently been associated with community acquired infections as well. The hospital strain is multi-resistant while the community strain has been found to be resistant only to β–lactam antimicrobials. It has been predicted that the prevalence of MRSA within the community may be as high as 25% by the year 2010 based upon historical data with penicillin resistance (Chambers,2001).

*S. aureus* is a facultative anaerobic, non-motile, Gram-positive coccus. This bacterium is normally present on human skin and mucous membranes. 10-20% of healthy adults are colonized with *S. aureus*, but colonization rates are higher among immunocompromised patients and IV drug abusers.

Infections arise when the protective barrier is broken, allowing staphylococci access to the bloodstream or surrounding tissue (Lowy,1998).

Virulence factors. Once infection is established, *S. aureus* can cause a wide spectrum of disease including cutaneous infections, sepsis, food poisoning and toxic shock syndrome. Many virulence factors contribute to the pathogenicity of *S. aureus*, including a variety of secreted proteins and adhesins. These factors contribute to staphylococcal pathogenicity by allowing evasion of the host immune system or by causing tissue damage (Foster,2005). Some virulence determinants present on the 2.8 Mb chromosome include secreted enzymes and cytotoxins such as hemolysins, nucleases, proteases, hyuluronidase and collagenase. These proteins degrade host tissue for nutrients that sustain bacterial growth (Lowy,1998).

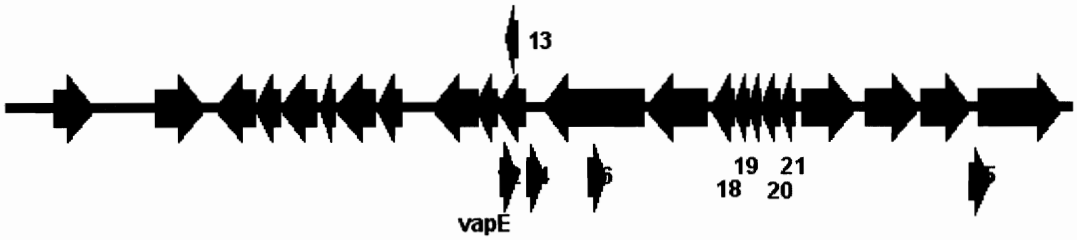
Some staphylococcal strains also contain mobile elements that carry virulence determinants. DNA may be transferred between staphylococcal strains by lateral transfer of transposons, plasmids, pathogenicity islands or bacteriophages (Holden *et al.*, 2004). Lateral gene transfer in staphylococci has been associated with prophage induction during the SOS response resulting from exposure to UV, quinolones (Ubeda *et al.*, 2005) and β-lactams (Maiques *et al.*, 2006). A number of the genes encoded by these elements, such as enterotoxins B and C and antimicrobial resistance genes, are associated with virulence (Holden *et al.*, 2004). The gene products include

exoproteins that stimulate the host immune response to *S. aureus*, such as the toxic shock syndrome toxin (TSST-1), staphylococcal enterotoxins, the exfoliative toxins and leukocidin. The TSST-1 and enterotoxins are also pyrogenic toxin superantigens (Dinges *et al.*, 2000).

SaPI Pathogenicity islands. *Staphylococcus aureus* Pathogenicity Islands (SaPI) are a family of related genetic elements that carry genes for superantigens, including TSST-1 and several enterotoxins (reviewed in Novick ,2003). Other common features of this family of genetic elements include a similarity in size (15-20 kb) and in gene organization, a lack of essential genes, flanking direct repeats, and the presence of phage-like integrase and small terminase subunit genes. SaPI integration is phage-like, occurring via site-specific recombination at specific sequences within the *S. aureus* genome which is catalyzed by the SaPI-encoded integrase. Laboratory studies suggest that transmission of SaPI is associated with lytic multiplication of a helper phage. Mobilization, replication and high frequency transfer of SaPI occurs in the presence of specific staphylococcal bacteriophages (Lindsay *et al.*, 1998).

General properties of SaPI1. SaPI1 is the prototypical member of the SaPI family. Sequence analysis of the 15,233 bp element (Fig 1) has identified two characteristic phage-like genes encoding integrase and a small terminase subunit. SaPI1 carries five genes encoding homologues of known or putative

**Figure 1. SaPI1 genomic map.** SaPI1 genomic map was based on GenBank U96388. Identified genes include the putative virulence genes *ear*, *tst, vapE, sel,* and *sek* and the phage-like ORFs *ter* and *int.* Genes were predicted with GeneMark.hmm PROKARYOTIC (Version 2.4a) predictive software. Arrows indicate predicted genes encoding proteins of at least 50 amino acids. Blue arrows indicate ORFs annotated in GenBank and predicted by GenMark, red arrows annotated ORFs only in GenBank and the gray arrow is an ORF predicted only by GenMark .

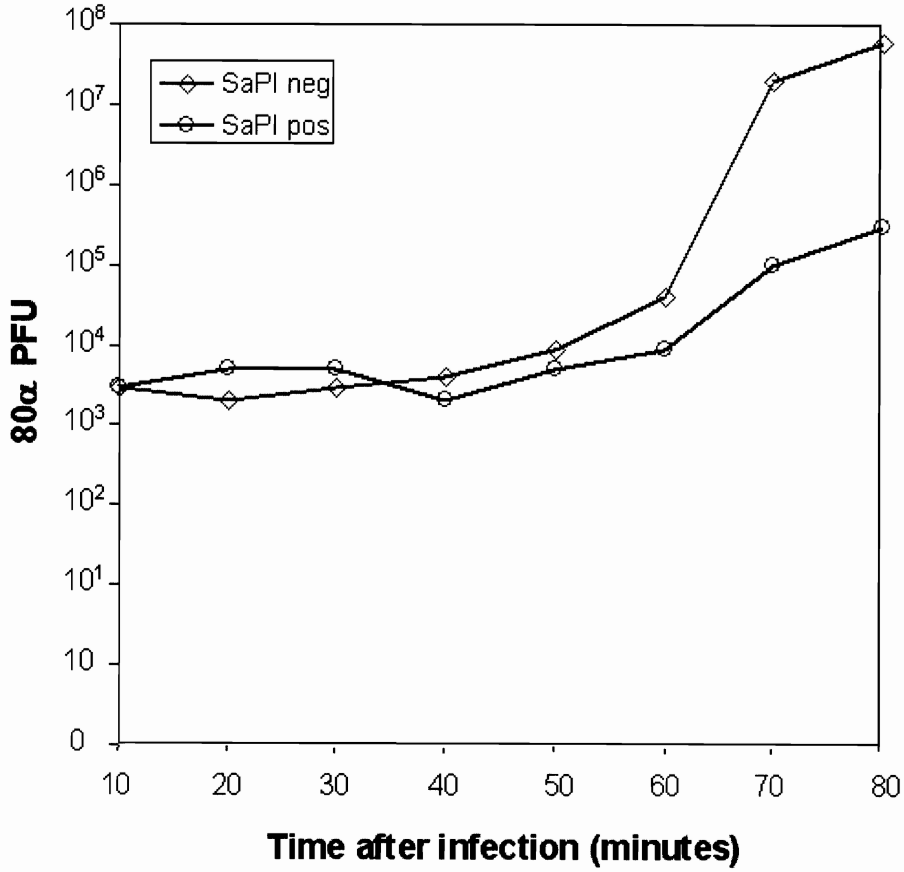

13
19
21
18
20
vapE

virulence factors, including *tst, sek, vapE* and truncated copies of *sel* and *ear* (Novick,2003). There are approximately 20 ORFs with unknown function; many of these lack significant homology to genes or proteins in the public databases other than those on related SaPI1s.

SaPI1 is excised from *S. aureus* genomic DNA during vegetative growth of staphylococcal phages ϕ13 and 80α, presumably by site-specific recombination between flanking *att* repeats. SaPI1 excision following ϕ13 infection results in conversion to a circular duplex but no amplified genome-sized SaPI DNA is observed and there is no SaPI1 transduction (Lindsay *et al.*, 1998). However, following 80α infection, excised SaPI1 converts to a linear replicative form, is amplified, and is efficiently packaged into smaller capsids one-third the size of helper phage capsids (Ruzin *et al.*, 2001). The smaller capsid size accommodates the 15kb SaPI1 genome but not the 44kb 80α genome. SaPI1 transduction frequency by 80α is high, $1 \times 10^{-1}$, as compared to the generalized

transduction frequency of chromosomal markers, $1 \times 10^{-7}$. The presence of SaPI1 has been shown to interfere with 80α lytic growth, reducing the production of progeny phage by about 2 orders of magnitude (Lindsay *et al.*, 1998) (Fig 2). Once SaPI1 is transferred, it integrates into the chromosomal $att_c$ site of the recipient staphylococcal genome by means of the SaPI1-encoded integrase (Ruzin *et al.*, 2001).

**Figure 2. One step growth curve of 80α.** One-step growth curve of 80α in SaPI1 positive and negative *S. aureus* strains (RN7045 and RN450 respectively). Cells were infected at t=0 at MOI = 0.1 and aliquots were removed at 10 minute intervals and titered on a SaPI1 negative indicator strain.


SaPI neg
SaPI pos
80α PFU
Time after infection (minutes)
0
10
$10^1$
$10^2$
$10^3$
$10^4$
$10^5$
$10^6$
$10^7$
$10^8$
10
20
30
40
50
60
70
80

Staphylococcal phages. Clinical staphylococcal isolates commonly carry multiple prophages (Bae *et al.*, 2006). For example, early studies with clinical strain NCTC8325 identified 3 prophages, Φ11, Φ12, and Φ13, which are members of the *Siphoviridae* family. Staphylococcal phages of the have been further classified based on their lytic activity, serological properties and virion morphology, i.e., capsid and tail. Cross-reactivity among antibodies raised against staphylococcal phages allowed classification of these phages into five serogroups A, B, F, L and G. Of the five, serogroup B is most often associated with virulence determinants and contains most of the generalized transducing phages, including Φ11 and phage 80α (Iandolo *et al.*, 2002). Some transducing phages have been shown to enable a staphylococcal cell to become competent in transformation and transfection, allowing uptake of foreign DNA that can enhance bacterial virulence (Pantucek *et al.*, 2004). Additionally, some of the temperate phages, including ΦPVL and phage ETA, can mediate lysogenic conversion of staphylococcal genomes through carriage of virulence determinants. Negative lysogenic conversion occurs if phage integration disrupts a host gene. Positive lysogenic conversion occurs if the host acquires a phage-borne virulence gene following phage integration. Virulence genes identified on temperate phages include enterotoxins, exfoliative toxin A and Panton-Valentine leukocidin (summarized by Firth and Skurray in Fischetti *et al.*, 2006).

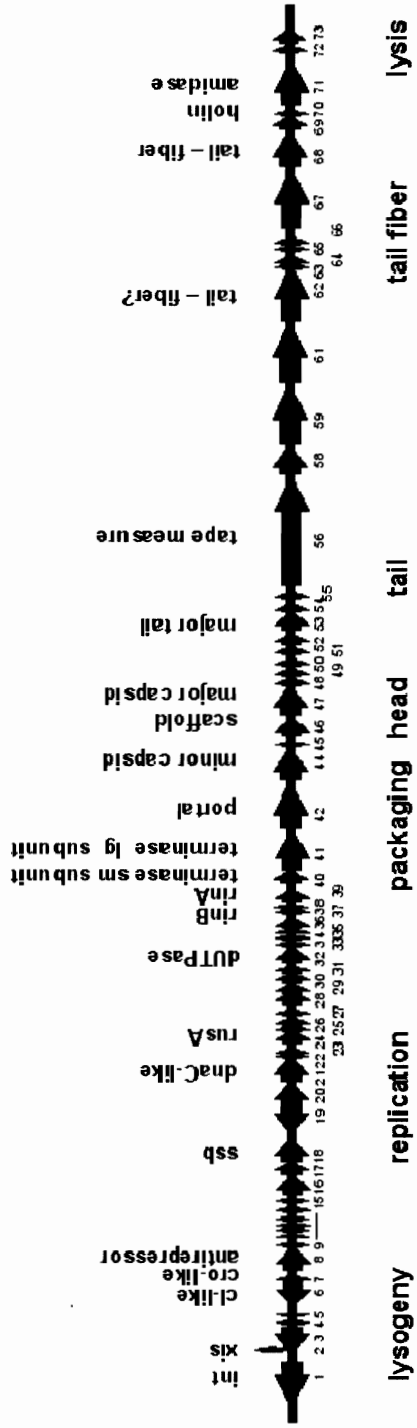
A recent study determined the genome sequence of 27 dsDNA staphylococcal phage genomes and established three classes of phages based on genome size, similarity in genomic organization and similarity in virion morphology (Kwan *et al.*, 2005). The smallest phages, class I, have genomes of about 20 kb and belong to the *Podoviridae* family. Class II phages (40 kb) belong to the *Siphoviridae* family and include all characterized members of the International Typing Strain Set as well as the generalized transducing phages. The largest staphylococcal phages, class III, have very large genomes (>125 kb) and belong to the *Myoviridae* family. The phages within each class show nucleotide sequence relatedness, but are not generally related to phages from the other classes. This collection of sequenced phages revealed that 49% of the staphylococcal phage ORFs were similar only to other staphylococcal phages (Kwan *et al.*, 2005).

Genomic organization of Φ11 and 80α. One of the best-characterized staphylococcal phages is temperate phage Φ11, a member of the Class II *Siphoviridae*. The 45kb genome of Φ11 is 5% terminally redundant and 42% circularly permuted (Lofdahl *et al.*, 1981). The organization of its genome is modular, with genomic cassettes arranged similarly to those characterized in streptococcal phages: lysogeny, DNA replication, transcriptional regulation, packaging, head, tail, tail fibers, and lysis. These cassettes can easily recombine with closely related regions from other phages, resulting in extensive

genetic mosaicism. Genes in the lysogeny cassette that have been assigned functions include ORFs thought to encode integrase, a cI-like repressor and a cro-like repressor. The DNA replication module includes homologues for DNA polymerase, single stranded binding protein and helicase. The next module encodes transcriptional regulators *rinA* and *rinB,* found in both Φ11 and 80α (Iandolo *et al.*, 2002). RinA and RinB activate the transcription of integrase (Ye *et al.*, 1993). Continuing downstream are the putative packaging and structural genes, including the ORFs for the small and large terminase subunits, portal protein, scaffolding protein, major capsid protein, tape measure protein, major tail protein and tail fibers. The last module is the lysis module, encoding holin and amidase (Iandolo *et al.*, 2002). A genomic map of 80α, constructed from the recently determined 80α sequence (GenBank DQ517338) showed that the genomic organization of 80α parallels that of Φ11 (Fig 3). A BLAST search comparing the genomic sequences of 80α and Φ11 shows significant similarity between the genes encoding the excisionase, repressor and antirepressor associated with lysogeny; rinA, rinB, dUTPase and resolvase found in the replication region and the late genes corresponding to the packaging, head, tail, and lysis modules. However, Φ11 cannot mobilize SaPI1 (Lindsay *et al.*, 1998).

Properties of model system P2 P4. The exploitation of 80α by SaPI1 is an example of what has been termed "molecular piracy". This phenomenon has

**Figure 3. 80α genomic map.** 80α genomic map based GenBank DQ517338. Functional modules are indicated below the map, and are consistent with the gene arrangement found in other staphylococcal phages. Arrows indicate predicted genes encoding proteins of at least 50 amino acids. Genes for which functional assignments have been made based on sequence similarity are indicated above the map. Blue arrows represent genes for which the assigned function is supported by protein analysis reported in this study. Genes were predicted with GeneMark.hmm PROKARYOTIC (Version 2.4a).


lysogeny
replication
packaging
head
tail
tail fiber
lysis
int
xis
cI-like
cro-like
antirepressor
ssb
dnaC-like
rusA
dUTPase
rinB
rinA
terminase sm subunit
terminase lg subunit
portal
minor capsid
scaffold
major capsid
major tail
tape measure
tail – fiber?
tail – fiber
holin
amidase

been well characterized in enterobacteria, where satellite phage P4 exploits temperate phage P2 as a helper for lytic growth (reviewed in (Lindqvist *et al.*, 1993). In the absence of helper, P4 can exist as a multicopy plasmid or as a lysogen. In the presence of a P2 helper phage, P4 can undergo lytic growth and is packaged into virions assembled from P2-encoded proteins, but with smaller capsids that accommodate the smaller P4 genome, similar to what is seen with SaPI1.

The mechanism for P4 capsid size determination has been extensively characterized and is well understood (reviewed by Lindqvist et al. 1993). Briefly, the P4 gpSid (size determination) interacts with hexamers of the P2 major capsid protein gpN and promotes the formation of 5-fold rings that then assemble into the smaller capsids, with gpSid forming an external scaffold that is subsequently removed during capsid maturation. Typically a P2 capsid of T=7 would contain 12 pentamers and 60 hexamers of gpN, in contrast to a P4 capsid of T=4 with 12 pentamers, but only 30 hexamers (Dokland *et al.*, 1992). The target of P4 gpSid was identified by the isolation and characterization of P2 mutants called "*sir*" that were insensitive to P4 interference (Six *et al.*, 1991).

A second structural protein, Psu, is also expressed from the $P_{sid}$ transcript. This protein is a decoration protein attached to the capsid. It is assumed that the protein stabilizes the smaller capsid since $Psu^-$ P4 particles are less heat stable compared to wild type P4 particles. However, the protein is nonessential for infectious particles (Isaksen *et al.*, 1992).

Activation of a P4 prophage following P2 infection involves multiple interactions that allow P4 to take advantage of the helper. P4 is derepressed by a P2-encoded protein, Cox, that activates transcription from the P4 $P_{LL}$ promoter. The genes transcribed from this promoter include the P4 excisionase (Cali *et al.*, 2004) and DNA replication genes (Saha *et al.*, 1987) A second P2 protein, Ogr, activates the expression of the P4 δ protein and P4 structural genes Sid and Psu from the $P_{sid}$ promoter. The P4 δ protein can also activate the P2 late promoters, stimulating transcription of the morphogenetic genes required for lytic growth by both P2 and P4.

Comparison of model system with 80α and SaPI1. Little is known about the regulatory interactions between SaPI1 and 80α, but there are fundamental differences in the way SaPI1 exploits its helper. Unlike P4, SaPI1 requires the helper for both excision and replication. There are differences in DNA packaging as well. P4 replication is autonomous (independent of helper phage) and depends only on P4-encoded gene products and host functions. The DNA substrate required for P4 packaging is closed circular DNA with cohesive ends (Liu *et al.*, 1996) and is based upon recognition and cleavage at a 19 bp *cos* site which is shared in P2 and P4 (Bowden *et al.*, 1985). SaPI1 DNA is thought to be packaged by a headful mechanism in which the terminase begins DNA packaging by binding and cutting the concatamer at a specific *pac* site (Ruzin *et al.*, 2001), followed by introduction of a second cut in the concatamer once the

capsid is full. The presence of a conserved small terminase subunit in the SaPIs suggests that the pathogenicity islands replace the phage encoded small terminase with a SaPI-specific one that directs substrate recognition to the SaPI DNA.

Dissertation goals. The goal of this project was to investigate the roles of 80α-encoded proteins in SaPI1 transduction. Specifically, we set out to determine whether SaPI1 transducing particles were assembled from 80α encoded proteins and to identify helper phage genes required for SaPI1 mobilization.

The first phase of this study demonstrates that the SaPI1 transducing particle is comprised entirely of helper phage encoded proteins. No SaPI1-encoded proteins were identified in the mature virions. The second phase of this study reports the selection and characterization of two phage mutants resistant to SaPI1 interference, in order to begin to elucidate SaPI1-helper interactions. DNA sequence analysis of these mutants revealed point mutations in two adjacent phage genes. One gene, essential for helper phage replication, was not required for SaPI1 mobilization. The second gene is essential for SaPI1 excision.

# Chapter 2 Materials and Methods

Bacterial culture methods. Bacterial strains used in this study are listed in Table 1. *S. aureus* was plated on phage agar (Novick,1991) (0.3% (wt/vol) Casamino acids, 0.3% (wt/vol) yeast extract, 100 mM NaCl, 1.5% agar (wt/vol), 0.5 mM $CaCl_2$, pH 7.8) or on Tryptic Soy Agar (TSA) (Remel, Lenexa, KS) and incubated overnight at 30°C. *S. aureus* strains positive for SaPI1*tst::tetM* were plated on GL agar (Novick,1991) (0.3% (wt/vol) Casamino acids, 0.3% (wt/vol) Yeast extract, 100 mM NaCl, 0.33% (vol/vol) Sodium lactate, 60% syrup, 25% (vol/vol) Glycerol, 1.5% (wt/vol) agar, 0.17 mM sodium citrate, pH 7.8) with 5 μg/ml tetracycline.

*E. coli* strains were cultured in Luria Bertani (LB) (Difco, Franklin Lakes, NJ) broth or plates supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) as required.

Studies with the shuttle vector pMAD (Arnaud *et al.*, 2004) required media supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (200 μg/ml) (American Bioanalytical, Natick, MA) and erythromycin (5 μg/ml) for staphylococcal strains or ampicillin (100 μg/ml) for *E. coli*.

Growth of phage. An overnight broth culture of a phage-sensitive *S. aureus*

**Table 1. Bacterial strains and plasmids used or created in this study.**

| *S. aureus* strain | Description | Reference or source |
|---|---|---|
| RN450 | NCTC8325 cured of Φ11, Φ12 and Φ13 | Novick, 1967 |
| RN4220 | Restriction defective derivative of RN450 | Novick, 1991 |
| RN7045 | RN450 (SaPI1 *tst*::*tetM*) | Sloane, 1991 |
| RN10616 | RN4220 (80α) | R. Novick |
| RN10628 | RN4220 (80α)( SaPI1 *tst*::*tetM)* | R. Novick |
| ST18 | RN4220 (80αΔORF21) | This study |
| ST20 | RN4220 (80αΔORF22) | This study |
| ST21 | RN4220 (80αΔORF22)(SaPI1 *tst*::*tetM*) | This study |
| ST22 | RN4220 (80αΔORF21) (SaPI1 *tst*::*tetM*) | This study |
| ST23 | RN4220 (SaPI2 *tst*::*tetM*) | This study |
| Plasmids | | |
| pMAD | Shuttle vector derivative of pE194 replication thermosensitive mutant | Arnaud, 2004 |
| pCRT7/NT-TOPO | Cloning vector | Invitrogen, Carlsbad, CA |
| Blunt TOPO | Cloning vector | Invitrogen, Carlsbad, CA |
| pSMT153 | Derivative of pMAD with cloned ORF22 deletion | This study |
| pSMT331 | Derivative of pMAD with cloned ORF21 deletion | This study |

strain was diluted 1:100 in CY-GL broth (Novick,1991) (1% (wt/vol) Casamino acids, 1% (wt/vol) Yeast extract, 30 mM glucose, 100 mM NaCl, 60 mM β-glycerophosphate) for incubation at 30°C on an orbital shaker at 200 rpm until it reached a density of $OD_{550}$ = 0.6 (Klett = 50), which is approximately 3 X $10^8$ CFU/ml. The culture was diluted with an equal volume of SA Phage Buffer (Novick,1991) (1mM $MgSO_4$, 4 mM $CaCl_2$, 0.05 M Tris-HCl pH 7.8, 100 mM NaCl, 0.1% gelatin) and infected with bacteriophage at a multiplicity of infection (MOI) equal to 0.1 unless otherwise indicated. The infected culture was incubated at 30°C on an orbital shaker at 100 rpm and monitored for lysis until the optical density stopped decreasing. Alternatively, a lysogen grown overnight in CY-GL broth was diluted 1:100 in CY-GL broth and incubated on an orbital shaker at 200 rpm until it reached a density of Klett= 30. The culture was centrifuged for 30 minutes at 5000 rpm 4°C, resuspended in the same volume of SA phage buffer, exposed to UV light (70 $Jcm^{-2}$) for 20 seconds, diluted 1:1 with CY-GL broth, and incubated at 30°C, 200 rpm until lysis.

Samples used in timed-course studies were cultured and infected with phage as described above, but following phage infection the culture was incubated at room temperature for 10 minutes and the unadsorbed phage were removed by centrifugation at 10,000 rpm 4°C for 10 minutes. The pellet was resuspended to twice the original volume in CY-GL broth and SA phage buffer (1:1) and incubated at 30°C with shaking at 100 rpm. Aliquots (2 ml) were removed at appropriate time intervals, and either filtered with 0.45 μM syringe

filters (Millipore, Billerica, MA) and stored at 4°C for titering or frozen on dry ice in 2 ml microcentrifuge tubes and stored at -20°C for subsequent DNA preparation.

Phage titers were determined by plating serial dilutions of phage on a sensitive indicator strain. Phage were diluted in SA phage buffer, and 100 µl aliquots of each phage dilution were added to 100 µl of an overnight culture of indicator cells, mixed with 3 ml of SA top agar (Novick,1991) (0.3% (wt/vol) Casamino acids, 0.3% (wt/vol) Yeast extract, 100 mM NaCl, 0.5% agar (wt/vol), 0.5 mM $CaCl_2$, pH 7.8) and quickly poured onto the surface of a phage agar plate. The plates were incubated overnight at 30°C and plaques were counted to determine the phage titer. To determine the transducing titer of lysates prepared from SaPI1 *tst*::*tet*M strains, serial dilutions of lysates were mixed with indicator cells as described above and spread onto the surface of GL agar plates with tetracycline (5 µg/ml). Colonies were counted following a 48-hour incubation at 30°C.

PEG precipitation. Phage and SaPI lysates were transferred to centrifuge bottles and centrifuged at 7000 rpm for 20 minutes at 4°C to remove cellular debris. Polyethylene glycol 8000 (PEG) (0.1% wt/vol) and NaCl (0.5 M) were dissolved in the supernatant and incubated overnight at 4°C. The lysate was centrifuged for 20 minutes at 7000 rpm at 4°C, the supernatant was decanted and the PEG precipitate was removed from the sides of the bottle and

resuspended in SA phage buffer (9 ml per liter of lysate) using a cell scraper or rubber policeman. The resuspended PEG pellet was transferred to a sterile Corex tube, allowed to incubate overnight at 4°C, and centrifuged 10 minutes at 10,000 rpm at 4°C. The supernatant was transferred to a sterile tube and the remaining pellet was resuspended in SA phage buffer (3 ml) and centrifuged a second time with the same settings. The second supernatant was pooled with the first supernatant.

Phage and SaPI1 purification. Viruses were purified from the resuspended PEG supernatant by centrifugation in a cesium chloride (CsCl) step gradient, followed by equilibrium ultracentrifugation in CsCl. Cesium chloride of different densities was prepared in SA phage buffer as follows: to a final volume of 25 ml in SA phage buffer, CsCl 10.1 g ($\rho$ =1.3), 13.47 g ($\rho$ =1.4), 16.87 g ($\rho$ =1.5), or 20.2 g ($\rho$ = 1.6) was added. A step gradient was prepared in an Ultra clear centrifuge tube (Beckman, Fullerton, CA) 1 x 3 ½ inches as follows: (bottom to top layer) 2 ml $\rho$ =1.6, 3 ml $\rho$ =1.5, 4 ml $\rho$ =1.4, and 4 ml $\rho$ =1.3. The pooled phage supernatant was carefully pipetted on top. The gradient was centrifuged in a Beckman SW28 rotor for 2.5 hours at 15°C 24,000 rpm with no brake. The phage band, visible at the 1.4-1.5 interface, was collected by puncturing the tube and aspirating with a syringe, and transferred to a Beckman ½ x 2 inches polyallomer tube for equilibrium banding. The banded phage was mixed with enough CsCl $\rho$ = 1.5 to fill the tube and centrifuged in a Beckman 55Ti rotor 24

hours at 15°C 42,000 rpm with no brake. Bands were collected by puncturing the tube and aspirating with a syringe. The double-banded sample was dialyzed at 4°C with four changes of SA phage buffer using a Slide-A-Lyzer dialysis cassette (Pierce Biotechnology, Rockford, ILL) with a 10,000 molecular weight cut off (MWCO).

Further purification, when required, included addition of DNase I (10 µg/ml) (Sigma, St. Louis, MO) and RNase A (10 µg/ml)(Worthington Biochemical Corporation, Freehold, NJ) to the double-banded dialyzed sample, with overnight incubation at 4°C. An additional dialysis step was used to recover phage and SaPI particles in EM Buffer (Dokland personal communication) (10 mM Tris-HCl, pH 7.8, 20 mM NaCl, 1 mM $MgCl_2$). Further concentration, when necessary, was accomplished by pelleting the phage. The dialyzed samples were transferred to a Beckman ½ x 2 inches polyallomer tube and centrifuged in a Beckman 55Ti rotor 1 hour at 50,000 rpm at 10°C. The buffer was decanted, 100 µl of EM buffer was added without disturbing the pellet and the pellet was incubated overnight at 4°C.

Electron microscopy. Negative staining of samples for Transmission Electron Microscopy (TEM) was carried out using double-banded dialyzed phage and SaPI1 stocks. A 200 mesh, formvar-coated copper grid was exposed to UV light (70 $Jcm^{-2}$) for 2 hours to ensure that the grid was hydrophilic and 8 µl of the pelleted particles resuspended in EM buffer were applied to the grid while

holding the grid with forceps. The sample was wicked after 1 minute using 3MM Whatman filter paper, and 8 µl 1% Phosphotungstic Acid (Electron Microscopy Sciences, Hatfield, PA) (wt/vol, pH 6.5-7.0) were applied and wicked with filter paper after 1 minute. The grids were dried overnight and examined with a JEOL-JEM-1230 TEM (Jeol, Peabody, MA) equipped with an UltraScan 4000SP 4K x 4K CCD camera (Gatan, Pleasanton, CA). Images were captured at 10,000 magnification.

Protein gels and mass spectrometry. Proteins present in purified phage and transducing particles were resolved by denaturation and electrophoresis on a 10% polyacrylamide gel. Pelleted phage samples resuspended in EM buffer were added to XT Sample Buffer (12.5 µl) (BioRad, Hercules, CA), XT Reducing Agent (2.5 µl) (BioRad, Hercules, CA), and water for a total volume of 50 µl. The mixture was heated to 95°C for 15 minutes, vortexed on a multi-sample platform vortex mixer for 20 minutes, and centrifuged for 10 seconds. The denatured protein samples (45 µl) were applied to a Criterion XT 10% Bis-Tris (Bio-Rad, Hercules, CA) pre-cast acrylamide gel. The proteins were separated under constant voltage at 200V for 50 minutes in 1X MOPS (Bio-Rad, Hercules, CA) running buffer. A Precision Plus Protein Standard unstained marker (10 kDa-250 kDa) (Bio-Rad, Hercules, CA) and a Precision Plus Protein Standard dual color marker (10 kDa-250 kDa) (Bio-Rad, Hercules, CA) were run with samples to estimate the molecular weight of the protein

bands. After electrophoresis the gel was rinsed in MilliQ water (Millipore, Billerica, MA) and stained overnight with Sypro Ruby protein stain (Bio-Rad, Hercules, CA) protected from the light and with gentle shaking. The gel was fixed for one hour in methanol (10% vol/vol) and acetic acid (7% vol/vol) and then rinsed in MilliQ water for 15 minutes. The gel was visualized under UV light and individual bands were excised. T. B. Langston, in the laboratory of Richard Moran, performed subsequent processing and mass spectrometry. Briefly, individual bands were cut, diced, washed with 100mM $NH_4HCO_3$, and subsequently reduced with dithiothreitol (DTT) and alkylated with iodacetamide to prevent disulfide bond formation (Shevchenko *et al.*, 1996a). Gel pieces were trypsinized overnight and then extracted with acetonitrile. The supernatant was dried in a SpeedVac and reconstituted in 3% acetonitrile. Extracted peptides were loaded onto a C-18 precolumn and washed extensively. The precolumn was connected to an integrated nano-HPLC analytical column/ESI emitter, fabricated as described by Martin et al. (2000) and analyzed by nano-HPLC ESI ion trap mass spectrometry (Shevchenko *et al.*, 1996b). The peptides were eluted using a linear 3-80% acetonitrile gradient at a flow rate of 25 or 50 nL/min. The LCQ Deca Max mass spectrometer (ThermoFinnigan) acquired, using dynamic exclusion, a full scan (400-2000 m/z) followed by MS/MS spectra on the three most abundant ions. Using the SEQUEST algorithm in Bioworks 3.1, SR1, the MS/MS spectra were compared to a database (50,719 entries) of non-redundant proteins of *S. aureus*, and all

bacteriophage. Peptides were matched using the following rigorous criteria Cross correlation vs Charge, +1>1.9, +2>2.7, +3>3.5; Δ correlation>0.1. The presence of at least two unique peptides was required for protein identification.

Whole particle samples were denatured as described in ((da Poian *et al.*, 1995) with the following modifications. The particles were disassociated in 6M urea followed by 6 freeze-thaw cycles (-80°C to 37°C). The disassociated particles were centrifuged in a Nanosep (Pall Corp, East Hill, NY.) 100K MWCO centrifugal device and the eluate was diluted to 0.75M urea and digested overnight with 36 ng of trypsin. The remainder in the cup was washed and reconstituted in 100 mM $NH_4CO_3$, digested overnight with 36 ng of trypsin. Peptides and DNA were separated with a 10K MWCO centrifugal device. Approximately 25 µl of each eluate was loaded onto the pre-column.

*S. aureus* genomic DNA extraction. Cone ml aliquots of liquid cultures were centrifuged at 14,000 rpm for 5 minutes to pellet the cells. The pellet was resuspended in 100 µl TES buffer (20 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA pH 7.5) with RNase A (20 µg/ml) and lysed with lysostaphin (0.5 mg/ml) (Sigma, St. Louis, MO) at 37°C for one hour. Following addition of 20 µl of 20% SDS the lysate was incubated for an additional 30 minutes at 37°C. Salt concentration was adjusted by the addition of 12 µl 3 M sodium acetate (pH 5.5). DNA was extracted from the lysed cells with 200 µl of a 25:24:1 mixture of TE saturated phenol:chloroform:isoamyl alcohol (Sigma, St. Louis, MO) and

centrifuged to pellet the cellular debris. The aqueous phase was carefully added to 2 volumes of room temperature 95% ethanol, mixed and centrifuged to pellet the precipitated DNA. The DNA pellet was rinsed by adding one volume of cold 70% ethanol and centrifuging 5 minutes at 14,000 rpm. The ethanol was decanted and the pellet was allowed to air dry and finally resuspended in 40 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

DNA manipulations. DNA was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) as described by the manufacturer. All restriction enzymes, specific buffers and BSA used for restriction enzyme digests of miniprep DNA or PCR fragments were purchased from New England Biolabs (Ipswich, MA) and used as recommended by the supplier.

Removal of phosphoryl groups from the 5' ends was accomplished with Antarctic Phosphatase (New England Biolabs, Ipswich, MA) using the supplied buffer.

Ligations used either T4 DNA Ligase and Ligase Buffer (New England Biolabs, Ipswich, MA) or the Ligate-IT Rapid Ligation Kit (Amersham Biosciences, Piscataway, NJ).

Plasmid screening. A quick-check method (Akada,1994) was used to screen colonies for the plasmids with insert. Each colony was resuspended in 100 µl of sterile water and 50 µl of phenol/chloroform (1:1) plus 10 µl loading dye (0.25%

bromophenol blue and 40% glycerol) were added. The tubes were vortexed for 10 seconds and centrifuged for 3 minutes at 13,000 rpm. An aliquot of (5 µl) from each sample was loaded onto a 0.8% agarose gel and compared to a super-coiled DNA ladder (Invitrogen, San Diego, CA).

Plasmid DNA purification. Plasmid mini-preps were prepared from individual *E. coli* transformants that were grown overnight in 5 ml of LB broth with ampicillin, using a NucleoSpin Plasmid Kit (ClonTech, Mountain View, CA) as described by the manufacturer.

Agarose gels. Agarose minigels (1%) were prepared using 20 ml of 1X TAE buffer (Fisher Scientific, Pittsburgh, PA) and 0.2 grams of agarose. Generally, 3 µl of DNA samples were mixed with 2 µl 5X loading dye (50% glycerol, 0.25% bromophenol blue and 0.25% Xylene cyanol). Gels were run in 1X TAE at 80 volts for approximately 45 minutes, stained with ethidium bromide (0.1µg/ml) and visualized under UV light.

Agarose gels (0.75%) used for Southern blot transfer were prepared with 100 ml 1XTAE buffer and 0.75 grams of agarose. Gels were run in 1X TAE at 100 volts for approximately 5 hours, stained with ethidium bromide (0.1µg/ml) and visualized under UV light.

Polymerase Chain Reaction (PCR). PCR reactions were performed in a TGradient thermocycler (Whatman Biometra, Goettingen, Germany). Primers, listed in Table 2, were designed from 80α sequence or SaPI1 sequence (GenBank DQ517338 and U93688, respectively) and were purchased from Integrated DNA Technologies, Coralville, IA and Invitrogen, Carlsbad, CA Primer stock solutions were prepared in HPLC grade water (Mallinckrodt Baker, Phillipsburg, NJ) at 1000 pmol/µl and working solutions were diluted to 20 pmol/µl. Standard PCR reactions were 50 µl with the following components: 10X PCR buffer (5 µl), 50mM $MgCl_2$ (2.5 µl), 10 mM dNTP mix (2.5 mM each, 4 µl), 20 pmol/µl forward primer (2.5 µl), 20 pmol/µl reverse primer (2.5 µl), template DNA (1 µl), distilled water (32.5 µl), 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR products used for sequencing and cloning were amplified with high fidelity polymerase AccuPol (GeneChoice, Frederick, MD) in 50 µl PCR reactions with the following components: 10X Ammonium buffer (5 µl), 10 mM dNTP mix (2.5 mM each, 4 µl), 20 pmol/µl forward (2.5 µl), 20 pmol/µl reverse (2.5 µl), template DNA, prepared as described below, (1 µl), distilled water (34 µl), AccuPol DNA Polymerase (2.5U). Conditions for PCR were as follows, one initial cycle at 95°C for 2 minutes followed by 30 cycles of: 95°C 1 minute to denature, annealing temperature ($T_M$ – 3) one minute and 72°C one minute per kb for extension, with a final extension at 72°C for 10 minutes. Bacterial template DNA was prepared from single colonies or broth cultures. Colonies were suspended in

**Table 2. Primers used in this study.**

| Primer | Sequence | Nucleotide position | DNA template |
|---|---|---|---|
| SMT19 | 5' AACGAGAATTCCTGTAATCAATCCTCAA CGGTATATCATCCA 3' | 2971-3002 | SaPI1 |
| SMT20 | *5' AACGAGGATCCGGCAATTGAGTGTG GAGGTGATAG* 3' | 3561-3548 | SaPI1 |
| SMT24 | 5' GTCAGTTCGAAAGTTGACACA 3' | 18070-18090 | 80α |
| SMT25 | *5' TCTGTGATAGGCATGCGTTC 3'* | 18842-18861 | 80α |
| SMT26 | 5' GGATTGAAGCTTACACCACG 3' | 18794-18813 | 80α |
| SMT27 | *5' CCGCGTTCTTCCCAATATTC 3'* | 19685-19704 | 80α |
| SMT28 | 5' GACCCTAGAGACATCAATGATGA 3' | 19589-19611 | 80α |
| SMT29 | *5' CGTGAACACGTGCCGATT 3'* | 20370-20370 | 80α |
| SMT30 | 5' CGAAGGAACAGCACGACA 3' | 20317-20334 | 80α |
| SMT31 | *5' GTTCGTCCGGATCATCTGAT 3'* | 21058-21077 | 80α |
| SMT32 | 5' TCCAAAACGTGCTGATGACAT 3' | 20938-20958 | 80α |
| SMT33 | *5' GTGCCATCTTTCTTTTCGTGC 3'* | 21737-21757 | 80α |
| SMT38 | 5' AAGGATCTATGTGGGTTGGC 3' | 16323-16342 | 80α |
| SMT39 | *5' GCACATGTCCCAAATACCG 3'* | 17097-17115 | 80α |
| SMT40 | 5' TCCTAGGCGTATACTATGGC 3' | 17010-17029 | 80α |
| SMT41 | *5' TTGTACAACCGAGCCCTTC 3'* | 17847-17865 | 80α |
| SMT42 | 5' GGATTGGACTTTGGCTACG 3' | 17602-17620 | 80α |
| SMT43 | *5' TCCCCGTAGATCTGTATCTG 3'* | 18147-18166 | 80α |
| SMT44 | 5' CAACTATGCATGTAGCATTGC 3' | 22522-22542 | 80α |
| SMT45 | *5' GCATGGTATCGCATCATAAG 3'* | 23354-23373 | 80α |
| SMT46 | 5' AAAAGGAAGGGCTGTGTTC 3' | 23245-23263 | 80α |
| SMT47 | *5' ACGTTTCGTGTCTGTATATCC 3'* | 23522-23542 | 80α |
| SMT48 | 5' ATCTTACCCTGTTAGCCACG 3' | 23463-23482 | 80α |
| SMT49 | *5' AGCATCTACTTTTTCGCCAG 3'* | 24378-24397 | 80α |
| SMT63 | 5' GAGCTAGGTTTACAAGCAACG 3' | 11349-11369 | 80α |
| SMT64 | *5' AACCAGTACGGATCACGT 3'* | 12283-12300 | 80α |
| SMT65 | 5' CGTAAAGAGGCTGAGCTAAG 3' | 12221-12240 | 80α |
| SMT66 | *5' CATAAATCTCCACACCGTGC 3'* | 13160-13179 | 80α |
| SMT67 | 5' GCAGATGTGTGCCTACCAA 3' | 12923-12941 | 80α |
| SMT68 | *5' CGTCTTATGATTTCGTTCGGGC 3'* | 13941-13962 | 80α |
| SMT69 | 5' GGGATTATGGCAGGTCAAGTTG 3' | 13845-13866 | 80α |
| SMT70 | *5' GCATCAGTGCGATTACGCC 3'* | 15137-15155 | 80α |
| SMT71 | 5' GTGTGGGAAATGACGGAGGTTA 3' | 14637-14658 | 80α |
| SMT72 | *5' CAGCCAACCCACATAGATCC 3'* | 16325-16344 | 80α |
| SMT80 | 5' CAGAAACTCATAGACGAGGGC 3' | 11046-11064 | 80α |
| SMT81 | 5' *CTGCCAGCGCTTCTTTTTC 3'* | 11888-11906 | 80α |
| SMT82 | 5' GGTCATGTGTGGAAAGACGA 3' | 11700-11719 | 80α |
| SMT83 | *5' ACCTTGCCAATCTGGTACG 3'* | 12481-12499 | 80α |
| SMT84 | 5' GGGCTATGTTAAGAATGATGCC 3' | 15020-15041 | 80α |
| SMT85 | *5' CTGACTGATCGCTCAACTTC 3'* | 15579-15598 | 80α |
| SMT86 | 5' CAAATATAGGCGGGGAGTTTG 3' | 15934-15954 | 80α |

| | | | |
|---|---|---|---|
| SMT87 | *5' GACTTGCTAAAGACTCTGCTG 3'* | 16501-16521 | 80α |
| SMT88 | 5' CTGACGGTAGAGAAGACACA 3' | 5002-5021 | 80α |
| SMT89 | *5' AATTCGGTGGTGACAGTGAC 3'* | 5678-5697 | 80α |
| SMT90 | 5' CATATCTTGTTGAGCTTTTAAGC 3' | 5567-5589 | 80α |
| SMT91 | *5' GTATTGATATGCCCATATGACC 3'* | 6266-6287 | 80α |
| SMT92 | 5' CGGAGGAAGTCAAGATGTAT 3' | 6174-6193 | 80α |
| SMT93 | *5' AATAGCCTTCTGGTGTAGC 3'* | 6870-6888 | 80α |
| SMT94 | 5' CACAGTAAAAGTACACGAGT 3' | 6803-6822 | 80α |
| SMT95 | *5' CCAATGACCCTTTGGATA 3'* | 7488-7505 | 80α |
| SMT96 | 5' CTAACGCTCAAGGAGAACGT 3' | 7407-7426 | 80α |
| SMT97 | *5' TGTCATTGTAGAACGTCCAGTC 3'* | 8073-8094 | 80α |
| SMT98 | 5' TACTACGGCTATGATGAACG 3' | 7995-8014 | 80α |
| SMT99 | *5' TGCTCCTCAGTTTTTAAATCAC 3'* | 8736-8757 | 80α |
| SMT100 | 5' GTTTCTAGGTTGTGTCCTGAC 3' | 8650-8670 | 80α |
| SMT101 | *5' GGGGGTGAATAATTATGGATCC 3'* | 9367-9388 | 80α |
| SMT102 | 5' GACAAGTTCCCAAGTTTTAGA G 3' | 9282-9303 | 80α |
| SMT103 | *5' TTATAGGGAATGGAAGACACC 3'* | 9900-9920 | 80α |
| SMT104 | 5' TGGTAAATCGCATACTATA 3' | 9801-9820 | 80α |
| SMT105 | *5' GTTTTTGACTGTTGCATCTC 3'* | 10472-10491 | 80α |
| SMT106 | 5' ATCTATGAGGAATATAAGCG 3' | 10393-10412 | 80α |
| SMT107 | *5' CACTCCTTAATATTCGACGA 3'* | 11137-11156 | 80α |
| SMT108 | 5' GCAGTAGAGACTACAGACGAG 3' | 10723-10743 | 80α |
| SMT109 | *5' CATAGGTGCCTCGAACGT 3'* | 11421-11438 | 80α |
| SMT110 | 5' ACAGGATCCGGAAGTATATTGTC GGGCAAC 3' | 9874-9894 | 80α |
| SMT111 | *5' CAAGAATTCTTTGGTTACCATGCGTC 3'* | 10987-11012 | 80α |
| SMT112 | 5' GTCGAATTCTAAGGAGTGTTAAA AAGCCG 3' | 11139-11168 | 80α |
| SMT113 | *5' GATCCATGAGTATGAGCCACTC GC 3'* | 12427-12446 | 80α |
| SMT123 | 5' AAAATAGGATCCATAATTATTCACCC CC 3' | 9361-9388 | 80α |
| SMT124 | *5' GTCTCGCTCTAGTGGTTTCATAATATCC CC 3'* | 10210-10230 | 80α |
| SMT125 | 5' ATGAAACCACTAGAGCGAGACG CATGGTAACCA 3' | 10981-11001 | 80α |
| SMT126 | *5' ATGGATCCGGTTTACCGTTAATC TTCACGACAC 3'* | 11991-12015 | 80α |
| SMT127 | 5' TCAGCATTGTTGATAACAGAGTAGG 3' | 10829-10853 | 80α |
| SMT128 | *5' CGTCGCTGAAATGGGC 3'* | 11265-11280 | 80α |
| SMT154 | *5' ACGCCACTCATACTAGTTACTGGG 3'* | 219-242 | SaPI1 |
| SMT155 | 5' CCATTCCGACCACCGTACAACGCT 3' | 14996-15019 | SaPI1 |
| SMT156 | 5' TCGTAAGTGCCGGTGATGTTCACT 3' | 815155-815178 | NCTC8 325 |
| SMT157 | *5' GTCTGCCATAATAAATGCCTCCTCGG 3'* | 816008-816034 | NCTC8 325 |
| pMADMCS1 | 5' GATTCTTCTCGCTTCCGGC 3' | * | pMAD |
| pMADMCS2 | *5' TACGTTACACATTAACTAGACAGATCTT CC 3'* | ** | pMAD |

| | | | |
|---|---|---|---|
| SP6 | 5' ATTTAGGTGACACTATAG 3' | Universal primer | |
| T7 | 5' TAATACGACTCACTATAGGG 3' | Universal primer | |

*pMADMCS1 pBR322 (GenBank J01749.1) nt#1006-1024
**pMADMCS2: Staphylococcus aureus subsp. aureus NCTC 8325, complete genome (GenBank CP000253.1) nt# 882468-882448
Primers in italics were used as reverse primers. Restriction sites are underlined.

100 µl of sterile water, heated to 95°C for 5 minutes, and transferred to an ice bath. Alternatively, bacterial cells from overnight broth cultures (100 µl) were pelleted at 14,000 rpm for 5 minutes and the pellet was washed two times with 900 µl of sterile water and finally resuspended in 100 µl of sterile water. Phage templates were prepared by diluting lysates 1:100 in sterile water or CsCl banded phage stocks 1:1000 in sterile water.

Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-11-dUTP) (Roche Applied Science, Mannheim, Germany) labeled probes were prepared for analysis of Southern blots. Dig-11-dUTP was randomly incorporated into the PCR product in place of dTTP. PCR conditions were the same as above for Taq except for the following changes: in place of the 10 mM dNTP mix, 2 µl of a dig-dNTP mix (2 mM dATP, dCTP and dGTP, 1.3 mM dTTP) and 3.5 µl of 70 µM DIG-11-dUTP were added to a 50 µl PCR reaction.

SOEing. The splicing by overlap extension technique (Horton *et al.*, 1990) was used to create the insert for ORF21 inactivation. PCR reactions were performed as described above with AccuPol. The first PCR reaction amplified sequence 1kb upstream of the start codon (SMT123, SMT124) and 1kb downstream of the stop codon (SMT125, SMT126). Small products and primers were eliminated from the first reaction products with SureClean and the size was verified on a minigel. A second PCR was performed using 1 µl each

of the amplified upstream and downstream fragments with the flanking primers (SMT123, SMT126).

TOPO cloning. PCR products were cloned in the Topo vector pCRT7/NT-TOPO™ (Invitrogen, Carlsbad, CA) as described in the kit insert. Following incubation, the mixture was added to an aliquot of chemically competent *E. coli* and incubated on ice for 5 minutes. The mixture was shifted to a 42°C water bath for 30 seconds and returned to ice, and immediately 250 µl SOC was added. The culture was incubated at 37°C with shaking for 1 hour, aliquots (25 µl and 225 µl) were spread to pre-warmed LB plates supplemented with ampicillin and incubated overnight at 37°C.

A similar procedure was used for cloning reactions with the Zero Blunt PCR Kit™ (Invitrogen, Carlsbad, CA). Aliquots of 50 µl and 100 µl were spread to LB agar supplemented with kanamycin.

Electrocompetent cell preparation. Electrocompetent *E. coli* cells were prepared from a 100ml culture grown in LB Lennox (Difco, Franklin Lakes, NJ) at 37°C with shaking. The culture was grown to a Klett= 80, then chilled on ice for 20 minutes, transferred to a pre-chilled centrifuge bottle and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was poured off and the pellet was resuspended in 200 ml of pre-chilled sterile water, centrifuged, resuspended in 100 ml of sterile water and centrifuged again. The pellet was

resuspended in 8 ml of 10% pre-chilled glycerol and transferred to a pre-chilled Corex tube and centrifuged at 10,000 rpm for 10 minutes. The pellet was resuspended in 2 ml of pre-chilled 10% glycerol and 40 µl aliquots were pipetted into 1.6 µl microcentrifuge tubes and placed on dry ice. The aliquots were stored at –80°C.

Electrocompetent *S. aureus* cells were prepared from a 100 ml BHI culture grown to a Klett of 95 and chilled on ice for 15 minutes. The culture was transferred to a pre-chilled centrifuge bottle and centrifuged at 5000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 10 ml of pre-chilled 0.5 M sucrose, transferred to a pre-chilled Corex tube and incubated on ice for 15 minutes. Centrifugation and resuspension were repeated two more times, reuspending the pellet with 5 ml and finally 2 ml 0.5 M sucrose. Aliquots of 50 µl were pipetted to chilled microcentrifuge tubes and placed on dry ice. The aliquots were stored at –80°C.

Plasmid electroporation. Aliquots of electrocompetent *E. coli* (40 µl) were incubated on ice with 3 µl of a vector-PCR ligation reaction for one minute, transferred to a pre-chilled electroporation cuvette (0.2 cm) (Bio-Rad, Hercules, CA) and pulsed one time using the MicroPulser (BioRad, Hercules, CA) *E. coli* setting EC2 (2.5 kV, 5 msec, 25 µF). Following electroporation, one ml of pre-warmed SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added, mixed and transferred

to a microcentrifuge tube, and incubated one hour with shaking at 37°C. The cells were centrifuged, the pellet was resuspended in 100 µl of LB broth, plated on LB plates supplemented with ampicillin and Xgal, and incubated overnight at 37°C.

Miniprep DNA purified from *E. coli* was used in the transformation of *S. aureus.* Purified DNA (7 µl) was added to 50 µl of electrocompetent RN4220 derivatives and incubated at room temperature for 10 minutes. The mixture was transferred to an electroporation cuvette (0.2 cm) and pulsed one time using the MicroPulser (BioRad, Hercules, CA) pre-set *S. aureus* setting Sta (1.8 kV, 2.5 msec, 25 µF). Following electroporation, one ml Brain Heart Infusion (BHI) broth (Remel, Lenexa, KS) was added then transferred to a microcentrifuge tube and incubated one hour with shaking at 30°C. The culture was centrifuged and the pellet was resuspended in 200 µl BHI. Aliquots (100 µl and 20 µl) were plated on TSA supplemented with erythromycin and Xgal and incubated at 30°C for 48 hours.

Allelic exchange. A single blue colony carrying the appropriate pMAD derivative was picked from TSA supplemented with erythromycin and Xgal, transferred to TSB supplemented with erythromycin, and incubated overnight with shaking at 44°C to select cointegrates. The following day, 100 µl of the culture was inoculated to pre-warmed TSB with erythromycin and placed on the orbital shaker for 3 hours at 44°C. Serial dilutions were prepared with pre-

warmed TSB and 100 µl aliquots were spread to pre-warmed TSA supplemented with erythromycin and Xgal and incubated at 44°C for 48 hours. Blue colonies were streaked for isolation to the same media and incubated 48 hours at 44°C. For cointegrate resolution, individual blue colonies were picked and transferred to TSB and incubated with shaking at 30°C until the broth was turbid, approximately 5 hours. Serial dilutions with pre-warmed TSB were prepared and 100 µl aliquots were plated on pre-warmed TSA with Xgal and incubated at 44°C for 48 hours to cure the cells of the plasmid. White colonies were streaked for isolation on TSA with Xgal and incubated at 30°C for 48 hours. An isolated colony was transferred to TSB, incubated with shaking at 30°C. DNA was extracted from an aliquot of the culture (100 µl) and used for a PCR reaction with a primer pair that would confirm a deletion in 80α. Once the deletion was confirmed, a second independent PCR reaction was performed with AccuPol and sent to Retrogen for sequencing.

Southern blot transfer. After staining, gels were trimmed and photographed under UV light. DNA was depurinated by incubating the gel in 0.25 N HCl for 10 minutes, and then rinsed with distilled water. The gel was soaked in a denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 45 minutes, rinsed with distilled water and soaked in a neutralization solution (1 M Tris-HCl pH 7.4, 1.5 M NaCl) for 30 minutes, rinsed in distilled water and soaked in fresh neutralization solution for 15 minutes. A positively charged nylon membrane

(ICN, Irvine, CA or Roche Applied Science, Mannheim, Germany) and two pieces of Whatman 3MM paper were cut slightly larger than the gel. The nylon membrane was submerged in distilled water, and once completely wet was transferred to 10X SSC buffer. A reservoir tank filled with 10X SSC was assembled using a glass plate wrapped in Whatman 3MM paper which served as a wick during the transfer. The gel was removed from the neutralization solution, centered upon the wet Whatman 3MM paper and surrounded with a parafilm barrier. The nylon membrane, the two pieces of Whatman 3MM paper wet with 2X SSC, a 5 cm stack of paper towels, a glass plate and a weight of approximately 500 g were layered on top of the gel. The transfer by capillary action required 24 hours after which the gel and nylon membrane were recovered. The nylon membrane was soaked for 15 minutes at room temperature in 6X SSC to remove agarose. The nylon membrane was then placed on Whatman 3MM paper saturated in 10X SSC and DNA was fixed to the membrane by UV-irradiation in a UV Stratalinker 1200 (Stratagene, La Jolla, CA) at 120,000 μjoules/$cm^2$. The gel was visualized under UV light to ensure DNA was transferred.

Hybridization and detection. Reagents were prepared using the DIG Wash and Block Buffer Set (Roche Applied Science, Mannheim, Germany) that contains concentrated buffers used in hybridization and detection. The appropriate volume (20 ml/100 $cm^2$ nylon membrane) of hybridization buffer (5X SSC, 0.1%

N-lauroyl sarcosine, 0.2% SDS and 1% blocking solution) was pre-warmed to the hybridization temperature (45°C) and placed into a hybridization bottle. The nylon membrane was placed into the bottle and incubated 30 minutes at 40°C with gentle agitation. The Dig-11-dUTP labeled probe (3 µl) was placed into a microcentrifuge tube with 50 µl of deionized water and boiled for 5 minutes, then rapidly cooled on ice. The denatured probe was mixed with pre-warmed hybridization buffer (3.5 ml/100 $cm^2$ nylon membrane). The pre-hybridization solution was decanted and replaced with the hybridization-probe solution and incubated for 16 hours at 45°C. After hybridization, the nylon membrane was washed two times for 5 minutes at room temperature in a high salt buffer (2X SSC, 0.1% SDS) followed by washing two times for 15 minutes each at 65°C in a lower salt buffer (0.1X SSC, 0.1% SDS). After washes, the membrane was placed into a clean tray with 1X Washing buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5, 0.3% (v/v) Tween20) for 5 minutes at room temperature with gentle agitation on an orbital platform. The solution was aspirated from the tray and replaced with 100 ml of 1X Blocking solution for 30 minutes with gentle agitation. Subsequently, the Blocking solution was replaced with (20 ml) of 1X Blocking solution containing Anti-digoxigenin-AP (75 mU/ml) and incubated for 30 minutes with gentle agitation. The antibody-containing solution was removed and the membrane was equilibrated with 20 ml of 1X Detection buffer for 5 minutes with gentle agitation. The membrane was placed into an opened hybridization bag and 1 ml of CSPD working solution (diluted 1:100 with 1X

Detection buffer) was applied to the membrane. The solution was spread evenly across the membrane by closing the hybridization bag and incubated at room temperature for 5 minutes. The excess liquid was squeezed from the bag, which was then sealed and incubated for 15 minutes at 37°C. The membrane was exposed to X-ray film (Kodak, Rochester, NY) and developed in an X-O-Mat (Kodak, Rochester, NY).

Computer analysis of nucleotide and protein sequences. Genomic and protein homology searches were performed with the BLAST program (Altschul *et al.*, 1997) on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Conserved domains were compared with the CDD-Search (Marchler-Bauer *et al.*, 2004) program, also on the NCBI website.

Protein secondary structures were predicted with PSIpred (Jones,1999) and the sequences were aligned with T-Coffee and M-Coffee (Notredame *et al.*, 2000). The software programs were found on the ExPASy Proteomics Server (http://au.expasy.org).

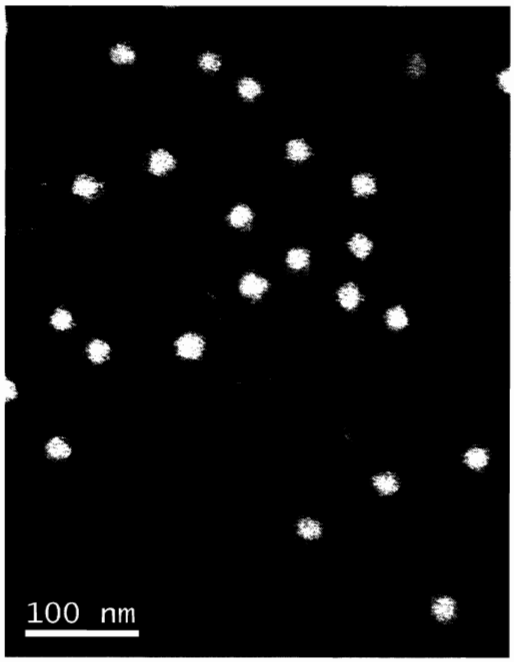
# Chapter 3 Identification of proteins in 80α virions and SaPI1 transducing particles

The genomic sequence of SaPI1 (GenBank U93688) does not reveal genes for any obvious structural proteins. Based on the structural similarities between helper phage and transducing virions observed in the EM (Ruzin *et al.*, 2001) and (Fig 4) and the similarity of this system to that described for satellite phage P4 and its P2 helper (Dokland,1999), it was hypothesized that SaPI1 virion proteins were encoded by the helper phage. To test this hypothesis and to determine whether there are any SaPI1-encoded proteins in transducing particles, peptides from virion proteins were analyzed and compared. The transducing particles and phage were concentrated from lysates by PEG 8000 precipitation and purified by CsCl step gradients. The samples were further purified by equilibrium centrifugation and each band was analyzed by comparing plaque titers, transducing titers, electron microscopy, and protein composition.

To ensure that the SaPI1 samples contained only one type of virion, a second equilibrium sedimentation was performed. Samples were then dialyzed and analyzed by titers and electron microscopy . The titers of each sample are indicated in parenthesis. The EM figures (4a-c) show a representative field for

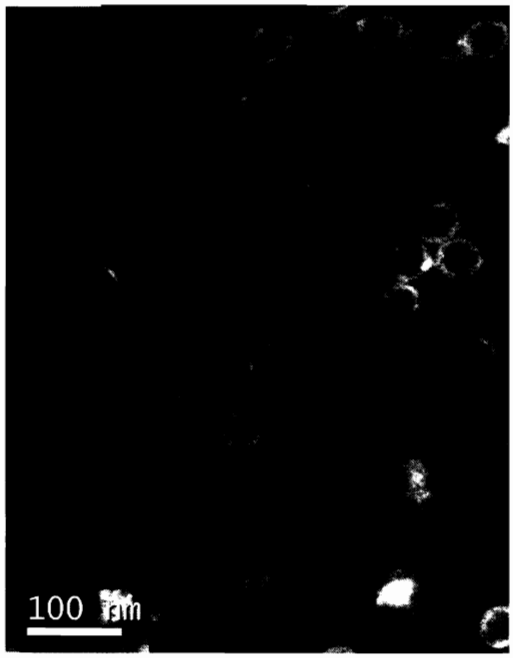
**Figure 4a-c. Electron micrographs of 80α and SaPI1**. Purified preparations are stained with 1% PTA on formvar-coated copper grids and magnified 10,000X using JEOL-JEM-1230 TEM and equipped with UltraScan 4000SP 4K X 4K CCD Gatan camera. (a) SaPI1 transducing particles; (b) phage 80α infectious particles; (c) phage capsids without DNA or tails.

a.




b.


c.

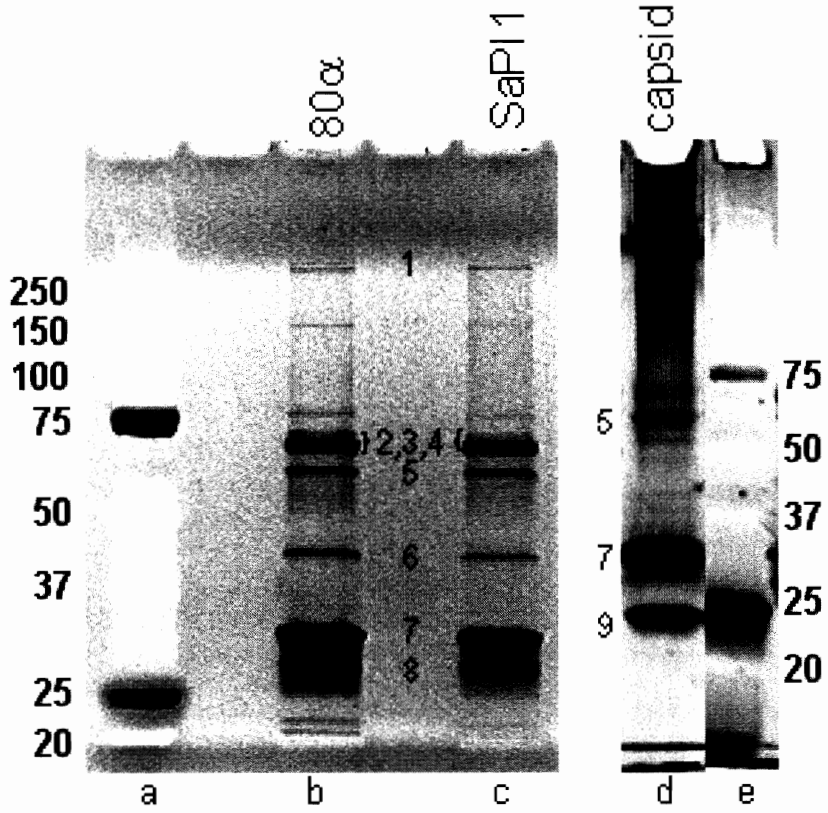


for each sample. Negative staining of double-banded and dialyzed samples confirmed that the phages and transducing particles have a similar morphology, with the SaPI1 capsid (approx 35 μm in diameter) being about 1/3 the volume of the 80α capsid (approx 50 μm in diameter). The enriched SaPI1 sample (SaPI1 titer=2 X $10^9$, phage titer=3X$10^8$) (4a) appears homogenous and no phage-sized capsids were detected. The EM of 80α particles (titer=3 X $10^{11}$) (Fig 4b) shows a field of homogenous phage particles consistent in size and morphology with previous results (Ruzin *et al.*, 2001). A band from the phage lysate that migrated higher in the CsCl gradient was also purified and analyzed. The electron micrographs of this fraction (titer=4X$10^8$) revealed only capsids of variable sizes (Fig 4d).

Virion proteins from the three samples described above were resolved by electrophoresis on SDS polyacrylamide gels. The banding patterns of 80α and SaPI1 virions were indistinguishable (Fig 5, Lanes b and c). The capsid fraction contained three predominant bands, one of which was unique (Fig 5, Lane d).

In order to confirm the identity of the 80α and SaPI1 proteins of the same electrophoretic mobility, and to allow assignment of virion proteins to the genes that encoded them, individual bands were excised, subjected to trypsin in-gel digestion, extracted and analyzed using nano-HPLC ESI ion trap mass spectrometry. The mass spectrometry data confirmed that the proteins derived from the transducing particles were identical to the helper phage-encoded proteins. No unique SaPI1-encoded proteins were detected. Nine proteins

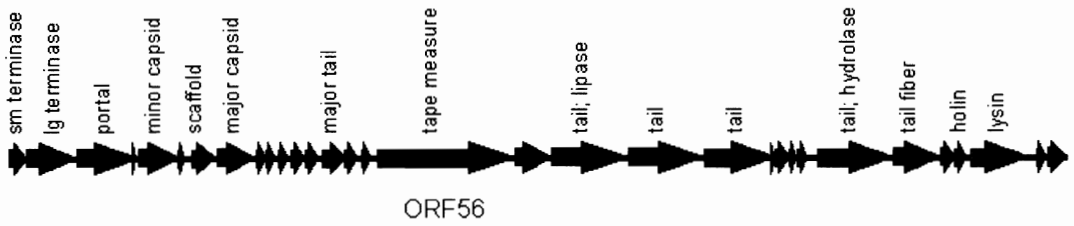
**Figure 5. SDS-PAGE gel.** Negative image of a Sypro-Ruby stained 10% SDS-PAGE gel with protein profiles of 80α and SaPI1-enriched virion preparations. Lane a and e, Precision Plus Protein Standard (10 kD-250 kDa); Lane b, 80α; Lane c, SaPI1 Lane d, 80α capsid fraction. Numbers indicate those bands excised (1-8 from both 80α and SaPI1 samples) and (5, 7, 9 from capsid fraction) and analyzed as described in the text.


80α
SaPI1
capsid
250
150
100
75
50
37
25
20
1
1,2,3,4
5
6
7
8
5
7
9
75
50
37
25
20
a
b
c
d
e

were assigned to predicted phage gene products based on the presence of multiple predicted tryptic peptides. These included proteins that are likely to be the major capsid subunit, portal protein, major tail tube, scaffolding protein, tape measure protein, and tail fiber/baseplate proteins.

Protein 1. Protein 1 on the gel (Fig 5) (>250 kDa) corresponded to the predicted protein encoded by ORF56 (1155 amino acids; 126 kDa) (Fig 6). This protein is the largest protein encoded by the phage genome and present in relatively low abundance and therefore is predicted to be the tail tape measure or tail size determination protein. It is absent from the capsid fraction (Fig 5, lane d). Migration of this polypeptide is extremely aberrant, it has a predicted mass of 126kDa but migrates with an apparent $M_r$ in excess of 250 kDa. We cannot account for this discrepancy at present, it may be an artifact due to protein conformation or it may represent a dimmer that is crosslinked or otherwise resistant to dissociation by SDS. The gene that encodes this protein is in a location characteristic of tape measure genes, i.e. downstream of the major tail subunit gene with two small intervening, overlapping reading frames containing a potential -1 translational frameshift (Xu *et al.*, 2004). Additionally, a CDD-Search detected two conserved domains. The N-terminus contains weak similarity to a variety of motifs that form coiled-coil structures, including eukaryotic SMC proteins and the myosin tail that assemble into

**Figure 6. Tape measure protein.** The genomic map representing the late genes is shown with the tape measure protein indicated by a blue arrow. Protein amino acid sequences are given, with tryptic peptides identified by mass spectrometry underlined and italicized.




ORF56

VTEYKIKATIEASVAKFKRQIDSAVKSVQRFKRVADQTKDVELNANDKKLQKTIKVAKKSLDAFSNKNVK
AK*LDASIQDLQQKILESNFELDKLNSK*EASPEVKLQKQKLTKDIAEAENKLSELEKKR*VNIDVNADNSK*F
NRVLKVSKASLEALNRSKAK*AILDVDNSVANSK*IKRTKEELKSIPNKTRSRLDVDTRLSIPTIYAFKKSL
DALPNKKTTKVDVDTNGLKKVYAYIIKANDNFQRQMGNLANMFRVFGTVGSNMVGGLLTSSFSILIPVIA
SVVPVVFALLNAIKVLTGGVLALGGAVAIAGAGFVAFGAMAISAIKMLNDGTLQASSATNEYKKALDGVK
SAWTDIIK*QNQSAIFTTLANGLNTVK*TAMQSLQPFFSGISRGMEEASQSVLKWAENSSVASRFFNMMNTT
GVSVFNKLLSAAGGFGDGLVNVFTQLAPLFQWSADWLDRLGQSFSNWANSAAGENSITRFIEYTKTNLPI
IGNIFKNVFAGINNLMNAFSGSSTGIFQSLEQMTAKFREWSEQVGQSQGFKDFVSYIQTNGPLIMQLIGN
IARGLVAFATAMAPIASAVLRVAVAITGWIANLFEAHPATAQLVGVIITLVGAFRFLIAPILAVMDFLGP
LAAR*LVALVTK*FGWAKTGTLVLSKAMTSLKGPIK*LVTAIFQLLFGK*IGLIRNAITGLVTVFGILGGPITI
VIGVIAALIAIFVLLWNKNEGFRNFIINAWNAIKTFMVNVWNVLKAVASVVWNAILTAITTAVSNVYNFI
MIVWNQIVAYLQGLWNGIIAIATTVWNLLVTIITTVFTTIMTIVMTIWTAIWTFLSTIWNTIITIATTIW
NLLVTVITTVFTTIMTIAMTIWNAIWTFLQTLWNTIVTVATKVWNAITTAISTALQAAWSFISNIWNTIW
SFLSGILTTIWNK*VVSIFTQVVSTISDK*MSQAWNFIVTKGMQWVSTITSTLINFVNR*VIQGFVNVVNK*VS
QGMTNAVNKIKSFIGDFVSAGADMIRGLIRGIGQMAGQLVDAAKNVAKKALDAAKSALGIHSPSREFMDV
GMYSMLGFVKGIDNHSSKVIRNVSNVADKVVDAFQPTLNAPDISSITGNLSNLGGNINAQVQHTHSIETS
PNMKTVKVEFDVNNDALTSIVNGRNAKRNSEYYL

macromolecular filaments. The C-terminus contains several regions of similarity to COG5412, a phage-related protein domain of unknown function. This protein is 94% identical to the presumptive tape measure protein of staphylococcal phage Φ11, and highly conserved among staphylococcal phages.

Proteins 2,3,4. These three virion proteins were not sufficiently resolved by SDS-PAGE to do mass spectrometry on individual bands. Nevertheless, analysis of tryptic peptides allowed assignment of these proteins to predicted genes. One of the proteins is predicted to be encoded by ORF59 (633 amino acids; 71 kDa) (Fig 7a). A second predicted protein corresponded to a minor tail protein encoded by ORF61 (636 amino acids; 74 kDa) (Fig 7b). The third protein from this group is predicted to correspond to a protein encoded by ORF62 (607 amino acids; 67 kDa) (Fig 7c).

These proteins were less abundant in the 80α capsid fraction (Fig 5, lane d), indicating that these proteins are components of the phage tail. They are similar to hypothetical proteins in other staphylococcal phages. Two of the proteins, encoded by ORF61 and ORF62, contain no known motifs. The protein encoded by ORF59 contains two domains identified by CDD-Search. The N-terminus shows weak similarity (4 e-06) to DUF1142, a domain of unknown function found in phage and bacterial proteins. The C-terminus contains a

**Figure 7a-c. Minor tail proteins.** The genomic map representing the late genes is shown. The genes encoding the proteins are indicated with blue arrows. Protein amino acid sequences are given, with tryptic peptides identified by mass spectrometry underlined and italicized. (a) Protein encoded by ORF59; (b) Protein encoded by ORF61; (c) Protein encoded by ORF62.


**a.**

MTITIKPPKGNGAPVPVETTLVKKVNADGVLTFDILENKYTYEVINAIGKRWIVSHVEGENDKKEYVITV
IDRKSEGDRQLVECTAREIPIDKLMIDRIYVNVTGSFTVERYFNIVFQGTGMLFEVEGKVKSSKFENGGE
GDTRLEMFKKGLEHFGLEYKITYDKKKDRYKFVLTPFANQK*ASYFISDEVNANAIKLEEDASDFATFIR*G
YGNYSGEETFEHAGLVMEAR*SALAEIYGDIHAEPFK*DGKVTDQETMDKELQSRLKKSLKQSLSLDFLVLR
ESYPEADPQPGDIVQIKSTK*LGLNDLVR*IVQVKTIRGINNVIVK*QDVTLGEFNR*EQRYMKKVNTAANYVS
GLNDVNLSNPSKAAENLKSKVASIAKSTLDLMSRTDLIEDKQQKVSSK*TVTTSDGTIVHDFIDK*SNIKDV
KTIGTIGDSVARGSHAKTNFTEMLGKKLKAKTTNLARGGATMATVPIGKEAVENSIYRQAEQIRGDLIIL
QGTDDDWLHGYWAGVPIGTDKTDTKTFYGAFCSAIEVIRKNNPASKILVMTATRQCPMSGTMIRRKDTDK
NKLGLTLEDYVNAQILACSELDVPVYDAYHTDYFKPYNPAFRKSSMPDGLHPNERGHEVIMYELIKNYYQ
FYG

**b.**

MDNKLITDLSRVFDYR*YVDENEYNFK*LISDMLTDFNFSLEYHRNK*EVFAHNGEQIK*YEHLNVTSSVSDFL
TYLNGRFSNMVLGHNGDGINEVKDARVDNTGYDHKTLQDR*LYHDYSTLDAFTK*KVEKAVDENYKEYRATE
YRFEPKEQEPEFITDLSPYTNAVMQSFWVDPRTKIIYMTQARPGNHYMLSRLKPNGQFIDRLLVKNGGHG
THNAYRYIGNELWIYSAVLDANENNKFVRFQYRTGEITYGNEMQDVMPNIFNDRYTSAIYNPIENLMIFR
REYKASERQLK*NSLNFVEVR*SADDIDKGIDKVLYQMDIPMEYTSDTQPMQGITYDAGILYWYTGDSK*PAN*
*PNYLQGFDIK*TKELLFKR*RIDIGGVNNNFK*GDFQEAEGLDMYYDLETGRK*ALLIGVTIGPGNNR*HHSIYS
IGQRGVNQFLKNIAPQVSMTDSGGR*VKPLPIQNPAYLSDITEVGHYYIYTQDTQNALDFP*LPKAFRDAGW
FFDVLPGHYNGALRQVLTRNSTGRNMLKFER*VIDIFNK*KNNGAWNFCPQNAGYWEHIPKSITKLSDLK*IV*
*GLDFYITTEESNR*FTDFPKDFKGIAGWILEVKSNTPGNTTQVLRR*NNFPSAHQFLVR*NFGTGGVGKWSLF
EGKVVE

**c.**

MVVDNFSKDDNLIELQTTSQYNPVIDTNISFYESDR*GTGVLNFAVTK*NNRPLSISSEHVKTFIVLKTDDY
NVDR*GAYISDELTIVDAINGRLQYVIPNEFLK*HSGK*VHAQAFFTQNGSDNVVVER*QFSFNIENDLVSGFD
GITKLVYIK*SIQDTIEAVGK*DFNQLKQNMADTQTLIAKVNDSATK*GIQQIEIKQNEAIQAITATQTSATQ*
*AVTAEFDKIVEK*EQAIFER*VNEVEQQINGADLVK*GNSTTNWQKSKLTDDYGKAIESYEQSIDSVLSAVNT
SR*IIHITSATDAPSFKDIGTVDTPKEDGVDDGSDIPVAPNTLGKSGVLVVYVVDDSTAR*ATWYPDDSNDE
YTKYKISGTWYPFYKKNDGDLTK*QFVEETSNNALNQAK*QYVDDKFGTTSWQQHKMTEANGQSIQVNLNNA
QGDLGYLTAGNYYATR*VPDLPGSVESYEGYLSVFVK*DDTNK*LFNFTPYNSKK*IYTRSITNGRLEQQWTVP
NEHKSTVLFDGGANGVGTTINLTEPYTNYSILLVSGTYPGGVIEGFGLTALPNAIQLSKANVVDSDGNGG
GIYECLLSKTSSTTLR*IDNDVYFDLGK*TSGSGANANKVTITKIMGWK

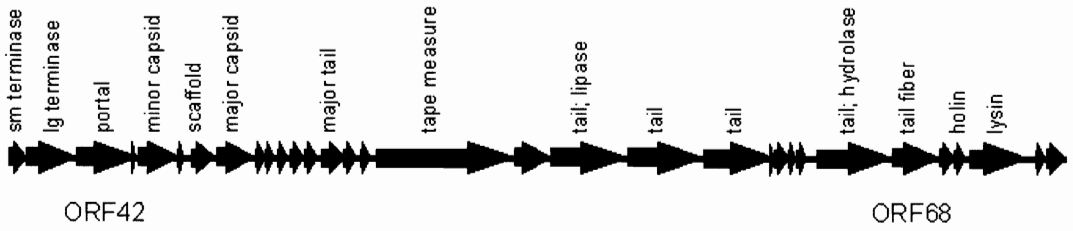
domain with high similarity (2e-40) to the SGNH_hydrolase subfamily of lipases and esterases. Given the enzymatic activities among members of this family, the precise function of this domain is unclear, but we speculate that this activity plays a role in the degradation of the cell wall components to allow infection.

Protein 5. Protein 5 from the gel (63 kDa) corresponds to the predicted protein encoded by ORF42 (511 amino acids; 60 kDa) (Fig 8a). This protein is a component of the phage procapsid, based on its presence in the virions and the 80α capsid fraction. A CDD-Search reveals that this protein shows significant similarity (3e-75) to the SPP1 Gp6-like family of phage portal proteins. It is highly conserved among other staphylococcal phages.

Protein 6. Protein 6 from the gel (45 kDa) corresponds to the predicted protein encoded by ORF68 (390 amino acids; 44 kDa) (Fig 8b). This protein is a component of the phage tail, based on its absence from the 80α capsid fraction, and is similar to a large number of putative tail fiber proteins in other staphylococcal phages. A CDD-Search did not reveal the presence of any known motifs.

Protein 7. Protein 7 from the gel (32 kDa) corresponds to the predicted protein encoded by ORF 47 (324 amino acids; 37 kDa) (Fig 9a). This protein was identified as the major capsid protein based on its high level of abundance in

**Figure 8. Portal Protein and Tail Protein.** The genomic map representing the late genes is shown. The genes encoding the proteins are indicated with blue arrows. Protein amino acid sequences are given, with tryptic peptides identified by mass spectrometry underlined and italicized. (a) Protein 5, encoded by ORF42; (b) Protein 6, encoded by ORF68



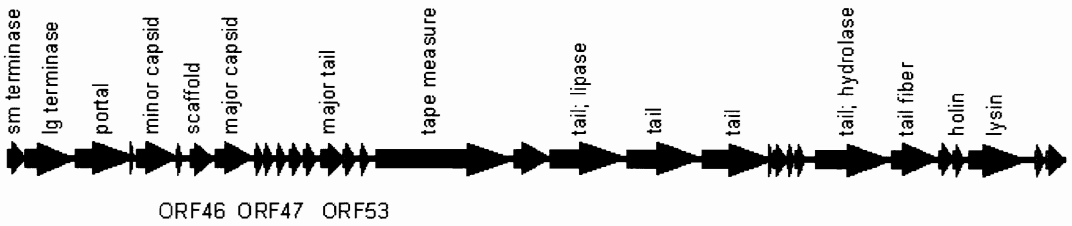

**a.**

MLK*VNEFETDTDLR*GNINYLFNDEANVVYTYDGTESDLLQNVNEVSKYIEHHMDYQRPRLK*VLSDYYEGK*
TK*NLVELTR*RKEEYMADNRVAHDYASYISDFINGYFLGNPIQYQDDDKDVLEAIEAFNDLNDVESHNR*SL*
*GLDLSIYGK*AYELMIRNQDDETRLYK*SDAMSTFIIYDNTVER*NSIAGVRYLRTKPIDKTDEDEVFTVDLF
TSHGVYRYLTNRTNGLKLTPR*ENSFESHSFERMPITEFSNNER*RKGDYEK*VITLIDLYDNAESDTANYMS*
*DLNDAMLLIKGNLNLDPVEVR*KQKEANVLFLEPTVYVDAEGR*ETEGSVDGGYIYKQYDVQGTEAYKDRLN*
*SDIHMFTNTPNMK*DDNFSGTQSGEAMKYK*LFGLEQR*TKTK*EGLFTK*GLRRRAK*LLETILK*NTRSIDANKD
FNTVRYVYNRNLPK*SLIEELK*AYIDSGGKISQTTLMSLFSFFQDPELEVKKIEEDEKESIKKAQKGIYKD
PRDINDDEQDDDTKDTVDKKE

**b.**

MYKIKDVETR*IKNDGVDLGDIGCRFYTEDENTASIR*IGINDKQGRIDLKAHGLTPR*LHLFMEDGSIFKNE*
*PLIIDDVVKGFITYK*IPKKVIKHAGYVRCK*LFLEKEEEKIHVANFSFNIVDSGIESAVAK*EIDVK*LVDDA*
*ITR*ILK*DNATDLLSK*DFK*EKIDKDVISYIEK*NESRFKGAKGDKGEPGQPGAKGEAGKKGEQGAPGK*NGTV*
*VSINPDTK*MWQIDGK*DTDIKAEPELLDKINIANVEGLEDK*LQEVEKIKDTTLNDSKTYTDTKIAELVDSA
PESMNTLR*ELAEAIQNNSISESVLQQIGSK*VSTEDFEEFKQTLNDLYAPKNHNHDER*YVLSSQAFTK*QQA
DSLYQLKSASQPTVK*IWTGTENEYNYIYQKDPNTLYLIK*G

**Figure 9. Major Capsid Protein, Major Tail Protein and Scaffolding Protein.** The genomic map representing the late genes is shown. The genes encoding the proteins are indicated with blue arrows. Protein amino acid sequences are given, with tryptic peptides identified by mass spectrometry underlined and italicized. (a) Protein 7, encoded by ORF47;(b) Protein 8, encoded by ORF53; (c) Protein 9, encoded by ORF46.




**a.**

MEQTQKLKLNLQHFASNNVKPQVFNPDNVMMHEKKDGTLMNEFTTPILQEVMENSKIMQLGKYEPMEGTE
K*KFTFWADKPGAYWVGEGQK*IETSKATWVNATMRAFK*LGVILPVTK*EFLNYTYSQFFEEMKPMIAEAFYK
*KFDEAGILNQGNNPFGKSIAQSIEK*TNKVIK*GDFTQDNIIDLEALLEDDELEANAFISK*TQNRSLLRKIV
DPETKERIYDR*NSDSLDGLPVVNLKSSNLKRGELITGDFDKLIYGIPQLIEYKIDETAQLSTVK*NEDGTP
VNLFEQDMVALR*ATMHVALHIADDK*AFAKLVPADKRTDSVPGEV

**b.**

MANMKNSNDR*IILFR*KAGEKVDATKMLFLTEYGLSHEADTDTEDTMDGSYNTGGSVESTMSGTAKMFYGD
DFADEIEDAVVDR*VLYEAWEVESR*IPGKNGDSAKFKAK*YFQGFHNK*FELKAEANGIDEYEYEYGVNGRFQ
R*GFATLPEAVTK*KLKATGYR*FHDTTKEDALTSEDLTAIPQPK*VDSSTVTPGEV

**c.**

MEENKLKFNLQFFADQSDDPDEPGGDGKKGNPDKK*ENDEGTEITFTPEQQKKVDEILER*RVAHEKKKADE
YAKEKAAEAAKEAAKLAKMNKDQKDEYEREQMEKELEQLRSEKQLNEMRSEAR*KMLSEAEVDSSDEVVNL
VVTDTAEQTKSNVEAFSNAVK*KAVNEAVKVNAR*QSPLTGGDSFNHSTKNKPQNLAEIAR*QKRIIKN

gels of both the virion and capsid fractions. No conserved domains were revealed by a CDD-Search. A BLAST search revealed similarities to putative major capsid proteins from phages of a number of Gram-positive bacteria, including staphylococci, bacilli and listeriae.

Protein 8. Protein 8 from the gel (28 kDa) corresponds to the predicted protein encoded by ORF 53 (193 amino acids; 22 kDa) (Fig 9b). This protein is predicted to be the major tail protein, based upon its high abundance in virions and its absence from the capsid fraction. No conserved domains were revealed by a CDD-Search. Similar proteins identified in a BLAST search include putative major tail proteins characterized in phages isolated from other Gram-positive bacteria including streptococci, enterococci and lactococci.

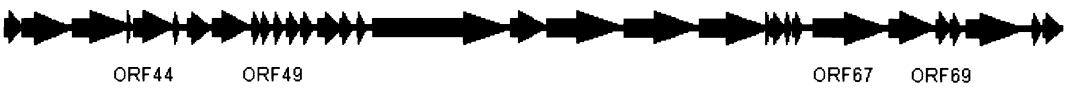
Protein 9. Protein 9 from the gel (24 kDa) corresponds to the predicted protein encoded by ORF46 (206 amino acids; 23 kDa) (Fig 9c). This protein is abundant in the capsid fraction but absent from mature virions. This suggests that it is the capsid scaffolding protein. It contains no known conserved domains. A BLAST search reveals high conservation of this protein among a number of staphylococcal phages.

ORF44. The protein encoded by ORF44 was identified on an 18% resolving gel (data not shown) and whole phage digestion (Table 3, Fig 10). The

**Table 3. Peptides identified by whole particle digestion with trypsin.**

| ORF number | Identification, MW and number of residues | Peptides |
|---|---|---|
| ORF44 | Minor head protein | AIENELKR* |
| $M_r$ = 39kDa | MW =38 kDa | EINAFIVK |
| | 331 amino acids | IIDEFDVK |
| | | LVENKDFSDR |
| | | VFSDQAGILGEGVQVAK |
| | | VHAQSSIDSMEKEISPEGYYMYIAK |
| | | ILNMMIK |
| | | YGDFAGVTLQEAQK |
| | | EQMLKIQIEFLIAYATAQTELSMR |
| | | SLLYTESAR |
| | | GLNGEIFK |
| ORF49 | Structural protein | IGAEGMTSEAVDGR |
| $M_r$ = 13kDa | MW =13 kDa; 110 amino acids | SNAYELNDFKEYEAIIDNYFNAR |
| ORF67 | Tail fiber, hydrolase domain | TWGNADAMAQK |
| $M_r$ = 72kDa | MW =72 kDa | NAQTMSSVESLYNSR |
| | 632 amino acids | HNIWAPR |
| | | VDDSIWR |
| | | VMVDNK |
| | | QPQIITETSPYTFK |
| | | YQGVSVSALNK |
| | | GIYFTR |
| | | YTISSQEVNYPEYIYHFIVEGNR |
| | | YKQDVEYPHFYVDR |
| | | YEIPLSIENLK |
| | | DKYEALEK |
| | | NADIANVR |
| | | KSNAWGWANATR |
| | | NKGWTSPAK |
| ORF69 | Structural protein | NKYPDSIEESTAK |
| $M_r$ = 15kDa | MW =14 kDa; 131 amino acids | WTINGVEPNKSYQVTIENVR |

**Figure 10. Proteins identified from whole phage digestion**. The genomic map representing the late genes is shown. The genes encoding the proteins from whole phage digestion are indicated with blue arrows.


ORF44
ORF49
ORF67
ORF69

predicted protein (331 amino acids; 38 kDa) was similar to a putative minor head protein. A CDD-Search revealed that this protein has similarity (7e-10) to the Phage_Mu_F domain. Members of this family are found in dsDNA phages and some bacteria. A member of this protein family is required for viral head morphogenesis in phage SPP1. It is highly conserved among other staphylococcal phages.

ORF49. The product of ORF49 was identified only in the whole phage tryptic digest. The peptides identified by mass spectrometry (Table 3, Fig 10) of the predicted protein (110 amino acids; 13 kDa) correspond to a conserved protein in staphylococcal phages. A protein BLAST revealed high similarity to conserved staphylococcal phage proteins with unknown function and 30% identity to a putative DNA packaging protein in *Lactobacillus* phage ΦJL-1.

ORF67. The product of ORF67 was identified only in the trypsin digested whole phage preparation. The peptides identified by mass spectrometry (Table 3, Fig 10) of the predicted protein (632 amino acids; 72 kDa) correspond to a peptidoglycan hydrolase and is a highly conserved staphylococcal phage protein. A CDD-Search identified two putative enzymatic domains. The N-terminus is similar to (2e-14) to the CHAP domain which has an amidase activity that is found in many Gram positive autolysins and phage endolysins and which catalyzes cleavage of the amide bond between N-acetylmuramic

acid and L-alanine in bacterial cell-wall peptidoglycan (Bateman *et al.*, 2003) (Rigden *et al.*, 2003). This domain of ORF67 shares 21% amino acid identity with the CHAP domain of the well characterized phage Φ11 endolysin, which has been shown to hydrolyze staphylococcal cell walls (Donovan *et al.*, 2006) (Sass *et al.*, 2007). The C-terminus is similar (9e-51) to the LytD domain, which has β-N-acetyl-glucosaminidase activity and cleaves the glycosidic bond between the long glycan strands of N-acetyl-glucosamine and N-acetyl-muramic acid. This domain is commonly found in staphylococcal autolysins, and the LytD domain of ORF67 shares 43% amino acid identity with the corresponding domain in the major autolysin from *S. aureus* NCTC8325 (Foster,1995). The role of this protein is presumed to be a virion-associated hydrolase that plays a role in genome penetration of the infected cell. A highly similar protein is encoded by staphylococcal phage Φ11, which has been shown to have a virion associated protein with peptidoglycan hydrolytic activity (Moak *et al.*, 2004). However, the Φ11 activity migrated with an apparent $M_r$ of approximately 47 kDa. If the product of ORF67 is responsible for this observed activity, the protein must be subject to proteolytic processing, with only one of the two domains retaining activity independent of virion association. Alternatively, this activity could be associated with ORF68 (band 6, Fig 5) which is also highly conserved between 80α and Φ11. If this is the case, it would represent a novel motif for peptidoglycan hydrolysis, since this protein contains no identified domains.

ORF69. The product of ORF69 was identified in the whole phage tryptic digest. The peptides identified by mass spectrometry (Table 3, Fig 10) of the predicted protein (131 amino acids; 14 kDa) correspond to similar proteins of unknown function which are conserved among staphylococcal phages.

In summary, 80α and SaPI1 particles were compared to determine the origin of the virion structural proteins in the smaller SaPI1 transducing particles. All virion proteins of sufficient abundance to visualize by SDS-PAGE were identical, demonstrating that SaPI1 transducing particles are comprised of virion proteins encoded by 80α. An 80α fraction containing empty capsid-like structures was also examined; the presence of an abundant protein absent from mature virions suggests that these are capsid precursors and allowed identification of the presumptive scaffolding protein. Whole virion digests were processed to identify proteins of low abundance or proteins that were too small to be resolved on SDS-PAGE. Three phage proteins that were not identified from gel electrophoresis were identified by this method.

All proteins were analyzed by mass spectrometry, confirming their identity and allowing correlation to 80α genes. No unique SaPI1 proteins were identified in the mature virion.

## Chapter 4 Selection and Characterization of Phage Mutants

The strategy for investigation of interactions between SaPI1 and its helper phage, 80α, was based on seletion of phage mutants resistant to interference by SaPI1. In order to select the mutants, wild type 80α was plated on a SaPI1 positive strain (RN7045). SaPI1 interference reduces the production of 80α by 2 orders of magnitude in a one-step growth curve (Fig 2). This reduction is sufficient to block plaque formation completely when plated on a lawn of a SaPI1-containing host. The rare plaque found on a lawn of a SaPI1-positive strain is potentially resistant to interference from the pathogenicity island. Individual plaques were isolated and plaque purified once on a SaPI1 negative strain, RN450, and then checked for equivalent efficiency of plating on both strains. Equivalent plating indicates a potential "*sir*" mutant. A plate lysate of each potential mutant was then prepared on a SaPI1 negative strain and checked again for equivalent plating. The plate stock was then used to inoculate larger cultures of a SaPI1 negative strain. Each phage mutant's resistance to SaPI1 interference and ability to transduce SaPI1 was tested. After initial characterization, two candidate mutants were chosen for further detailed analysis and designated 80α *sirE* and 80α *sirB.*

Effects of mutants on SaPI1 transduction. A typical phage burst from a SaPI1 positive *S. aureus* strain infected with 80α will contain both transducing particles and phage. The results from the 70-minute time point of one-step growth curve experiments (Fig 11) show the phage titers in isogenic *S. aureus* strains with and without SaPI1. The phage titer is reduced by at least 2 orders of magnitude in the SaPI1 positive strain, similar to what has been reported previously (Lindsay *et al.*, 1998). The SaPI1 transducing titer is within an order of magnitude of the PFU in a SaPI1 positive strain, as seen in Figure 11. To compare the 80α *sir* mutants, a ratio representing SaPI1 interference with phage lytic growth was calculated by dividing the number of plaques counted on RN7045 (SaPI1 positive) by the number of plaques counted on RN450 (SaPI1 negative). The *sirE* mutant and the *sirB* mutant show no interference by SaPI1, as indicated by a ratio close to or equal to 1 (Table 4). Initial characterizations of the effects of the *sir* mutations on SaPI1 transduction are also presented in Table 4. The SaPI1 transduction frequency is calculated by dividing the number of tetracycline resistant colonies, i.e., SaPI1 transductants, by the plaque forming titer in the same lysate, when plated on a SaPI1-negative indicator strain. The transducing titer of 80α *sirE* was decreased by two orders of magnitude compared to 80α and the transducing titer for 80α *sirB* was decreased by 4 orders of magnitude compared to 80α (Table 4). The apparent lack of SaPI1 interference and lower SaPI1 transduction rate motivated further evaluation.

**Figure 11. SaPI1 interference and transduction with a wild type helper phage.** Shown is the 70-minute time point from a one-step 80α growth curve experiment of 80α infection in SaPI1 negative (RN450) and positive (RN7045) strains. Phage were titered on RN450. The reduction in PFU seen in infection of RN7045 is due to interference by SaPI1. SaPI1 transducing units (TU) are measured by the number of tetracycline resistant colonies or transductants obtained following infection of RN450 with the lysate obtained on RN7045.

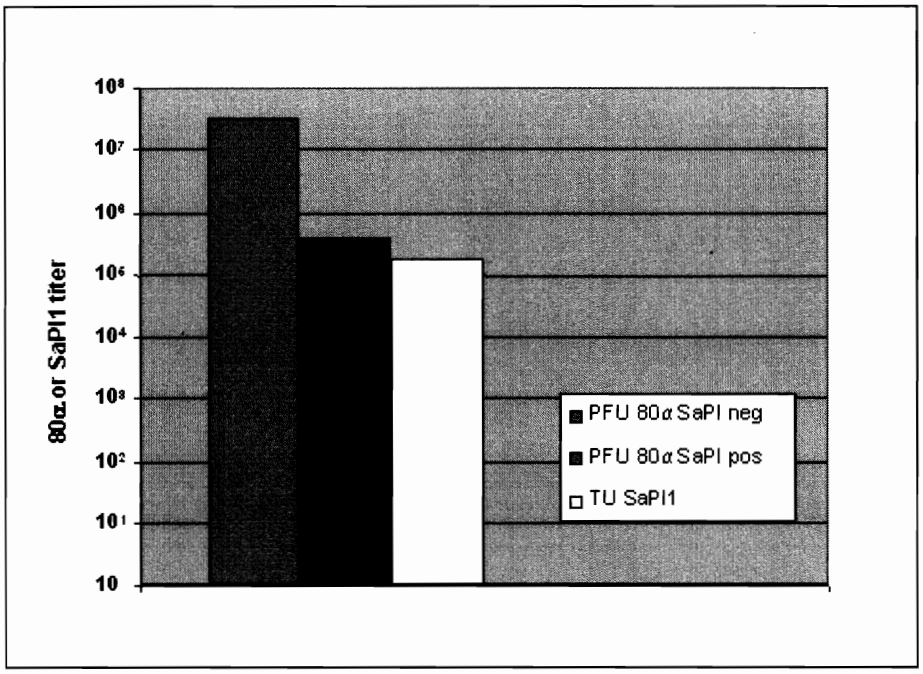

80α or SaPI1 titer
10⁸
10⁷
10⁶
10⁴
10³
10²
10¹
10
PFU 80α SaPI neg
PFU 80α SaPI pos
TU SaPI1

**Table 4 Initial characterization of 80α *sir* Mutants.**

| Phage | SaPI1 plating interference[a] | SaPI1 transducing frequency[b] |
|---|---|---|
| 80α | $2 \times 10^{-6}$ | $1 \times 10^{-1}$ |
| *sirE* | .966 | $1.5 \times 10^{-3}$ |
| *sirB* | 1 | $2 \times 10^{-5}$ |

[a] $\frac{\text{80α plaques on SaPI}^+ \text{ lawn}}{\text{80α plaques on SaPI}^- \text{ lawn}}$

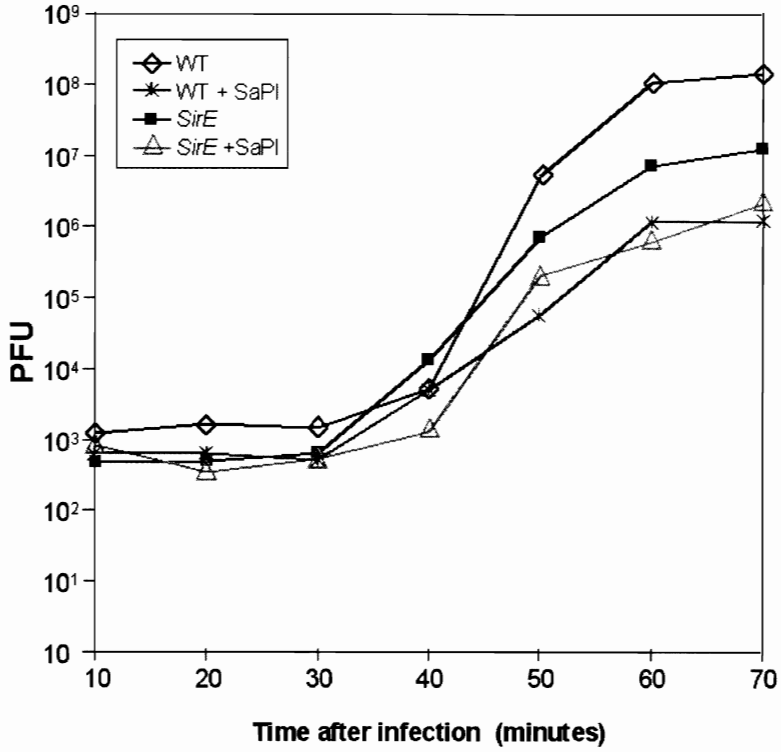
[b] $\frac{\text{SaPI TU on SaPI}^- \text{ lawn}}{\text{80α PFU on SaPI}^- \text{ lawn}}$

Growth of 80α *sir*E. One mutant, selected and designated *sirE*, showed little or no interference from SaPI1 as demonstrated by equivalent efficiency of plating on SaPI1 negative and positive strains and retained a reduced level of SaPI1-specific transduction. One step growth experiments were used to further evaluate the growth of 80α and the *sirE* mutant in isogenic SaPI1 negative and positive strains of *S. aureus* (Fig 12a). The *sirE* mutant phage yield in the SaPI1 negative strain is about an order of magnitude lower than the yield of 80α in the same strain. This was surprising, since there was no apparent difference in plaque morphology or size between the wild type and *sirE* mutant phage. One possible explanation is that even with a lower burst size, the phage lysins degrade the surrounding host cells giving what should be a smaller sized plaque the appearance of a normal sized one. In the SaPI1 positive strain the *sirE* mutant phage titer and the 80α titer are equivalent. This represents a 100X reduction in the presence of SaPI1 for 80α but only a 10X reduction in the case of the *sirE* mutant . Thus, *sirE* does appear to be more resistant to SaPI1 interference than does 80α. There is a discrepancy between the plating results, where we observed a decrease in efficiency of SaPI1 transduction, and the one-step growth experiment, where we have the same number of transducing particles as wild type (Fig 12b). We cannot account for this difference.

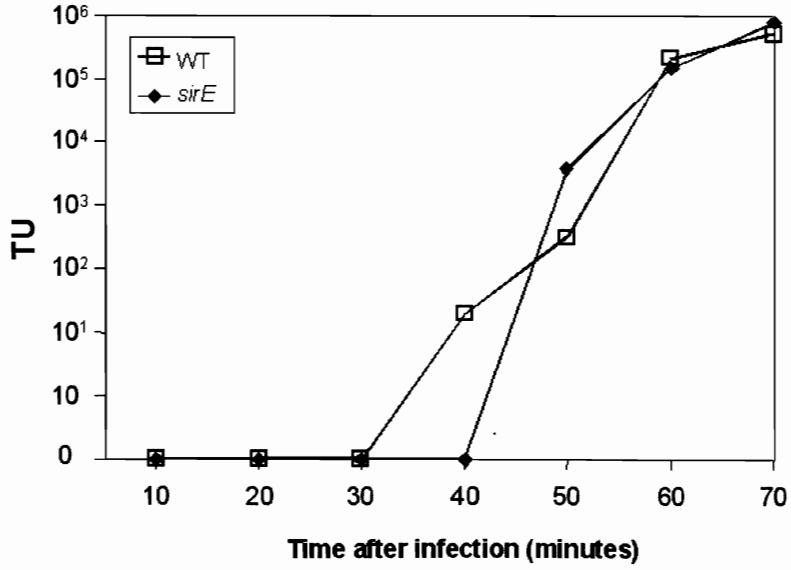
Growth of 80α *sirB*. A second phage mutant, designated *sirB*, also showed no SaPI1 interference as demonstrated by equivalent plating and a much greater

**Figure 12. One-step growth curves of 80α and 80α *sirE*.** The top panel represents the phage titers and the lower panel represents the transducing titers. (a) Phages were grown in a SaPI1 negative or SaPI1 positive host and titered at 10-minute intervals on SaPI1 negative strain RN450. (b) Transducing titers of phages grown on SaPI1 positive strain RN7045 and used to transduce RN450 to tetracycline resistance.

**a.**


WT
WT + SaPI
SirE
SirE +SaPI
PFU
10
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
10
20
30
40
50
60
70
Time after infection (minutes)

**b.**


WT
sirE
TU
0
10
10¹
10²
10³
10⁴
10⁵
10⁶
10
20
30
40
50
60
70
Time after infection (minutes)

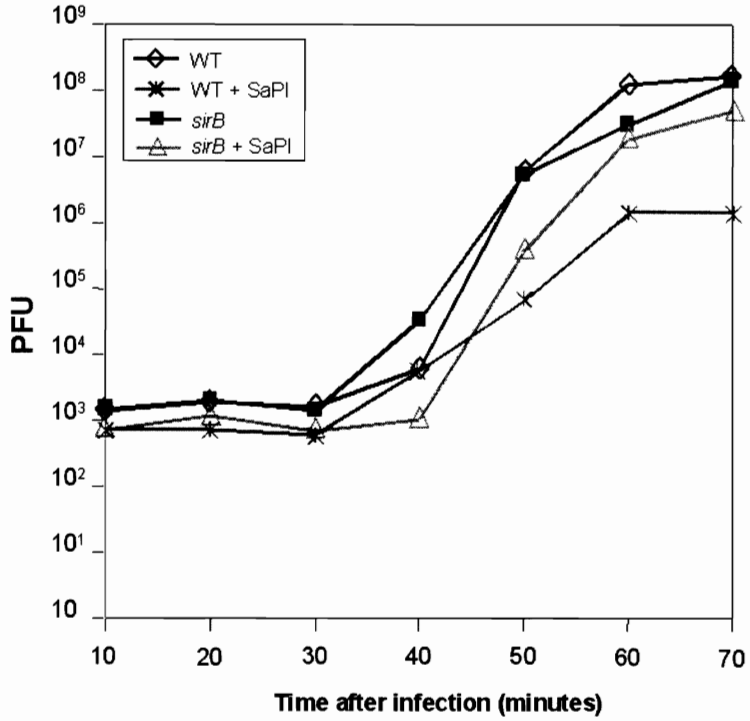
reduction in SaPI1 transduction. This mutant also showed no SaPI1 interference in one-step growth curves (Fig 13a). In addition, the *sirB* mutation does not appear to impair phage growth. The SaPI1 transducing titer following a *sirB* mutant infection was at least 4 orders of magnitude less than the transducing titer following wild type 80α infection (Fig 13b). This level of transduction is characteristic of the rate of generalized transduction seen with chromosomal markers (Lindsay *et al.*, 1998) indicating that SaPI1 is no longer mobilized by the 80α *sirB* mutant.

Identification of the *sir* mutations. Based on what is known or predicted about SaPI1 mobilization, there are several possibilities for the mechanism of interference by SaPI1 of 80α lytic growth. SaPI1 interference could be due to the change in capsid size, which would prevent phage DNA from being packaged into smaller capsids. Interference could also be due to a change in DNA packaging specificity conferred by the SaPI1-encoded small terminase subunit. A third possible mechanism for interference could be due to a SaPI1-imposed block to helper phage replication.

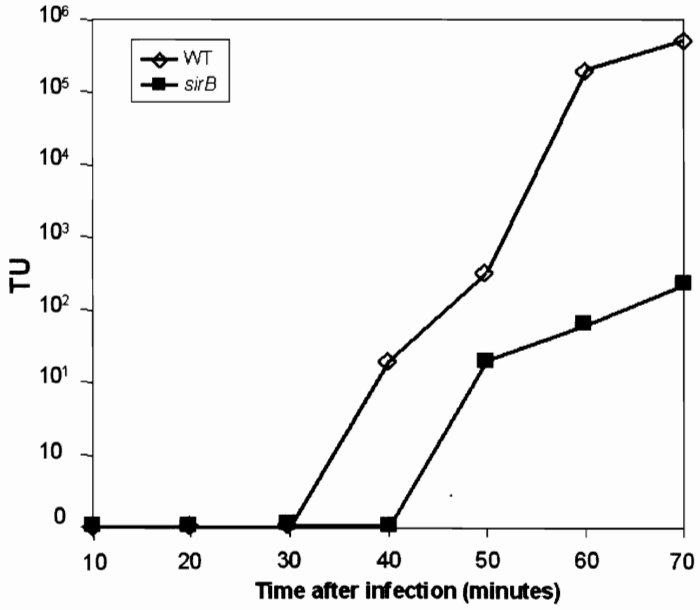
In the absence of phage mutants that could be used for genetic mapping, we chose direct DNA sequence analysis and sequenced the genomes of the mutant phages from the putative replication region through the capsid cluster. Sequence analysis of DNA amplified from ORF8-39 revealed point mutations in

**Figure 13. One-step growth curves of 80α and 80α *sirB*.** The top panel represents the phage titers and the lower panel represents the transducing titers. (a) Phages were grown in a SaPI1 negative or SaPI1 positive host and titered at 10-minute intervals on a SaPI1 negative strain RN450. (b) Transducing titers of phages grown on SaPI1 positive strain RN7045 and used to transduce RN450 to tetracycline resistance.

**a.**


WT
WT + SaPI
sirB
sirB + SaPI
PFU
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10
10
20
30
40
50
60
70
Time after infection (minutes)

**b.**

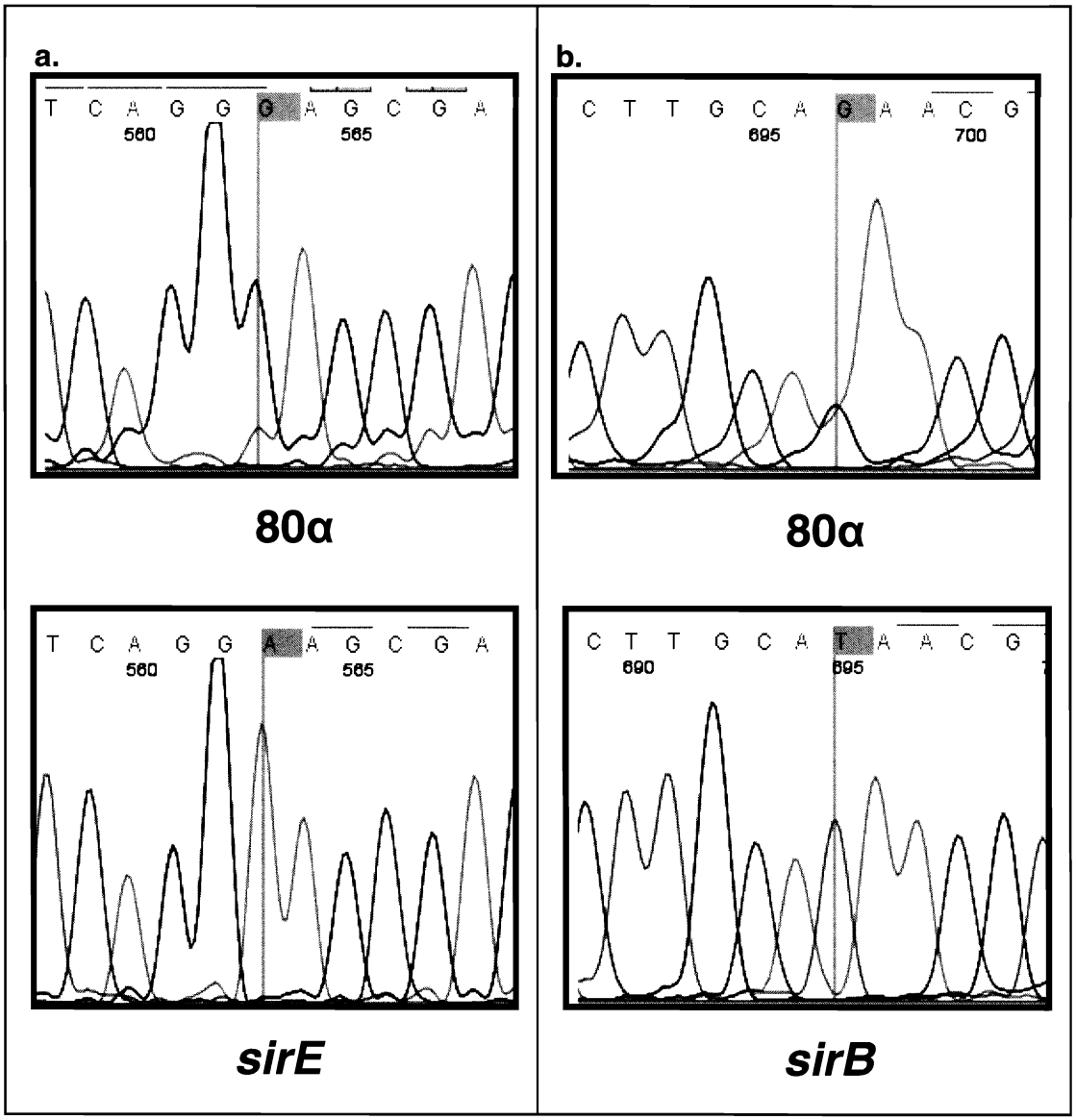

WT
sirB
TU
10⁶
10⁵
10⁴
10³
10²
10¹
10
0
10
20
30
40
50
60
70
Time after infection (minutes)

two adjacent genes. The *sirE* mutant contained a point mutation in ORF21, while the *sirB* mutant contained a point mutation in ORF22. These point mutations were confirmed with sequence analysis of an independent PCR product using a different pair of primers. In total, roughly 42% of the phage genomes, from nucleotides 5002-23482 (corresponding to ORF8-ORF50), was sequenced and compared.

ORF21 (10219-10998), which contains the *sirE* mutation, has a predicted length of 780 nucleotides, encoding a protein of 259 amino acids. A BLAST search with the nucleotide sequence revealed homology to other staphylococcal phages. The top 5 hits ranging from 98-99% similarity include ΦETA2, Φ13, ΦSLT, ΦNM4, and ΦNM2. A CDD-Search with the predicted protein sequence showed similarity to a conserved domain COG1484, a DnaC-like protein thought to be involved in DNA replication, recombination and repair. The 80α *sirE* point mutation was found at nucleotide 10985, and changed the sequence from G to A (Fig 14a). This point mutation results in an amino acid change from a glutamic acid residue to lysine (amino acid 255). The gene product must be important for phage growth, as indicated by the lower titers of the *sirE* mutant in both SaPI1 negative and positive staphylococcal strains, but is not required by SaPI1 since the transducing titer is similar to the titer seen in an 80α infection in the same strain (Fig 12b).

ORF22, which contains the *sirB* mutation, is a predicted gene of 159

**Figure 14. Identification of mutations in the 80α *sirE* and 80α *sirB* mutants.** Chromatograms of the DNA sequencing reactions for each *sir* mutant and the equivalent region of the wild type phage are shown. The nucleotide that was changed is highlighted. (a) 80α *sirE* ORF22. (b) 80α *sirB* ORF21.


a.
T C A G G G A G C G A
560
565
80α
b.
C T T G C A G A A C G
695
700
80α
T C A G G A A G C G A
560
565
sirE
C T T G C A T A A C G
690
695
sirB

nucleotides encoding a predicted protein of 52 amino acids. A BLAST search identified a similar gene in a number of staphylococcal phages. The product of this gene from phage 77, ORF104, has been shown to interact with the host helicase loader DnaI, thereby blocking bacterial replication (Liu *et al.*, 2004). The point mutation was a G to T change at nucleotide 11115, which generates a premature stop codon (Fig 14b). This would truncate the gene product to 42 amino acids, and the final product is likely not a functional protein. This gene product must be essential for SaPI1 mobilization, since transduction by this mutant is reduced to the level of generalized transduction (Fig 13b). Growth of the *sirB* mutant phage in both SaPI1 negative and positive strains is similar, which suggests that ORF22 is not essential for phage growth (Fig 13a).

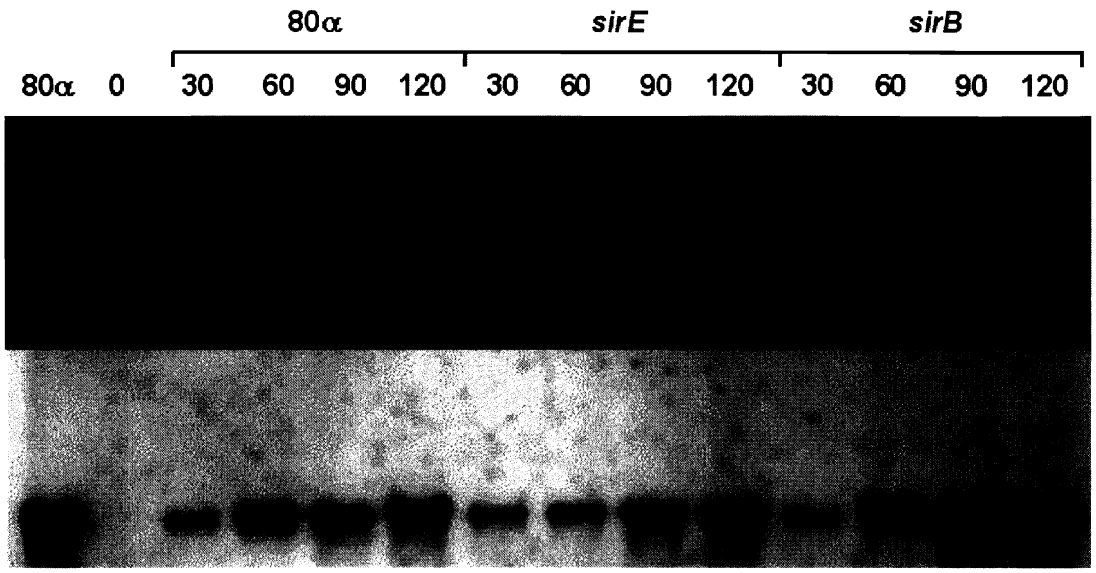
Evaluation of phage and SaPI1 DNA replication. The genes affected in the SaPI1 interference-resistant mutants both mapped to the phage replication region. This implicated phage DNA replication as a possible target of SaPI1 interference. To evaluate more directly phage and SaPI1 replication, DNA was extracted and compared at different time points after phage infection of SaPI1 negative and positive strains. Southern blots were probed with either 80α or SaPI1 specific DIG-11-dUTP labeled probes. The 80α probe is specific for the putative phage replication region between nucleotide coordinates 7995-8757. The SaPI1 probe is specific for the small terminase subunit, nucleotide

coordinates 2971-3561. Every attempt was made to ensure equal loading of DNA samples in each lane of the gel.

The first blot (Fig 15) shows phage replication following infection with wild type 80α and the *sirE* and *sirB* mutants. An increase in phage DNA is detected at 30 minutes in the 80α infection, and remains fairly constant at later time points. The same time points from the *sirE* mutant infection are similar to the matching time points in 80α infection. There does appear to be a slight delay in replication, as evident in the 60-minute time point. However, this result was hard to reproduce. Phage replication in the *sirB* mutant is similar to wild type 80α, but a more intense band is seen at 90 and 120 minutes post infection. While this mutant did not yield a higher phage titer in one-step growth experiments, an increased yield of phage particles was consistently evident following CsCl step-gradient purification of 80α *sirB* lysates. The volume and intensity of the phage band was markedly increased compared to that obtained in the same amount of lysate from the wild type phage. We cannot account for the discrepancy between the apparent increase in both phage DNA and phage particles and the lack of increase in plaque-forming units.

Evaluation of SaPI1 replication in the presence of 80α and the *sirE* and *sirB* mutants was also performed (Fig 16). The SaPI1 specific probe hybridizes to bulk DNA detected as the predominant upper band seen on the gel and the blot. A high molecular weight (presumably concatameric) form also migrates

**Figure 15. Southern blot analysis of phage replication**. DNA was extracted at 30-minute intervals after infection of a SaPI1 negative staphylococcal strain with the indicated phages. The upper panel is an agarose gel stained with ethidium bromide and the lower panel is an autoradiogram of the Southern blot hybridized with an 80α DIG-11-dUTP probe and exposed for one hour. 80α virion DNA was used as a control.


80α
sirE
sirB
80α 0 30 60 90 120 30 60 90 120 30 60 90 120

*sirB* mutants was also performed (Fig 16). The SaPI1 specific probe hybridizes to bulk DNA detected as the predominant upper band seen on the gel and the blot. A high molecular weight (presumably concatameric) form also migrates with the bulk DNA. The lower band detected on the gel and the blot is the extrachromosomal band. This was originally predicted to represent an excised and replicating form of SaPI1, (Lindsay *et al.*, 1998) but is now believed to represent mature linear SaPI1 DNA (Novick, 2007). Extrachromosomal SaPI1 is detected in the 80α phage infection at 60 minutes post-infection, but is delayed 30 minutes in the 80α *sirE* infection. This is similar to the change in kinetics observed in replication of the *sirE* mutant phage itself, and correlates with a similar 10X reduction in titer. There is a slight reduction in the bulk DNA signal. No extrachromosomal SaPI1 replication was detected in the 80α *sirB* infection (Fig 16), consistent with the transduction only at the level of a chromosomal marker (Fig 13b). In addition, no increased signal was observed in the bulk DNA band.

To investigate SaPI1 interference with phage DNA replication, isogenic SaPI1 negative and SaPI1 positive staphylococcal strains were infected with wild type 80α or one of the 80α mutants. The blots were hybridized with an 80α DIG-11-dUTP probe. Phage replication is evident in all three phages and is almost equivalent in the corresponding time points for each phage. The SaPI1 interference does not appear to be at the level of DNA replication (Fig 17 a-c).

**Figure 16. Southern blot analysis of SaPI1 replication.** DNA was extracted at 30-minute intervals after infection of a SaPI1 positive staphylococcal strain with the indicated phages. The upper panel is an agarose gel stained with ethidium bromide and the lower panel is an autoradiogram of the Southern blot hybridized with a SaPI1 DIG-11-dUTP probe and exposed for 10 minutes. SaPI1 virion DNA was used as a control. The upper arrow in each panel indicates the predominant band of bulk DNA. The lower band indicated by the arrow represents the extrachromosomal copy of SaPI1.

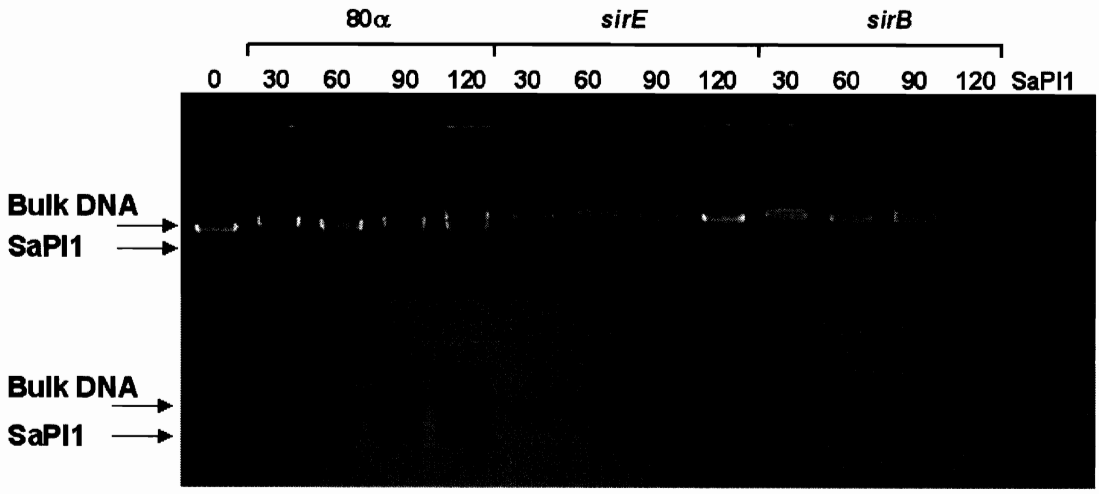
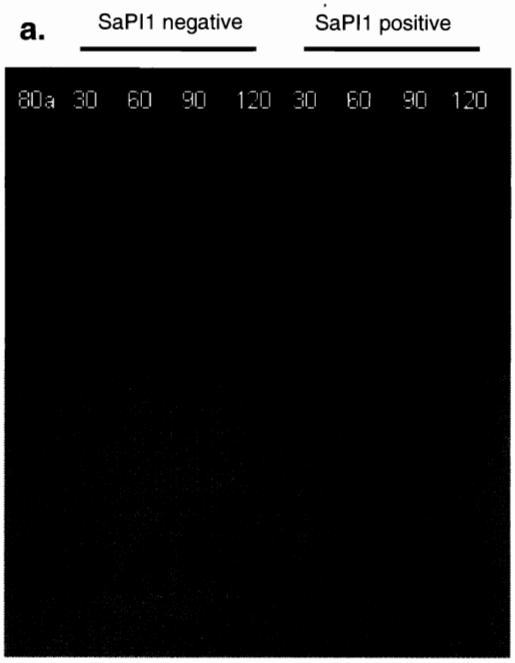
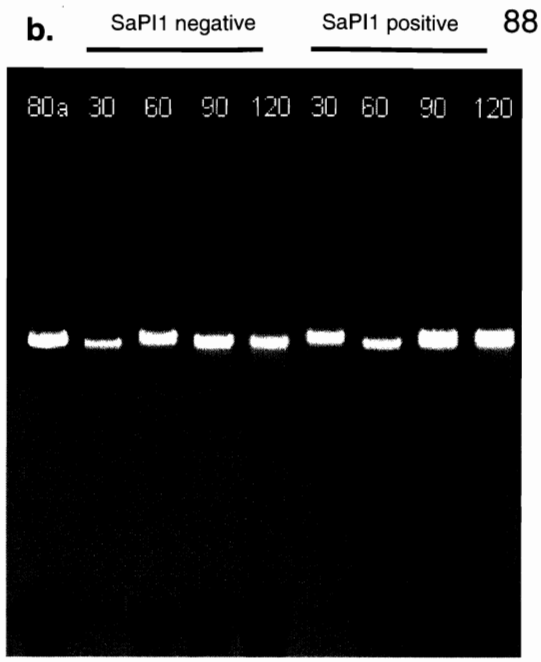

80α
sirE
sirB
0 30 60 90 120 30 60 90 120 30 60 90 120 SaPI1
Bulk DNA
SaPI1
Bulk DNA
SaPI1

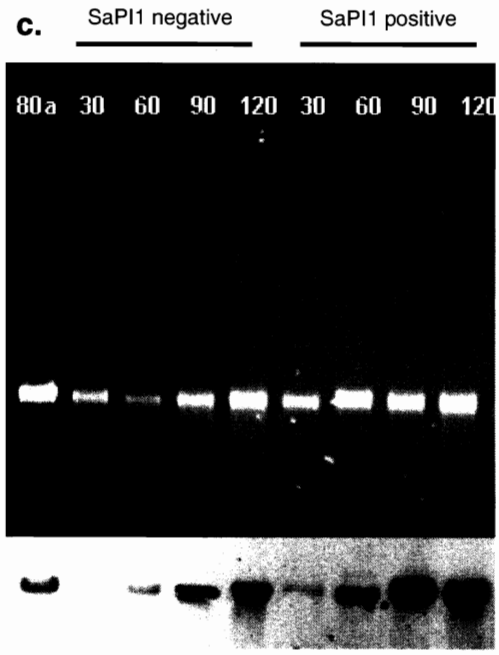
**Figure 17. Effect of SaPI1 on 80α replication.** Shown are agarose gels and blots comparing phage replication during a time course experiment in SaPI1 negative and positive staphylococcal strains. Blots were probed with an 80α DIG-11-dUTP probe and exposed one hour. Genomic 80α was used as a control. (a) Wild type 80α (b) 80α *sirE* (c) 80α *sirB.*




80α



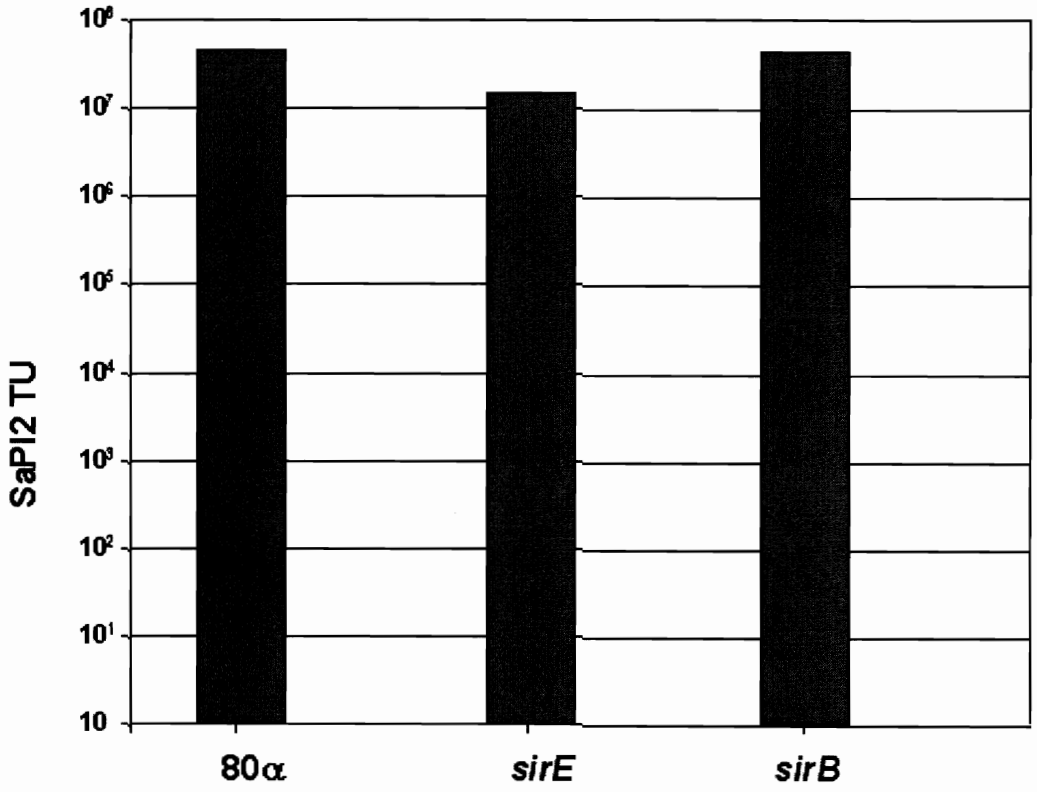

80α *sirE*




80α *sirB*

A variety of phages have been tested for their ability to excise, replicate and mobilize SaPIs. SaPI1 is excised from the genome during vegetative growth of Φ13 and 80α and transduced at high frequency by 80α but not Φ13. SaPI2, another pathogenicity island carrying the toxic shock gene, is excised by phage 80, 29 and 52 (Lindsay *et al.*, 1998) and also by 80α at least in RN4220. A SaPI2 carrying strain, ST23, was infected with 80α, 80α *sirE*, and 80α *sirB* to determine if these mutations effect SaPI2 transduction. The transducing titers are almost equivalent and above the level of generalized transduction for both mutants indicating that SaPI2 is mobilized by these phages (Fig 18). In contrast, the transducing titers for SaPI1 were similar for 80α and the *sirE* mutant (Fig 12b), while the *sirB* mutant failed to mobilize SaPI1 (Fig 13b), which indicates this gene is specific for this pathogenicity island and not SaPI2.

**Figure 18. SaPI2 transducing titers.** Shown are the transducing titers of 80α, 80α *sirE* and 80α *sirB* lysates grown on a SaPI2 *tst*::*tetM* positive (ST23) strain. Transducing particles were titered on RN450.


SaPI2 TU
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10
80α
sirE
sirB

## Chapter 5 Construction and Testing of Lysogens with Deletion of ORF21 or ORF22

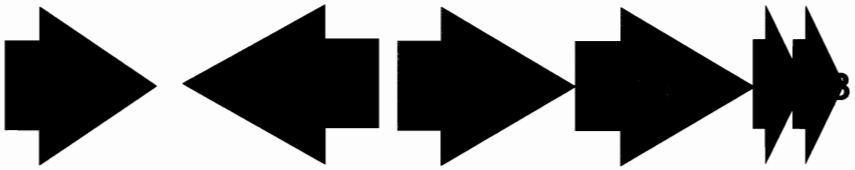
The SaPI1 resistant phage mutants required further study to assess the effect of each gene in phage replication, SaPI1 replication and SaPI1 interference. The initial findings suggested that ORF21 was involved in helper phage replication and ORF22 was nonessential for 80α, but required for mobilization of SaPI1. In order to confirm that the point mutations were responsible for the observed phenotypes and to provide additional insight into the role(s) of these genes, each affected gene was directly inactivated by deletion. An in-frame deletion of each ORF was constructed in order to avoid possible polar effects. Furthermore, since at least one of these genes was likely to be essential for lytic growth of the helper phage, the deletions were constructed in prophage copies of 80α in SaPI1 negative and positive strains. Phage and SaPI1 replication and SaPI1 interference were evaluated in the newly constructed strains following induction of the mutant prophages.

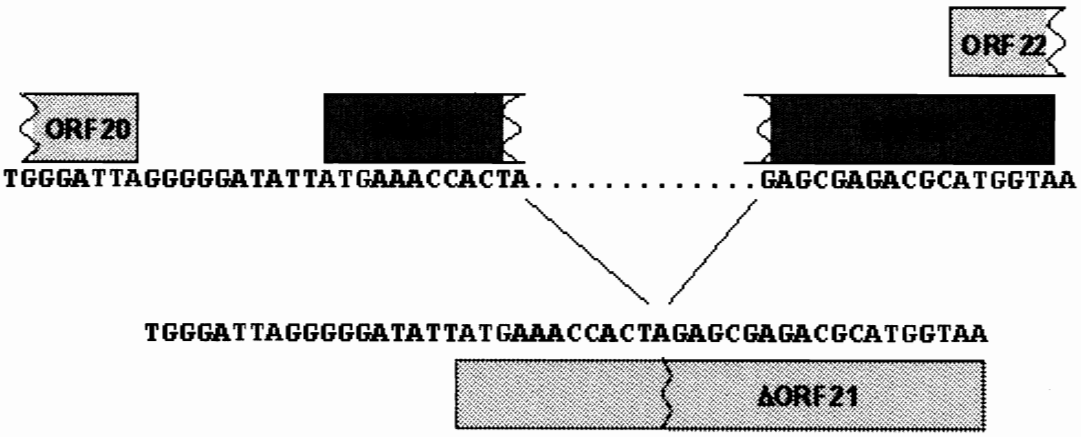
Properties of pMAD. The temperature sensitive plasmid pMAD (Arnaud *et al.*, 2004) was used as the allelic exchange vector. This vector, a gift from Kimberly Jefferson, contains a temperature sensitive *ori* for replication in staphylococci derived from pE194 (Iordanescu,1976) and an *E. coli ori* derived from pBR322

(Bolivar *et al.*, 1977). Other relevant features include a multiple cloning site, selectable antibiotic resistance markers for both *S. aureus* and *E. coli*, and a constitutively expressed promoter placed upstream of the *bgaB* gene from *Bacillus stearothermophilus* that encodes a thermostable β-galactosidase. This allows identification of plasmid-containing strains by blue/white screening on media supplemented with Xgal. *E. coli* strains carrying pMAD were screened on media supplemented with ampicillin (100 μg/ml) and staphylococcal strains carrying pMAD were screened on plates supplemented with erythromycin (5 μg/ml).

Deletion of ORF21. The phenotype of the *sirE* mutant suggested that this gene is required for phage replication, but is not required for SaPI1 replication. In order to delete the gene, regions of about 1kb flanking ORF21 were amplified and joined using the splicing by overlap extension (SOEing) PCR technique (Horton *et al.*, 1990). The external primers were designed to add a BamHI restriction site matching the cloning site in pMAD. The initial PCR reactions amplified the regions downstream of the start codon and upstream of the stop codon flanking ORF21. The two products were used in a second PCR reaction to create the in-frame ORF21 deletion (Fig 19). The insert was cloned into the Zero Blunt Topo vector, purified and digested from the vector with BamHI. The BamHI digested insert was ligated to pMAD digested with the same enzyme.

**Figure 19. Sequence of the ORF21 in-frame deletion.** ORF21 and flanking ORFs are shown. Start codons are highlighted in green and stop codons are highlighted in red. Upstream and downstream sequence from ORF21 was amplified by PCR. The SOEing technique was used to splice the two sequences and create the in-frame deletion. The lower sequence depicts the ΔORF21 construct.



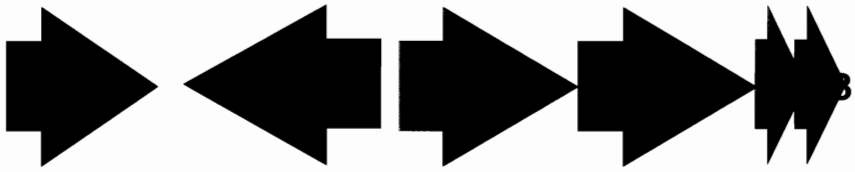

ORF22
ORF20
TGGGATTAGGGGGATATTATGAAACCACTA.....GAGCGAGACGCATGGTAA
TGGGATTAGGGGGATATTATGAAACCACTAGAGCGAGACGCATGGTAA
ΔORF21

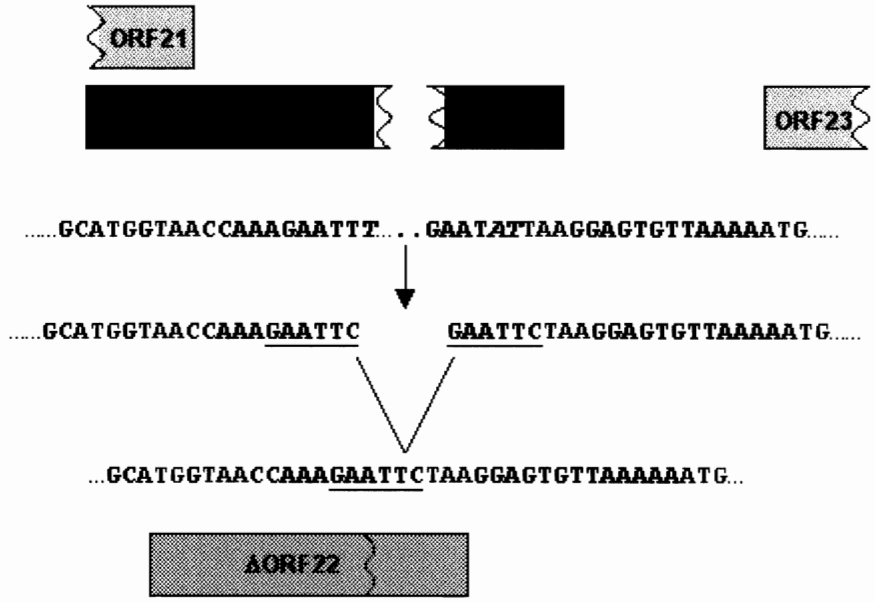
*E. coli* was transformed with the resulting plasmid, pMADΔORF21. The plasmid was purified and introduced by electroporation into the desired staphylococcal strains for allelic exchange.

Deletion of ORF22. The phenotype of 80α *sirB* suggested that ORF22 was nonessential for 80α, but is required for SaPI1 interference and high frequency transduction. As with ORF21, an in-frame deletion of the gene was prepared using PCR products of 1kb flanking regions that retained the ORF22 start and stop codons (Fig 20). In this case however, the primers used for amplification introduced an internal EcoRI site and an external BamHI site. The upstream and downstream regions were amplified in a PCR reaction, each PCR product was digested with BamHI and ligated to the same restriction site in the Topo vector pCRT7/NT. The two plasmids, one carrying the upstream fragment and the second carrying the downstream fragment, were purified, digested with EcoRI, and joined together to create the deletion of ORF22. The fragment carrying the deletion was excised with BamHI, ligated with BamHI-digested pMAD, and used to transform *E. coli*. The resulting plasmid, pMADΔORF22, was purified from *E. coli* and introduced into 80α staphylococcal lysogens negative (RN10616) and positive for SaPI1 (RN10628) for allelic exchange.

Allelic exchange. The transformed staphylococcal strains were subjected to temperature shifts to promote allelic replacement. Antibiotic resistant

**Figure 20. Sequence of the ORF22 in-frame deletion.** ORF22 and flanking ORFs are shown. Start codons are highlighted in green and stop codons are highlighted in red. Upstream and downstream sequence from ORF21 was amplified by PCR and an EcoRI site was added. The nucleotides that were changed are italicized and the EcoRI site is underlined. The two sequences were ligated at this junction creating the ΔORF22 in frame deletion depicted in the lower sequence.

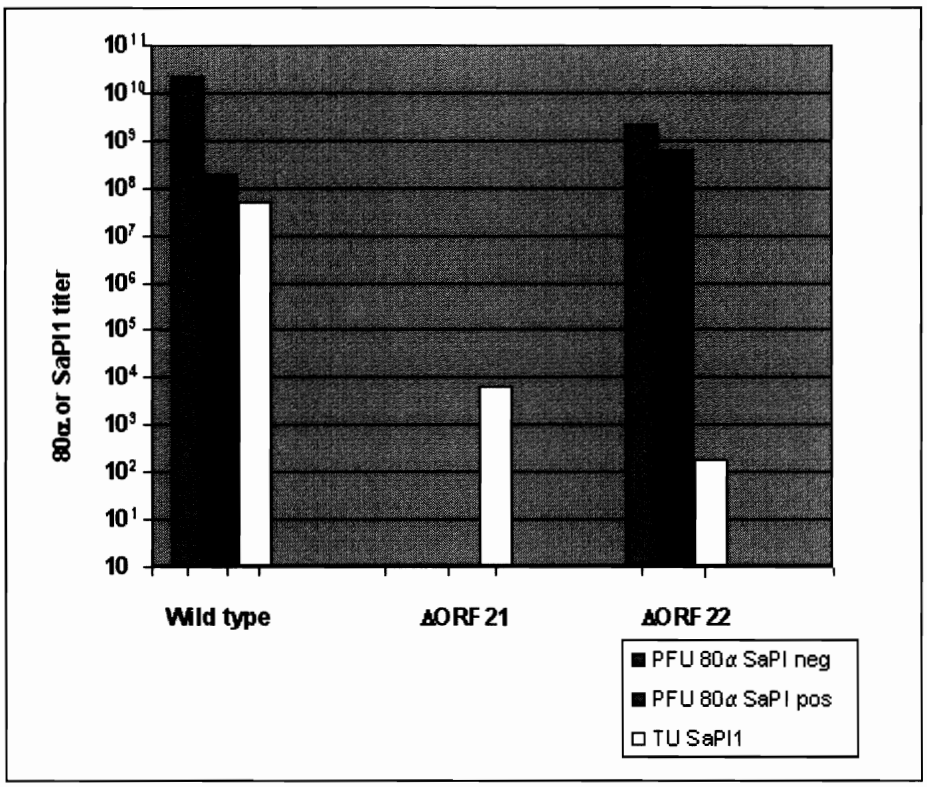



ORF21
ORF23
.....GCATGGTAACCAAAGAATTT...GAATATTAAGGAGTGTTAAAAATG.....
.....GCATGGTAACCAAAGAATTC GAATTCTAAGGAGTGTTAAAAATG.....
...GCATGGTAACCAAAGAATTCTAAGGAGTGTTAAAAAATG...
ΔORF22

co-integrates were selected at the non-permissive temperature, and subsequent resolution was enhanced by growth at the permissive temperature in the absence of antibiotic selection. Strains cured of the plasmid were then isolated by plating at the non-permissive temperature without antibiotic selection and screening for white colonies. PCR was used to detect the presence or absence of the desired deletion in the genomic prophage DNA and deletions were confirmed by sequencing. The two newly created strains carrying the ORF21 deletion (Table 1) are designated ST18 (SaPI1 negative) and ST22 (SaPI1 positive). The two newly created strains carrying the ORF22 deletion (Table 1) are designated ST20 (SaPI1 negative) and ST21 (SaPI1 positive).

Growth of phage mutants containing deletions. The newly created lysogens carrying the ORF21 and ORF22 deletions were evaluated for plaque forming titers and transducing titers following UV induction. Wild type lysogenic strains, RN10616 (SaPI1 negative) and RN10628 (SaPI1 positive), were evaluated by the same methods. The results in Figure 21 demonstrate that the wild type strains are consistent with previous results showing SaPI1 interference with 80α growth. However, no progeny phage were produced in either of the ΔORF21 strains, ST18 and ST22, confirming that this gene is essential for phage growth. The presence of SaPI1 transducing particles following induction in ST22 is

**Figure 21. Effect of deletions on phage growth, SaPI1 interference and transduction.** Shown is the 70-minute time point from a one-step growth curve experiment following UV induction. Plaque titers (PFU) and transducing titers were determined using RN4220 as the indicator strain.

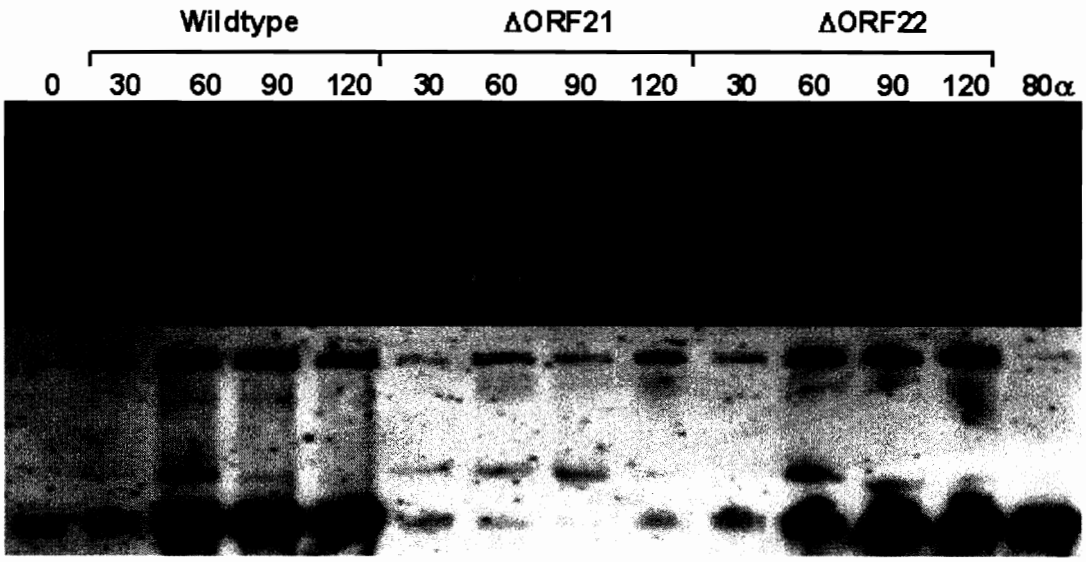

80α or SaPI1 titer
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10
Wild type
ΔORF 21
ΔORF 22
PFU 80α SaPI neg
PFU 80α SaPI pos
TU SaPI1

reduced from the wild type levels, but greater than generalized transduction. This result suggests that helper phage lytic growth is not required for SaPI1 mobilization and that at least a limited amount of the helper phage products required for SaPI1 growth are available. The results of Figure 21 also show the plaque forming titers and transducing titers following UV induction of the two strains carrying the ORF22 deletion ST20 (SaPI1 negative) and ST21 (SaPI1 positive). The phage and transducing titers from the ΔORF22 strains are similar to what was observed for the *sirB* point mutant. The burst size in both deleted strains is similar, consistent with a lack of interference from SaPI1. The transducing titer from the ΔORF22 strain is reduced to the same level as generalized transduction of chromosomal markers, confirming that ORF22 is required for mobilization of SaPI1.

Evaluation of phage and SaPI1 DNA replication. The effects on replication of deleting the ORFs were also examined. DNA from wild type 80α lysogens and the newly created deletion strains was isolated and analyzed at various times after induction. Aliquots were removed at 30-minute intervals following UV induction and analyzed by Southern hybridization with either 80α or SaPI1 specific DIG-11-dUTP labeled probes as described in the previous chapter.

The effects of deletion of ORF21 and ORF22 on phage replication can be seen in Figure 22. The wild type strain, RN10616, clearly shows an increase

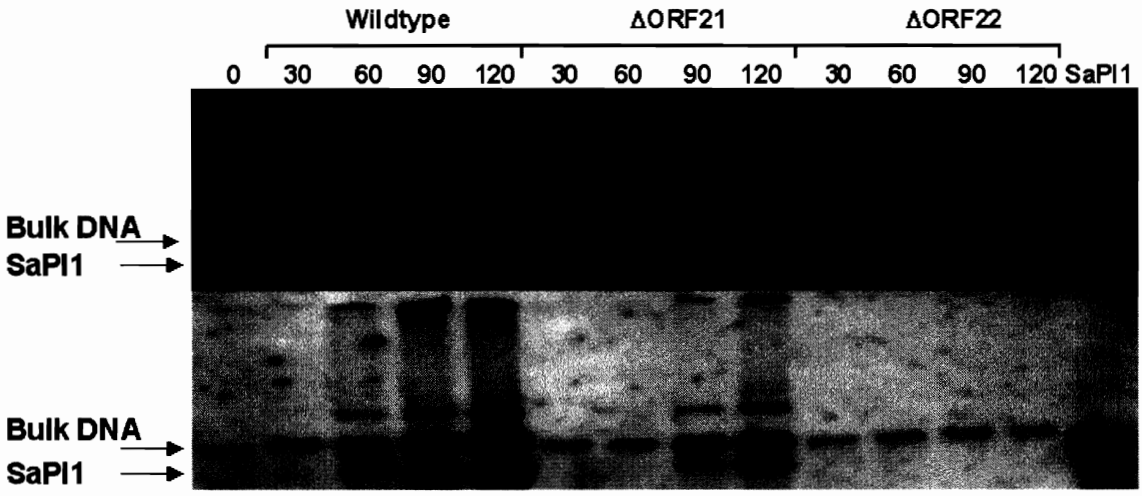
**Figure 22. Effect of deletions on 80α replication.** Upper panels are agarose gels stained with ethidium bromide and lower panels are blots hybridized with 80α DIG-11-dUTP probe and exposed overnight. DNA was extracted from aliquots taken at 30-minute intervals following UV induction. Virion 80α DNA was used as a control. Lanes 1-5, RN10616 (80α); lanes 6-9, ST18 (80α ΔORF21); lanes 10-13, ST20 (80α ΔORF22); lane 14, virion 80α.


Wildtype
ΔORF21
ΔORF22
0
30
60
90
120
30
60
90
120
30
60
90
120
80α

in phage DNA between the 30 and 60-minute time points and the level is maintained in the next two time points. Both the kinetics of appearance and the amount of DNA are similar in the ΔORF22 strain. This is consistent with what was observed in the *sirB* point mutant and confirms that ORF22 is not essential for phage replication. In contrast, an increase in phage DNA is not detectable in the ΔORF21 strain at any time point, unlike the *sirE* point mutant (Fig 15) where replication was delayed but later time points were equivalent to wild type phage levels. The gene product from ORF21 is clearly required for phage replication. In this case the phenotype of the deletion mutant, i.e., no phage replication, is much different than the *sirE* point mutant phenotype of a slight delay in replication.

SaPI1 replication is also affected in both 80α deletion mutants, as shown in Figure 23. Extrachromosomal SaPI1 DNA increases over time after induction of wild type 80α as observed in previous experiments. Appearance of both the signal in bulk DNA and the extrachromosomal band is delayed and is not as intense in the 80α ΔORF21 mutant. A similar delay was seen with the 80α *sirE* point mutant. SaPI1 transducing titers were 4 orders of magnitude lower following UV induction of the ΔORF21 mutant compared to wild type (Fig 21). Since 80α in the ORF21 deletion mutant is not replicating and SaPI1 replication still occurs, albeit somewhat delayed, we can conclude that phage replication is not required for SaPI1 replication. The reduced helper phage gene dosage and

**Figure 23. Effect of deletions on SaPI1 replication.** Upper panel is an agarose gel stained with ethidium bromide and lower panel is a blot hybridized with a SaPI1 DIG-11-dUTP probe and exposed for 10 minutes. SaPI1 virion DNA was used as a control. Excised SaPI1 is seen as the extrachromosomal band migrating below the bulk DNA as indicated with arrows. Lanes 1-5, RN10628 (80α SaPI1); lanes 6-9, ST22 (80α ΔORF21SaPI1); lanes 10-13, ST21 (80α ΔORF22 SaPI1); lane 14, virion SaPI1.


Wildtype
ΔORF21
ΔORF22
0 30 60 90 120 30 60 90 120 30 60 90 120 SaPI1
Bulk DNA
SaPI1
Bulk DNA
SaPI1

fewer phage gene products available for SaPI1 replication likely explain the replication delay and lower titers of SaPI1 with the ΔORF21 helper phage.

The extrachromosomal band is absent following UV induction of the ΔORF22 strain and there is no increase in the signal in bulk DNA, (Fig 23) as we observed for the 80α *sirB* point mutant (Fig 16), confirming that SaPI1 replication or excision is dependent upon the gene product from ORF22.

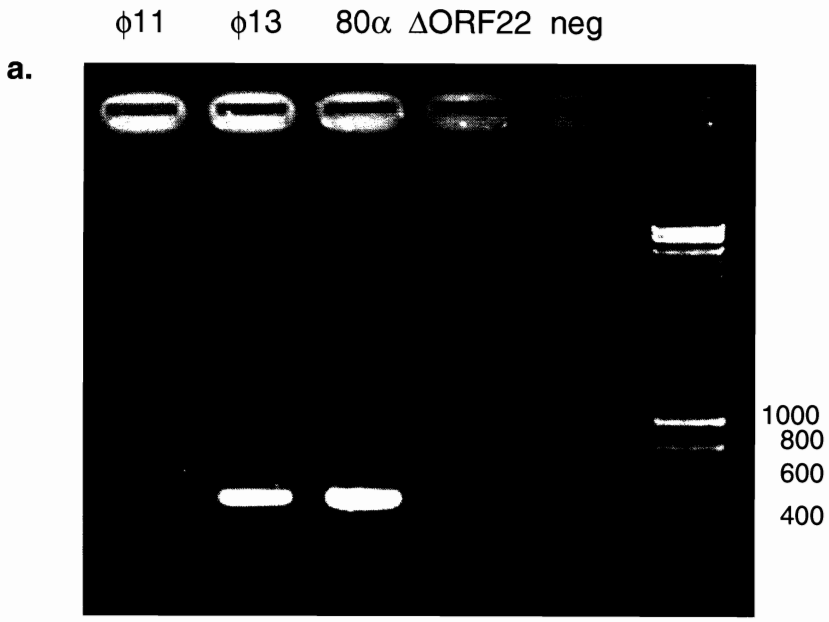
The 80α ΔORF22 strain cannot transduce SaPI1 with a high level of frequency and replicating SaPI1 was not detected by our Southern blot analysis. It was uncertain whether the block imposed by the ORF22 deletion was at the level of SaPI1 excision or subsequent replication. This was resolved by a combination of PCR reactions that amplified the attachment site junction of *S. aureus* and SaPI1. The SaPI1 *att* sequence (Lindsay *et al.*, 1998) was used to BLAST the staphylococcal genome (AF424783) and one perfect match was identified. One set of primers was designed to amplify 400bp upstream (SMT156) and 480bp downstream (SMT157) of the staphylococcal attachment site. A second pair was designed to amplify the ligated ends of the covalently closed circular form or replicating concatameric form of SaPI1, SMT154 near ORF1 and SMT155 near ORF26 or integrase. We verified that the primers were correct by generating expected junction fragments in a PCR reaction from SaPI1-containing genomic DNA (data not shown).

The extrachromosomal SaPI1 band was not detected following phage infection with the 80α *sirB* mutant or following UV induction of 80α ΔORF22.

Southern blotting may not have been sensitive enough to detect low levels of the extrachromosomal band and so PCR was employed to verify the presence or absence of the extrachromosomal copy of SaPI1. The SaPI1 junction primers were used to test uninduced and induced staphylococcal strains carrying 80α ΔORF22 as well as control phages known to excise SaPI1 or incapable of SaPI1 excision. The correct sized product was detected in the lanes from induced phage Φ13 (lane2) and 80α (lane 3), but not Φ11 or 80α ΔORF22 (Fig 24a). This verified the results seen on the blots (Fig 16, 23) and confirmed that SaPI1 replication or excision is dependent upon the gene product from ORF22.

A PCR reaction with the primers designed to amplify the staphylococcal-SaPI1 junction site was used to address excision. A PCR product with this primer pair will be generated if SaPI1 is not integrated, but no product will be obtained if SaPI1 remains integrated in the staphylococcal chromosome following UV induction of the phage. Induced and uninduced lysogens were tested in PCR reactions and RN4220 was tested as a control. The correct sized PCR product was detected from RN4220 (lane 1), induced Φ13 (lane 7), and 80α (lane 9), but an amplified product was not detected in the uninduced Φ13 or 80α (Fig 24). No PCR product was detected in Φ11 or 80α ΔORF22. This demonstrates that SaPI1 is excised during vegetative growth of Φ13 and 80α, but not Φ11 or 80αΔORF22. The product of ORF22, therefore, appears to be required for SaPI1 excision.

**Figure 24. Appearance of extrachromosomal SaPI1 DNA and SaPI1 excision.** Agarose gel stained with ethidium bromide of PCR amplified products from lysogens positive for SaPI1. (a) The appearance of extrachromosomal SaPI1 is shown. Template DNA was prepared from RN4220 lysogens collected 60 minutes post UV induction. Lane 1, Φ11; lane 2, Φ13; lane 3, 80α; lane 4, 80α ΔORF22; lane 5 no template control; lane 6, marker with basepairs as indicated. (b) SaPI1 excision was demonstrated with PCR reactions of template DNA prepared from uninduced lysogens or collected 60 minutes post UV induction. Lane 1, marker with sizes in base pairs as indicated; lane 2, RN4220; lane3, RN4220 (SaPI1); lane 4, Φ11 uninduced; lane 5, Φ11 induced; lane 6; Φ13 uninduced; lane 7, Φ13 induced; lane 8, 80α uninduced; lane 9, 80α induced; lane 10, 80αΔORF22 uninduced; lane 11, 80α ΔORF22.


ϕ11
ϕ13
80α
ΔORF22
neg
a.
1000
800
600
400


1 2 3 4 5 6 7 8 9 10 11
b.
1000
800
600
400

# Chapter 6 Discussion

SaPI1 is a pathogenicity island integrated in some *S. aureus* strains and mobilized by helper phage 80α. SaPI1 is excised, amplified and transduced at high frequency during vegetative growth of 80α. Previous studies established that SaPI1 transducing particles have an icosahedral head smaller than 80α but with identical tails to 80α. Lack of predicted phage structural genes in the SaPI1 genomic sequence (GenBank U93688) led to the hypothesis that the smaller particles seen in electron micrographs are comprised of phage-encoded proteins (Ruzin *et al.*, 2001).

The protein analysis presented in this study confirmed that SaPI1 virions are comprised of phage-encoded proteins. A total of 12 virion proteins, identified from in-gel and whole phage tryptic digests, were present in both phage and SaPI1 virions. No SaPI1 encoded proteins were identified. An additional scaffolding protein was identified in a phage capsid precursor fraction, but we have not obtained SaPI1 precursors in sufficient yield to determine its presence there. Mass spectrometry analysis allowed unambiguous assignment of gene function to the following phage-encoded proteins: tape measure protein, major capsid protein, major tail protein, portal protein, minor capsid protein, scaffolding protein and minor tail proteins.

The exploitation of a helper phage encoded virion by SaPI1 resembles the P2-P4 system found in *E. coli*. P4 is a dsDNA satellite virus dependent upon helper phage P2 for lytic growth. P4 capsids are one-third the volume of helper phage capsids and are comprised of proteins encoded by P2 (Lindqvist *et al.*, 1993) similar to SaPI1 and 80α. P4 encodes two unique structural proteins. One is the Sid protein that interacts with P2 major capsid protein N to alter the size of the capsid during assembly. The second protein, Psu, is found in the mature virion and is thought to stabilize the smaller capsid (Lindqvist *et al.*, 1993). No unique SaPI1 proteins were found in our protein analysis. We would not have expected to find a Sid-like protein in the mature virion. Future experiments should analyze procapsids for SaPI1 proteins that may interact with the helper phage capsid precursors during capsid assembly.

To investigate SaPI1 and helper phage interactions we selected two apparent SaPI1 interference resistant (*sir*) mutants. This led to the identification of point mutations in two adjacent genes in the phage replication region, ORF21 and ORF22. Through allelic exchange we inactivated each of these candidate genes in a prophage copy of 80α, which allowed us to characterize gene function and effects on SaPI1 mobilization.

ORF21 is a DnaC candidate. The gene product from ORF21 is required for phage growth and replication. This gene is homologous to ORFs in a number of staphylococcal phages and a BLAST search of proteins showed high

similarity (3e-16) to the carboxyl end of DnaC, a replication protein thought to be a helicase loader. 80α gp21 is identical to the putative DnaC or helicase loader in Φ13 (AAL82346.1). A sequence alignment of the putative DnaC proteins from 80α and Φ13 with *E. coli* DnaC shows significant similarity (Fig 25a). An evolutionary study showed that the C-terminus of the helicase loader from Φ13 is predicted to have a nucleotide binding domain of the Rossmann-fold type. This protein structural motif is generally comprised of three or more parallel β-stranded regions connected with α-helical regions. The interactive site of the molecule is the binding site for the helicase. This site is in an α-helical region close to the Walker A and Walker B motifs that function in ATP binding (Weigel *et al.*, 2006) (Fig 25b). The consensus sequence of the Walker A binding motif is (G/A)XXXXGK(T/S) and the consensus sequence of the Walker B motif has four hydrophobic residues followed by and aspartic acid residue (Walker *et al.*, 1982).

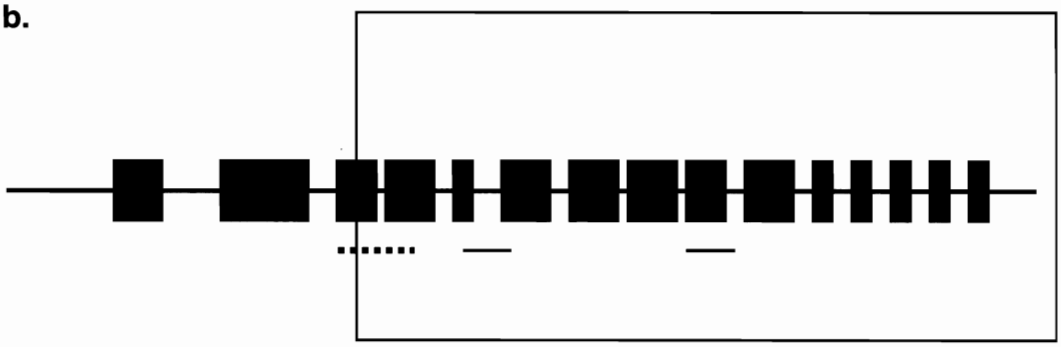
The helicase loader is thought to perform a number of functions including interaction with the helicase and replication machinery (Weigel *et al.*, 2006). DnaC is required for replication in two distinct steps as revealed by studies with *E. coli* mutants (Davey *et al.*, 2002). The first step is to load the helicase onto single stranded DNA in preparation for replication and the second step is to hydrolyze ATP to ADP removing the inhibition of the helicase and allowing replication at *oriC* to proceed. Additionally, six molecules of DnaC are required

**Figure 25. The alignment of putative DnaC proteins and a predicted secondary structure.** (a) Shown is a comparison of amino acid sequences of putative DnaC proteins from 80α and *E. coli* (GenBank ABF71592 and AAG59544.1, repsectively). An asterisk indicates amino acids that are identical; a colon indicates a conserved substitution; and a period indicates a semi-conserved substitution. The predicted interactive site is indicated by a thin line on top of the sequences; the predicted Walker A motif is within a box; and the predicted Walker B motif is underlined with a thick line. Alignment was performed with T-coffee and M-coffee available from European Bioinformatics Institute (EBI). (b) The predicted secondary structure of 80α gp21 fashioned after Weigel et al., 2006. The green color represents α-helical region, blue represents β-stranded region, and the long solid line represents unstructured regions. The area within the box represents the structural elements of the Rossmann fold. The approximate position of the interactive site is indicated by the dotted line and the Walker A and Walker B motifs are indicated by short solid lines. Secondary structure was predicted with PSIPRED V2.4 (Jones,1999).

**a.**

```
80α      MKPLFSEKINESLKKYQPTHVEKGLKCERCGSEYDLYKFAPTKKHPNGYEYKDGCKCEIY
E. coli  MKNVG--DLMQRLQKMMPAHIKPAFKTG---------------EELLAWQKEQGAIRSAA
         ** :   .: : *:*  *:*:: .:*                :.  .:: ::*.  .

80α      EEYKRNKQRKINNIFNQSNVNPSLRDATVKNYKPQNEKQVHAKQTAIEYVQGFSTKEPKS
E. coli  LE-RENRAMKMQRTFNRSGIRPLHQNCSFENYRVECEGQMNALSKARQYVEEFDGN-IAS
          * :.*:  *::. **:*.:.*  ::.:.:**: : * *::* ..* :**: *. :   *

80α      LILQGSYGTGKSHLAYAIAKAVKAKGHTVAFMHIPMLMDRIKATYNKNAVETTDELVRLL
E. coli  FIFSGKPGTGKNHLAAAICNELLLRGKSVLIITVADIMSAMKDTF-RNSGTSEEQLLNDL
         :*:.*. ****.*** **.: :  :*::* :: :. :*. :* *: :*:  : ::*:. *

80α      SDIDLLVLDDMGVENTEHTLNKLF--SIVDNRVGKNN--IFTTNFSDKELNQNMNWQRIN
E. coli  SNVDLLVIDEIGVQ-TESKYEKVIINQIVDRRSSSKRPTGMLTNSNMEEMTKLLG-ERVM
         *::****:*::**: ** . :*::  .***.* ..:.   : ** . :*:.: :. :*:

80α      SRMK-HNARKVRVIGDDFRERDAW---
E. coli  DRMRLGNSLWVIFNWDSYRSRVTGKEY
         .**:  *:  * .  *.:*.* :
```

**b.**



per hexamer of DnaB (helicase) (Davey *et al.*, 2002). DnaC is essential for cell growth in *E. coli* (Ludlam *et al.*, 2001).

Effect of ORF21 on SaPI1. Our data established that ORF21 is required for 80α growth and replication, but it was not required for SaPI1 growth or replication. This is similar to what is seen in P2 replication mutants. Studies with P2 replication mutants showed that deletion of either of the two replication genes, *A* or *B*, was lethal for the helper phage, but not for satellite phage P4. P2 mutants defective in either of the replication genes caused a 20-minute delay in P4 growth compared to wild type. The P4 phage yield in a P2 lysogen carrying a defective *A* gene was similar to wild type levels. However, the P4 yield in the P2 *B*⁻ lysogen was approximately 30% lower than wild type levels (Six,1975). The P2 *B* gene corresponds to the helicase loader (Odegrip *et al.*, 2000).

Our results with the ORF21 deletion strain are similar to those seen for the P2 *B* mutant. Phage replication is abolished, SaPI1 replication is delayed and the SaPI1 titer is reduced. The reduction is much greater in SaPI1 than in P4, presumably due to the fact that unlike P4, SaPI1 cannot replicate autonomously. The replication-defective 80α helper is still able to provide functions essential for SaPI1 excision, replication and encapsidation, albeit at a reduced level. This implies that either phage late gene (morphogenetic) functions can be expressed independently of phage DNA replication, or that

SaPI1 can directly activate expression of the helper genes it requires for transduction.

Integration and Excision. Excision of SaPI1 depends on the helper phage, and our findings established that SaPI1 excision does not occur when the helper is defective for ORF22. Excision of SaPI1 is believed to be phage-like and the reciprocal of integration. Integration of a temperate phage into the bacterial genome is a site-specific recombination event occurring between the phage attachment site ($att_P$) and a specific host attachment site ($att_B$). Integration generates two sequences ($att_L$ and $att_R$) flanking the linearized phage genome. Integration is mediated by a phage-encoded integrase for cleavage and rejoining of the sequence (Carroll *et al.*, 1995). The attachment site sequence is the core sequence and is the site of recombination. Once the phage has integrated, the host chromosome attachment site is split. The resulting hybrid sites are designated the *P* arm and the *B* arm. The *P* arm in λ contains 5 integrase binding sites, 2 excisionase binding sites and 3 binding sites for a required host protein Integration Host Factor (IHF). The *B* arm is not as important in recombination specificity and only has 2 integrase binding sites (Lee *et al.*, 1986).

Excision requires integrase and a second phage-encoded protein, Xis or excisionase. Phages Φ11, L54a and 80α also encode an excisionase found

upstream of the integrase. No homologues of excisionase were found in the SaPI1 sequence (Lindsay *et al.*, 1998).

The best characterized excisionase or recombination directionality factor (RDF) is found in *E. coli* phage λ. The excisionase reaction requires recombination between $att_L$ and $att_R$ to regenerate the phage and host genomes. Interaction between Xis and Int determines the direction of recombination by altering the specific protein-DNA architectures. Once Xis is bound to its specific recognition sites, sharp bends are introduced into the DNA. The sharp bends that occur in $att_R$ promote recombination and in $att_L$ prevent confirmation required for integration (Lewis *et al.*, 2001).

The right end of the integrated SaPI1 sequence contains the $att_R$ site, Int and a regulatory sequence for integrase expression that is conserved among staphylococcal phages including Φ11, Φ13, Φ42, L54a and 80α. The SaPI1 *att* site is 17bp and is 76% A+T (Lindsay *et al.*, 1998). SaPI1 integrase is 48% identical to phage L54a integrase by BLAST (Lindsay *et al.*, 1998) and has many conserved residues as seen with a global alignment (Fig 26a). L54a integrase is a member of the tyrosine family of site-specific recombinases (Lewis *et al.*, 2001). Two proteins, RinA and RinB, bind to the conserved region and regulate the expression of integrase in Φ11 and 80α. Phage L54a is reported to encode only RinA (Iandolo *et al.*, 2002), however, the integrase gene of L54a has two translational start sites. The protein translated from the first 142 nucleotides may serve as RinB (Ye *et al.*, 1993). No homologues

**Figure 26. Sequence alignments of Int and DnaI.** Shown are amino acid sequences of SaPI1 candidates compared to proteins of known function. An asterisk indicates amino acids that are identical; a colon indicates a conserved substitution; and a period indicates a semi-conserved substitution. (a) Comparison of amino acid sequences of putative integrase from staphylococcal phage L54a and SaPI1 (GenBank AAW37525.1 and AAC28969.1, respectively) (b) Comparison of amino acid sequences of *S. aureus* DnaI with SaPI1 repressor (ORF22) (GenBank AAW36835 and AAC28967.2, respectively). A bold line indicates the DNA binding region and the putative protein interaction site is boxed. Alignments were performed with T-coffee and M-coffee available from European Bioinformatics Institute (EBI).

**a.**

```
SaPI1 Int   MWFEKFKNKNKETKYRYYEKYKDPLINKWRRVSVVLNKNGKQSQKEAQRLLNERIEAKLN
L54a Int    MWFEKFKNKNNETKYRYYEKYKDPYTDKWKRVSVVLNKNTKQSQKEAMFRLEEKIKEKLN
            **********:************** *:**:********** *******   *:*:*: ***

SaPI1 Int   DKTPTTLKSLTFHAASDEWFQNYIKTSGSKRTTIKTKLSKLNTLKKFVDEDILINKITLS
L54a Int    NKSSSELKTLTFHALLDEWLEYHIKTSGFKVTTLDNLKTRIKNIKKNSSQNLLLNKIDTK
            :*:.: **:*****  ***:: :***** * **:..  ::::.:**  .:::*:***  .

SaPI1 Int   YAQQVFDEMDSKGYVYQVNKDALSIFKNVFEYTRRIYKLQDLEFLKDITLNKRIKSYDEV
L54a Int    YMQTFINELSNVYSANQV-KRQLGHMKEAIKYAVKFYNYPNEHILNSVTLPKKSKTIEDI
            * * .::*:..   . ** *  *. :*:.::*: ::*:  : .:*:.:** *: *: :::

SaPI1 Int   K---AKRNKYLELNEIQSIIKDINMKAQKMHSGIHKRFYLFVALMTEFQALNGMRIGEML
L54a Int    EKEEAKMYNYLEMEQVIQ-IRDFILNDNNM----QYRARILVAGAVEVQALTGMRIGELL
            :   **  :***:::: . *:*: :: ::*    : *  ::**  .*.***.******:*

SaPI1 Int   AIQNEDIDFDNKSLNINGTIHWFHDESGGFGVKDTTKTESSYRTIGLSSRSCEILKKAIL
L54a Int    ALQVKDVDLKNKTIDINGTIHRIKCNA-GFGHKDTTKTAGSKRKIAINSRIANVLKKIML
            *:* :*:*:.**:::****** :: :: *** ****** .* *.*.:.** .::*** :*

SaPI1 Int   ENKKDSKWNDGYLNRNFVFTNHKGNPMQTERFNKILREAAKDVGIDKEVSSHILRHSHIS
L54a Int    ENKKMQQWEPSYVDRGFIFTTCQGNPMQGSRINKRLSSAAESLNINKKVTTHTLRHTHIS
            **** .:*: .*::*.*:**. :****** .*:** * .**:.:.*:*:*::* ***:***

SaPI1 Int   LLSQQGVSLKAIMDRVGHSDHRTTLSIYSHVTEQMDKDMMNKLEQLKLG
L54a Int    LLAEMNISLKAIMKRVGHTDEKTTIKVYTHVTEKMDRELEQKLEKLVY-
            **:: .:******.****:*.:**:.:*:****:**::: :***:*
```

**b.**

```
S aureus DnaI   --MKQFKSIINTSQDFEKRIEKIKKEVINDPDVKQFLEAHRAELTNAMIDEDLNVLQEYK
   SaPI1 gp22   MIYMTFGEILKKE-RVSWKLSVKELSTLSGVSQTYISKLENGKRNFPSLETIFNLLIGFK
                     * .*::..  .. ::.  : ..:.. . . : : ...: . . ::  :*:*  :*

S aureus DnaI   DQQKHYDGHKFADCPNFVKGHVPELYVDNNRIKIRYLQCPCKIKYDEERFEAELITSHHM
   SaPI1 gp22   THIEYKMG---SESPFYEINNS---YLDE--ILIMFINSSNSTISDRD--PNELITQFNE
                 : ::  *   ::.* :  .:    *:*:  * * :::.. .   *.:    ****..:

S aureus DnaI   QRDTLNAKLKDIYMNHRDRLDVAMAADDICTAITNGEQVKGLYLYGPFGTGKSFILGAIA
   SaPI1 gp22   YYDVTIKKKQNE--NSKIESDIFSNKIKLVKGTTKKEVIEKPY----------FDLNWLL
                  *.   * ::   * : . *:     .: .. *: * ::  *          * *. :

S aureus DnaI   NQLKSKKVRSTIIYLPEFIRTLKGGFKDGSFEKKLHRVREANILMLDDIGAEEVTPWVRD
   SaPI1 gp22   TQNEYE-----VFFDRSFL-------LDNNFLNKKHF-TEKDMYYYNVLNDNDLKT-IKD
                .* : :     :::  .*:        *..* :* *   * ::   : :. :::.. ::*

S aureus DnaI   EVIGPLL-HYRMVHELPTFFSSNFDYSELEHHLAMTRDGEEKTKAARIIERVKSLSIPYF
   SaPI1 gp22   LIVVFLLNKYNYIKNKDDFFN------------IFTNSEDDKTKRDALYKILYE------
                 ::  ** :*. :::   **.             :*.. ::***   : : : .

S aureus DnaI   LSGENFRNN
   SaPI1 gp22   -------TD
                       .:
```

of *rinA* or *rinB* were detected in the SaPI1 sequence (Lindsay *et al.*, 1998).

Potential roles of 80α ORF22. Our experimental data established that in the absence of 80α gp22, there is no detectable SaPI1 excision or replication. If excision is a necessary prerequisite to replication it is possible that 80α gp22 is just required for SaPI1 excision. However, recent results from the Novick lab have shown that a SaPI integrase mutant, which cannot excise, still appears capable of some replication *in situ*. Since no similar *in situ* SaPI1 replication is seen in the 80αΔORF22 mutant strain, it seems more likely that the gene product of ORF22 may be involved in inactivation of a SaPI1 encoded repressor that controls both excision and replication.

Previous studies have demonstrated that a homologue of 80α gp22 binds to the staphylococcal helicase loader, DnaI. This protein-protein interaction blocks host DNA replication (Liu *et al.*, 2004). One likely role of 80α gp22 for the phage is interference with host replication during phage lytic growth. This host shutdown is not necessary for 80α lytic growth, at least under the growth conditions we tested, since deletion of this gene had no effects on the phage. It is not clear how eliminating interference with host replication would directly impair SaPI1 excision or replication, unless the host DnaI interferes somehow with SaPI1.

The most likely possibility is that 80α gp22 directly derepresses SaPI1. Multiple roles for a small regulatory protein in helper-satellite interactions is not

without precedent. P2 encoded Cox is a multifunctional protein required by the prophage for excision and by P4 satellite phage for derepression (Saha *et al.*, 1987). Two divergent genes encoding proteins with helix-turn-helix binding motifs that are members of the Cro/CI family are found in the SaPI1 genome. The product of SaPI1 ORF22 is predicted to encode the SaPI1 repressor. It is possible that 80α gp22 functions as an antirepressor by interacting with SaPI1 repressor. We compared the amino acid sequence of *S. aureus* DnaI (GenBank AAW36835), the known binding partner of 80α gp22, to that of SaPI1 repressor (GenBank AAC28967.2). A sequence alignment of the two predicted proteins revealed a region with five identical residues and several conserved residues that may be a protein binding site for 80α gp22 (Fig 26b). This putative binding site is in the N-terminal half of the protein, but distal to the predicted DNA binding region.

Another formal possibility is that 80α ORF22 is the SaPI1 excisionase, although this is unlikely. Generally, the excisionase or RDF is encoded within 50 bp of the cognate recombinase. Attempts to search the NCBI databse to identify RDFs is difficult because the proteins are small, usually <100 residues, and have few highly conserved residues (Lewis *et al.*, 2001). A BLAST search with the genomic and protein sequence of ORF22 (GenBank ABF71593) produced significant alignments to other staphylococcal phages, but not to predicted RDfs. One RDF group that interacts with the tyrosine integrase family of site-specific recombinases includes the L54a group. This group of RDFs

includes staphylococcal phage L54a, streptococcal phage T12 and plasmid pXO1 from *Bacillus anthracis* (Lewis *et al.*, 2001). The gene product from ORF22 shares some similarities with the L54a group of RDFs, including an acidic pI which is a unique characteristic of this group. RDFs as a general rule have a predicted helix-turn-helix motif, but gp22 has a helix-coil-helix motif. A global alignment of 80α ORF22 with the putative excisionase from T12 shows a region with a potentially significant number of conserved residues (Fig 27a).

Experiments with plasmid constructs carrying the SaPI1 integrase and attachment site found that while SaPI1 encoded integrase is sufficient for integration it is not sufficient for excision, even in the presence of helper phage (Ruzin *et al.*, 2001). Thus it appears that an additional SaPI1-encoded gene product not present on the plasmid must be necessary for excision of the element, even if the excisionase is provided by the helper phage. The SaPI1 sequence used to create the plasmid included sequence from nucleotide coordinates 12436-15254 and 1-1763, spanning the junction of circular SaPI1. This plasmid construct included the integrase, the conserved regulatory region and the attachment core sequences required for integration (Ruzin *et al.*, 2001). The missing factor may be a regulatory protein that activates expression of integrase, which by analogy to phage L54a should be a RinA homologue.

A multiple sequence alignment (Fig 27b) of putative RinA proteins from 80α, Φ11 and L54a with candidates from SaPI1 identified gp20 as a possible

**Figure 27. Sequence alignments of Xis and RinA.** Shown are amino acid sequences of 80α or SaPI1 candidates compared to proteins of known function. An asterisk indicates amino acids that are identical; a colon indicates a conserved substitution; and a period indicates a semi-conserved substitution. (a) Comparison of amino acid sequences of *Streptococcus pyogenes* phage T12 putative excisionase and 80α ORF22 (GenBank AAC48866 and ABF71593, respectively). (b) Comparison of amino acid sequences of putative RinA from phages L54a, Φ11 and , 80α with SaPI1 (GenBank AAW37571, AAL82256.1, ABF71610, AAC28966, respectively). Alignments were performed with T-coffee and M-coffee available from European Bioinformatics Institute (EBI).

**a.**

```
80α gp22  ----------MVTKEFLKIK-LECSDMYAQKLIDEAQGDENKLYDLFIQKLAERHTRPAI
T12 Xis   MFHGWNYNSYKANNNLLEIRHLEDIVKYNTIMRDQYKKGSNE-YNAFQQDIEQDKNQIAL
                  ..:::*:*: **    *   : *: : ..*: *: * *.: : :.: *:

80α gp22  ----------VEY
T12 Xis   LFNEHQLLENIDN
                  ::
```

**b.**

```
L54a RinA   MGKASYDIKPGTFKYIESEIYNLQENKKEINRLRMEILNPTKELDTNIVYGPLQKGEPVR
ϕ11 RinA    MTKKKYGLKLSTVRKLEDELCDYPNYHKQLEDLRSEIMTPWIPTDTNIG-GEFVPSNT-S
80α RinA    MTKKKYGLKLSTVRKLEDELCDYPNYHKQLEDLRSEIMTPWIPTDTNIG-GEFVPSNT-S
SaPI1 gp20  MPRTK--------------LQDFPLKENTVTEPKQVVVNPLFAKPNALA-GIFGISY---
            * : .             : :   .: :   :  ::.*    . :  * :  .

L54a RinA   TTELMATRLLTNKMLRNLEEMVEAVESEYLKLPEDHKKVIRLKYWNKDKKLKIEQIGDAC
ϕ11 RinA    KTEMAVTNYLCSIRRGKILEFKSAIERIINSSSRKEREFIQEYYFNKKELVKV---CVDI
80α RinA    KTEMAVTNYLCSIRRGKILEFKSAIERIINTSSRKEREFIQEYYFNKKELVKV---CDDI
SaPI1 gp20  ----------------------SSVNRILKEWEKDHKGIDDLYYSLSSSMIVI-------
                               .:::      ...: .   *  ... : :

L54a RinA   HMHRNTVTTIRKNFVKAIAYHAGIK
ϕ11 RinA    HISDRTAHRIKRKIISRLAEELGED
80α RinA    HISDRTAHRIKRKIISRLAEELGEE
SaPI1 gp20  --------SIPRFEEYMKARHKKWM
                    * :     * .
```

RinA protein (GenBank ABF71610, AAL82256.1, AAW37571, AND AAC28966, respectively). Based on its location in the genome, expression of this gene would likely be under SaPI1 repressor control.

This study has provided insights into the interactions between SaPI1 and 80α that begin to address functions involved in replication, excision and potential transactivation. The phenotype of the *sirE* point mutation in ORF21 suggests that changes in the balance between the two numbers of replicons can alter the ratio of virions produced; a similar phenomenon has been documented for P2 and P4 (Six,1975). The ability of SaPI1 to form transducing particles even when the helper phage has a deletion of ORF21 implies that gene products essential for SaPI1 morphogenesis can be expressed even in the absence of phage replication. The product of ORF22 is likely involved in SaPI1 derepression, which is a necessary prerequisite to interference. A similar class of P2 mutants, in the *cox* gene, was obtained in selection for mutants overcoming resistance to P4 (Six *et al.*, 1991). In order to obtain mutants in the direct target of SaPI1 interference, SaPI1 dependence on the helper for excision and replication needs to be bypassed. In order to accomplish this, an origin for autonomous replication could be inserted within the SaPI1 genome. Mutants resistant to interference by this element would then be expected to lie in the helper phage morphogenetic functions targeted by SaPI1 during assembly of small capsids and SaPI1 encapsidation.

**Figure 26. Sequence alignments of Int, and RinA.** Shown are amino acid sequences of 80α or SaPI1 candidates compared to proteins of known function. An asterisk indicates amino acids that are identical; a colon indicates a conserved substitution; and a period indicates a semi-conserved substitution. (a) Comparison of amino acid sequences of putative integrase from staphylococcal phage L54a and SaPI1 (GenBank AAW37525.1 and AAC28969.1, respectively) (b) Comparison of amino acid sequences of putative RinA from phages L54a, Φ11 and , 80α with SaPI1 (GenBank AAW37571, AAL82256.1, ABF71610, AAC28966, respectively). Alignments were performed with T-coffee and M-coffee available from European Bioinformatics Institute (EBI).

**a.**

```
SaPI1 Int   MWFEKFKNKNKETKYRYYEKYKDPLINKWRRVSVVLNKNGKQSQKEAQRLLNERIEAKLN
L54a Int    MWFEKFKNKNNETKYRYYEKYKDPYTDKWKRVSVVLNKNTKQSQKEAMFRLEEKIKEKLN
            **********:************** *:**:********* *******   *:*:*: ***

SaPI1 Int   DKTPTTLKSLTFHAASDEWFQNYIKTSGSKRTTIKTKLSKLNTLKKFVDEDILINKITLS
L54a Int    NKSSSELKTLTFHALLDEWLEYHIKTSGFKVTTLDNLKTRIKNIKKNSSQNLLLNKIDTK
            :*:.: **:***** ***:: :***** * **:..  ::::.:**  .:::*:***  .

SaPI1 Int   YAQQVFDEMDSKGYVYQVNKDALSIFKNVFEYTRRIYKLQDLEFLKDITLNKRIKSYDEV
L54a Int    YMQTFINELSNVYSANQV-KRQLGHMKEAIKYAVKFYNYPNEHILNSVTLPKKSKTIEDI
            * * .::*:..   . ** *  *. :*:.::*: ::*:  : .:*:.:** *: *: :::

SaPI1 Int   K---AKRNKYLELNEIQSIIKDINMKAQKMHSGIHKRFYLFVALMTEFQALNGMRIGEML
L54a Int    EKEEAKMYNYLEMEQVIQ-IRDFILNDNNM----QYRARILVAGAVEVQALTGMRIGELL
            :   **  :***:::: . *:*: :: ::*    : *  ::**  .*.***.******:*

SaPI1 Int   AIQNEDIDFDNKSLNINGTIHWFHDESGGFGVKDTTKTESSYRTIGLSSRSCEILKKAIL
L54a Int    ALQVKDVDLKNKTIDINGTIHRIKCNA-GFGHKDTTKTAGSKRKIAINSRIANVLKKIML
            *:* :*:*:.**:::****** :: :: *** ****** .* *.*.:.** .::*** :*

SaPI1 Int   ENKKDSKWNDGYLNRNFVFTNHKGNPMQTERFNKILREAAKDVGIDKEVSSHILRHSHIS
L54a Int    ENKKMQQWEPSYVDRGFIFTTCQGNPMQGSRINKRLSSAAESLNINKKVTTHILRHTHIS
            **** .:*: .*::*.*:**. :***** .*:** * .**:.:.*:*:*::* ***:***

SaPI1 Int   LLSQQGVSLKAIMDRVGHSDHRTTLSIYSHVTEQMDKDMMNKLEQLKLG
L54a Int    LLAEMNISLKAIMKRVGHTDEKTTIKVYTHVTEKMDRELEQKLEKLVY-
            **:: .:******.****:*.:**:.:*:****:**::: :***:*
```

**b.**

```
L54a RinA   MGKASYDIKPGTFKYIESEIYNLQENKKEINRLRMEILNPTKELDTNIVYGPLQKGEPVR
ϕ11 RinA    MTKKKYGLKLSTVRKLEDELCDYPNYHKQLEDLRSEIMTPWIPTDTNIG-GEFVPSNT-S
80α RinA    MTKKKYGLKLSTVRKLEDELCDYPNYHKQLEDLRSEIMTPWIPTDTNIG-GEFVPSNT-S
SaPI1 gp20  MPRTK--------------LQDFPLKENTVTEPKQVVVNPLFAKPNALA-GIFGISY---
            * : .              : :    .: :   :  ::.*     . :  * :  .

L54a RinA   TTELMATRLLTNKMLRNLEEMVEAVESEYLKLPEDHKKVIRLKYWNKDKKLKIEQIGDAC
ϕ11 RinA    KTEMAVTNYLCSIRRGKILEFKSAIERIINSSSRKEREFIQEYYFNKKELVKV---CVDI
80α RinA    KTEMAVTNYLCSIRRGKILEFKSAIERIINTSSRKEREFIQEYYFNKKELVKV---CDDI
SaPI1 gp20  ----------------------SSVNRILKEWEKDHKGIDDLYYSLSSSMIVI-------
                                .:::       ...: .    *  ... : :

L54a RinA   HMHRNTVTTIRKNFVKAIAYHAGIK
ϕ11 RinA    HISDRTAHRIKRKIISRLAEELGED
80α RinA    HISDRTAHRIKRKIISRLAEELGEE
SaPI1 gp20  --------SIPRFEEYMKARHKKWM
                    * :      * .
```

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## Literature Cited

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# VITA

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