2008

Role of the Small Terminase Subunit Encoded by Staphylococcus Aureus Pathogenicity Island SaPI1 in Formation of SaPI1 Transducing Particles

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ROLE OF THE SMALL TERMINASE SUBUNIT ENCODED BY *STAPHYLOCOCCUS AUREUS* PATHOGENICITY ISLAND SAPI1 IN FORMATION OF SAPI1 TRANSDUCING PARTICLES

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Richmond, Virginia
May 2008

Acknowledgement

I would like to acknowledge all of the wonderful people at Virginia Commonwealth University that helped to get me this far. I am grateful for the unending patience and support of my committee members William Eggleston Jr., Jeff Elhai, Michael McVoy, and especially my advisor Gail Christie. I cannot imagine another group of individuals that I would have entrusted to oversee my research.

I am particularly thankful for the assistance and input from Adriana Rosato and Kimberly Jefferson, without their assistance my project would have never gotten off the ground. I am also grateful for the patience and assistance from Judy Williamson at the VCU Department of Neurobiology & Anatomy Microscopy Facility as she taught our lab how to use the transmission electron microscope that provided the stunning image of 80α and SaPI1 enclosed in this work.

I am particularly grateful for the unending support and collaboration of the Christie lab members. Without their selfless contributions and genuine encouragement this body of work might not exist. A special thanks goes to my lab partner Sandy Tallent whose assistance on countless occasions allowed me to complete my research and whose guidance constantly reminded me that my degree was within reach.

Dedication

I would like to dedicate this effort to my grandparents, parents, siblings, and my cousin Pamela. Their belief in me allowed me to complete my research. Their unflinching support during the times of hopelessness and despair reassured me that the end was in sight.

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List of Abbreviations

A	adenine
bp	base pair
C	cytosine
cm	centimeter
CsCl	cesium chloride
CY-GL	casamino acids yeast extract glycerophosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine 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	guanine
kb	kilobase pair
LB	Luria-Bertani
M	molar
Mg	milligram
ml	milliliter
mM	millimole
nm	nanometer
NMR	nuclear magnetic resonance
ºC	degrees centigrade
OD	optical density
ORF	open reading frame
pac	packaging site
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol

PFU	plaque forming units
RCSB	Research Collaboratory for Structural Bioinformatics
rpm	revolutions per minute
SA	*Staphylococcus aureus*
S. aureus	*Staphylococcus aureus*
SaPI	*Staphylococcus aureus* pathogenicity island
SaPI1	*Staphylococcus aureus* pathogenicity island one
SaPIbov1	*Staphylococcus aureus* pathogenicity island bovine one
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate buffer
T	thymine
TAE	Tris acetate EDTA
TE	Tris EDTA
terL	large terminase subunit
terS	small terminase subunit
Tris	Tris hydroxymethylaminoethane
TSA	Tryptic soy broth
tst	toxic shock toxin
tetM	tetracycline resistance marker
TU	transducing units
U	uracil
UV	ultraviolet
vol/vol	volume per volume
wt/vol	weight per volume
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µg	microgram
µl	microliter

Abstract

ROLE OF THE SMALL TERMINASE SUBUNIT ENCODED BY *STAPHYLOCOCCUS AUREUS* PATHOGENICITY ISLAND SAPI1 IN FORMATION OF SAPI1 TRANSDUCING PARTICLES

By Nicholas Paul Olivarez, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Dr. Gail E Christie
Professor, Microbiology & Immunology

Staphylococcus aureus pathogenicity island SaPI1 is a genomic element that is mobilized and transduced at high frequency by helper phage 80α. SaPI1 encodes a small terminase protein that belongs to the phage small terminase subunit family. The presence of SaPI1-encoded small terminase suggests that it plays a role in SaPI1-specific packaging into transducing particles by complexing with the 80α large terminase subunit and redirecting recognition to a *pac* site on SaPI1 DNA from 80α DNA. The effects of deleting the small

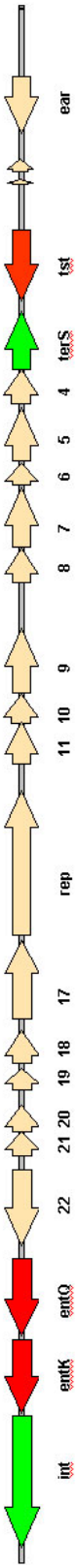
terminase genes in SaPI1 and in a prophage copy of 80α are consistent with this hypothesis. Induction of the 80α small terminase deletion mutant produces wild type levels of SaPI1 transducing particles, demonstrating that SaPI1 small terminase can replace that of 80α in SaPI1 packaging. Southern blot analysis of virion DNAs isolated from the deletion mutants confirms that SaPI1 redirects packaging of its DNA into SaPI1-sized capsids.

CHAPTER 1 Introduction

Characteristics of *S. aureus*. The Gram-positive bacterium *Staphylococcus aureus* is a major cause of hospital-acquired infections. It has become an important community acquired pathogen, largely because of its increasing resistance to methicillin and vancomycin (Lowy, 1998). Its ubiquitous presence combined with increased antibiotic resistance and the spread of its virulence has made it more difficult for hospitals to treat (Shukla, 2005). *S. aureus* virulence is due to a variety of toxins, many of which are encoded by prophages, pathogenicity islands, and other mobile elements in the bacterial chromosome. *S. aureus* superantigen pathogenicity islands, SaPIs, comprise a family of related pathogenicity islands that encode several staphylococcal enterotoxins, including toxic shock toxin-1 (Novick, 2005). Most known SaPIs encode an integrase and a phage-like small terminase subunit, thought to play a role in their integration into the genome and packaging of their DNA into helper phage capsids, respectively. SaPIs exhibit an unusual mode of horizontal transfer that is dependent upon infection with a helper phage.

Properties of SaPI1. SaPI1 (~15 kb, Fig. 1), a prototype element of the SaPI family, is excised, amplified, and transduced only in the presence of the generalized transducing helper phage 80α, or *S. aureus* phage ϕ53, from which 80α was derived (Kwan *et al*., 2005). Like other related pathogenicity islands, SaPI1 is flanked by direct repeats and

Figure 1. SaPI1 genomic map. Genomic map of *S. aureus* pathogenicity island SaPI1 determined from GenBank U93688. The map is inverted relative to the GenBank sequence, to conform to current convention. Genes associated with pathogenicity are highlighted in red; these include the genes for toxic shock toxin (*tst*), enterotoxin K (*entK*), and enterotoxin Q (*entQ*). Phage-like genes present in SaPI1 are highlighted in green. Integrase (*int*) is required for chromosomal integration, while the subject of this study, the small terminase subunit (*terS*), is required for packaging of SaPI1 DNA into capsids. ORFs 8 through *terS* constitute a putative morphogenetic operon. Current evidence suggests that the products of ORFs 6 and 7 are involved in diverting the helper phage capsid assembly process to produce small capsids (Úbeda *et al*., 2007). ORF 22 encodes a repressor, analogous to phage immunity repressors (Úbeda *et al*., 2008).


int
entK
entQ
22
21
20
19
18
17
rep
11
10
9
8
7
6
5
4
terS
tst
ear

encodes an integrase necessary for site-specific integration (Lindsay *et al*., 1998). Besides the integrase, the only other recognizable phage-like gene encodes a small terminase subunit, thought to be essential for the headful packaging of SaPI1 DNA into helper phage capsids (Ruzin *et al*., 2001). Electron micrographs of 80α virions and SaPI1 transducing particles reveal that the only apparent morphological difference between them is that the SaPI1 particles have capsids that hold one-third the volume of 80α capsids (Fig. 2, unpublished transmission electron micrograph courtesy of Sandra Tallent). It is believed that the smaller capsids prevent complete packaging of 80α DNA, but accommodate the smaller SaPI1 genome. Analysis of both virions by MALDI-TOF confirmed that all of the proteins in SaPI1 transducing particles are identical to 80α virion proteins (Tallent *et al*., 2007).

Properties of 80α. *S. aureus* helper phage 80α (45 kb, Fig. 3) is a temperate generalized transducing phage that belongs to the family *Siphoviridae* (Stewart *et al*., 1985). Restriction map analysis of 80α DNA reveals that it has a partially circularly permuted genome, like the SaPI1 it mobilizes (Ruzin *et al*., 2001). This is consistent with phages that utilize a headful DNA packaging in addition to initiation cleavage at a packaging site or *pac* site. The genomic organization of helper phage 80α is essentially identical to that of other staphylococcal *Siphoviridae* (Kwan *et al*., 2005) and also similar to that of typical of other Gram-positive DNA phage, including those of streptococci and lactococci. These phage genomes are arranged into gene clusters based on when they are expressed during

Figure 2. Electron micrograph of SaPI1 and 80α particles. This electron micrograph clearly shows the capsid size differences between the two particles in the center of the image. The electron-dense particles with larger capsid are 80α virions (labeled **A**). The electron-dense particles with smaller capsids are SaPI1 transducing particles (labeled **B**). Also shown are virions with empty phage capsids (Unpublished transmission electron micrograph courtesy of Sandra Tallent).

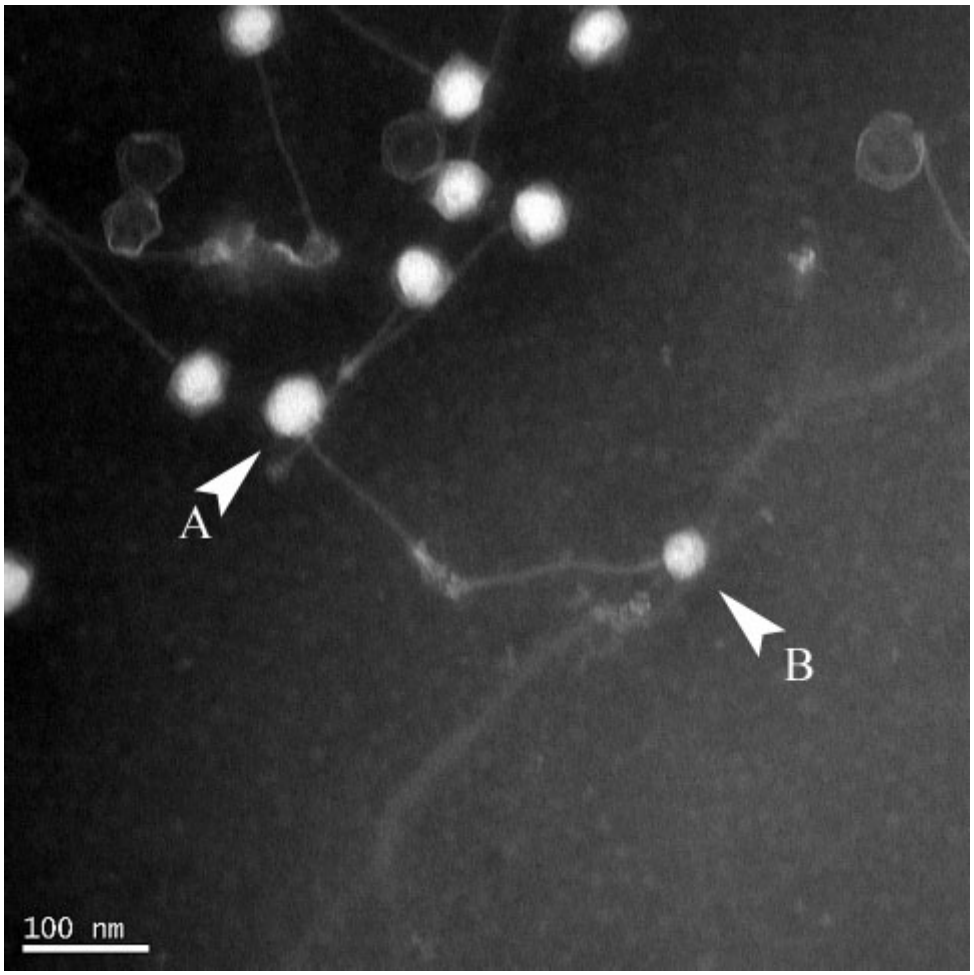
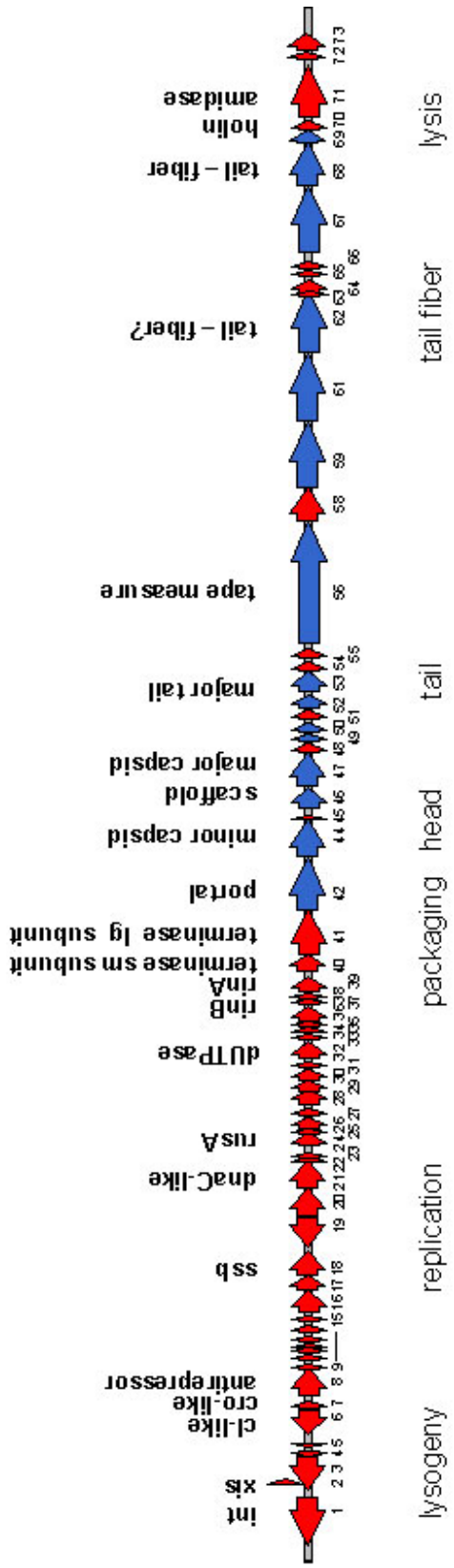

A
B
100 nm

Figure 3. 80α genomic map. Genomic map of 80α determined from GenBank DQ517338. Blue highlighted ORFs encode proteins present in 80α virions (Tallent *et al*. 2007). Additional functional assignment of ORFs is based on protein sequence similarity.


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
lysogeny
replication
packaging
head
tail
tail fiber
lysis

the life cycle of infection. Genes expressed early during infection, like the genes responsible for the establishment of lysogeny, are located to the left of the replication and regulation genes. Genes expressed late during the infection, like the structural genes and gene clusters necessary for lysis, are located to the right of the early genes (Canchaya *et al*., 2003). This genomic organization exemplifies one of defining characteristics of phage, their modular genomes, this unifying theme points to a common ancestor that modern phage have evolved from (Casjens, 2005). This modular nature of their genomes is a result of the intense selective pressure phage are under to have a functional organized genome packed in as little space possible (Hendrix, 2003). Identical gene clusters are found present in closely and distally related phage, often sharing 100% sequence identity. One example is ϕ11 and 80α, which share gene clusters for structural components and lysis that are virtually identical, although ϕ11 cannot mobilize SaPI1.

Phage DNA packaging. Double-stranded DNA (dsDNA) phages generally replicate their DNA as concatamers prior to packaging. Insertion of DNA into capsids is carried out by a DNA packaging enzyme called terminase (Fujisawa & Morita, 1997). The terminase is a complex comprised of copies of two protein subunits. The large subunit contains ATP-binding, DNA cleavage and translocation activities, while the small subunit confers DNA binding specificity (Catalano *et al*., 1995). The general function of the terminase is the same for all dsDNA phages, however the manner in which the terminase complex interacts with the DNA determines whether the packaged genomes are terminally redundant and whether they are permuted (Fujisawa & Morita, 1997).

DNA packaging is categorized by how the concatemeric DNA is cleaved before packaging (initiation cleavage) and after complete packaging into a capsid (termination cleavage). Phage λ is an example of cohesive end DNA packaging; initiation and termination cleavage occur at specific *cos* sites (Catalano *et al*., 1995). These *cos* sites are nicked at each cleavage event creating cohesive ends that enable the genome to circularize after the virus successfully infects a host (Catalano *et al*., 1995). In contrast, the headful mechanism by which T4 processes its DNA for packaging utilizes random initiation and termination cleavage events, but always packaging more than 100% of its genome. This allows the genome to circularize via homologous recombination once inside a host. T4 DNA is recognized at a packaging site located within the small terminase gene, though the initiation cleavage site is random (Lin & Black, 1998). This mode of packaging results in virion DNAs that are terminally redundant and circularly permuted. Another example of headful DNA packaging is provided by Salmonella phage P22, which differs from T4 in that initiation cleavage occurs near the packaging recognition (*pac*) site, but like T4, termination cleavage is random (Susskind & Botstein, 1978). This results in virion DNAs that are terminally redundant, but only partially circularly permuted. Since this is similar to what is observed in 80α, we have used the P22 model as a starting point to explain how the terminase is involved in DNA packaging for 80α and SaPI1.

The general roles of the large and small terminase subunits during DNA packaging is widely agreed upon, though disagreements remain about specific *in vivo* details (Catalano, 2005). The small terminase subunit associates with the replicated DNA and recognizes a specific sequence, after which the large terminase subunit cleaves the DNA.

For the purposes of this discussion, focus will be given to headful DNA packaging of phage with *pac* sites, like P22 (Fujisawa & Morita, 1997). The mature terminase complex associated with the cleaved DNA binds to the portal of an empty capsid to begin packaging DNA into the capsid and cleaves the DNA at a nonspecific site once the capsid is full of DNA (Fujisawa & Morita, 1997). The P22 terminase complex packages 3-5 chromosome lengths, each consisting of 104% of the genomic sequence, into successive capsids before it dissociates from the DNA to find another *pac* site. Restriction map analysis of the resulting packaged DNA yields a discrete number of submolar fragments flanking the *pac* site, indicative of a partially circularly permuted genome (Casjens *et al*., 1992).

Terminase structure and function. The structures of the terminase complex and its subunits have proven extremely difficult to visualize using X-ray crystallography or NMR. Currently, only two partial structures of the small terminase subunit are publicly available (RCSB PDB). In 2007 Benini *et al*. posted the structure of a phage SF6 small terminase subunit monomer using X-ray diffraction, revealing a completely α-helical secondary structure (unpublished). This structure differs from the DNA binding domain of λ small terminase subunit, which is comprised of both α-helices and β-strands (de Beer *et al*., 2002).

In the absence of structural data, the number of individual proteins that comprise each subunit in the mature terminase complex has been calculated based on electron microscopy, sedimentation analyses, and mass spectrometry data from *in vitro* experiments.

A recent study of the P22 large terminase subunit has determined, from sedimentation experiments and electron micrographs, that *in vitro* the large terminase subunit exists as a monomer. The predicted secondary structure is comprised of 36% β-strands and 36% α-helix based on Raman spectral analyses (Němeček *et al*., 2007). These findings concur with studies of T4 and λ large terminase subunits, suggesting that a similar secondary structure might be found in the 80α large terminase subunit (Sun *et al*., 2007; Maluf & Feiss, 2006).

Studies of small terminase subunits suggest that these proteins self-associate to form ring structures. The T4 small terminase subunit is comprised of 8 subunits arranged in a ring approximately 8 nm in diameter (Lin *et al*., 1997). It was also shown for phage SPP1 that the small terminase is comprised of 10 subunits arranged in a cylinder with an approximate diameter of 9 nm (Chai *et al*., 1995). The small terminase subunit of P22 was reported to form a 9-subunit ring with an α-helical secondary structure and a diameter of approximately 10 nm. A 2 nm diameter hole is present in the center of the ring structure. Whether DNA is threaded through the hole in the center of the small terminase complex or whether the DNA is wrapped around the outside of the ring remains unresolved (Němeček *et al*., 2007).

Recent electron micrograph studies on the organization and structure of the P22 small terminase subunit in virions has provided essential data to construct a detailed DNA packaging model for P22 (Němeček *et al*., 2007). Based on the DNA packaging similarities shared between 80α and P22, we have adapted and extended that packaging model to incorporate the complex relationship between SaPI1 and its helper phage.

DNA packaging model for 80α and SaPI1. Restriction map analysis of SaPI1 and 80α virion DNA reveals that both are terminally redundant and partially circularly permuted, consistent with several cycles of headful packaging after initial cleavage at a specific *pac* site. Both 80α and SaPI1 encode small terminase subunits, while the only large terminase subunit is encoded by the helper phage. 80α-encoded small and large terminase subunits are thought to bind specifically to the *pac* site and to translocate 80α DNA into the capsid, respectively. The presence of a small terminase homolog in SaPI1 suggests that SaPI1 small terminase associates with 80α large terminase, forming a hybrid terminase complex capable of redirecting packaging specificity from 80α DNA to SaPI1 DNA. The high rate of transduction of SaPI1 (10^{-1}-10^{0}) is accompanied by a two order of magnitude decrease in 80α titers (Lindsay *et al*., 1998). Packaging specificity and reduced capsid size are two possible explanations for the decrease in the helper phage titer.

Bacteriophage that package DNA utilizing a *pac* site commonly have their *pac* site located within the small terminase gene. The *pac* site of P22 has been located in the small terminase gene by directed mutational analysis (Wu *et al*., 2002). *S. aureus* phage ϕ11 is closely related to 80α and the small terminase genes of the two phages share 99% sequence identity. The *pac* site in ϕ11 has been mapped to a restriction fragment that also includes the ϕ11 small terminase gene (Bachi *et al*., 1980). The restriction map analysis of 80α DNA is consistent with that of ϕ11, leading us to predict that the *pac* site of 80α is located within the 80α small terminase gene (Christie, unpublished data). In contrast,

restriction mapping of SaPI1 by Ruzin *et al*. (2001) indicates that the SaPI1 *pac* site is located outside the small terminase gene at the opposite end of the genome.

We propose a DNA packaging model for SaPI1 based on the information above. In the presence of 80α, SaPI1 DNA is excised and replicates alongside 80α DNA during lytic growth. Helper phage 80α encodes the structural components of the virion, while unidentified SaPI1 proteins alter the size of the capsids during assembly. The SaPI1 small terminase subunit and the 80α small terminase subunit recognize their respective *pac* sites. The 80α large terminase subunit associates with SaPI1 and 80α small terminase subunits, enabling DNA cleavage followed by the association of the DNA/terminase complex with the capsid portal where the terminase complex begins the process of translocating DNA into capsids. What is unknown in this model is whether the 80α large terminase subunit indiscriminately packages SaPI1 and 80α DNAs into both large and small capsid sizes.

Thesis goals. The goals of this project are to elucidate the role of SaPI1 small terminase in the packaging of SaPI1 DNA in relation to its helper phage 80α and to determine the extent of SaPI1 packaging specificity.

In order to address both goals we have deleted *terS* from both SaPI1 and 80α. Since 80α *terS* was expected to be an essential gene for lytic growth, this deletion was introduced into an 80α prophage. By measuring the titers of 80α virions and SaPI1 transducing particles from the deletion mutants after prophage induction and comparing them to wild type titers we have shown how the absence of each small terminase affects

both SaPI1 transduction and 80α propagation. We have further examined the role of each *terS* in DNA packaging by probing DNA isolated from virions from the mutant strains. These studies indicate that SaPI1 *terS* preferentially packages SaPI1 DNA into capsids.

CHAPTER 2 Materials & Methods

Bacterial culture methods. Staphylococcal strains listed in Table 1 were 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 unless stated otherwise. Staphylococcal colonies were inoculated 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) and incubated at 30°C on an orbital shaker at 200 rpm unless stated otherwise. Media were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (American Bioanalystical, Natick, MA) (200 μg/ml), tetracycline (5 μg/ml), or erythromycin (5 μg/ml) as needed. All *E. coli* strains were plated on Luria-Bertani agar (LBA) (Difco, Franklin Lakes, NJ) supplemented with ampicillin (100 μg/ml) or Xgal (200 μg/ml) as needed.

Phage growth and titering. Overnight cultures of *S. aureus* strains containing prophage were diluted 1:100 in CY-GL broth and incubated at 30°C at 200 rpm on an orbital shaker until Klett = 30. Bacterial cultures were pelleted at 4,000 rpm at 4°C for 10 minutes. Supernatants were discarded and pellets were resuspended in an equal volume of *S. aureus*

Table 1. Bacterial strains and plasmids.

S. aureus strain and plasmids	Description	Reference or source
RN4220	Restriction defective derivative of RN450	Novick, 1991
RN10616	RN4220 (80α)	R. Novick
RN10628	RN4220 (80α)(SaPI1 *tst*::*tetM*)	R. Novick
ST16	RN10628 (80αΔterS)(SaPI1 *tst*::*tetM*)	This study
ST17	RN10628 (80α)(SaPI1 *tst*::*tetM* Δ*terS*) (3548 C to A; 4444 G to T)	This study
ST24	RN10616 (80αΔterS)	This study
ST27	RN10628 (80αΔterS)(SaPI1 *tst*::*tetM* Δ*terS*) (3548 C to A; 4444 G to T)	This study
ST37	RN10628 (80α)(SaPI1 *tst*::*tetM* Δ*terS*)	Poliakov *et al*., in press
Plasmids		
PMAD	Shuttle vector derivative of pE194 replication thermosensitive mutant	Arnaud *et al*., 2004
pNO258	Derivative of pMAD with cloned 80α *terS* deletion	This study

pST278	Derivative of pMAD with cloned SaPI1 *terS* deletion (3548 C to A; 4444 G to T)	This study, provided by S. M. Tallent
pPD11	Derivative of pMAD with cloned SaPI1 *terS* deletion	Poliakov *et al.*, in press

phage buffer (SA phage buffer) (Novick, 1991) (1 mM $MgSO_4$, 4 mM $CaCl_2$, 0.05 M Tris-HCl pH 7.8, 100 mM NaCl, 0.1% gelatin) before being transferred to petri dishes. Petri dishes containing the resuspended cells were exposed to a 15 Watt UV light source at a distance of 30 cm on a rocking platform for 20 seconds to induce lysis of the prophage. The irradiated cells were diluted 1:1 with CY-GL broth and incubated at 30°C at 200 rpm until lysis.

Lysates were passed through 0.45 μm filters and diluted in SA phage buffer. Titering of phage was accomplished by plating serial dilutions of filtered lysates on the indicator strain RN4220. Aliquots of 100 μl of each phage dilution were mixed with 100 μl of an overnight culture of RN4220 and incubated for 10 minutes at room temperature. Then 3 ml of *S. aureus* Top Agar (Novick, 1991) (0.3% (wt/vol) Casamino acids, 0.3% (wt/vol) Yeast extract, 100 mM NaCl, 0.5% (wt/vol) agar, 0.5 mM $CaCl_2$, pH 7.8) was poured into the phage dilution mixture and poured onto Phage Agar plates. SaPI1 *tst*::*tetM* transducing particles were titered by mixing as described above and spreading the mixture onto GL agar plates supplemented with tetracycline (5 μg/ml). Plates were incubated at 30°C overnight and 48 hours for the phage titers and SaPI1 transducing titers, respectively.

Agarose gel electrophoresis. Agarose gels (1%, 0.8%, and 0.75%) were made using agarose in 1X TAE buffer (Fisher Scientific, Pittsburgh, PA) containing ethidium bromide (0.1 μg/ml) and run at 100 Volts in 1X TAE Buffer. Sample loading dye (1X) (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was mixed with DNA samples prior to loading in the gel.

Plasmid quick-check screening. Colonies were initially screened for the presence of a plasmid carrying the desired deletion fragment by the quick-check method (Akada, 1994). Each colony was inoculated in 5 ml of LB broth for *E. coli* (CY-GL for *S. aureus*) and incubated overnight at the desired temperature. An aliquot of 100 µl from each overnight was mixed with 50 µl of phenol:chloroform (1:1) plus 10 µl of 1X loading dye (0.25% bromophenol blue and 40% glycerol) and vortexed for 10 seconds followed by 1 minute centrifugation at 13,000 rpm. Screening of *S. aureus* required an initial incubation with lysostaphin (0.5 mg/ml) (Sigma, St. Louis, MO) at 37°C for 30 minutes prior to the addition of phenol:chloroform. A 5 µl aliquot of the aqueous phase was loaded onto a 0.8% agarose gel alongside Supercoiled DNA Ladder (Invitrogen, San Diego, CA), and visualized under UV light.

Plasmid purification. Plasmids were purified from *E. coli* strains using the NucleoSpin Plasmid Kit (ClonTech, Mountain View, CA) as recommended by the manufacturer. Purification of plasmids from *S. aureus* required a modification of the protocol, adding a 1-hour incubation at 37°C with lysostaphin (0.5 mg/ml) after the initial resuspension of the cell pellet in A1 Buffer.

Polymerase Chain Reaction (PCR). High fidelity PCR amplification was performed using AccuPol polymerase (GeneChoice, Frederick, MD), following the manufacturer's recommended protocol. Primers used for amplifications are shown in Table 2.

Table 2. List of primers. Underlined nucleotides designate restriction sites added to the resulting amplicon.

Primers	Sequence	Template
SMT15	5’ AACGAGGTACCTCATTCTATCACCTCCACAAT 3’	SaPI1
SMT16	5’ AACGAGGATCCTAAAAGTGGTGTAACAATGGATA 3’	SaPI1
SMT46	5’ AAAAAGGAAGGGCTGTGTTC 3’	80α
SMT47	5’ ACGTTTCGTGTCTGTATATCC 3’	80α
SMT51	5’ ACACCATGGGCATACAGATATTCTCTGGA 3’	SaPI1
SMT52	5’ ACAAAGCTTGTGGATGATATACCGTTAGAG 3’	SaPI1
SMT53	5’ ACAAAGCTTACGCGCTTGTTTTGC 3’	SaPI1
SMT54	5’ ACACCATGGCAATATGCAGGAGATTTCAAG 3’	SaPI1
SMT55	5’ GCCAGATCTCCGGTCTTATACGAAGTAAAG 3’	80α
SMT56	5’ GCCAAGCTTTGCTGAAATTGCTGCTTT 3’	80α
SMT57	5’ GCCAAGCTTGGTGAGTACGATGACGAA 3’	80α
SMT58	5’ GCCAGATCTACCCTTGTACAACCGAG 3’	80α
SMT98	5’ TACTACGGCTATGATGAACG 3’	80α
SMT99	5’ TGCTCCTCAGTTTTTAAATCAC 3’	80α

Labeled probes for Southern blots were made by amplifying the target sequence in the presence of digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-11-dUTP) (Roche Applied Science, Mannheim, Germany), randomly incorporating the label into the amplicon in place of dTTP. An aliquot of 2 μl from the DIG-dNTP mix (2 mM dATP, dCTP, and dGTP, 1.3 mM dTTP) and 3.5 μl of 1000 μM DIG-11-dUTP were used in place of the 10 mM dNTP mix in a 50 μl PCR reaction. The SaPI1 ORF 4 probe was created using the primer pairs SMT 15/SMT 16. The 80α upstream probe was specific for the putative phage replication region between nucleotides 7995-8757 and was created using SMT 98/SMT 99. The 80α downstream probe was specific for the major capsid region between nucleotides 23244-23542 and was created using SMT 46/SMT 47.

Creation of *terS* deletions. Non-polar, in-frame deletions were made by amplifying regions of approximately 1 kb upstream and downstream of the gene and introducing internal restriction sites for the ligation of the two amplicons. External restriction sites were also introduced to allow for ligation to the vector. CsCl double-banded SaPI1 virions served as the template for SaPI1 primers, filtered lysates containing 80α virions served as the template for 80α primers. A 1 kb region upstream of 80α *terS*, which includes the region encoding the first 25 amino acids of 80α *terS*, was amplified using the SMT 55/SMT 56 primer pair. A 1 kb region downstream of 80α *terS*, including sequence encoding the last 7 amino acids of 80α *terS*, was amplified using the SMT 57/SMT 58 primer pair. Both fragments were cleaned using SureClean (Bioline, Taunton, MA) as directed by the

manufacturer. Both amplicons were digested with *Hin*d III, cleaned with SureClean, and ligated together using T4 DNA Ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. A 1 μl aliquot of the ligation reaction was used as template for a second PCR using the outer SMT 55/SMT 58 primer pair. The resulting 80α*Δter*S fragment was digested with *Bgl* II, cleaned and ligated with *Bam*H I-digested pMAD allelic exchange vector (Table 1). Electrocompetent *E. coli* cells were transformed with the ligation mixtures, plated on LB plates containing ampicillin and Xgal for blue-white screening and incubated overnight at 37°C.

Deletion of SaPI1 *terS* was accomplished using a strategy similar to that used for 80α*ΔterS*. The SMT 53/SMT 54 primer pair was used to amplify the upstream fragment, including sequence encoding the first 9 amino acids. The SMT 51/SMT 52 primer pair was used to amplify the downstream fragment, including sequence encoding the last 8 amino acids of SaPI1 *terS*. *Hin*d III was used to digest the amplicons, and *Nco* I was used to digest the pMAD vector in addition to the ligated amplicon of the SaPI1 *terS* deletion fragment.

Allelic exchange. Replacement of the genomic wild type sequence with the plasmid borne deletions was accomplished by allelic exchange. Each pMAD plasmid containing a deletion fragment was introduced into electrocompetent *S. aureus* strains RN10628 and RN10616 by electroporation, plated on TSA containing erythromycin and Xgal, and incubated at the permissive temperature of the temperature sensitive origin or replication on pMAD (30°C) for 48 hours (Arnaud *et al*., 2004). Individual blue colonies were

inoculated into TSB with erythromycin and incubated overnight at the nonpermissive temperature (44°C) on an orbital shaker at 200 rpm to select for cointegrates. The overnight culture was diluted (1:100) into pre-warmed TSB with erythromycin and incubated at 44°C on an orbital shaker for 3 hours. Serial dilutions were made and 100 µl aliquots were plated onto pre-warmed TSA plates containing erythromycin and Xgal and incubated at 44°C for 48 hours. Blue colonies were streaked for isolation onto the same prewarmed media and incubated at 44°C for 48 hours. To promote cointegrate resolution, blue colonies were picked and inoculated into TSB and incubated at 30°C on an orbital shaker for 5 hours until the broth was slightly turbid. Serial dilutions were made and 100 µl aliquots were plated onto pre-warmed TSA containing Xgal and incubated at 44°C for 48 hours to cure the cells of the plasmids. White colonies were streaked for isolation on pre-warmed TSA containing Xgal at 30°C for 48 hours. Verification of the allelic exchange was performed by pelleting 100 µl of overnight cultures of candidate white colonies, rinsing in sterile MQ H2O, and using 1 µl as template for PCR amplification with the primer pairs SMT 55/SMT 58 for 80α *terS* and SMT 51/SMT 54 for SaPI1 *terS*. The RN10628 strain with the 80α *terS* deletion was designated ST16. The RN10628 strain with the SaPI1 *terS* deletion was designated ST17. An additional SaPI1 *terS* deletion in RN10628, constructed by P.K. Damle, was designated ST 37. The RN10616 strain with the 80α *terS* deletion was designated ST24.

DNA sequence verification of the ST17 SaPI1Δ*terS* mutant failed to consistently match template sequence (3548 C to A; 4444 G to T). Position 3548 corresponds to a

region at the end of the ORF 4 coding sequence. This mutation changes the last glutamate to a stop codon. Position 4444 corresponds to the intergenic region between ORF 5 and ORF 6. While it seemed unlikely that either of these two point mutations would affect the results of this study, an additional SaPI1Δ*terS* mutant (ST37) was created whose sequence consistently matched the template DNA. Ultimately, the data obtained from the two mutants were the same.

Isolation of phage DNAs. Cultures of RN10616, ST16, ST17, ST37 and RN10628 grown overnight were inoculated 1:100 into 500 ml CY-GL broth and incubated at 30°C in an orbital shaker at 200 rpm until OD_{540} = 0.4. An equal volume of SA phage buffer was added to the cultures, and ciprofloxin (0.4 μg/ml) was added to induce the prophage. Incubation of the cultures was continued until lysis occurred. The lysates were treated with DNase I (1 μg/ml) at 37°C on an orbital shaker at 200 rpm for 30 minutes to remove non-encapsidated DNA, followed by centrifugation at 7,000 rpm for 15 minutes at 4°C to remove cellular debris. Virions were precipitated by slowly dissolving polyethylene glycol 8000 (PEG) (10% (wt/vol) and NaCl (0.5 M) in the lysates, followed by incubation overnight at 4°C. The lysates were centrifuged at 7,000 rpm for 20 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 9 ml SA phage buffer and stored overnight at 4°C. The resuspended pellet was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was layered on top of a glycerol step gradient composed of 3 ml of 40% glycerol (diluted in SA phage buffer) and 3 ml of 5% glycerol (diluted in SA phage buffer). The gradients were loaded in a Beckman SW41 TI rotor and centrifuged

at 35,000 rpm for 1 hour at 4°C in a Beckman XL-70 Ultra centrifuge. The supernatant was decanted and the phage pellet was resuspended overnight at 4°C in 100 µl of SA phage buffer. DNA was isolated from the phage by adding 100 µl of a 25:24:1 mixture of TE saturated phenol:chloroform:isoamyl alcohol (Sigma, St. Louis, MO) to each resuspended phage pellet, mixing by inversion for 1 minute and centrifuging at 13,000 rpm for 1 minute at 4°C. The aqueous phase was transferred into a sterile tube, then 100 µl of SA phage buffer was added to the resuspended phage pellet and centrifuged again. The aqueous phase was pooled with the previous one and cold 3 M sodium acetate pH 7.0 (1/10 the volume of the aqueous solution) was added and mixed by inversion for 1 minute. Two volumes of cold 100% ethanol were added and mixed by inversion for 1 minute, incubated at -20°C for 1 hour, and centrifuged at 13,000 rpm at 4°C. The supernatant was aspirated off and the DNA pellet was dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The phenol/chloroform extraction was repeated until the resulting DNA migrated as discreet bands on an agarose gel when visualized.

Southern blot. Equal concentrations of DNA were brought to a final volume of 10 µl with 1X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) and loaded on a 0.7% agarose gel containing 0.1 µg/ml ethidium bromide. Electrophoresis was carried out at 100 volts for approximately 5 hours in 1X TAE to ensure complete separation of 80α-sized and SaPI1-sized DNAs. The agarose gel was photographed under UV light and trimmed. DNA samples in the agarose were depurinated by soaking the gel in

0.25 N HCl for 10 minutes and rinsing twice in MQ H_2O. The agarose gel was then soaked in a denaturation solution (1.5 M NaCl, 0.5 NaOH) for 45 minutes at room temperature on an orbital shaker, followed by two rinses in MQ H_2O. The agarose gel was then soaked in a neutralization solution (1 M Tris-HCl pH 7.4, 1.5 M NaCl) for 30 minutes at room temperature on an orbital shaker, rinsed twice in MQ H2O and soaked in fresh neutralization solution for an additional 15 minutes at room temperature on an orbital shaker. During this incubation a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany) and two pieces of Whatman 3MM paper (Whatman, Kent, United Kingdom) were cut slightly larger than the gel. The nylon membrane was completely wetted with MQ H_2O and then equilibrated in 10X SSC buffer (1.5 M NaCl, 0.15 M $Na_3C_6H5O_7$). A glass reservoir was filled with 10X SSC buffer and a glass plate support was placed on top of the reservoir wrapped with Whatman 3MM paper that acted as a wick during the transfer to the gel to the nylon membrane. The gel was placed on the Whatman 3MM paper on top of the glass support and surrounded with Parafilm M (Pechiney Plastic Packaging Inc., Chicago, IL) to ensure that the transfer could not bypass the nylon membrane. The nylon membrane was placed on top of the agarose gel, followed by two pieces of Whatman 3MM paper wetted in 2X SSC, a 5 cm stack of paper towels, a glass plate and a weight (approximately 500 g). A 24-hour incubation at room temperature allowed capillary action to transfer the DNA in the agarose gel onto the nylon membrane. The nylon membrane was removed and soaked in 6X SSC for 15 minutes at room temperature to remove residual agarose. DNA was fixed to the nylon membrane by placing

the nylon membrane onto Whatman 3MM paper soaked in 10X SSC and irradiated in a UV Stratalinker 1200 (Stratagene, La Jolla, CA) at 120,000 μjoules/cm^2.

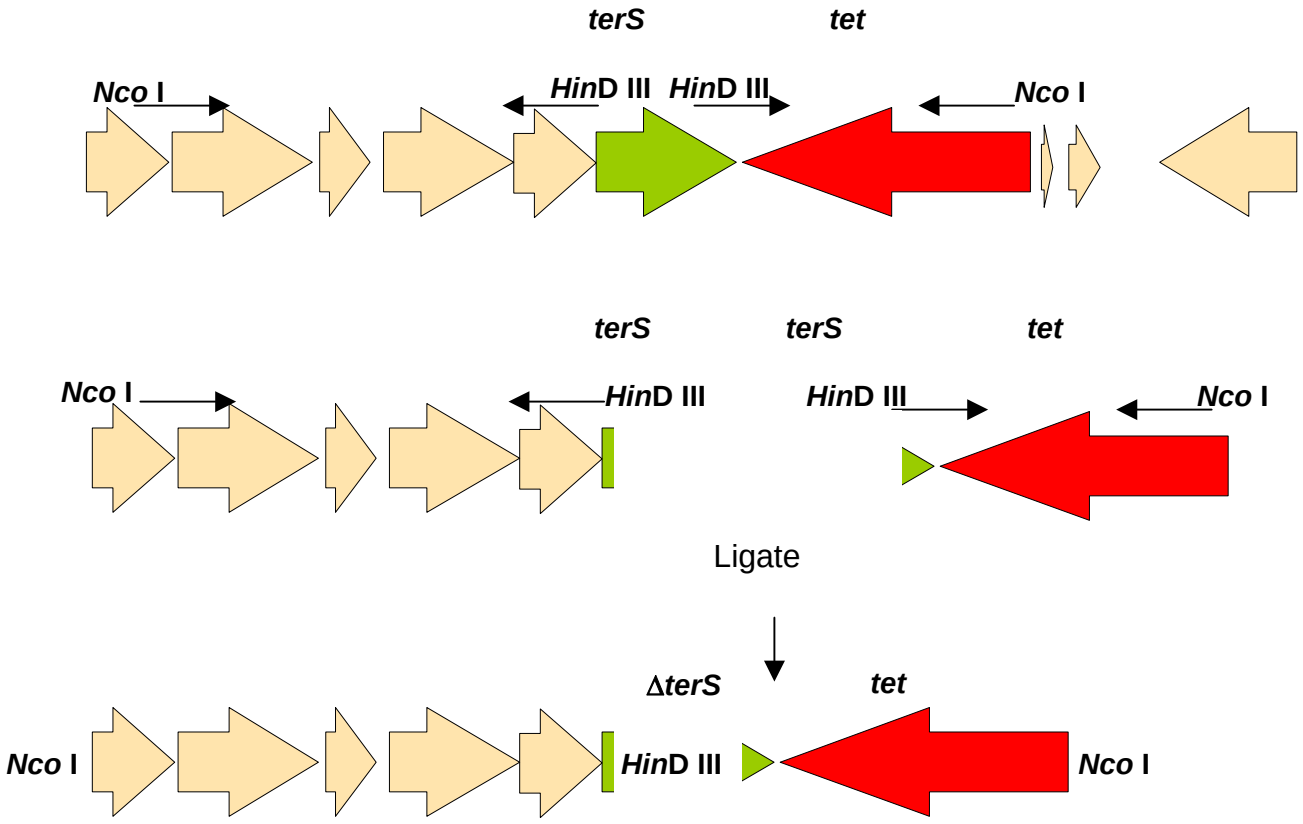
Hybridization of probes was accomplished using the DIG Wash and Block Buffer Set (Roche Applied Science, Mannheim, Germany) as directed by the manufacturer. After exposure to the Detection buffer, the membranes were sealed in hybridization bags and the chemiluminescent DIG signal was detected by exposure to X-ray film (Kodak, Rochester, NY)

CHAPTER 3 Characterization of SaPI1 and 80α *terS* deletions

The proposed DNA packaging model for SaPI1 and 80α postulates that the small terminase subunits recognize their respective *pac* sites while the 80α large terminase subunit binds to the small terminases, forming the functional terminase complex. During this process the DNA is cleaved at the *pac* site before the terminase associates with the portal of the capsid allowing the DNA to be translocated into the capsid. By deleting the small terminase genes we have analyzed the role of each in SaPI1 transduction and 80α propagation. The approach used to create the deletions of each *terS* and introduce them by allelic exchange focused on preserving the flanking coding sequences in-frame to prevent polar effects. This was especially critical for 80α, because *terS* appears to lie at the start of an operon. A frameshift would potentially inhibit expression of the downstream genes. Additionally, it was necessary to introduce the 80α *terS* deletion into a prophage, since the deletion was expected to be lethal to the phage (Fig. 4). Since the prophage remains inactive in the genome of the host, it can be maintained over the course of the study and induced to study effects on lytic growth.

Biological analyses of the small terminase subunit deletions were performed by inducing lytic growth of 80α and SaPI1 by exposure to a UV light source and measuring the quantity of 80α and SaPI1 particles produced. A typical UV induction of a *S. aureus*

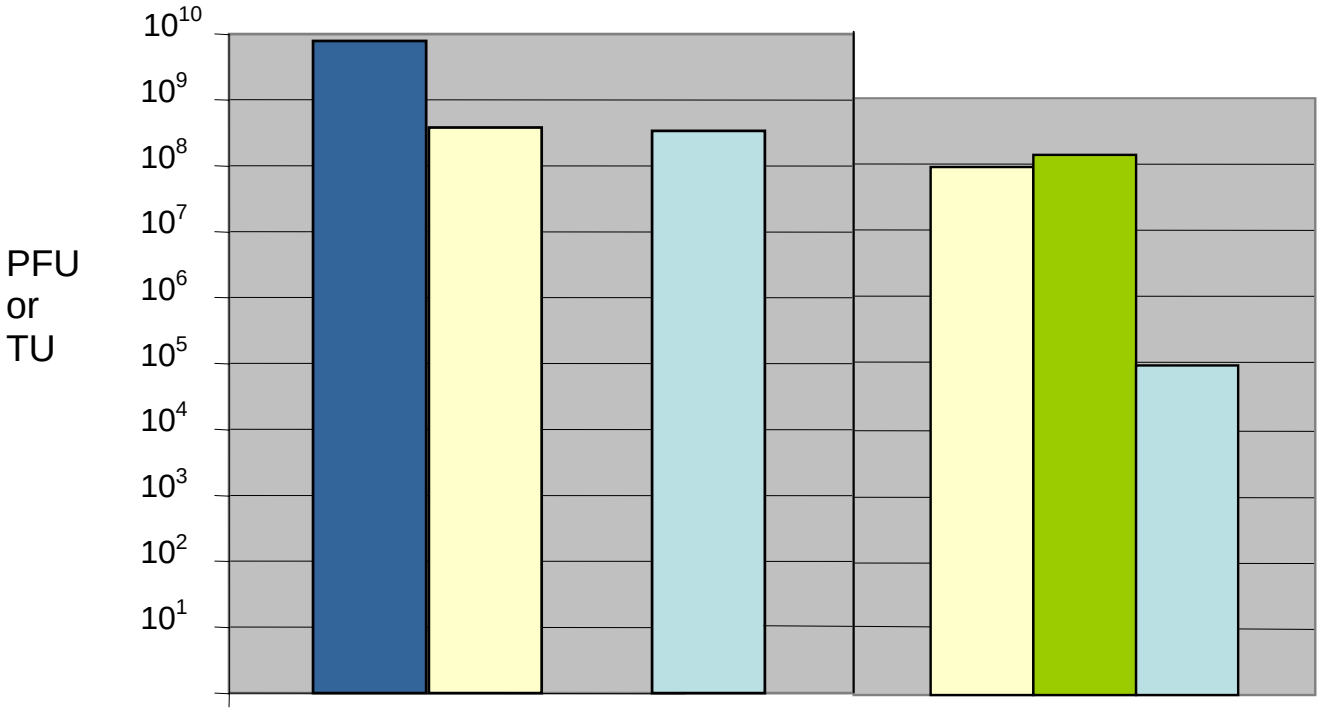
Figure 4. In-frame deletion of the *terS* genes. Non-polar, in-frame deletions were made by amplifying approximately 1 kb regions upstream and downstream of each gene and introducing internal restriction sites for the ligation of the two amplicons. External restriction sites were also introduced to allow for ligation to the vector. SaPI1Δ*terS* was created using *Hin*d III for the internal restriction sites and *Nco* I for the external restriction sites. 80αΔ*terS* was created using the same strategy using *Hin*d III for the internal restriction sites, but *Bgl* II for the external restriction sites.


terS
tet
Nco I
HinD III
HinD III
Nco I
terS
terS
tet
Nco I
HinD III
HinD III
Nco I
Ligate
ΔterS
tet
Nco I
HinD III
Nco I

strain containing SaPI1 and an 80α prophage produces a lysate containing SaPI1 particles in addition to 80α particles. Measurement of SaPI1 transduction is facilitated by the use of a SaPI1 variant that has a tetracycline resistance marker (*tetM*) inserted into the toxic shock toxin gene (*tst*). The addition of this marker increases the genomic size to 18,129 bp (Ruzin *et al*., 2001). SaPI1 transduction is measured by incubating serial dilutions of the filtered lysate of the UV induced culture with an indicator strain, RN4220, that has neither SaPI1 or 80α, and plating onto GL agar supplemented with tetracycline. Measurement of 80α phage titers is accomplished by plating serial dilutions of the filtered lysate with the same indicator strain, RN4220, in a soft agar overlay. The number of tetracycline resistant colonies and plaques were counted to ascertain the SaPI1 transducing titers and 80α phage titers, respectively.

80α *terS* is nonessential for SaPI1 transduction. The complex relationship that SaPI1 shares with 80α involves many facets; it was essential to determine what effects, if any, that 80α *terS* had on SaPI1 transduction. This was addressed by deleting the 80α *terS* in strains containing both 80α and SaPI1. Deletion of 80α *terS* had no effect on transduction of wild type SaPI1 (Fig 5, SaPI1 transduction). There is no significant difference in SaPI1 transducing titers in the presence or absence of a functional 80α small terminase. This was the expected outcome if SaPI1 is utilizing its own small terminase in place of its helper phage.

Figure 5. Effects of *terS* mutations on phage growth and SaPI1 transduction. The genotype of 80α and SaPI1 present in each strain is depicted below the graphs. Wild type *terS* is designated as "wt." The deletion of *terS* is designated as "Δ*terS*." The presence or absence of a genotype is depicted beneath each column of data as a "+" and "-", respectively. Titers obtained after induction of 80α and its *terS* mutant were determined by plating serial dilutions of each lysate on indicator strain RN4220. Wild type and mutant SaPI1 transductants were measured by incubating serial dilutions of each lysate with RN4220 in soft agar supplemented with 5 μg/ml tetracycline.



wt 80α	+	+	-	+	-	+	-	+	-
wt SaPI1	-	+	- or +	-	-	+	+	-	-
80αΔ*terS*	-	-	+	-	+	-	+	-	+
SaPI1Δ*terS*	-	-	- or +	+	+	-	-	+	+

80α *terS* is essential for 80α propagation. Deletion of 80α *terS* enabled us to test whether the small terminase subunit of 80α was an essential gene product for 80α propagation, as was expected from its predicted role in DNA packaging. Deletion of 80α *terS* in either the absence or presence of SaPI1 eliminated production of 80α plaque-forming units, demonstrating the requirement of this gene and the *pac* site for 80α growth (Fig 5, 80α growth). This result also suggests that SaPI1 *terS* cannot substitute for 80α *terS* in packaging of 80α DNA. However, since any resulting packaged 80α would still be mutant and unable to plate on the indicator strain, direct examination of packaged DNA (see Chapter 4) is necessary to investigate this point.

SaPI1 *terS* is not responsible for SaPI1 interference with 80α growth. As mentioned earlier, the presence of SaPI1 in the chromosome of a host strain results in a two order of magnitude decrease in 80α titers compared to the 80α yield obtained in the same strain in the absence of SaPI1 (Fig. 5, 80α growth). However, there was no significant difference in 80α titers in the presence of either wild type SaPI1 or SaPI1Δ*terS* during growth of wild type 80α, suggesting that the SaPI1 *terS* has no inhibitory effect on 80α titers.

80α *terS* transduces SaPI1 in the presence of SaPI1Δ*terS*. The results described above established that 80α *terS* was not necessary for high frequency SaPI1 transduction, but was essential for 80α propagation. We expected SaPI1 *terS* to be similarly essential in the transduction of SaPI1. However, the deletion of SaPI1 *terS* resulted in only a three order of

magnitude decrease in SaPI1 transduction in the presence of wild type 80α compared to the transduction frequency seen for wild type SaPI1 (Fig. 5, SaPI1 transduction). It should be noted that a recent study of a close SaPI1 relative, SaPIbov1, showed a similar decrease in transduction of the pathogenicity island following deletion of the SaPI1 small terminase gene (Úbeda *et al*., 2007). Results from these studies indicate that while *terS* from SaPI1 and SaPIbov1 are essential for high frequency SaPI transduction, the residual level of SaPI1 transduction in the absence of SaPI1 *terS* is two to three orders of magnitude higher than would be expected for generalized transduction by the helper phage. Further testing revealed that the reduced transduction of SaPI1Δ*terS* was completely eliminated in the presence of 80αΔ*terS* (Fig 5, SaPI1 transduction), clearly demonstrating that 80α *terS* was responsible for the residual transduction seen in SaPI1Δ*terS* (Fig. 5, SaPI1 transduction).

CHAPTER 4 Capsid specificity of SaPI1 and 80α *terS*

Data from protein analyses show that mature SaPI1 particles are comprised of the same structural proteins as mature 80α virions (Tallent *et al*., 2007). Additionally, a recent study of a close SaPI1 relative, SaPIbov1, has identified two genes that may be involved in diverting 80α capsid assembly to create SaPI-sized capsids (Úbeda *et al*., 2007). From these studies we know that two capsid sizes, comprised of identical capsid proteins, form during the lytic multiplication of 80α in the presence of SaPI1. The smaller of the two capsids appears to be the result of SaPI1-encoded size determining proteins that act during capsid assembly but are not present in the mature capsid.

Our initial studies revealed that SaPI1 *terS* was essential for high frequency SaPI1 transduction. We observed that 80α *terS* facilitates transduction of SaPI1 at a reduced efficiency, although SaPI1 *terS* did not facilitate production of 80α plaques. This biological analysis, however, could not answer if SaPI1 DNA was being packaged into both large and small capsids, nor could it reveal if SaPI1 *terS* was packaging fragments of 80α DNA into small capsids. Also, the biological analysis could not detect any packaging of 80α DNA by SaPI1 *terS* in the 80αΔ*terS* mutant since the resulting progeny phage would not be viable. Most current phage DNA packaging models propose that the large terminase subunit interacts with the portal of the capsid, while the small terminase subunit

is bound to the phage DNA (Catalano *et al*., 1995). It should be noted, however that there is disagreement over which proteins directly bind the capsid portal protein and few definitive results to support any one model. Two models for P22 published recently show either the large (Lander *et al*., 2006) or the small (Němeček *et al*., 2007) terminase subunit associating with the portal protein. It would be expected that the large terminase subunit of 80α would associate with both large and small capsids during 80α and SaPI1 propagation. For this reason we chose to analyze by Southern blot the virion DNA packaged by the 80αΔ*terS* and SaPI1Δ*terS* mutants alongside their wild type counterparts as controls. The large and small capsids carry approximately 45 kb and 18 kb of DNA, respectively, which can easily be separated on a 0.7% agarose gel. Probes for both virions were created using PCR that randomly incorporated DIG-11-dUTP enabling visualization by Southern blot.

If 80α DNA is packaged like P22 DNA, where only a small number of virions are filled after cleavage at an initial *pac* site, we expected to see a bias in packaging of fragments into small capsids. The part of the genome within 18 kb downstream of the *pac* site in the direction where packaging proceeds should be present in a large excess as compared to the part of the genome on the opposite side of the *pac* site. We therefore used two different 80α probes on either side of *terS*. These probes enabled us to confirm that packaging started near or in *terS* and to determine in which direction 80α packaging occurred.

Interpretation of the Southern blot data is complicated by a number of factors. During an 80α infection of a SaPI1 containing host, two capsid sizes are produced, but approximately 95% are small capsids. When visualized by electron microscopy it is also

seen that only a portion of the capsids have been filled with DNA, though this could be an artifact of preparing the samples for electron microscopy. This bias resulting from small capsid prevalence must be accounted for when analyzing the data.

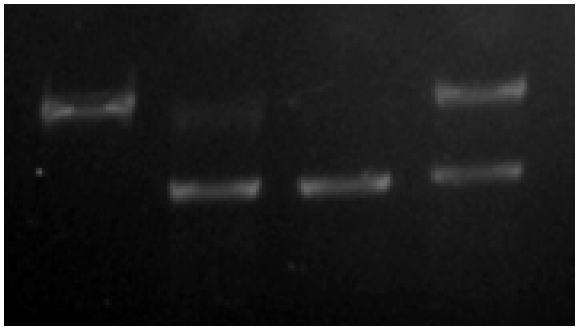
DNA packaging direction of 80α. When probing the virion DNAs isolated from the wild type 80α/SaPI1 strain, RN10628, with the 80α downstream probe we saw nearly equal amounts of DNA present in both capsid sizes (Fig. 6b, lane 2). In contrast, DNA from the same strain probed with the 80α upstream probe showed substantially lower hybridization to the DNA present in the smaller capsids (Fig. 6c, lane 4). This difference is even more apparent in the SaP1Δ*terS* strain (Fig. 6c, lane 4). This suggests that 80α DNA packaging initiates at a *pac* site near 80α *terS*, and confirms that the direction of packaging is downstream of the site (towards the remaining morphogenetic genes).

DNA packaging into large capsids. The Southern blot of the virion DNAs probed with the SaPI1 ORF 4 probe indicates the presence of SaPI1 DNA of both 18 kb and 45 kb sizes (Fig. 6d and 6e). In a wild type infection we see an intense hybridization signal corresponding to SaPI1 sized DNA and a much less intense band that corresponds to 80α sized DNA (Fig. 6d, lane 2). Examination of the distribution of these two bands in the two small terminase mutants, in conjunction with a consideration of the prevalence of SaPI1 capsids, enables us to determine the contributions of each small terminase.

Figure 6. Southern blot analysis. Southern blot hybridization of DIG labeled primers specific for 80α and SaPI1 DNA to DNAs isolated from virions present in the lysates of RN10628 (80α/SaPI1), ST16 (80αΔ*terS*/SaPI1), and ST17 (80α/SaPI1Δ*terS*). Lane 1 contains 45 kb DNA purified from CsCl double-banded 80α virions in the absence of SaPI1. (a) Agarose gel containing equivalent amounts of DNAs purified from virions and purified CsCl double-banded 80α DNA. (b) 30 minute exposure of DIG labeled probe specific for 80α capsid region located downstream of 80α *terS*. (c) 30 minute exposure of DIG labeled probe specific for 80α replication region located upstream of 80α *terS*. (d) 30 minute exposure of DIG labeled probe specific for SaPI1 ORF 4. (e) Lane 4 after 4.5 hour exposure of DIG labeled probe specific for SaPI1 ORF 4.

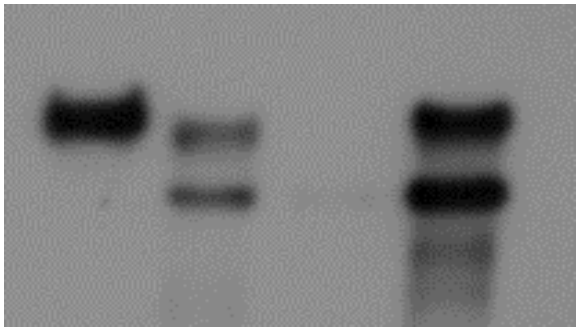
Lanes	1	2	3	4
wt 80α	+	+	-	+
wt SaPI1	-	+	+	-
80αΔ*terS*	-	-	+	-
SaPI1Δ*terS*	-	-	-	+

A.



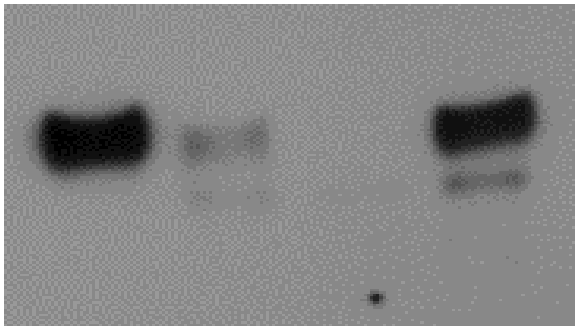
B.

Probe:
80α downstream



C.

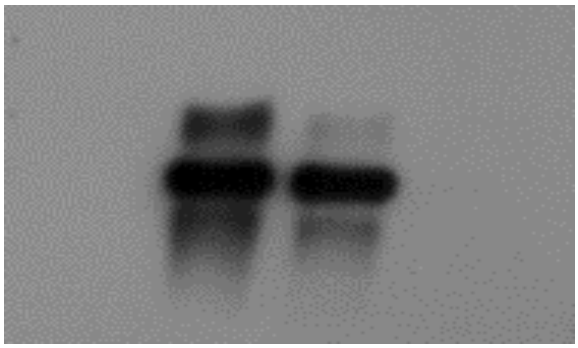
80α upstream



E.

D.

SaPI1



Compared to the distribution of bands present during a wild type infection, the 80αΔ*terS* mutant shows a dramatic decrease in 45 kb SaPI1 DNA present (Fig. 6d). However, it is crucial to note that there is still a small amount of 45 kb SaPI1 DNA detected, this strongly suggests that SaPI1 *terS* is capable of packaging a headful (approximately three genomes worth) of SaPI1 DNA into large capsids, although with reduced efficiency.

Deletion of SaPI1 *terS* shows no SaPI1 DNA of any size detected in the blot after the standard 30 minute exposure time (Fig. 6d, lane 4). However, we know from the biological data that SaPI1 is transduced under these conditions, with a decrease in efficiency of about three orders of magnitude. A four hour overexposure of the blot, where the adjacent lanes were shielded with tape to prevent the signal from bleeding into the other lanes, clearly reveals that in the absence of SaPI1 *terS* there is a small amount of SaPI1 DNA being packaged into capsids of both sizes (Fig. 6e). This demonstrates that at least in the absence of SaPI1 *terS*, 80α *terS* is capable of packaging of SaPI1 DNA into both large and small capsids with reduced efficiency.

The fate of 80α DNA is of equal interest in this study. The wild type infection reveals both 18 kb and 45 kb sized DNA bands in agarose and when probed with either one of the 80α probes. If we just consider the probe downstream of the pac site, we see that in the SaPI1 *terS* deletion mutant, there are bands of both 18 kb and 45 kb sizes (Fig. 6b, lane 4). The bands of the SaPI1 *terS* mutant are significantly more intense than those from wild type, indicating a lack of interference by SaPI1 by some mechanism that remains unclear and that is not reflected in the phage titers. The equivalent intensity of the bands in both

lanes indicates that 80α must package its DNA preferentially into large capsids, since small capsids are in great excess during these infections.

Examination of the 80α *terS* deletion probed with the 80α downstream probe reveals a faint 18 kb sized band (Fig. 6b). The virtual absence of a 45 kb band indicates an absence of packaging of 80α DNA into large virions in the absence of 80α *terS*.

DNA packaging into small capsids. The Southern blot using the SaPI1 probe shows that SaPI1 DNA is predominantly found in the smaller band that corresponds to the small capsid size (Fig. 6d). This same figure also reveals a faint 45 kb band in the 80αΔ*terS* mutant that indicates that SaPI1 can also package its DNA into large capsids. The bias towards packaging into small capsids may be due, at least in part, to the excess of small capsids relative to large capsids in this infection. In order to determine whether SaPI1 packaging is really specific for small capsids in the same way that 80α packaging appears to be specific for large capsids would require analysis of packaged DNA using 80α mutants that were unable to form the small SaPI1-directed capsids. Nevertheless, the demonstration of a mobile genetic element capable of redirecting DNA packaging is a completely novel discovery.

CHAPTER 5 Discussion

Previous work has established that SaPI1 propagation and spread relies upon the presence of helper phage 80α. SaPI1 hijacks the structural components of 80α virions for its own propagation. Based on electron micrographs of 80α and SaPI1 transducing virions, it was proposed that SaPI1 encodes capsid size determining factor(s) responsible for altering 80α capsid assembly, producing smaller capsids that only accommodate a single SaPI1 genome.

The presence of a small terminase subunit homolog in SaPI1 genomes suggested a method by which SaPI1 could selectively package its DNA into small capsids by creating a hybrid terminase complex with the large terminase of its helper phage 80α. In this study we sought to characterize the function of SaPI1 small terminase and its role in the relationship between SaPI1 and 80α.

Role of SaPI1 *terS*. The exploitation of a phage by a genetic element present in the host genome is not a novel mechanism. This relationship is perhaps best known in the well-studied helper phage P2 and satellite phage P4 system. The similarities and differences between P2/P4 and 80α/SaPI1 have allowed us to target specific SaPI1 genes for study. P2/P4 utilize the previously described *cos* site mechanism for packaging their DNA, an

important distinction from 80α and SaPI1. Helper phage P2 and satellite phage P4 utilize an identical *cos* site (Bowden *et al*., 1985). This means that satellite phage P4 relies upon the terminase complex of its helper phage to package P4 DNA. Also, the interference imposed on helper phage 80α by SaPI1 is similar to that seen in P2/P4. P4 encodes proteins that redirect capsid assembly to generate a smaller capsid that results in a several fold decrease in helper phage P2 titers (reviewed by Lindqvist *et al*., 1993; and by Christie & Calendar, 1990). The differences between these two systems led us to speculate that one reason SaPI1 encodes its own small terminase subunit would be that SaPI1 possesses its own unique *pac* site. The deletion of the SaPI1 small terminase subunit allowed us to determine the role of this protein in the propagation of both SaPI1 and 80α. In addition, deletion of the 80α *terS* allowed us to examine packaging of genomes by terminase complexes carrying only the SaPI1 *terS*.

Since SaPI and helper phages both use the pac/headful mechanism, a change in packaging specificity could increase the frequency with which SaPI is packaged – and could also provide an additional level at which helper genomes are excluded. However, the results obtained in this study rule out a major contribution of SaPI1 *terS* in interference with 80α titers. The SaPI1 *terS* mutant still shows the same two order of magnitude decrease in 80α titers as is seen for wild type SaPI1 (Fig. 5, 80α growth). The results of the Southern blot indicate that more 80α DNA is packaged in the absence of wild type SaPI1 *terS* (Fig. 6b, lane 4).

SaPI1 *terS* is required for efficient packaging of SaPI1 DNA. This is evident in the Southern blot, where packaging of SaPI1 DNA is unaffected by deletion of the 80α small terminase, but dramatically reduced by deletion of the SaPI1 small terminase. The transduction assay shows that deletion of SaPI1 *terS* resulted in a three order of magnitude decrease in SaPI1 transduction (Fig. 5, SaPI1 transduction). The residual transduction of SaPI1 seen in this mutant is still several orders of magnitude higher than would be expected from generalized transduction, but is clearly attributable to the 80α terminase since it is eliminated when 80α *terS* is deleted as well. Possible explanations for this observation are discussed below. SaPI1 *terS* is also sufficient for packaging of SaPI1 DNA, since the transduction frequency is unchanged when the small terminase of the helper phage is eliminated. These observations are consistent with the proposed role for SaPI1 small terminase in redirecting the packaging machinery of the helper phage to specifically package SaPI1 DNA.

Additionally, the 80αΔ*terS*/SaPI1 wild type strain when hybridized to the 80α downstream or upstream probe shows little to virtually no 80α DNA present in either capsid size (Fig. 6b and 6c, lane 3). This low level of DNA hybridization by either probe suggests that 80α DNA without a *pac* site is packaged randomly by SaPI1 *terS* at a very low level.

Role of 80α *terS*. Phage 80α is a generalized transducing phage. We expected, therefore, to see a low level of SaPI1 transduction in the SaPI1 small terminase mutants due to a

small amount of nonspecific packaging of SaPI1 DNA. The residual packaging we observed was several orders of magnitude higher than expected, as discussed previously.

Deletion of the 80α *terS* eliminated packaging of 80α DNA by 80α *terS*, as expected. Interpretation of this result is complicated, however, by the fact that the 80α *pac* site is also expected to lie within the 80α *terS* gene. Thus, it is not possible to dissect the contributions from the loss of *terS* and the loss of the *pac* site – both would presumably result in the observed loss of phage packaging. There does not seem to be any significant nonspecific packaging of 80α DNA by the terminase carrying the SaPI1 small subunit.

The SaPI1 *terS* mutant transduced SaPI1 at two to three orders of magnitude greater than generalized transduction and SaPI1 DNA was present in both capsid sizes with reduced efficiency, suggesting that 80α *terS* is responsible for SaPI1 transduction with reduced frequency. However, the expected properties of generalized transduction by 80α only account for a fraction of the SaPI1 transduction observed. There are several possibilities that account for the observed higher residual level of SaPI1 transduction in the presence of 80α *terS*. There are a few regions of sequence homology between SaPI1 and 80α that would permit homologous recombination. In theory, this would increase the frequency of transduction. It has been observed that regions of 80α DNA as small as 100 bp can dramatically increase transduction of foreign genetic elements (Novick *et al*., 1986). The longest stretch of sequence identity, 52 bp of identical sequence homology, lies downstream of the integrase gene in both 80α and SaPI1. The largest homologous region has an interrupted stretch of 163 bp with 94% identity. It is possible that these regions

provide sufficient sequence homology to allow for homologous recombination between the two genomes, which would integrate SaPI1 into the concatameric 80α packaging substrate and result in a reduced frequency of SaPI1 packaging by 80α small terminase. These regions of homology might explain the continued presence of SaPI1 DNA seen in Figure 6c as well as the titer of SaPI1 transducing particles in Figure 5 of the SaPI1Δ*terS* mutant in the presence of wild type 80α. An additional explanation of the residual SaPI1 transduction would be the increased number of SaPI1 copies present during phage replication. It has been asserted that plasmids with high copies numbers are transduced with greater frequency than genomic DNA (Novick *et al*., 1986). Currently, the mechanism(s) behind the low frequency SaPI1 transduction by 80α *terS* remain unclear.

It is important to note that the electron micrographs of the virions from the 80α/SaPI1Δ*terS* strain appeared identical to electron micrographs from the wild type strain, RN10628. This indicates that the deletion of SaPI1 *terS* has no influence on the redirection of capsid assembly to produce small capsids.

Capsid specificity. Although the primary role of the SaPI1 *terS* in specific recognition of SaPI1 DNA is established by this study, the results of the Southern blot have raised additional questions regarding a secondary role the small terminase may have in packaging DNA into 80α and SaPI1-sized capsids. Further studies are needed to determine if trimers of SaPI1 DNA are packaged into large capsids by 80α result in defective transductants. However, studies of other SaPI family members have demonstrated that they can transduce

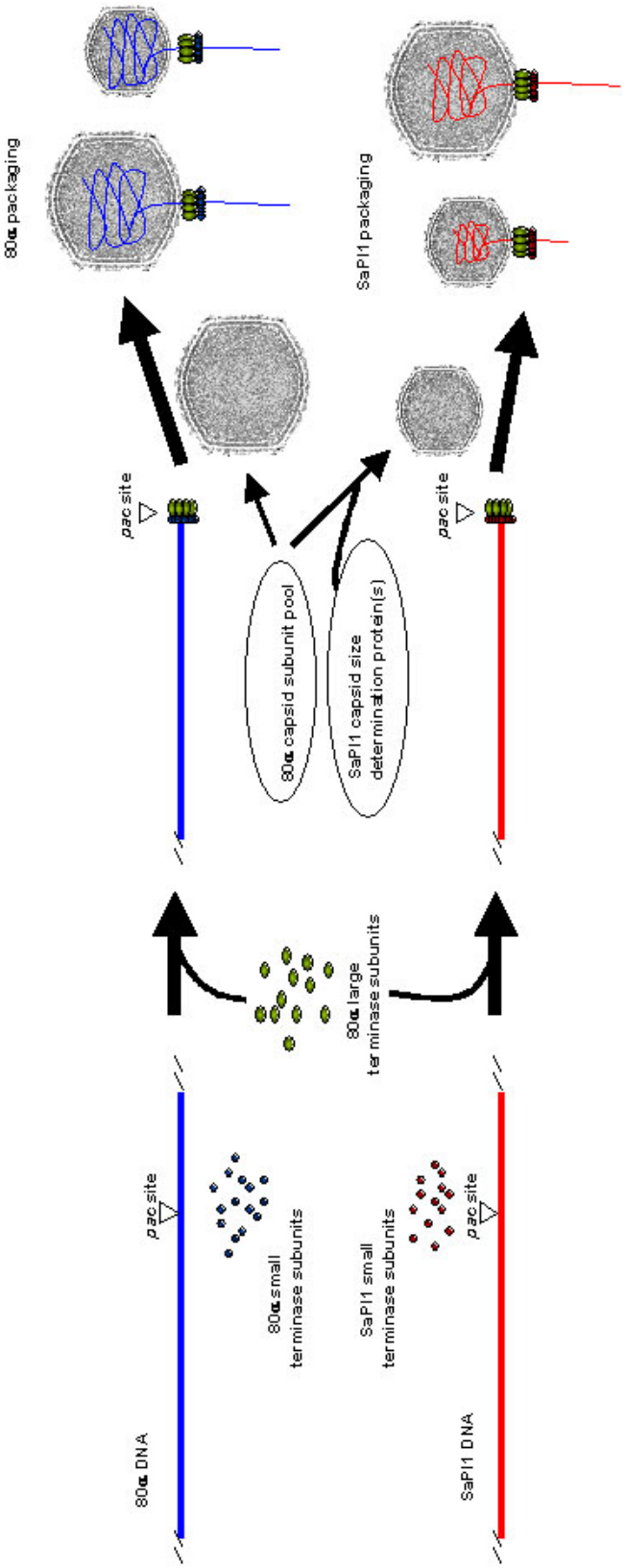
at high frequency by packaging SaPI DNA into the large helper phage capsids (Maiques *et al*., 2007). Southern blot analysis of the 80α *terS* mutants provided data that strongly indicates that 80α is capable of preferentially packaging DNA into large capsids. Considering the intimacy of the relationship between SaPI1 and 80α, it is no surprise that both may exhibit capsid specificity.

The results of capsid size determining gene mutants in SaPIbov1 indicate that SaPI1 subversion of capsid assembly does not result in the interference in helper phage titers. It should be noted that SaPIbov1, despite sharing a few similarities with SaPI1, is different than SaPI1. This recent work established that the separate deletion of SaPIbov1 ORFs 8 and 9 yield no SaPIbov1-sized DNA (Úbeda *et al*., 2007). They assert that these two ORFs encode key proteins in subverting capsid assembly of the helper phage to produce small capsids. Only the large capsids were observed in electron micrographs of the virions obtained from the SaPIbov1 ORF 9 deletion. However, this deletion did not result in reduced SaPI titers. SaPIbov1 ORFs 8 and 9 are homologous to SaPI1 ORFs 6 and 7. Current work is underway to create deletions of these SaPI1 genes to determine if the reduction of 80α titers can be restored in the presence of these deletions. If this is true, then SaPIs have coevolved with their helper phages and developed a system where fewer helper phage-sized capsids are produced, decreasing the phage yield, while specifically increasing the yield of SaPI1 transductants by forcing the formation of small capsids that helper phage cannot propagate in.

This study of the SaPI1 small terminase subunit and its role in DNA packaging in the presence of its helper phage 80α has shown that SaPI1 *terS* is essential for specific

SaPI1 DNA packaging into SaPI1-sized capsids, revealing a novel system by which a pathogenicity island is capable of preferentially packaging its DNA into smaller capsids that it produces by subverting the capsid assembly process of the helper phage (Fig. 7). Analysis of the effects of SaPI1Δ*terS* has concluded that the role of the gene matches those of phage small terminase genes. SaPI1 *terS*, now determined to be a functional small terminase subunit, could in future studies prove to be an integral player in a novel capsid recognition mechanism.

Figure 7. Revised SaPI1/80α packaging model. Packaging of SaPI1/80α begins with the recognition of their respective *pac* sites by the small terminase subunit and is followed by recruitment of the 80α large terminase subunit to form the terminase complex and cleavage of the *pac* site. The terminase complex associates with an empty capsid and translocates DNA inside. SaPI1 selectively packages its DNA into small capsids, while 80α packages its DNA into either size capsid. (Not shown is 80α packaging SaPI1 DNA into either size capsid).


80α packaging
SaPI1 packaging
pac site
pac site
80α capsid subunit pool
SaPI1 capsid size determination protein(s)
80α large terminase subunits
pac site
pac site
80α small terminase subunits
SaPI1 small terminase subunits
80α DNA
SaPI1 DNA

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