Transcriptional crosstalk between helper bacteriophages and Staphylococcal aureus pathogenicity islands

Kristin Lane
Virginia Commonwealth University

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TRANSCRIPTIONAL CROSSTALK BETWEEN HELPER BACTERIOPHAGES
AND STAPHYLOCOCCUS AUREUS PATHOGENICITY ISLANDS

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

by

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Dedication

I cannot thank my family and friends enough for their support, tragic comedy and the random extra cup of coffee they brought me. A very special thank you to my excellent husband and parents. I wouldn’t be here without you.
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<th>Description</th>
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<tbody>
<tr>
<td>-RT</td>
<td>minus reverse transcriptase enzyme</td>
</tr>
<tr>
<td>+RT</td>
<td>plus reverse transcriptase enzyme</td>
</tr>
<tr>
<td>5' RACE</td>
<td>5' random amplification of cDNA ends</td>
</tr>
<tr>
<td>B-PER</td>
<td>bacterial protein extraction reagent</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>coding deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CsCl</td>
<td>cesium chloride</td>
</tr>
<tr>
<td>CSPD</td>
<td>disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-5'-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate</td>
</tr>
<tr>
<td>CY-GL</td>
<td>casamino acids yeast extract glycerophosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxygenin-11-dUTP</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>dUMP</td>
<td>deoxyuridine monophosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>g</td>
<td>guage</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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GL beta-glycerophosphate disodium salt pentahydrate
GST Glutathione-S-transferase
int integrase
IPTG Isopropyl-B-D-1-thiogalactopyranoside
Jcm\(^{-2}\) Joules per centimeter
Klett Klett-Summerson units
kV kilo volt
LB lysogeny broth
M molar
MC Mitomycin C
MCS multiple cloning site
ml milliliter
mM millimolar
MOI multiplicity of infection
MOPS 3-(N-morpholino)propanesulfonic acid
MQ MilliQ filtered water
ms millisecond
N Normal
NaCl sodium chloride
OD optical density
orf open reading frame
PBS phosphate buffered saline
PCR polymerase chain reaction
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>q.s.</td>
<td>quantum satis (Latin: sufficient quantity)</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>semi-quantitative real-time reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>SaPI</td>
<td><em>Staphylococcus aureus</em> pathogenicity island</td>
</tr>
<tr>
<td>SaPI6Δ</td>
<td><em>Staphylococcus aureus</em> pathogenicity island 6 delta</td>
</tr>
<tr>
<td>SCC</td>
<td>saline sodium citrate buffer</td>
</tr>
<tr>
<td>SCC</td>
<td>staphylococcal cassette chromosome</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOC</td>
<td>super optimal broth with catabolite repression (SOB plus glucose)</td>
</tr>
<tr>
<td>SOS</td>
<td>Save Our Ship, sequence of events initiated following DNA damage</td>
</tr>
<tr>
<td>TAP</td>
<td>tobacco acid pyrophosphatase</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′′-Tetramethylethane-1,2-diamine</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptic soy agar</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TU</td>
<td>transduction units</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µJ</td>
<td>microjoules</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>vol/vol</td>
<td>volume to volume</td>
</tr>
<tr>
<td>wt/vol</td>
<td>weight to volume</td>
</tr>
<tr>
<td>WTA</td>
<td>wall-associated teichoic acid</td>
</tr>
<tr>
<td>X</td>
<td>times</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-3-indoyl-B-D-galactopyanside</td>
</tr>
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Abstract

TRANSCRIPTIONAL CROSSTALK BETWEEN HELPER BACTERIOPHAGES AND STAPHYLOCOCCUS AUREUS PATHOGENICITY ISLANDS

By Kristin Downie Lane

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

Virginia Commonwealth University, 2013

Major Director: Gail E. Christie, Ph.D.
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Acquisition of a superantigen pathogenicity island (SaPI) significantly increases virulence in Staphylococcus aureus. Horizontal transfer of SaPIs occurs at high frequency and depends upon a helper bacteriophage, either through direct infection or SOS-mediated induction of a lysogen. SaPIs hijack the packaging machinery of the helper phage, leading to the formation of SaPI-containing transducing particles that can introduce the pathogenicity island into neighboring SaPI-negative cells. All SaPIs contain a conserved core of genes, some of which are co-transcribed as an operon and encode functions involved in helper
exploitation. The goal of this study was to more fully understand the intricate relationships between the SaPI elements and their helper bacteriophages, specifically any regulatory crosstalk that might occur between them. We demonstrated phage-host interactions in 80 and 80α, and SaPI1 and SaPIbov1-mediated crosstalk with helper phage 80α. The phage Sri protein was shown to be a bi-functional protein that both derepresses SaPI1 and interferes with host chromosome replication. Incoming SaPI1 experiments showed that SaPI1 modulates the levels of the N-terminal part of orf14 mRNA. Induction experiments using the 80α ΔrinA phage as a genetic tool, reveal several new phage genes that SaPI1 targets for expression modulation. Finally, a novel SaPI1 interference mechanism was identified. In an 80α ΔrinA mutant, which cannot activate its late operon, SaPI1 can directly turn on expression of the packaging and structural genes in a noncanonical manner, initiating from the 2nd gene in the operon, the large terminase subunit.
Chapter 1. Introduction

*Staphylococcus aureus pathogenicity and mobile genetic elements*

The sheer number and variation of virulence mechanisms encoded by *Staphylococcus aureus* comprises a serious arsenal for causing disease in humans and other mammals. An opportunistic pathogen, the bacteria colonize humans on the skin and in the nares, rarely causing disease until presented with an opportunity. The disease spectrum exhibited by *S. aureus* infection is varied, and can range from simple boils and pustules to life-threatening cardiac and pulmonary infections. The economic burden of both human and food animal infections in the United States cannot currently be measured. However it is sure to exponentially rise as *S. aureus* has been steadily acquiring resistance to all antibiotics approved for use in the United States.

The evolution and adaptation of the currently circulating *Staphylococcus aureus* strains on a global scale has been driven by the horizontal transfer of mobile genetic elements, mediated predominately by bacteriophage (Christie *et al.*, 2010, Novick *et al.*, 2010, Ubeda *et al.*, 2009). Genes acquired in this manner include toxins found on integrated prophages, the superantigen pathogenicity islands (SaPIs), staphylococcal cassette chromosome (SCC), and plasmids and transposons (McCarthy *et al.*, 2012), the majority of which encode virulence factors and resistance genes (Lindsay, 2010, McCarthy *et al.*, 2012).

At least five of the known SaPIs are induced by endogenous prophages, and can therefore be mobilized in their naturally occurring host strains under
conditions that lead to prophage induction. Not only are prophages induced by a number of commonly used antibiotics including ciprofloxin, (Maiques et al., 2006, Ubeda et al., 2005), but several studies of consecutive isolates from the same patient indicate prophage acquisition, loss and/or movement occurs during the course of infection (Goerke et al., 2006, Moore & Lindsay., 2001). Two additional recent results further highlight the potential importance of this mechanism in the context of S. aureus in a polymicrobial community. The first is the demonstration of intergeneric mobilization of SaPI1 from S. aureus into L. monocytogenes (Chen & Novick, 2009, Winstel et al., 2013). The second is the finding that hydrogen peroxide at levels produced by pneumococci induces the SOS response and prophage derepression in S. aureus (Selva et al., 2009), indicating that this competitive strategy employed by bacteria colonizing the same environmental niche could lead to SaPI mobilization and high frequency transfer of virulence determinants. These observations underscore the importance of elucidating the molecular mechanisms involved in SaPI mobilization.

**S. aureus Pathogenicity Island (SaPI) biology**

SaPIs are a family of molecular pirates that prey on helper bacteriophage for mobilization into a new cell. The majority are 14-17 kilobases in size with a conserved core genome arranged in a modular architecture (Lindsay et al., 1998, Novick et al., 2010). With the known exception of SaPI6Δ thus far, all
Fig. 1. Genetic map of SaPI1 (U93688). The integration, regulation, replication and operon 1 modules are labeled. Numbered genes have no discerned function. The superantigen genes are sek, seq and tst. Phage-like genes include: integrase (int) and the small terminase subunit homolog (terS). Regulation of the island occurs via stl (master repressor). Str also has characteristics of a transcriptional regulator, but has no known function. Genes involved in phage interference: ppi (phage packaging inhibitor), cpmA and cpmB (capsid size redirection), and terS (packaging redirection). Genes in blue were deleted as part of the deletion mutant panel in Chapter 6. Map is shown in the conventional SaPI orientation, which is the reverse of the way the sequence is displayed in GenBank.
SaPIs encode at least one superantigen gene. SaPI1, the prototypical clinical SaPI, encodes three superantigens: toxic shock syndrome toxin (tst), enterotoxin Q and enterotoxin K (seq, sek) (Fig. 1). At the extreme left of the integrated SaPI1 genome is a phage-like gene, integrase (int), which maintains SaPI as an integrated element and serves as an essential protein for the excision process (Mir-Sanchis et al., 2012, Ubeda et al., 2009). The pair of divergent orfs, stl and str, are regulated by their divergent promoters which control leftward and rightward transcription respectively. All SaPIs encode this pair of divergent promoters that resemble the classic temperate phage regulatory switch. In phage, this switch determines lysis or lysogeny. In SaPI1 and SaPIbov1 this region has also been shown to function in maintenance of “lysogeny”. Under normal circumstances, expression of Stl from the stl promoter results in repression of the rightward transcript and stable integration in the host chromosome (Tormo-Mas et al., 2010, Ubeda et al., 2008). Stl can be removed by interactions with a helper phage encoded antirepressor. The known antirepressors are encoded in the phage early/middle gene cluster (Novick et al., 2010, Ubeda et al., 2008). This ties SaPI derepression to phage early/middle expression, which ensures productive piracy of the phage virions. Removal of the Stl master repressor results in SaPI1 derepression and transcription from the SaPI1 rightward promoter (Harwich MD, 2009). In SaPIbov1, electrophoretic mobility shift assays showed that Stl binds the str promoter and that dUTPase competes with this interaction (Tormo-Mas et al., 2010).
Replication occurs autonomously once the SaPI is derepressed, and middle and late SaPI1 gene expression results in phage exploitation. None of the SaPIs encode any virion components; therefore, a failure to interfere with a helper phage results in loss of SaPI mobilization. SaPIs possess numerous functions to exploit the helper phage. All SaPIs, including SaPI1, encode a phage-like small terminase subunit homolog, TerS. This subunit is substituted into the phage-encoded terminase holoenzyme, which is composed of small and large terminase subunits (TerS, TerL). TerS$_{\text{SaPI}}$ recognizes the SaPI genome, complexes with phage-encoded TerL and specifically packages SaPI DNA into small or large capsids. Exploitation has recently been divided into two types based on (1) capsid size redirection (Cpm-mediated) or (2) packaging redirection (Ppi-mediated) (Ram et al., 2012). Different SaPIs use one or both mechanisms depending on the helper phage. SaPI1 interference with 80$\alpha$ is predominately Cpm-mediated; the expression of two SaPI1 encoded proteins, CpmA and CpmB, redirects the 80$\alpha$ capsid assembly process to make capsids small enough to fit SaPI1 and too small for the entire 80$\alpha$ genome. In contrast, SaPIbov2 interference is on 80$\alpha$ is Ppi-mediated. SaPIbov2 lacks the $cpmA$ and $cpmB$ genes and cannot make small capsids. The SaPIbov2 Ppi protein binds to the 80$\alpha$ TerS subunit, which blocks phage genome packaging, presumably by destabilizing the holoenzyme formed by the TerS/TerL (small terminase subunit/large terminase subunit) complex. SaPIbov1 exploitation of 80$\alpha$ uses both mechanisms. Having packaged themselves into phage-derived capsid at the
expense of the helper phage, SaPIs are released from the host cell when 80α initiates lysis.

Helper phage interference results in a practical matter of how to distinguish between SaPI and phage containing virions that are mixed in a lysate. Our lab works with SaPI1 and SaPIbov1, each containing a tetM cassette inserted into the tst locus. In order to quantitate the numbers of phage or SaPI containing virions, we mix dilutions of the lysate with recipient cells, plate them and incubate overnight. We use selective media to inhibit phage replication supplemented with tetracycline to select for recipient cells into which SaPI has stably transduced. Similarly, we can use selective media to encourage phage replication in order to select for recipient cells into which 80α has transduced.
Fig. 2. Genetic map of 80\(\alpha\) (DQ917338) showing putative transcription units. The \textit{cl} and \textit{cro}-like genes make up the genetic switch region which regulates lysis and lysogeny. The replication module is not well defined but initiates with either the single strand DNA binding protein (ssb) or one of the small upstream orfs with no defined function. The late operon begins at the small terminase (\textit{terS}) gene and is transcribed through the endolysin gene at the far right.
**Helper bacteriophages**

To date, all staphylococcal helper bacteriophage sequenced are temperate and belong to the *Siphoviridae* family. They have non-contractile tails with icosahedral capsids, and mosaic genomes with an ordered, modular arrangement (Christie *et al.*, 2010). 80α (DQ917338) is a prototypical helper bacteriophage that has been found to mobilize at least five SaPIs thus far (Christie *et al.*, 2010, Novick *et al.*, 2010). The 80α genome is 43,864 base pairs in length, containing 73 open reading frames, the majority of which are arranged in a single long operon, the late operon (Fig. 2). The genome can be divided into numerous modules including integration, regulation, replication, packaging and structural genes and the lysis cassette. Integration results from integrase (*int*) expression and site-specific recombination into the *S. aureus* chromosome between *rpmF* and the iron regulated cell wall anchored protein SirH (Christie *et al.*, 2010). The regulatory module is composed of the two divergent orfs that are characteristic of temperate phages and resemble that of the *E. coli* phage lambda. The *cl*-like gene has a helix-turn-helix motif and an SOS inducible cleavage motif, suggesting that it binds DNA and is cleaved following RecA activation. The *cro*-like gene also has a helix-turn-helix motif and its promoter mediates rightward transcription. The replication module boundaries are unclear. There are several small orfs that are conserved among staphylococcal phages but have no defined function. We choose to use the *ssb* gene as the initial marker for replication. The 80α late operon is activated by the phage-encoded RinA protein, which binds to the *terS* promoter to regulate transcription (Ferrer *et
Late operon expression is not tied directly to phage replication in contrast to what has been seen in other phages (Harwich MD, 2009). The first genes in the late operon, terS and terL, encode the hetero-oligomeric terminase holoenzyme. The terminase complex has several tasks: it must recognize the viral DNA in a pool of DNA, which includes the host chromosome; it must associate with and cleave the viral concatamer; finally it must associate with the procapsid portal protein and translocate the DNA into the viral shell cleaving the end of the genome (Feiss & Rao, 2012). Packaging substrates are usually concatamers of phage DNA formed by replication and/or recombination. Generally, recognition of the viral genome occurs at a specific site, called the pac site. After recognition, the TerL subunit makes an initial cut on the concatamer and the holoezyme packages the nucleic acid in an ATP-dependent manner. When packaging is complete, the terminase complex makes the final cut on the genome, ending the packaging process. Tails are attached to the capsid after packaging, producing an infectious particle. SaPIs and their helper phages use the headful packaging mechanism, by which a headful (100+% of the genome is packaged. The signal indicating the head is full is both sequence-independent and unknown. Following phage particle assembly, the host cell is lysed by an accumulation of holin and lysin.

Recently the host cell wall receptor for several staphylococcal Siphoviridae was discovered. The related helper phage ϕ11 uses glycosylated wall-associated teichoic acids (WTA) (Xia et al., 2011). This anionic polymer is one of the most abundant structures on the cell surface. Phi 11 and other
serotype B *Siphoviridae* phages, including 80 (DQ908929), use 1,5 ribitol phosphate WTA decorated with \(\alpha\)-N-acetylglucosamine for adherence (Xia *et al*., 2010, Xia *et al*., 2011). In a *tarM* mutant, which makes but cannot glycosylate WTA, both \(\phi\)11 and phage 80 cannot adsorb. However, complementation by plasmid expression of *tarM* restores the adsorption phenotype. An NMR analysis of WTA from *S. aureus* strain RN4220 demonstrated that it primarily produces a short form, the K-type, caused by overexpression of *tarK*, which is generally associated with low cell density and negatively regulated by the *agr* locus (Swoboda *et al*., 2010). Interestingly, WTA glycosylation is species-specific in *Listeria*, which likely contributes to the ability of 80\(\alpha\) to infect several *L. monocytogenes* species and integrate into the chromosome (Chen & Novick, 2009, Xia *et al*., 2010, Xia *et al*., 2011).
Chapter 2. Materials and Methods

Note: unless otherwise specified, all reagents listed came from standard suppliers including, but not limited to Fisher Scientific (Pittsburgh, PA) and Sigma Aldrich (St Louis, MO).

2.1 Bacterial Culture Methods. Table 1 lists bacterial strains used or created during this work. *Escherichia coli* strains were cultured with 200 rpm shaking in Brain Heart Infusion (BHI) media (Remel; Lenexa, KS) supplemented with either Ampicillin (100 µg/ml), Kanamycin (50 µg/ml), or Chloramphenicol (12.5 µg/ml) overnight at 37ºC (unless otherwise indicated) and plated on Luria-Burtani plates (LB), again supplemented with the appropriate antibiotic. *Staphylococcus aureus* strains were cultured with 200 rpm shaking in BHI media supplemented with Tetracycline (15 µg/ml), Erythromycin (5 µg/ml), Kanamycin (50 µg/ml), or Chloramphenicol (30 µg/ml) as appropriate. *S. aureus* cultures were grown overnight at either 32ºC or 37ºC unless otherwise indicated. *S. aureus* strains were plated on tryptic soy agar plates or phage agar (0.3% wt/vol Casamino acids, 0.3% wt/vol yeast extract, 100 mM NaCl, 1.5% wt/vol agar, pH 7.8, 0.5 mM CaCl$_2$ added after autoclaving) (Novick., 1991). *S. aureus* strains containing *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 syrup (60%), 25% vol/vol glycerol, 1.5% wt/vol agar, pH 7.8, 0.17 mM sodium citrate and 15 µg/ml tetracycline were added after autoclaving. 5-bromo-4-chloro-3-
indolyl-β-D-galactopyranoside (Xgal; Gold Biotechnology Inc; St Louis, MO) was added to agar (200 µg/ml) for blue/white screening of appropriate vectors. Isopropyl-β-D-1-thiogalactopyranoside (Gold Biotechnology Inc; St Louis, MO) was added to some cultures to induce protein expression.
Table 1: Bacterial strains used or created for this study

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2.2 Phage Propagation Methods: Inductions. Phages $80\alpha$ or $\phi 11$ were isolated from a lysogenic S. aureus strain by SOS pathway induction using either UV light or Mitomycin C (MC, Sigma Aldrich; St Louis, MO). An overnight culture of the lysogenic strain was diluted 1:200 in BHI and grown until Klett=30 ($OD_{600}=0.3$ or approximately $2.28 \times 10^8$ cells/ml). The cells were pelleted, then resuspended in 5 ml of S. aureus phage buffer ($0.1 \text{ mM MgSO}_4$, $0.4 \text{ mM CaCl}_2$, $2.5 \text{ M Tris-HCl pH 7.8}$, $100 \text{ mM NaCl}$, $0.1\%$ gelatin) (Novick. 1991), exposed to UV light (70 Jcm$^{-2}$) for 30 seconds and diluted 1:1 with CY+ GL 0.6 M $\beta$-glycerophosphate disodium salt pentahydrate (GL). CY is composed of 1% wt/vol Casamino Acids, 1% wt/vol Yeast Extract, 0.5% wt/vol Glucose, 0.59% wt/vol NaCl. (Novick, 1991). Aeration was reduced to 100 rpm and the cultures were allowed to lyse at 32ºC. MC induction followed the same procedure until Klett=30, when the culture was diluted 1:1 with phage buffer and MC was added to 2 $\mu$g/ml. In the event that the MC had been resuspended more than 30 days prior to use, the induction concentration was increased to 4 $\mu$g/ml to account for the lack of stability of the antibiotic. The cells were returned to 32ºC until lysis or until the optical density ceased to drop.

2.3 Phage Propagation Methods: Infections. S. aureus strains without a prophage were infected with either 80, $80\alpha$, or $\phi 11$ at a MOI equal to 0.1-1 for strains without SaPIs or MOI=5 for strains containing SaPIs, unless otherwise indicated. Briefly, an overnight culture of the strain to be infected was diluted
1:200 in fresh media and grown at 32°C with 200 rpm shaking until Klett=30 (OD$_{550}$=0.3 or approximately $2.28 \times 10^8$ cells/ml). The culture was diluted 1:1 with phage buffer and the appropriate amount of phage was added to achieve the desired MOI. The phage cell mixture was allowed to adsorption at room temperature for 10 minutes, then returned to 32°C with reduced shaking (100 rpm) until lysis occurred or until the optical density ceased to drop.

2.4 Phage Transduction. Most staphylococcal strains possess an intact restriction system and thus require transduction for moving SaPI mutants and plasmids into them from RN420. In this study, the generalized transducing phages 80, 80α or φ11 were used. Strains containing the entity to be moved were grown to Klett=30, diluted 1:1 with phage buffer and infected with phage at a MOI=1-5. The infected strains were grown at reduced shaking (100 rpm) until lysis or until the Klett readings ceased to drop any further. The resulting lysate was pelleted and the supernatant was sterile filtered using a 0.45 µM PVDF syringe filter. The filtered lysate was diluted in phage buffer and 100 µl was mixed 1:1 with the destination strain, allowed to adsorb for 5 minutes and plated on GL plates supplemented with the appropriate antibiotic.

2.5 Titering: Phage and SaPI. Phage titers were quantified by plating serial dilutions on appropriate indicator cells. Phage lysate was diluted in S. aureus phage buffer to make 10-fold dilutions. Unless otherwise indicated, 100 µl of phage dilution was mixed with 100 µl of RN420 indicator cells and allowed to
stand at room temperature for 5 minutes. Three milliliters of *S. aureus* top agar supplemented with 5 mM CaCl$_2$ was added to the phage-cell mix, poured on to *S. aureus* phage agar plates, allowed to solidify, then incubated overnight at 37°C at which time plaque forming units (PFU) were quantified. Phage top agar is 0.3% wt/vol Casamino acids, 0.3% wt/vol yeast extract, 100 mM NaCl, 0.75% wt/vol agar, pH 7.8, with 5 mM CaCl$_2$ added after autoclaving (Novick, 1991).

A modified titer protocol was used to quantify SaPI transduction following phage infection or induction. All SaPI derivatives used in this study contain a *tetM* cassette inserted into the *tst* gene; therefore, selection for transduction was done on GL plates supplemented with 5 µg/ml tetracycline. Serial dilutions were made following the titer assay protocol; 100 µl of lysate dilution was mixed with 100 µl of RN4220 indicator cells and allowed to stand at room temperature for 5 minutes. The lysate-indicator cell mixture was poured onto a GL agar plate and evenly distributed by spread plate technique. The plates were incubated at 37°C for 24-48 hours, at which time colonies or transducing units (TU) were quantified.

Strain sensitivity to phage was confirmed by spot plating. 100 µl of an overnight culture of the strain in question was mixed with 3 ml of top agar supplemented with 5 mM CaCl$_2$. The cell-agar mix was poured onto a phage agar plate and allowed to harden. Serial dilutions of phage lysate were made in phage buffer and 10 µl aliquots were spotted onto the prepared plate. The plate was incubated for 24 hours at 37°C and examined for plaque formation.
2.6 Large-Scale Phage Induction. An overnight liquid culture was diluted 1:200 and grown in 2 x 500 ml of either CY-GL or BHI media supplemented with antibiotics as appropriate and grown at 32°C with 200 rpm shaking. At OD_{550}=0.6 the cultures were diluted 1:1 with phage buffer. Strains without a prophage were infected with an MOI of 0.1 for non-SaPI1 strains or MOI of 5 for SaPI1 strains. Cultures containing lysogens were induced with Ciprofloxin (1 µg/ml, Sigma Aldrich, St Louis, MO). Both infected and induced cultures were allowed to lyse with shaking reduced to 100 rpm. At lysis, the culture was centrifuged at 7000 rpm for 20 minutes at 4°C in a Sorvall GS-3 rotor to pellet the cellular debris. The supernatant was decanted into sterile Fernbach flasks, 0.1% wt/vol PEG8000 (Fisher, Pittsburgh, PA) and 0.5M NaCl were added. Following overnight incubation at 4°C, the lysates were centrifuged at 7000 rpm for 20 minutes at 4°C, the supernatant decanted, and the PEG precipitate collected from the walls of the centrifuge bottles by resuspension in phage buffer (9 ml per liter culture). The resuspended PEG pellet was transferred to a sterile 15 ml Corex tube and stored overnight at 4°C. The precipitate was centrifuged at 10,000 rpm for 10 minutes and the supernatant decanted into a clean Corex tube. Three ml phage buffer was added to the remaining precipitate and respun; this supernatant was collected and pooled with the first.

A cesium chloride (CsCl) step gradient was poured in an ultra clear (1 x 3.5 inches) centrifuge tube (Beckman; Fullerton, CA). Four densities of CsCl were
prepared, each in 25 ml phage buffer: ρ=1.3 (10.1g), ρ=1.4 (13.47g), ρ=1.5 (16.87g), ρ=1.6 (20.2g). The step gradient was formed by layering from bottom to top: 2 ml ρ=1.6, 3 ml ρ=1.5, 4 ml ρ=1.4, 4 ml ρ=1.3. The precipitate was carefully layered on the CsCl gradient and centrifuged in a Beckman SW28 rotor for 2.5 hours at 15°C 24,000 rpm with no brake. After centrifugation, a visible phage band was present at the 1.4-1.5 interface and was collected by puncturing the centrifuge tube with an 18g needle, bevel up, and aspirating into a 5ml syringe.

2.7 SaPI Particle Purification. Isolation of pure SaPI particles was achieved using a modified CsCl gradient procedure. The SaPI of interest was transduced into ST24, a strain lysogenic for 80α ΔterS, which is unable to package phage DNA and therefore yields a lysate of pure SaPI particles. Strains constructed in this manner were subjected to large-scale phage induction as described above. The CsCl fraction was added to an Amicon® Ultra-15 10,000 kDa molecular weight cut off spin column (EMB Millipore; Billerica, MA) and rinsed at least 3 times with 10-15 ml of phage buffer (without gelatin added) and spun at 4000 rpm to concentrate the volume down to less than 1 ml. After the final rinse, the particles were removed from the top of the column using a pipet, and titered.

2.8 DNA Methods: genomic DNA Extraction. Genomic DNA (gDNA) was extracted from growing strains at the time points indicated following induction or infection for use as a PCR template. Samples were taken from the culture, pelleted and the supernatant was decanted. The final pellet was frozen at -20°C
until processed. DNA extraction was performed using DNAzol® (Invitrogen™; Grand Island, NY) extraction. Isolation started with 250 µl of overnight culture, pelleted and resuspended in 100µl TES (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, SDS, 0.1% wt/vol) containing 2 µl lysostaphin (5 mg/ml, Sigma Aldrich; St Louis, MO) and incubated at 37°C for 30 minutes. DNAzol® (500 µl) was mixed in gently and incubated for 5 minutes at 65°C. Contents were transferred to a Qiagen miniprep spin column (Qiagen; Valencia, CA) and centrifuged 10,000 rpm for 1 minute to bind the DNA. The column was washed with 750 µl PE and centrifuged at 10,000 rpm for 1min. The flow through was discarded and the column was centrifuged again to ensure that all the PE was removed from the filter. The column was then washed with 750 µl 70% ethanol and centrifuged at 10,000 rpm for 1min. The flow through was discarded and the column was centrifuged again to ensure that all the ethanol was removed from the filter. DNA was eluted with 50 µl of prewarmed DNase-free water and quantitated on a Nanodrop 1000 (Thermo Scientific; Pittsburgh, PA).

2.9 DNA Methods: Virion DNA Extraction.

To extract DNA packaged into virions, large-scale inductions of the appropriate strains were prepared as in section 2.7. The volume of banded virions was measured and transferred to a 15 ml glass Corex tube; 1/15 volume 0.5M ethylene diamine tetraacetic acid (EDTA) and an equal total volume of formamide were added. The tube was sealed with parafilm and allowed to stand at room temperature overnight. An equal volume of TE (10 mM Tris-HCl, 1mM
EDTA, pH 8.0) and two volumes of 100% ethanol were added, and DNA was allowed to precipitate overnight at -70°C. The DNA was pelleted by centrifugation at 4°C for 5 minutes at 4000 rpm (or 10 minutes at 10,000 rpm), the supernatant was carefully decanted, and the pelleted DNA was allowed to dry. One ml TE was added and the tube gently tapped to mix and then allowed to rehydrate for ~10-15 minutes. The redissolved DNA was extracted with an equal volume of phenol, vortexed gently, and centrifuged at 4°C for 10 minutes at 10,000 rpm. A Pasteur pipet was used to transfer the upper aqueous phase to a 1.5 ml microfuge tube. This phase was centrifuged at 10,000 rpm for 1 minute to ensure maximal aqueous phase recovery coupled with minimal protein contamination. The upper aqueous phase was transferred to a clean Corex tube and 1/10 volume of cold 3M sodium acetate, pH 5.4 was added. Two volumes of 100% ethanol were added and a stringy precipitate was observed. After centrifugation for 1 min. at 10,000k rpm, the supernatant was decanted and mixed with 2 ml 75% ethanol. This was centrifuged for 1 min. at 10,000k rpm and the supernatant decanted. One ml 95% ethanol was added and the tube was rolled to dry the DNA, then carefully poured off. For small amounts of DNA an additional centrifugation for 1 min. at 10,000k rpm was added at this step. The tube containing the pelleted DNA was inverted and allowed to dry. The DNA was redissolved in 1 ml TE and stored at 4°C.
2.10 DNA Methods: Cloning. General cloning techniques were performed as in Sambrook and Russell, 2006 (Sambrook & Russell, 2006). Plasmid construction followed a simple formula: insert amplification by PCR, vector preparation (miniprep, restriction enzyme digestion, Antarctic Phosphatase treatment or gel extraction), insert-vector ligation using T4 ligase (New England Biolabs; Ipswich, MA) and sequence confirmation. Enzymes were purchased from New England Biolabs (NEB, Ipswich MA), except where otherwise indicated. Primers listed in Table 2 were purchased from Integrated DNA Technologies (Coralville, IA). PCR for cloning was done using Pfu Turbo, Pfu Ultra II (both Agilent Technologies; Santa Clara, CA) or Phire Hotstart II (Thermo Scientific; Pittsburgh, PA). PCR purification and Gel Extraction kits (Valencia, CA) were purchased from Qiagen. Kits and enzymes were used according to manufacturer's instructions for insert and vector clean up following restriction enzyme digestion. Alternatively, the Infusion HD kit (Clontech; Mountain View, CA), which uses complementary overhangs of the insert and vector and the Gibson Assembly method (Gibson, 2011) was used in lieu of insert-vector ligation. Plasmids were verified by sequence analysis, performed by Retrogen, ACGT or MWG Biotech. Plasmids made for this study are listed in Table 3.
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<td><strong>Cterm orf22 plus XhoI</strong></td>
<td>AATCTCGAG(\text{TTAATATTGCAGATAGCGG})</td>
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<tr>
<td><strong>Nterm orf22 plus BamHI</strong></td>
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<td><strong>KDL63</strong></td>
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<td>56.5</td>
<td>Staph Dnal</td>
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<tr>
<td><strong>KDL75</strong></td>
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<td><strong>KDL123</strong></td>
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<td>SaPI(\text{bov1})</td>
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<tr>
<td><strong>KDL124</strong></td>
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<td><strong>KDL125</strong></td>
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<tr>
<td><strong>KDL132</strong></td>
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<tr>
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Table 3: Plasmids used or created in this work

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<th>Plasmid Name</th>
<th>Description</th>
<th>Reference</th>
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<td>pKDL2</td>
<td>SaPI1 Pstr-pCN56</td>
<td>This work</td>
</tr>
<tr>
<td>pKDL3</td>
<td>SaPI1 PentQ-pCN56</td>
<td>This work</td>
</tr>
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<td>SaPI1 Porf19-pCN56</td>
<td>This work</td>
</tr>
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<td>This work</td>
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<td>SaPIbov1 Pstr-pCN56</td>
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<tr>
<td>pCN56</td>
<td>Promoterless transcriptional fusion vector, GFP</td>
<td>(Charpentier et al., 2004)</td>
</tr>
<tr>
<td>pGEX-4T1</td>
<td>Expression vector</td>
<td>GE Health Life Sciences</td>
</tr>
<tr>
<td>pRN9004</td>
<td>StlSaPI1-pCN51</td>
<td>(Ubeda et al., 2008)</td>
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2.11 Competent Cell Preparation: *E. coli* (Tang *et al*., 1994). Chemically competent *E. coli* were prepared by diluting an overnight liquid culture 1:100 in 50 ml fresh LB broth supplemented with antibiotics as appropriate and grown at 37°C with 200 rpm shaking until Klett=95-110. The culture was centrifuged at 4°C for 15 minutes at 4000 rpm and supernatant discarded. The pellet was resuspended in 10 ml of cold Solution A (80 mM CaCl₂ and 50 mM MgCl₂) and incubated on ice for 10 minutes. Cells were pelleted by centrifugation at 4°C for 10 minutes at 4000 rpm, resuspended in 10 ml of Solution A and incubated on ice for 10 minutes. Cells were pelleted again as before and resuspended in 6 ml of Solution B (0.1M CaCl₂, 6 ml filter sterilized 50% glycerol, q.s. to 50 ml sterile water). Samples were aliquotted (60-100 µl) for storage at -70°C. Alternatively, electrocompetent *E. coli* were used for some transformations (BioRad) A fresh overnight liquid culture was diluted 1:100 in fresh LB broth supplemented with antibiotics as appropriate and grown at 37°C with 200 rpm shaking until Klett ~95-110 (OD₆₀₀=0.5-0.8, exponential phase). The culture was incubated on ice for 20 minutes then pelleted at 4°C for 15 minutes at 4000 rpm and the supernatant was decanted. The cells were washed with 1 volume of ice-cold 10% glycerol, pelleted at 4°C for 5 min. at 4,000 rpm and the supernatant decanted. The culture was then resuspended with 0.5 volumes ice-cold 10% glycerol, pelleted at 4°C for 5 minutes at 4,000 rpm and the supernatant decanted. The cells were washed again with 10 ml ice-cold 10% glycerol, pelleted at 4°C for 5 min. at 4,000 rpm, then the supernatant was decanted and the pellet was
resuspended in a final volume of 0.5 ml 10% glycerol before aliquots were made for storage as above.

2.12 Competent Cell Preparation: *S. aureus* (Nickoloff., 1995). Electrocompetent *S. aureus* were prepared by diluting an overnight culture in 50 ml of fresh BHI media, supplemented with the appropriate antibiotic as necessary, and grown at 37°C with 200 rpm shaking until Klett ~95-110 (OD<sub>600</sub>=0.5-0.8, exponential phase). The culture was chilled on ice for 20 minutes, pelleted at 4°C for 15 minutes at 4000 rpm, and the supernatant was decanted. The cell pellet was washed with 25 ml of ice-cold sterile water, pelleted at 4°C for 5 min. at 4,000 rpm and the supernatant decanted; this cycle was repeated for a total of three times. Next the cell pellet was washed with 30 ml of ice-cold 10% glycerol, pelleted at 4°C for 5 min. at 4,000 rpm, then supernatant was decanted and the pellet washed with 15 ml of ice-cold 10% glycerol. The cells were pelleted, the supernatant decanted, and the final pellet was resuspended in 0.5 ml 10% glycerol. Aliquots (60 µl) were made for storage at -70°C.

2.13 Transformation of Cells. *E. coli* was transformed either by heat shock of chemically competent cells (Tang *et al.*, 1994) or electroporation of electrocompetent cells. Plasmid DNA (2-10 µl) was added to chemically competent *E. coli* cells and allowed to stand on ice for ≤10 minutes. The plasmid-cell mixture was incubated in a 42°C water bath for 30 seconds and then 0.25 ml SOC (2% wt/vol tryptone, 0.5% wt/vol yeast extract, 85.5 mM NaCl, 2.5 mM KCl,
10 mM MgCl$_2$, 20 mM glucose) was added immediately. The cells were allowed to recover for 1 hour at 37°C either with or without 200 rpm shaking.

Electrocompetent *E. coli* cells (60 µl) were allowed to thaw on ice and stand with 2-5 µl of plasmid DNA for ≤10 minutes before transfer into a chilled 0.2 cm cuvette (Bio-Rad; Hercules, CA) and pulsed in a MicroPulser™ (Bio-Rad; Hercules, CA) on the preset EC2 setting (1.80 kV, 1 pulse). Immediately post pulse, 0.5-1 ml of SOC was added to the cuvette, the cells were allowed to recover for 1 hour at 37°C and then plated on LB plates containing antibiotic as appropriate.

Electrocompetent RN4220 derivatives were transformed by electroporation. Briefly, ≤5 µl containing 0.5-1 µg plasmid DNA were added to 60 µl thawed cells and allowed to stand for 30 minutes at room temperature. The cells were transferred to a 0.2cm cuvette and electroporated on the preset STA setting (2.50 kV, 1 pulse, 2.5 ms) of a MicroPulser™. Immediately post pulse, 0.5-1 ml of BHI was added to the cuvette, the cells were allowed to recover for 2 hours at 37°C and then plated on GL, phage agar, or TSA plates containing antibiotic as appropriate Protocols for *E. coli* and *S. aureus* electroporation (BioRad.).

**2.14 RNA Methods: Isolation, DNase Treatment, cDNA Synthesis, qRT-PCR.**

In order to assess gene expression, semi-quantitative, real-time, reverse transcriptase PCR (qRT-PCR) was performed on strains as indicated. *S. aureus* strains (5-100 ml) were grown to mid exponential phase, either induced or
infected as described and the cell pellet was collected and stored at -70°C. Cell pellets, regardless of initial size, were thawed on ice and resuspended in 1 ml of TRIzol (Ambion®; Grand Island, NY), transferred to either a Lysing Matrix B tube (MP Biomedicals; Solon, OH) or a 2 ml screw top microfuge tube (USA Scientific; Ocala, FL) containing 0.5 ml of 0.1 mm glass disruption beads (Fisher Scientific; Pittsburgh, PA). Cell wall disruption was achieved by processing three times in a FastPrep FP120 (Thermo Scientific; Pittsburgh, PA) at speed 6.5 for 30 seconds each; samples were placed on ice between runs. The aqueous fractions were transferred to sterile RNase-free microfuge tubes; 200 µl chloroform was added, vortexed for 20 seconds and samples were allowed to stand at room temperature for 5 minutes. Following centrifugation at 4°C and 13,000 rpm for 15 minutes, the upper aqueous phase was transferred into a new, sterile RNase-free microfuge tube and precipitated with an equal volume of 100% ethanol overnight at -70°C. Following centrifugation, the pellets were either air dried at room temperature or dried in a 65°C heat block for 10-15 minutes; the RNA pellet was resuspended in 50 µl prewarmed (65°C) RNase-free MQ water.

Freshly isolated RNA samples were treated twice with TURBO DNA-free™ kit (Ambion®; Grand Island, NY) per manufacturer’s instructions and quantitated by Nanodrop 1000 (Thermo Scientific; Pittsburgh, PA). Random Hexamer primers and SuperScript® II RT synthesis kit (both Invitrogen™; Grand Island, NY) or Tetro cDNA Synthesis (Bioline; Taunton, MA) were used to synthesize +RT and –RT cDNA from 0.5-1 µg of RNA of each strain and/or condition. SensiMixPlus SYBR & Fluorescein Kit (Bioline; Taunton, MA) was
used in qRT-PCR reactions with primers designed to amplify specific SaPI or phage genes (Table 2). Primers were designed to amplify a 100-300 bp product and subjected to a temperature gradient qRT reaction to determine optimum temperature, followed by a primer efficiency qRT reaction to confirm amplification efficiency between 85-120+%. All qRT-PCR reactions were performed on an IQ5 Multicolor Realtime PCR Detection System (Bio-Rad; Hercules, CA) with a standard protocol of initial denaturation at 95°C for 7 minutes, 40 cycles of (a) 95°C/20 seconds, (b) optimized extension temperature/20 seconds, (c) 72°C/20 seconds followed by melt curve (72°C-95°C, 1 degree change every 30 seconds). Gene-specific reactions used a 1:100 dilution of cDNA in MQ. 16S reactions for normalization used a 1:10,000 cDNA dilution in MQ. qRT-PCR reaction mix was composed of 12.5 µl SYBR, 8.5 µl MQ, 1 µl each of forward [10 µM] and reverse primers [10 µM] and 2 µl cDNA dilution.

2.15 RNA Methods: Northern blots. RNA was isolated from cell pellets collected from uninduced control S. aureus strains at 30 and 60 minutes post induction or infection. Isolated RNA was run on 1.5% Agarose-LE RNase-free gels (Ambion®; Grand Island, NY) with either 1X Glyoxyl buffer or 1X Tris-Borate-EDTA (TBE) buffer. Glyoxyl buffer (1X) was diluted with MQ to 1X working concentration from 10X NorthernMax® Gly gel Prep/Running Buffer (Ambion®; Grand Island, NY); TBE (1X) was diluted with MQ from UltraPure™ 10X TBE (Invitrogen™; Grand Island, NY). RNA was mixed with NorthernMax®-Gly Sample Loading Dye for loading and the BrightStar® Biotinylated RNA Millennium™ Ladder (both from
Ambion®; Grand Island, NY) was used to size RNA species. Following electrophoretic separation, the gel was soaked for 45 minutes in 10X SSC and a positively charged nylon membrane (Roche Applied Science; Indianapolis, IN) was prewetted, first in deionized water, then in 2X SSC. RNAs were transferred to the membrane by capillary action overnight as described in Southern blot section 2.16, and crosslinked with 120,000 µJ/cm² UV. The 16S and 23S rRNAs were marked in pencil on the membrane, which was then sealed in a hybridization bag with 10 ml of prewarmed (to 50°C) UltraHyb or hybridization buffer (5X SSC, 0.1% N-lauroyl-sarcosine, 0.2% SDS and 1X Blocking buffer from the DIG Wash and Block buffer set). The bag was incubated at 50°C for 30 minutes to 2 hours with gentle shaking. A DIG-labeled (DIG-11-dUTP) probe was used to detect transcripts containing the sequences of interest. Probes (3.5-10 µl DIG-PCR product, 200-500 ng) were diluted in 50 µl of MQ and boiled for 5 minutes, then placed on ice to cool, and added to 7 ml UltraHyb. The prehybridization buffer was removed from the blot and the DIG probe-UltraHyb added. The extra air was removed and the bag was sealed and incubated overnight at 50°C with gentle shaking. The blot was removed from the incubator and washed twice for 10 minutes at room temperature with low stringency wash solution (2X SSC, 0.1% SDS in RNase-free water), then washed twice for 10 minutes with high stringency wash solution (0.1X SSC, 0.1% SDS in RNase-free water). The blot was incubated for 5 minutes at RT with wash buffer (0.1M maleic acid, 0.15M NaCl pH 7.5, 0.3% v/v Tween-20). The wash buffer was removed and the blot incubated in 100 ml 1X Blocking buffer (from the DIG Wash and
Block kit) at RT for 60 minutes with gentle shaking. Blocking solution was
decanted and replaced with 45 ml 1X Blocking solution premixed with 6 µl Anti-
digoxigenin-AP (750U/ml) and incubated at RT for 30 minutes. The antibody-
buffer solution was decanted and the membrane was washed twice at RT for 15
minutes each with 1X wash buffer. The membrane was incubated in 20 ml 1X
Detection buffer (from DIG Wash and Block kit) for 5 minutes with gentle shaking.
The membrane was put in a hybridization bag with 1 ml CSPD (Disodium 3-(4-
methoxySpiro{1,2-dioxetane-3,2'(5'-chboro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl
phosphate) working solution (1:100 CSPD: 1X Detection buffer, 1 ml total volume
per blot). The bubbles were carefully removed from the bag and it was sealed
providing optimal contact of CSPD to the face of the blot, and incubated for 15
minutes at 50°C. Blots were exposed to Blue Double Emulsion UltraRad Film
then developed in a Kodak X-O-Mat developer.
**Fig. 3. Capillary transfer of nucleic acids from an agarose gel to a nylon membrane for Northern blots.** A large sheet of Whatman paper was draped over the glass bridge such that the ends contact a reservoir of 10X SSC that is wicked towards the center. The gel was placed in the center of the bridge surrounded with an outline of parafilm and topped with the membrane, then three pieces of Whatman paper and finally an inch of paper towels. The paper wick was topped with a slab of glass, a volume of Methods of Enzymology and two bricks. This was allowed to remain undisturbed for 12+ hours.
2.16 RNA Methods: 5' RACE. 5' RACE was performed on RNA isolated from ST251 (SaPI1-RN4220(80α ΔrinA) using the FirstChoice® RLM-RACE kit (Ambion®; Grand Island, NY). RNA was isolated from cell pellets, DNase treated twice as previously described, then quantitated by a Nanodrop 1000. Ten µg of RNA was treated with 10 units Terminator™ 5'-Phosphate-dependent enzyme (Epicentre Biotechnologies; Madison, WI) to enrich for 5'-triphosphate RNA species that originated by de novo synthesis. The reaction was incubated at 30°C for 60 minutes, after which RNase-free MQ was added to a total volume of 200 µl and the reaction was terminated by extraction with an equal volume of 5:1 acid phenol:chloroform pH 4.5 (Ambion®; Grand Island, NY). The reaction was vortexed for 20 seconds, centrifuged at 13,000 rpm for 15 minutes at 4°C and the upper aqueous fraction transferred to a clean, RNase-free microcentrifuge tube. One tenth volume cold 3M sodium acetate pH 5.4 and 2.5 volumes 100% ethanol were added and the RNA was precipitated at -70°C for a minimum of 60 minutes. The RNA was pelleted by centrifugation 4°C and 13,000 rpm for 30 minutes, washed with 75% ethanol, repelleted, dried in a 65°C heat block for 10-15 minutes and resuspended in 11 µl of RNase-free MQ. Tobacco Acid Pyrophosphatase (TAP) treatment and RNA adapter ligation were performed according to the manufacturer’s instructions. Briefly, 5 µl of the Terminator-treated RNA was incubated with 1X TAP buffer, 2 µl TAP enzyme and 2 µl MQ, then incubated at 37°C for 60 minutes. The RNA adapter was ligated by mixing 2-5 µl of the Terminator-TAP-treated RNA, 1 µl of the 5' adapter, 1X Ligase
buffer, 5 units T4 ligase and MQ in a total volume of 10 µl, then incubated at 37°C for 60 minutes. Finally, cDNA was synthesized using M-MLV RT enzyme provided with the kit as follows: 2 µl ligated-RNA, 4 µl dNTPs, 2 µl Random Decamers, 1X RT buffer, 1 µL M-MLV RT enzyme and MQ to a total volume of 20 µl were incubated at 42°C for 60 minutes. The synthesized cDNA was amplified using the kit-provided 5’ outer adapter primer (sense) and a reverse primer designed to the antisense strand to amplify at least 200 bases of the presumed 5’ end of the transcript.

2.17 Protein Methods: GFP Assays. In order to assess the promoter activities of various putative SaPI1 promoters, fusion plasmids were constructed using pCN56 (Charpentier et al., 2004), a promoterless plasmid with transcriptional fusion of sequences encoding Green Fluorescent Protein (GFP) containing six stop codons, one in all reading frames, between the multiple cloning site and the GFP protein fusion site. A putative SaPI1 promoter was cloned using primers that amplify the template from the 3’ end of the upstream gene through the intergenic region and into the first several residues of the gene that the promoter activates. An aliquot (250 µl) of overnight culture was placed in a Costar® black opaque 96-well plate and fluorescence was read on a BIOTEX plate reader at 485 nm (excite wavelength) and 528 nm (emission wavelength) using GEN 5.11.11 software.
2.18 Protein Methods: Induction of Plasmid-Based Protein Expression. Protein-protein interactions were assessed by co-purification studies and electrophoretic mobility shift assays. The pGEX-4T1 (GE Healthcare Life Sciences: Picataway, NJ) overexpression plasmid was used in this work. For protein expression, an overnight culture was diluted 1:100 in 50-100 ml fresh BHI media supplemented with the 100 µg/ml ampicillin and grown for 3 hours at 37°C. Cultures were induced with 0.5-1 mM IPTG for 3 hours with a temperature shift to 30°C. Following induction, the cells were pelleted, supernatant decanted and the pellets stored at -70°C until processing. E. coli cells were resuspended in 5-7 ml of lysis solution (20 ml B-PER® (Thermo Scientific; Pittsburgh, PA), 150 mM NaCl, 2 µM β-mercaptoethanol, 1 tablet cOmplete™ EDTA-free protease inhibitor cocktail (Roche Applied Sciences; Indianapolis, IN)) and sonicated on ice 6 times using a sonicator ultrasonic processor W-225 (Heat Systems-Ultrasonics Inc, Farmingdale, NY) at power level 6 for 30 seconds each. The resulting cell debris was pelleted and the supernatant was clarified by filtration through a 0.45 µM PVDF syringe filter.

2.19 Protein Methods: Column Purification. A 0.5-2 ml 50% slurry of reduced glutathione-agarose resin (Pierce Biotechnology; Rockford IL) wt/vol with sodium azide was transferred to a 10 ml column and washed with 10 ml of MQ and then 10 ml 1X PBS in order to remove the sodium azide. Lysate of cells expressing GST-tagged proteins from genes cloned in pGEX-4T1 was applied to the resin. The flow through was collected, reapplied to the resin and the resulting flow
through was collected and stored on ice. The protein bound to the resin was washed 5 times with 10 ml of 1X PBS, each fraction was collected and stored on ice. After all the wash steps were completed, the resin was resuspended with 250-500 µl of 1X PBS. In order to cleave the GST tag from the recombinant protein, the protein-resin complex was transferred to a microfuge tube and incubated with 80U of thrombin (Sigma Aldrich, St Louis, MO) over night at RT. The resin was pelleted by centrifugation to separate the resin from the thrombin-cleaved protein, and the supernatant was reapplied to fresh reduced glutathione resin to remove the cleaved GST tag from the purified protein. 4X XT Sample Buffer and 20X XT Reducing buffer (Bio-Rad; Hercules, CA) were added to 100 µl aliquots of collected fractions to 1X final concentrations. Samples were boiled for 10 minutes and loaded on a Criterion Bis-Tris 12% polyacrylamide gel. Precision Plus Dual Color Protein Standards were loaded for size comparison. The gel was run in 1X MOPS buffer (Bio-Rad; Hercules, CA) at 200V for 45 minutes, then stained with 0.5% Coomassie Brilliant Blue G-250 (Bio-Rad; Hercules, CA), 50% methanol, 10% acetic acid for 1 hour and then destained with 45% methanol, 10% acetic acid.
Chapter 3. Derepression of SaPI1: 80α Sri is a bifunctional protein

Introduction

The SaPI lifecycle is a model of molecular piracy. It steals the structural proteins from a replicating helper phage in order to package its own genome at the expense of the phage and propagate its own spread. SaPIs invading naïve cells integrate at specific attachment sites in the host chromosome, residing there as stable entities until mobilized by helper phage induction or infection. SaPI repression is controlled by a pair of divergent promoters. The divergent orfs regulated by these promoters, stl and str, resemble the cl and cro regulators from the temperate coliphages in E. coli. All sequenced SaPIs encode a pair of stl and str genes, but these genes are highly divergent in sequence. This sequence diversity has implications for mechanisms that perturb the integrated state. Stl is the master repressor; deletion of this gene in SaPI1 or SaPIbov1 results in unregulated transcription from the rightward str promoter (Tormo-Mas et al., 2010, Ubeda et al., 2008). The SaPIbov1 master repressor, Stl, has been shown to bind the str promoter, preventing transcription of the genes essential for replication, phage interference and DNA packaging (Tormo-Mas et al., 2010, Ubeda et al., 2008).

Helper phage derepression of SaPIs is highly specific and occurs by an antirepressor mechanism in which a nonessential phage protein binds directly to SaPI Stl, resulting in a loss of affinity for the DNA by the protein. The repressor
dissociates from the nucleic acid and transcription proceeds from the *str*
promoter in a rightward direction (Tormo-Mas *et al.*, 2010, Ubeda *et al.*, 2008).

Previous work in our lab has established the existence and identity of the
SaPI1 and SaPIbov1 antirepressors encoded by the helper phage 80α (Harwich
mutants were isolated, and the mutations were located by DNA sequencing.
SaPIbov1-resistant mutants mapped to *orf32*, the dUTPase gene, while SaPI1-resistant
phage had mutations in 80α *orf22*, which was renamed *sri* (Tormo-Mas
*et al.*, 2010) (Tallent SM, 2007). In further studies on the SaPI1 antirepressor,
*sri*, was shown to be nonessential by construction of a clean deletion.
Transduction assays confirmed that 80α *Δsri* phage titers were unaffected by
SaPI1 and SaPI1 titers were reduced to generalized transduction levels (Harwich
MD, 2009, Tallent SM, 2007) Southern blots assessing SaPI1 replication and
packaging confirmed that deletion or mutation of 80α *sri* resulted in loss or
inhibition of SaPI1 replication and mobilization (Harwich MD, 2009, Tallent. SM,
2007). A PCR assay showed that SaPI1 excision did not occur with 80α *Δsri*
(Harwich MD, 2009). 80α *Δsri* transduced both SaPI2 and SaPIbov1 at high
frequency and both interfered with phage yield, confirming *sri* specificity for
SaPI1 (Tormo-Mas *et al.*, 2010).

*Sri* is not homologous to known excisionases, therefore we postulated that
it was the antirepressor. If Sri acted as a classic antirepressor, it should bind to
Stl, derepressing SaPI1, and activating transcription from *str*. We used qRT-PCR
to assess *str* expression. In the absence of 80α and in the presence of 80α *Δsri*,

39
str expression was nearly undetectable. However, after infection with WT 80α, SaPI1 str expression increased 20-fold. Therefore, sri was responsible for derepressing SaPI1 (Harwich MD, 2009). Finally, co-purification experiments in which GST-tagged Sri was used to pull down SaPI1 Stl demonstrated direct binding between the phage-encoded antirepressor and its target. The co-purified products were confirmed by mass spectrometry (Harwich MD, 2009).

**Sri mutants defective in SaPI1 derepression still bind Dnal**

Previous work had established that the SaPIbov1 antirepressor encoded by 80α, dUTPase, is a bifunctional, moonlighting protein and the dUTPase and derepression activities are genetically separable (Tormo-Mas *et al.*, 2010). 80α Sri also appeared to be a moonlighting protein. An Sri homolog, phage 77 ORF104, had been identified by a high throughput screen of phage proteins searching for those that inhibited staphylococcal growth. It had been further shown that ORF104 affected growth by binding the host replication helicase loader, Dnal (Liu *et al.*, 2004). This function was likely shared by the nearly identical protein, 80α Sri.

A related helper phage, 80 also encodes an Sri homolog, orf19, with 57% amino acid identity. 80 cannot mobilize SaPI1. It can, however, mobilize a constitutively derepressed mutant (Fig. 4) showing that the 80 Sri protein lacks the derepression activity. Based on toxicity, in both *E. coli* and *S. aureus*, noted during cloning of 80 sri, we hypothesized that it was likely still able to bind to Dnal.
Fig. 4. Phage 80 can mobilize SaPI1Δstl but not WT SaPI1 at HFT. 80 can mobilize a constitutively derepressed SaPI1 mutant (SaPI1 stl::tetM) at high frequency (433 fold increase, p<0.005), but wildtype SaPI1 only at generalized transduction levels indicating that the block to SaPI1 high frequency transduction is derepression. Cultures of SaPI1 and SaPI1 stl::tetM were grown to Klett=50, diluted 1:1 with phage buffer and infected with phage 80 (MOI=5), then transduction units were quantified. Each bar is the result of 3-4 independent experiments, error bars represent standard deviation. Students T-test was performed, **p<0.0005.
Our initial attempt to demonstrate that 80 and 80\textalpha{} Sri would both bind staphylococcal DnaI used a bacterial 2-hybrid system (BACTH, EuroMedex). The BACTH system uses a bait-and-prey system of plasmids encoding tags, which when brought in close proximity, result in a colorimetric change. Staphylococcal \textit{dnaI} was cloned into the high copy fusion plasmid, with the fusion proteins located at either at the N or C terminus of the \textit{dnaI} gene. Hoping to avoid toxicity problems while cloning \textit{sri}, we cloned both \textit{sri} variants into the cognate low copy plasmid, again with the fusion protein located at both the N-terminal and C-terminal ends of the gene. However, this approach failed to detect interaction even between 80\textalpha{} Sri and DnaI.

We then turned to a co-purification approach to show an interaction. Both \textit{sri} alleles were cloned into vector, pGEX-4T1, which has an N-terminal GST tag and a thrombin cleavage site to allow tag removal. Staphylococcal \textit{dnaI} from strain RN4220 was also cloned into the pGEX-4T1 vector. We overexpressed GST-tagged 80\textalpha{} Sri, 80 Sri and DnaI, and affinity purified each separately. The GST tags were removed from the Sri proteins by thrombin cleavage and an additional round of purification on reduced glutathione columns removed the cleaved tags. The purified tagless Sri proteins were applied to columns containing GST-DnaI bound to reduced glutathione resin. Samples of each resin were run on polyacrylamide gels to assess binding (Fig. 5A). Lanes 8 and 9 (Fig. 5A) clearly show GST-DnaI running at approximately 60 kDa and both Sri proteins co-purifying at the bottom of the gel (~6 kDa). Both Sri proteins (post thrombin
Fig 5. Both 80 Sri and 80α Sri bind the host protein Dnal. A. Overexpression of GST-Dnal (Lane 2), 80 and 80α sri (lanes 3,4) bound to reduced glutathione resin. The GST tag was removed (Lanes 5, 6) and purified Sri proteins were each added to a column containing bound GST-Dnal (Lane 8, 9). Lanes 1, 7 contain the 250 kDa ladder, Precision Plus Dual Color Molecular Weight Marker. B. Neither Sri protein nonspecifically binds to reduced glutathione resin. Lane 1 contains the Precision Plus Dual Color Ladder. Lanes 2, 3 contain 80 Sri-GST and 80α Sri-GST at approximately 32 kDa. Lanes 4, 5 contain Dnal-GST at approximately 60 kDa. Lanes 6, 7 contain lysate from 80 Sri and 80α Sri post thrombin cleavage of the GST tag. The cleaved GST tag runs at 25 kDa. The lower intense bands below 6 kDa are 80 Sri and 80α Sri. In lanes 8, 9, untagged 80 Sri and 80α Sri were column purified a second time to clear the GST tag post-cleavage. Lanes 10, 11 show Dnal-GST (~60 kDa), the GST tag (25 kDa) and co-purified Sri (~6 kDa)
**Fig 6. Sri alignment: residues important to SaPI1 derepression.** Residues in the N-terminus (amino acids 11-14, numbering relative to 80α) are potentially critical residues for SaPI1 derepression. The strains above the red line are known to derepress SaPI1 except 77 ORF104. 80 Sri and the 80α Sri mutants below the red line do not derepress SaPI1. 80 Sri binds DnaI (Fig 5A, 5B), the mutants are believed to bind DnaI based on toxicity observed during cloning. Aligned using Geneious® 6.1.5 software with Blosum 62 matrix, threshold =1.

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<tr>
<th>NM1 Sri</th>
<th>NM2 Sri</th>
<th>77 ORF104</th>
<th>80α Sri</th>
<th>80α Sri L11H</th>
<th>80α Sri C13S</th>
<th>80α Sri S14L</th>
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<td>AEHRTPAIV.</td>
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cleavage) in the absence of Dnal did not show any nonspecific binding to the resin (Fig 5B, lanes 8 and 9).

Our lab had previously identified several derepression mutants of 80α in our laboratory, which resisted SaPI1-mediated interference (Fig. 6). Two phages from clinical isolates, NM1 and NM2, had also been shown to mobilize SaPI1 (Dearborn & Dokland, 2012). Based on sequence homology, we speculated that NM1 and NM2 likely bind Dnal. An alignment of the Sri proteins known to derepress SaPI1 and variants that could bind Dnal but did not derepress SaPI1 (80α sri mutants and 80 sri) was constructed. Inspection of Fig. 6, shows that the residues important in SaPI1 derepression cluster in the N-terminus of the Sri protein between amino acids 11-14 relative to the 80α Sri sequence (Fig. 6). Sri is a small protein, only 52-53 residues, and the existence of variants which both derepress SaPI1 and bind Dnal and variants which only bind Dnal suggests two genetically distinct activity domains.

**Discussion**

80α encodes a small, nonessential protein, Sri, which functions as the SaPI1 antirepressor and is necessary and sufficient for SaPI1 derepression. This activity is specific to SaPI1, since Sri will not derepress the other SaPIs that are also mobilized at high frequency by 80α. The SaPIbov1 antirepressor encoded by 80α, dUTPase, is a bifunctional, moonlighting protein and the dUTPase and derepression activities are genetically separable (Tormo-Mas et al., 2010). We have demonstrated that the SaPI1 antirepressor, Sri, has both antirepressor
activity and, additionally, Sri directly binds to Dnal and likely inhibits host cell replication. The phage 80 Sri variant binds Dnal but does not derepress SaPI1. SaPI1-resistant mutants, affected in three clustered amino acids have been identified which do not derepress SaPI1, but presumably still bind Dnal based on toxicity noted during cloning. Given the relatively high frequency with which we were able to isolate mutants deficient in derepression activity, and the great difficulty we had in cloning said mutants, we are confident that the activities are separate. However, that remains to be demonstrated and should be the focus of new experiments on Sri.

The dual nature of both the SaPlbov1 and SaPI1 antirepressor proteins is important because it provides a clue into the SaPI-helper phage co-evolution process. Clearly, the primary activities of the antirepressors convey an advantage to the phage. The dUTPase activity of the SaPlbov1 antirepressor aids phage replication by catalyzing the hydrolysis of dUTP to dUMP, a precursor in the dTTP synthesis pathway. Reducing cellular levels of dUTP relative to dTTP is an important task as most DNA polymerases cannot distinguish between the two nucleotides and DNA uracilation increases mutagenesis and can lead to strand breaks and cell death. Recently it was demonstrated that the 80α dUTPase must bind dUTP in order to derepress SaPlbov1 and this activity is controlled by a motif VI, which is conserved across all staphylococcal phage dUTPases (Tormo-Mas et al., 2013). In the absence of bound dUTP, the enzyme is no longer able to bind Stl, indicating that binding of the dUTP substrate and cycling between active and inactive conformations are part of the
regulatory nature of the enzyme (Penades et al., 2013, Tormo-Mas et al., 2013). Given that S. aureus strains encode their own dUTPase functions, there must be another reason why this phage gene has been conserved. A hint has been provided by a paper linking the diversity observed in motif VI with potential regulatory functions during the phage lifecycle and indicating that dUTP might be an important second messenger (Penades et al., 2013).

Sri is a very small protein, about 4 times smaller than 80α dUTPase. We believe that the natural oligomeric state is a dimer (Harwich MD, 2009). We predict, based on the sequence alignment and small cluster of point mutations that abolish SaPI1 derepression activity, that the N-terminus is important for binding Stl (Fig. 6). The Sri protein is predicted to contain two small, antiparallel helices (about 8 residues each) connected by a four amino acid loop where the point mutations are located. This is followed by a three residue loop followed by a longer helix (28 residues). The C13S mutation eliminates the only cysteine residue and potentially destroys an intermolecular disulfide bond holding the dimer together. The other mutations, L11H and S14L, likely cause a steric hindrance problem (L11H) and disrupt potential stabilization contacts (S14L) when dimerized or when binding Stl.

SaPIs have co-opted the use of several phage middle proteins for derepression, tying their lifecycles directly to the phage’s replication and structural assembly cycles enabling productive interferences to occur. These phage derepression proteins have newly discovered moonlighting functions that
have important implications for the host cell and the phage life cycle as well.

Clearly we have more to learn from these tiniest of parasites.
Chapter 4. SaPI1 gene regulation

Introduction

Derepression is the first in a series of steps that must be accomplished in order to package SaPI DNA into phage-derived capsids before cell lysis occurs. Examination of the genomic architecture of SaPI1 (Fig. 1) shows an operon type organization where the genes are organized into functional modules. The promoters for the divergent orfs stl and str are where leftward and rightward transcription initiate.

Leftward transcription results in expression of the master repressor, which in SaPIbov1 has been shown to be autoregulated (Ubeda et al., 2008). We speculate that due to the nature of Stl as the master repressor, autoregulation is likely a conserved property of SaPI Stls. In SaPI1, a pair of superantigen genes lie downstream of stl, and our lab has shown these are constitutively transcribed (Harwich MD, 2009). The most distal leftward gene is integrase, which is necessary for both integration into the chromosome and for excision out of it (Ubeda et al., 2008). Regulation of SaPI1 int expression remains unclear. In SaPIbov1 int is co-transcribed with stl (Mir-Sanchis et al., 2012). Stl also regulate SaPI1 int expression, but the presence of two constitutively expressed toxin genes between stl and int argues against a single polycistronic mRNA.

Rightward transcription originates from the str promoter and is thought to proceed through the replication module, the phage interference functions and packaging machinery in operon 1. However, the actual length of the rightward
message is unknown. A perplexing problem has been that the \textit{str} promoter is responsible for regulating the expression of rightward genes necessary for lytic growth of SaPI, but a function for the Str protein has yet to be discovered. Deletion of \textit{str} slightly increases phage titers but does not have a significant effect on SaPI titers, in either SaPI1 or SaPIbov1 (Table 4). Beyond the genetic switch region containing the \textit{stl} and \textit{str} promoters, regulation of gene expression in SaPI1 is a fairly unexplored area.

SaPI operon 1 expression is thought to initiate from the \textit{str} promoter during helper phage infection (Harwich MD, 2009). It was established that a promoter for SaPIbov1 operon1 existed just upstream of the initial orf in the operon and that promoter was LexA-dependent (Ubeda \textit{et al.}, 2007). Our lab confirmed the existence of that promoter in SaPI1 and that it was also LexA-responsive (Harwich MD, 2009). The LexA-regulated promoter driving SaPI operon 1 expression would only be active during an induction not an infection. Potentially, this promoter is relevant in order to expedite phage exploitation gene expression in the prior to SaPI derepression.

The complex interactions between SaPIs and their helper phages during SaPI mobilization are intricate, requiring temporal transcription regulation in order to replicate and package the respective genomes prior to host cell lysis. Our current understanding of the molecular details of both SaPI and phage gene regulation is still vague. We sought to characterize SaPI1 gene regulation by first answering a few basic questions. How many promoters there are in SaPI1? Where do the transcriptional units start? Finally, what is the function of \textit{str}?
Table 4. SaPI str deletion affects PFU but not TU in SaPI1 and SaPIbov1. Cultures were grown to Klett= 30, diluted 1:1 with phage buffer and induced with 2 μg/ml of MC. The cultures were incubated until lysis at 32°C with reduced shaking, and sterile filtered to remove any remaining bacteria. Transduction frequency is the ratio of transduction units over phage particles. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.005, ***p<0.0005

<table>
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<tr>
<th>Strain</th>
<th>PFU/ml (x10^8)</th>
<th>TU/ml (x10^8)</th>
<th>TD frequency</th>
<th>TU Fold Change to WT SaPI1</th>
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<tbody>
<tr>
<td>RN4220(80α)</td>
<td>180±49.2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>RN4220(80α)-SaPI1</td>
<td>1.3±0.5</td>
<td>4.9±0.9</td>
<td>3.8</td>
<td>NA</td>
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<tr>
<td>RN4220(80α)-SaPI1Δstr</td>
<td>19.1±6.2**</td>
<td>8.5±0.9**</td>
<td>0.4</td>
<td>1.7</td>
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<tr>
<td>RN4220(80α)-SaPIbov1</td>
<td>8.8±3.2</td>
<td>9.7±1.0</td>
<td>1.1</td>
<td>NA</td>
</tr>
<tr>
<td>RN4220(80α)-SaPIbov1Δstr</td>
<td>27.6±8.7*</td>
<td>4.5±1.3**</td>
<td>0.2</td>
<td>0.5</td>
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Predicting the ends of SaPI1 and SaPIbov1 transcripts

To begin to answer these questions, we used predictive software to look for promoters located in intergenic regions larger than 40 nucleotides, indicating where transcription units might begin. The online Softberry program, BPROM, which recognizes bacterial sigma 70 sequences with approximately 80% accuracy and specificity (V. Solovyev & Salamov, 2011) was used to predict SaPI promoters. To identify the ends of transcription units, a combination of predictive softwares was used. Both the TransTerm (Kingsford et al., 2007) and ARNold prediction software (Gautheret & Lambert, 2001, Macke et al., 2001) were used to find rho-independent terminators in the SaPI1 and SaPIbov1 genomes and both programs needed to predict the terminator in order for us to accept it. Figure 7 shows where the predicted and established promoters and terminators are located in SaPI1 and SaPIbov1. SaPI1 is predicted to have, at most, seven promoters: two for leftward transcription stl and seq (Harwich MD, 2009), three for rightward transcription (str, orf19, orf8) (Harwich MD, 2009) and two in the accessory region tst and orf1 (Vojtov et al., 2002). There are four predicted terminators in SaPI1: one terminating integrase transcription, one terminating operon 1 transcription and two in the far right accessory region. Taken together, these data suggest that the majority of SaPI1 genes are transcribed as one of two operons. There are more predicted promoters in SaPIbov1. Leftward transcription is predicted to have two promoters, but recent data indicates that integrase expression is driven from the stl promoter (Mic-Sanchis et al., 2012). Transcription of the genes involved in the lytic cycle of
SaPIbov1 is predicted to initiate from four promoters. The initial rightward transcript originates from *str* which has been described but not mapped (Ubeda *et al.*, 2008). Additional transcripts are predicted to originate upstream from *orf17, ppi* and *orf10* (Ubeda *et al.*, 2007). Terminators in SaPIbov1 were only predicted for the rightward accessory genes, with the sole exception of a predicted terminator in the intergenic region upstream of operon 1 which is in the opposite orientation to the major rightward transcript. Our *in silico* method successfully predicted the *stl* and *str* promoters, as well as the operon 1 promoter, all of which had been previously described in the literature. Newly predicted SaPIbov1 promoters include promoters for: *int, orf17, ppi, tst, secbov* and *sel*. The SaPIbov1 *int* promoter is likely a false positive as recent data suggests that *int* is co-transcribed with *stl* (Mir-Sanchis *et al.*, 2012). The existence of the rest of the predicted promoters remains to be demonstrated.

An important caveat is that the promoter prediction softwares used can only predict sigma$^{70}$ promoters. Promoters that are recognized by alternate sigma factors would not be identified. Additionally, promoters that require alternate protein activators would also to be identified. Therefore the final pool of promoters likely has not been completely defined.
Fig. 7. SaPI1 and SaPIbov1 predicted promoters and terminators overlayed with amino acid homology. Intergenic regions larger than 45 bases were checked by BPROM for predicted promoters and by TransTerm and ARNold for predicted terminators, which would delineate the beginning and end of transcription units. The major leftward and rightward promoters, stl and str, are colored blue. Predicted promoters are depicted as arrows topping the boxed intergenic regions, arrow direction indicates the potential direction of transcription. Terminators are depicted as grey hairpin structures in the boxed intergenic regions, with an arrow indicating the direction of termination. Darker shading between SaPI1 and SaPIbov1 genes represents at least 50% amino acid identity, paler shading between ppi genes represents ~30%. Numbered promoters have been mapped and reported in the literature (1Harwich 2009, 2Vojtov et al., 2002, 3Ubeda et al., 2007).
**SaPI1 promoters are not sufficient to drive GFP expression in S. aureus**

We next asked, are these predicted promoters real? To assess promoters in SaPI1, we constructed a series of promoter fusion plasmids using the promoterless vector pCN56, which carries the $gfp_{mut2}$ gene fused to the multiple cloning site (MCS) with a series of in frame stop codons between the $gfp$ gene and the MCS (Charpentier et al., 2004). Putative promoters were amplified by PCR from sequence corresponding to the last 4-6 amino acids of the upstream gene through sequence corresponding to the first 4-6 amino acids of the gene driven by the promoter in question. Plasmids carrying the $str_{SaPI1}$ and $str_{SaPIbov1}$ promoters, and those carrying the SaPI1 putative promoters from int, entQ and orf19, were introduced into E. coli strains and analyzed for GFP production. Samples of overnight cultures from the plasmid-containing strains were collected and read on a BIOTEK 5 plate reader in black opaque plates to prevent fluorescence spillover from wells. Only the SaPI1 and SaPIbov1 $str$ promoters were strong enough to drive GFP expression in E. coli to levels above that of empty vector. We knew that the $str$ promoters should be constitutively active in E. coli due to a lack of the SaPI repressor, Stl, to block the promoter. However, none of the other promoters were active. We suspected that promoter activity in E. coli might be hampered by the lack of transcription factors encoded by SaPI, by the lack of helper phage activation proteins produced during an induction, or by a requirement for S. aureus-encoded transcription factors. Therefore we
Fig. 8. Only the SaPI promoters SaPl1 $P_{str}$ and SaPlbov1 $P_{str}$ are strong enough to drive GFP expression in *E. coli*. pCN56 derivatives containing the promoters indicated were transformed into *E. coli* and GFP production was measured from an aliquot of overnight culture in a BIOTEK 5 plate reader using an excitation wavelength of 485 and an emission wavelength of 520. The Gen 5.11.11 software reported fluorescence units. Bars represent 3 biological replicates, each performed with technical triplicates; error bars represent standard deviation.
transformed strain RN10628, which carries both 80α and SaPI1, with the collection of plasmids and assessed GFP expression. Overnight cultures were subcultured in fresh broth supplemented with the appropriate antibiotics and grown to early log phase, where uninduced samples were collected. The helper phage was induced with 2 µg/ml of MC and then cultures were sampled again after 3 hours of induction. SaPI promoter-driven GFP expression above that of the empty vector control was not detected either from the uninduced or the induced samples. The overnight cultures were then assessed and again, GFP expression was not detected. However, as the strain background contained SaPI1, Stl would be present in the uninduced cultures and expected to block at least the str promoters.

In the SaPI1-RN4220(80α) strain, MC induction results in culture lysis by 2-3 hours post treatment. We suspected that this is not sufficient time to produce detectable levels of GFP and/or that our promoters are not sufficiently strong to drive GFP expression. The latter explanation is less likely, since in E. coli cells, GFP expression was close to reaching maximum detectable threshold in at least two of the str biological replicates. The empty vector control and the SaPI1 Pstr construct in RN4220 (SaPI1 negative and 80α negative) and RN10616 (SaPI1 negative and 80α positive), again, failed to yield significant GFP expression. In the S. aureus strains we used, there was no SaPI Stl to repress the str promoter, suggesting that the SaPI promoters are not sufficient to drive GFP expression or that GFP is a poor reporter for this assay. Future work to elucidate SaPI promoter activity needs to use a more sensitive test. The pCN41 vector is a
promoterless plasmid constructed for promoter fusions in the same way as the pCN56 plasmid, except that pCN41 drives β-lactamase production, detectable by an assay using nitrocefin as a substrate (Charpentier et al., 2004). The multiple cloning sites from both plasmids are exactly the same, so the promoter could be restriction digested out of pCN56 and ligated straight into pCN41. This plasmid has previously been used to analyze expression from SaPIbov1 plasmids (Mir-Sanchis et al., 2012, Ubeda et al., 2008).

**Northern blot analysis of SaPI1 transcripts**

As an alternate approach to determine where the SaPI1 transcripts initiate, Northern blot analysis was used to assess transcript length. In SaPI1, there are two constitutively expressed superantigen genes located between the *stl* and *int* genes. There was a report in the literature of a novel staphylococcal sigma factor, σ^H*, which modulates levels of *Siphoviridae* phage integrase levels resulting in high rates of spontaneous excision (Tao et al., 2010). A consensus binding sequence, GGG TAG CCC GCC TAC CCT TAT TAT TTT TTG CCA ATT T, was conserved in 42 prophages examined, including 80α and φ11. This sequence was preceded by a predicted stem loop structure suggesting transcription factor involvement (Tao et al., 2010). A sigH consensus binding sequence was also present in SaPI1, just upstream of a very strong, predicted ribosome binding site spaced 10 bases upstream of the ATG start codon for integrase (Fig. 9). This spacing is consistent with other observed
**Fig. 9.** The *sigH* binding sequence in SaPI1 is just upstream of the integrase RBS and start codon. The left bar represents the beginning of the coding sequence of *int*; the gold arrow at the far right end is the stop codon of *sek*. The *sigH* consensus binding sequence, GGGTAGCCGCCTACCCCTATTATTATTGCCATTTT is represented by the purple arrow and a green arrow marks the putative RBS.
staphylococcal regulatory sequence spacing for the *int* RBS (Tao *et al.*, 2010).

We reasoned since the sigH binding site was upstream of SaPI1 integrase, it was regulated by sigH like the phage *int* genes were. We, therefore, carefully examined the leftward transcription in both RN4220 and RN4220 Δ*sigH*.

In the wildtype RN4220 background, integrase (*int*) expression was detected in the SaPI1 only control as well as in both infected samples (Fig. 10A, lanes 2-4). Over time, *int* expression increased from basal levels observed during the SaPI1 alone (uninduced) state, through 60 minutes post-80α infection. During the course of induction or infection (as in Fig. 10A), *int* levels would naturally rise via a gene dosage effect as the integrase copy number increased during replication (Fig. 10A, compare lane 3 to lane 4). The absence of any signal in lane 1, the RN4220 control lane, indicates that the DIG-labeled *int* probe is specific to the *int* message and is not cross-hybridizing with the 23S or 16S rRNA species or other host RNA species. The schematic in Fig. 10B shows the predicted length of all the possible transcripts containing *int*. The actual transcript size was smaller than predicted from the *stl* promoter and both species run at the same length as the 23S (2.9 kb) and 16S (1.5 kb) rRNAs.
Fig. 10. Northern blot analysis of SaPI1 int expression. A. RNAs from the indicated strains were probed with a DIG-11-dUTP labeled probe specific for SaPI1 integrase. (1) RN4220, (2) SaPI1 uninfected (3) SaPI1 30 minutes post-80α infection, (4) SaPI1 60 minutes 80α post-infection, (5) RN4220 ΔsigH, (6) SaPI1-RN4220 ΔsigH uninfected, (7) SaPI1-RN4220 ΔsigH 30 minutes post-80α infection, (8) SaPI1 60 minutes post-80α infection. B. Schematic representation of all potential transcripts containing the integrase message with predicted lengths, potential promoters and terminators.
There are two potential explanations for this: (i) the larger of the two transcripts appears to initiate just upstream from seq (~3 kB) and the smaller is either the int message post processing (~1.2 kB) or one arising from a promoter just upstream of int; (ii) the two int-containing transcripts have not been released from the rRNA subunits and are co-migrating. In the RN4220 ΔsigH background, int expression was not detected until 60 minutes post-80α infection and was not detected at all in the SaPI1 alone control lane. SaPI1 integrase transcription appears impaired in the RN4220 ΔsigH background. However stl levels needed to be examined to confirm this was integrase specific and not lower expression levels of the leftward operon. Decreased expression of integrase is not sufficient to drive SaPI1 excision from the host chromosome, both excisionase and integrase activities are required. Therefore, we would speculate that despite lower levels of integrase in the absence of sigH, SaPI1 would remain integrated in the host chromosome (Mir-Sanchis et al., 2012, Novick et al., 2010, Ubeda et al., 2009). Our lab had previously identified the SaPI1 excisionase gene as orf20 (J. Bento, unpublished), which is transcribed as part of the rightward transcript and should be unaffected by the sigH deletion.

Expression of stl, the master repressor, is known to be autoregulated in SaPIbov1 and we speculate that is a property conserved throughout the SaPI stl genes in order to maintain integration. We asked what effect the wildtype RN4220 background and the RN4220 ΔsigH background has on stl expression post-80α infection (Fig. 11A). RNA was isolated and processed as above, and following transfer to a nylon membrane, the fixed RNA was probed with a DIG-
labeled SaPl1 stl probe. Relatively equivalent amounts of stl-containing transcript were detected in both the wildtype and ΔsigH background. However, stl was not detected until 60 minutes post-infection when the copy number should be well over 100 as a result of the actively replicating genome (Novick, 2003). This suggests that prior to 60 minutes stl levels are too low to be detected by Northern blot, therefore either extensive optimization would be necessary or an alternate approach would need to be used. We did note the presence of a transcript running between 2.5-3 kB which would correspond to stl transcript terminating following transcription of sek, which was present in both backgrounds.
**Fig. 11. Northern blot analysis of SaPI1 stl expression.** A. RNAs from the indicated strains were probed with a DIG-11-dUTP labeled probe specific for SaPI1 stl. (1) RN4220, (2) SaPI1 uninfected (3) SaPI1 30 minutes post-80α infection, (4) SaPI1 60 minutes 80α post-infection, (5) RN4220 ΔsigH, (6) SaPI1-RN4220 ΔsigH uninfected, (7) SaPI1-RN4220 ΔsigH 30 minutes post-80α infection, (8) SaPI1 60 minutes post-80α infection. B. Schematic representation of all potential transcripts containing the integrase message with predicted lengths, potential promoters and terminators.
**SaPI1 produces a long transcript initiating at the str promoter**

During the course of $80\alpha$ infection, expression of the phage early/middle genes would result in Sri production and subsequent SaPI1 derepression and excision from the chromosome. Given the SaPI1 genomic architecture (Fig. 7), it is reasonable to assume that transcription from the *str* promoter would proceed through the end of operon 1 as one long transcription unit. SaPIb0v1 has a unique LexA-mediated promoter for operon 1, which can drive expression of the SaPI late genes following SOS pathway induction (Ubeda *et al.*, 2007). Our lab had identified and mapped the equivalent LexA-responsive operon 1 promoter in SaPI1 and deduced that it was active during SOS-mediated induction scenarios but not following phage infection (Harwich MD, 2009). This led us to speculate that transcription following induction resulted in two transcripts: a shorter transcript initiating from the operon 1 (*orf8*) promoter and a longer one initiating upstream of that (Fig. 7). The longer transcript potentially could initiate from either the predicted *ppi* promoter or as far upstream as the *str* promoter. We then asked if we could detect the production of the single long transcript terminating at SaPI1 *terS* during induction conditions. RNA was isolated from large cultures of RN4220 (SaPI1 negative, $80\alpha$ negative), SaPI1 alone (ST1) uninduced, SaPI1 alone at 60 minutes post-induction, SaPI1-RN4220(80$\alpha$) at 60 minutes post UV-induction and from SaPI1 at 60 minutes post-$80\alpha$ infection. These samples were processed as previously described for Northern blot transcript analysis and probed for genes encoded in the putative long transcript from *str* to *orf8* (marker
Fig. 12. (A-D) Northern blot analysis of rightward transcripts containing genes between str and operon 1 (orf8). Arrowheads indicate a large transcript detected by all four probes. Each panel contains RNA isolated 60 minutes after induction or infection, probed with a different DIG-labeled probe, as indicated: (A) str, (B) orf19, (C) ppi, (D) orf8. In each panel, lane assignments are: (1) RN4220 (no SaPI1 control), (2) SaPI1 uninduced, (3) SaPI1 induced (no phage), (4) SaPI1-80α 60 minutes post UV induction, (5) SaPI1-80α 60 minutes post infection. The locations of 23S and 16S RNA are marked in all panels, as are the positions of the size markers (Ambion® BrightStar® Biotinylated RNA Millennium™ Markers). Shown below each gel is a partial SaPI1 genetic map, including known (black arrows) promoters. Predicted lengths for potential transcripts detected by each probe are illustrated.
for operon 1). Fig. 12 A-D demonstrates that we were able to detect the predicted long str-driven transcript (indicated by the arrow) with the str, orf19, ppi and orf8 probes. This long transcript was only seen in the UV-induced lanes (Fig. 12 A-D lanes 4), never in the infected lanes (Fig. 12 A-D lanes 5). Additional transcripts of multiple lengths were detected in both the UV-induced (lanes 4) and 80α-infected (lanes 5) lanes, suggesting that SaPI1 mRNA is rapidly processed post-transcription. This complicated any further conclusions we could draw.

Finally, multiple attempts were made to map the 5' end of the str transcript. RNA was collected following induction of various strains containing SaPI1. Several were used for qRT-PCR experiments and shown to produce ample str expression. Following RNA isolation, genomic DNA was removed by DNase treatment. The RNA was enriched for transcripts containing 5'-end triphosphates and treated with TAP to remove the cap structure. An RNA adaptor was ligated to the 5'-end of the remaining transcripts and this adapter-ligated RNA was converted to cDNA. Multiple strategies for enriching the cDNA pool with str transcripts were devised. Briefly, random hexamer primers, random decamer primers and 3 different gene specific reverse primers were tried in various combinations and alone. The cDNA pools were treated with Taq to add adenine bases to the ends to allow TOPO cloning. TOPO clones were heat shocked into E. coli and the resulting colonies were screened by PCR for those containing an insert of the appropriate size. Finally, the individual clones were sequenced. Message corresponding to the 5' end of the str transcript was never able to be identified. Very few clones had transcript lengths of inappropriate size
and thus were never sent for sequencing. From the few that were sequenced, the data erroneously suggested that that the str transcript started a third of the way through the annotated gene. There could be multiple interpretations including: rapid processing of the 5’-end of the str transcript or initiation of an unmapped promoter within str that drives downstream transcripts.

Discussion

In the repressed SaPI1, gene regulation is simple: stl mRNA translation produces the Stl protein, which is thought to bind to the str promoter in a similar manner to SaPIbov1 Stl (Tormo-Mas et al., 2010) , repressing transcription of replication, excision, and phage interference genes. SaPIbov1 Stl is autoregulated, expression is upregulated by its own promoter (Ubeda et al., 2008) and we suspect that this might be a conserved regulatory function among the SaPIs. Examination of the genomic architecture revealed that SaPI1 appeared to employ an operon-based system for gene expression that is predicted to use very few promoters to regulate transcription from the genetic switch region where stl and str are located. In the SaPI1 leftward gene cluster, the superantigen genes seq and sek are constitutively expressed and fairly unresponsive to phage induction or infection (Harwich MD, 2009). The integrase gene is downstream of seq and sek, and we speculate it is expressed at a fairly constant and low level to maintain integration and coordinate with excisionase for island excision. This expression might be stochastic read through from the seq promoter. Indeed we found that in the wildtype RN4220 background, integrase
expression is detectable by Northern blot in the SaPI1 uninduced control and increases slightly following 80α infection to its highest levels at 60 minutes post-infection (Fig. 10A, lanes 2-4). We further reason that under the conditions we used for testing and in the wildtype RN4220 background, integrase expression is driven from either the stl or the seq promoter. We cannot, however, rule out the possibility that integrase expression can be activated from a sigH promoter located near the int ATG start codon (Fig. 9). Unexpectedly, the sigH consensus binding sequence was not found in the SaPI1bov1 genome. However, in the RN4220 ΔsigH background, SaPI1 int expression is not detectable until 60 minutes post-infection, indicating that in the absence of sigH something is perturbing either expression of int itself or the message is degraded. We do not believe the ΔsigH mutant has overall lower levels of transcription since in both the presence and absence of sigH, detected levels of stl were comparable. Further experiments would be required to elucidate the contribution, if any, of the sigH promoter to SaPI1 integrase regulation.

During SaPI1 derepression, Stl dissociates from the str promoter and rightward transcription begins. Both genomic architecture and promoter and terminator prediction analysis suggest that SaPI1 employs very few promoters and transcribes most rightward genes as a single long transcript. Our lab has previously identified a LexA-responsive promoter which turns on operon 1 gene expression following SOS pathway induction (Harwich MD, 2009). Our current hypothesis is that the operon 1-specific promoter is active in order to produce a large pool of phage interference proteins, e.g. cpmB, in order to out-compete the
phage for capsid assembly and packaging into virions. We have now demonstrated that a long transcript of approximately 8 kilobases, which could correspond to a message initiating from the \textit{str} promoter and terminating after \textit{terS}, was detected at 60 minutes following UV induction (Fig 11). We were able to walk down the transcript using probes designed to hybridize to \textit{str}, \textit{orf19}, \textit{ppi} and \textit{orf8}, which is a marker for the start of operon 1 (Fig. 12 A-D). However, we were unable to detect this transcript following 80\textalpha\ infection due to the extensive mRNA processing that was apparent by Northern blot. We cannot rule out that 60 minutes post induction or infection is an inappropriate time point in order to best measure leftward transcription, this simply might be too late.

During SaPI mobilization, the interactions between SaPIs and their helper phages are complex and interconnected, requiring temporal gene regulation in order to fulfill their destiny. Our current understanding of the molecular details of both SaPI and phage gene regulation is still vague. In this study, we sought to answer a few basic questions about SaPI1 gene regulation. We can conclude that for SaPI1 there are at least four promoters and perhaps as many as eight (Fig. 7, Fig. 9). Known promoters include the \textit{stl} and LexA-responsive operon 1 promoters (Harwich MD, 2009) and the \textit{tst} promoter (Vojtov et al., 2002), all of which have been mapped by 5' RACE. Predicted promoters include the \textit{sigH}-dependent promoter identified in this work, the \textit{entQ} promoter, the \textit{orf19} promoter (located in the replication region) and \textit{orf1} promoter located in the rightward accessory region. We demonstrated by Northern blot that we were able to detect and walk down a long transcript that included genes \textit{str} through \textit{terS}.
(Fig. 12 A-D). We also discovered that there was extensive message processing, which further complicated our use of Northern blots to determine all the primary transcripts produced in SaPI1. We were not able to demonstrate sigH dependent transcription of integrase. We speculate that int message might still be activated by sigH under certain conditions that we did not test. These results add more detail to our understanding of leftward and rightward transcription in SaPI1, but leave many questions still unanswered.
Introduction

Our laboratory has an extensive history working with the earliest described molecular pirates, the *E. coli* P4, and its helper phage P2. The precedent for transcriptional cross-talk and reciprocal-derepression between these two elements has been well described (Christie & Dokland, 2012). Like SaPIs, P4 is about one-third the size of P2 and does not encode genes for structural proteins. P4 is entirely dependent on P2 for mobilization. Both P2 and P4 can exist as an integrated element in the chromosome. Either can derepress the other phage. The P2 Cox protein derepresses the P4 lysogen, stimulating transcription from the \( P_{LL} \) promoter leading to transcription of the replication genes (Christie & Dokland, 2012). The P4 Epsilon protein binds the P2 master repressor, C, which results in early gene transcription. Exploitation of the P2 late genes by P4 requires a different set of interactions between the phages. P2 early gene transcription leads to expression of the P2 ogr gene, which has two functions. Ogr activates transcription of the P2 late operons that encode the structural genes. Ogr also activates the P4 late promoters resulting in expression of genes involved in redirecting P2 capsid assembly (Sid and Psu) and in production of Delta, a protein that can also activate the P2 late promoters (Christie & Dokland, 2012). During capsid assembly, the P4 Sid protein forms an external scaffold on P2-derived capsids to form small virions in which the larger P2 genome does not fit.
In the SaPI1/80α system, the phage protein Sri derepresses SaPI1 resulting in expression of two SaPI1 genes, \textit{cpmA} and \textit{cpmB}, that redirect the phage capsid assembly process, by way of an internal scaffold, to form smaller capsids that contain the much smaller pathogenicity island genome but not that of 80α. (Damle \textit{et al.}, 2012, Poliakov \textit{et al.}, 2008). Given the similarities between P2/P4 and the SaPI1/80α system, we wondered about potential crosstalk between SaPI1 and 80α. First we needed to know what happened when a SaPI1 entered both an 80α lysogen-containing cell and a nonlysogen cell. There were three possible outcomes: (i) SaPI would integrate into the host chromosome and not cross talk with 80a (ii) Incoming SaPI derepress the phage, resulting in culture lysis and producing huge amounts of phage progeny or (iii) Incoming SaPI activate only the phage late operon, producing phage heads, tails and finally, SaPI-filled virions that could infect neighboring cells.

We had a small number of 80α mutants that we were able to exploit as genetic tools in order to examine the effects of SaPI entering a cell. The first, an 80α \textit{ΔterS} mutant (ST24), allowed us to isolate lysates composed of pure SaPI particles. This was possible because the phage small terminase subunit (\textit{terS}) of the terminase holoenzyme is responsible for specifically recognizing phage DNA for packaging into virions. SaPIs encode their own small terminase subunit that is substituted into the terminase holoenzyme, redirecting packaging specificity to SaPI DNA, thus allowing efficient packaging of SaPI1 by the phage \textit{ΔterS} mutant (Novick \textit{et al.}, 2010, Ubeda \textit{et al.}, 2009). A second phage mutation, 80α \textit{Δ44} (ST64), affects a minor head protein that is thought to play a role in stabilizing
phage DNA inside the capsid (Damle et al., 2012). SaPI1 containing particles generated from this strain background are much more stable than SaPI particles generated from wildtype 80α (Dearborn et al., 2011). Cryo-electron microscopy an 80α ∆44 lysate shows the presence of intact, mature virions that rarely contain DNA. This suggests that the phage DNA is being packaged, triggering capsid maturation and then the DNA is slipping out of the mature heads. We used ST64 in order to generate mature phage particles that could not inject DNA into the cells in order to assess the effect of phage tails on staphylococcal cell walls.

**Pilot studies to determine endpoint assessment**

In order to assess a SaPI1-mediated effect on prophage in S. aureus strains, we first examined the spontaneous release rate of prophages to establish if measuring phage titer was an appropriate endpoint to determine derepression. Overnight cultures of an 80α lysogen in the RN450 (RN10359) background were pelleted and the supernatant was reserved. The cell pellet was washed with phage buffer and resuspended in fresh BHI. The supernatant and cell fractions were then titered for phage release on RN4220 indicator cells.
Fig. 13. The titer resulting from spontaneous release rate of Siphoviridae prophages is high. A. An aliquot of an overnight culture of an 80α lysogen was pelleted and the supernatant decanted into a sterile microcentrifuge tube. The pellet was washed with 1 ml phage buffer and the cells resuspended in 1 ml BHI. 100 μl of either the reserved supernatant or the washed cells were mixed with 100 μl RN4220 indicator cells and let stand for 10 minutes. Dilutions were made in phage buffer, plated on phage agar plates in top agar supplemented with 5 mM CaCl₂, and incubated overnight at 37°C.
The RN450(80α), supernatant titer was ~5x10^6 on RN4220 indicator cells (Fig. 13). The estimated number of cells/ml in the overnight culture was ~1x10^{11}, of which 10 µl (~1x10^9) was diluted and plated for phage release. The 80α supernatant titer was ~5x10^6, therefore about 0.5% of the cells (supernatant titer/fraction diluted) are spontaneously releasing phage. This is a low rate of spontaneous release, however, the burst size for 80α is ~600-900 phage released per infected cell. The high titer is due to the large burst size multiplied by relatively few lysing cells. To confirm that this was a phenomenon observed in staphylococcal Siphoviridae phage and not just 80α, several of our in-house lysogens were tested. The supernatant titer for φ53, φ11, φ85 and φ13 were all similarly high, indicating that spontaneous release of phage is a common occurrence (Fig 13).

Because release of phage from lysogens was so high, we wondered whether it was somehow induced by the indicator cells. S. aureus strains are known to release exosomes, which are membrane derived vesicles trafficking nucleic acids and effector proteins from cell to cell for communication or to lyse neighboring cells (Gurung et al., 2011). It has also been established that in a co-infection of S. aureus and S. pneumoniae, the pneumococci release hydrogen peroxide, inducing resident staphylococcal prophage, effectively killing off the competition (Selva et al., 2009). Therefore we assessed whether the indicator cells were releasing a phage activating substance.
Fig. 14. RN4220 does not secrete a lysogen-inducing factor. An aliquot (1 ml) of RN4220 overnight culture was pelleted, the supernatant was decanted and sterilized by filtration. An aliquot of an RN4220(80α) lysogen overnight culture was mixed 1:1 with either BHI or RN4220 supernatant. Both control (BHI) and 4220 treatments were then diluted 1:1 with phage buffer and allowed to stand for 15 minutes. Both BHI- and 4220-treated cells were plated immediately to assess spontaneous release of phage at 15 minutes (black and grey bars). The rest of the culture was incubated for 3 hours and then plated to assess phage release (blue bars). Results are from a single pilot experiment.
An overnight culture of RN4220 was pelleted, the supernatant sterile filtered and then used in our assay. Aliquots of an overnight culture of RN10616 [RN4220(80α)] were mixed 1:1 with either the RN4220 supernatant or fresh BHI, and incubated 15 minutes. Aliquots of both control (mock treated with BHI) and RN4220-supernatant treated were titered for pre-“lysis” activity. The remaining cultures were incubated for 3 hours, then the supernatant was titered for post-“lysis” phage activation. The control cells and those treated with RN4220-conditioned media released equivalent amounts of phage at both 15 minutes and 3 hours, indicating that the RN4220 cells did not secrete a phage activation factor (Fig. 14).

Finally, we investigated how long it takes an incoming SaPI1 genome to integrate into the host chromosome. The chromosomal and right SaPI1 attachment sites (attC and attR) were amplified by PCR at various time points after infection. RN4220 was grown to early log phase and infected with purified SaPI1 particles. DNA was extracted from samples collected every 5 minutes over a time course that ranged from 0-90 minutes. PCR amplification was performed to assess SaPI1 integration into the chromosome (Fig. 15). We first assessed integration in 30 minute intervals in order to determine when integration began post-infection. Integration was apparent in the 30, 60 and 90 minute samples (Fig. 15A, right panels); the attC PCR was used as a positive control since if even a single SaPI1 failed to integrate the site would be amplified (Fig. 15A., left panels). Next we examined earlier time points, and were able to observe integration by 15 minutes post-infection.
Fig. 15. Timing of SaPI1 integration into the host chromosome. A. The chromosomal \((att_C)\) and SaPI1 right attachment sites \((att_R)\) were detected at 30, 60 and 90 minutes post-infection with purified SaPI1 particles. Clear integration was observed by 30 minutes \((att_R)\) panels, \(att_C\) was used as a positive control. B. Shorter time course. SaPI1 integration is detected by 15 minutes post-SaPI1 infection. RN4220 was grown to early log phase and infected with SaPI1 particles. At the indicated time points, samples were taken and the DNA extracted. PCR was performed and the products visualized on a 1% agarose gel by ethidium bromide. PCR amplification detected either \(att_C\) (marker for unintegrated SaPI1) or \(att_R\) (marker for integrated SaPI1). Hyper ladder I \((L)\) was used to determine PCR products were the correct size.
**SaPI entering a lysogen negatively affects growth**

Next we devised an incoming SaPI1 assay to test what happens when SaPI1 enters *S. aureus* cells in the absence of exogenous phage. We were able to isolate a pure lysate of SaPI1 transducing particles by large-scale induction in a SaPI1(80α\_terS) lysogen (ST16) as described in Methods section. Normally, induction of an 80α lysogen carrying a SaPI results in equal numbers of infectious phage particles and SaPI transducing particles. Isolation of just SaPI particles required separation by sucrose gradient sedimentation. For SaPI1 infection, cells were grown to early log phase, washed to remove released phage, and resuspended in 2 volumes BHI-phage buffer (1:1). SaPI1 particles (MOI=1) or an equal volume of phage buffer (No SaPI control) were added, and then incubated at room temperature for 15 minutes. Growth was assessed at 15 minutes post-infection, and checked every hour thereafter for 4 hours. Samples were titered for released phage at 15 minutes, 2 hours and 3 hours post-infection. At 60 minutes post-infection, aliquots were plated for colony forming units (CFUs) to assess bacterial viability at that time point. At 3 hours post-infection, aliquots were titered for SaPI1 in the supernatant. Fig. 16A illustrates that SaPI1 clearly has a detrimental effect on RN4220(80α) growth. Fig. 16B shows that both the (-)SaPI and +SaPI1 cultures have a statistically significant difference in CFUs (5.8 fold). However the CFUs are high in the +SaPI1 cultures. This suggests that the incoming SaPI is negatively affecting cell growth at early time points but the cells are able to recover and thus are not dead. This effect could be explained by lysogen activation resulting in cell lysis. Fig. 16C shows
Fig. 16. Incoming SaPI1 affects growth of an 80α lysogen (RN10616). A. Growth curves show that SaPI1 entering RN10616 cells depress growth over 4 hours. RN10616 was grown to Klett=30, the cells were pelleted and washed to remove released phage. The pellet was resuspended in an equal volume BHI, and diluted 1:1 with phage buffer. SaPI1 particles (MOI=1) or equal volume phage buffer for no SaPI control was added. The cultures were grown for 3 hours with reduced shaking. B. Both cultures, (+)SaPI1 and (-) SaPI, were sampled at 60 minutes post-infection, the cells pelleted and resuspended. Dilutions were made and the cells were plated for CFUs on BHI plates. C. Cultures (+) SaPI1 and (-) SaPI released an equivalent amount of phage over the time course. Cultures from A were sampled every hour over the time course, the cells pelleted and dilutions of the supernatant were plated for free phage. D. Input SaPI1 was compared to output SaPI1 as measured by TU/ml. All figures represent results obtained from 3 independent replicates; error bars represent standard deviation. In A, p<0.0001, in C, D *p<0.0008
that both treatments result in comparable amounts of phage being released into
the supernatant (2-fold difference, p<0.0008) and this correlates with the
spontaneous release rate observed in Fig. 13. Our initial input of SaPI1 particles
was 1.14 x10^9, and output is calculated to be double the input number, or
approximately 3x10^9 (Fig 16.D). This suggests that by three hours, a fraction of
the incoming particles were able to replicate, induce phage late operon
expression, and induce cell lysis. Alternatively, a fraction of incoming SaPI1
particles could have entered cells in which the lysogen spontaneously released
with appropriate timing, such that they were able to be packaged and exit the cell
alongside the phage progeny. Increasing the MOI from 1 to 2 adversely affects
the growth of the recipient cell in a SaPI-concentration dependent manner (Fig.
17A).

The deleterious effect on cell growth had two potential origins: (i) SaPI1-
mediated activation of toxic genes on the resident prophage; or (ii) a direct
SaPI1-mediated toxicity effect on the host. We examined whether this negative
growth effect was lysogen dependent or if it was a recipient cell response to
incoming SaPI1 particles. In Fig. 17B, the incoming SaPI1 effect (MOI=1 and
MOI=2) was examined in RN4220 (phage negative) cells. Both of the No SaPI
controls in RN10616 and RN4220 exhibited a normal growth pattern, reaching a
maximum growth of ~350 Klett-Summerson units in four hours (Fig. 17B, blue
lines). Conversely, both incoming SaPI1 treatments (MOI=2), failed to grow
above ~50 Klett-Summerson units in over the same time period.
Fig. 17. Incoming SaPI1 particles inhibit growth of the recipient cells over a 4 hour time course regardless of prophage. A. The (-)SaPI1 80α lysogen (RN10616) optical density as measured by Klett units increased to ~350 over a 4 hour time course, while SaPI1 at MOI1 of 1 or 2 barely reach over 50. B. The (-)SaPI controls (RN10616, RN4220) reach a Klett ~ 350 by 4 hours. The RN10616 and RN4220 (+)SaPI1 (MOI=2) cultures barely reach Klett ~50 over the same time course. Graphs are a result of 3 independent experiments; error bars represent standard deviation.
Taken together the above data confirm that incoming SaPI1 particles have a deleterious effect on recipient cell growth an that this effect is not mediated by the presence of a phage lysogen but is likely due to a SaPI1-encoded factor(s) interacting with host cell factors.

The SaPI1 str promoter is responsible for activating rightward transcription. While the str gene product is predicted to be a transcriptional activator, a function for this protein has yet to be discovered. To date, no phenotype for the ∆str mutant has not been found; it is nonessential for SaPI1 mobilization and packaging. We tested this mutant to see whether str might play a role in the negative growth effect seen following SaPI1 infection. In Fig. 18, both SaPI1 and the SaPI1 ∆str mutant exhibit comparable growth inhibition and this effect is again MOI-dependent. Therefore, SaPI1 Str is not responsible for the negative growth associated with incoming SaPI1 particles.

SaPIbov1, the other prototypical SaPI, was assessed to determine if the growth defect was a more general SaPI phenomenon or specific to SaPI1. In Fig. 19A, incoming SaPIbov1 assays were performed exactly as previously described, and growth post-infection was monitored over time. At MOI=1 and MOI=2, recipient cells grew to the same optical density (OD) by four hours post-infection, which differed from the SaPI1 assay results in Fig. 17. Furthermore, they grew to approximately twice the final OD seen in SaPI1 strains using the same MOI as assessed by Klett-Summerson units.
Fig. 18. Inhibition of cell growth by SaPI1 $\Delta$str. SaPI1 infection was performed as described and growth was monitored over a 4 hour time course. Data are means of 3 independent experiments; error bars represent standard deviations.
Fig. 19. Inhibition of cell growth by SaPIbov1. A. Incoming SaPIbov1 assay using RN4220(80α) recipient cells (RN10616). SaPIbov1 particles were purified by large scale induction of a SaPIbov1-80α ΔterS mutant. Particles were titered to establish MOI and then the incoming SaPI1 assay was performed as previously described. B Incoming SaPIbov1 assay using nonlysogenic RN4220 cells. Graph represents 3 independent experiments; error bars are standard deviation.
As seen in Fig. 19B, growth of the recipient cells was reduced with incoming SaPIbov1, however, this effect is intermediate to the more dramatic SaPI1 effect. Growth comparisons of both the recipient cells (phage positive: RN10616, Fig. 19A; phage negative: RN4220, Fig. 19B) demonstrated that the incoming SaPIbov1 particles have a similar effect on both strains that is phage-independent, confirming that the target for the SaPI-mediated growth inhibition is host-derived.

Thus far, there is a clear impact on both phage-negative (RN4220) and phage-positive (RN10616) recipient cells when infected with pure SaPI lysates. The SaPI1-mediated growth defect is striking and extremely deleterious to the recipient, while the SaPIbov1-mediated effect is somewhat less severe. There remained the formal possibility that this effect was simply one of recipient cell damage caused by the tails of the SaPI particles puncturing the cell wall. In order to test this, a large-scale induction of ST64 RN4220(80αΔ44) was prepped in the same manner as the SaPI1 and SaPIbov1 particles. RN4220(80αΔ44) inexplicably cannot form plaques during a plaque assay, however it transduces SaPI1 at wildtype phage levels (Dearborn et al., 2011). To determine the number of 80αΔ44 particles necessary to approximate an MOI of 2-5, purified SaPIbov1 and mutant particles were boiled for 15 minutes, vortexed for 20
Fig. 20. Protein level comparisons between SaPlbov1 and 80α Δ44 particles. Banded particles of SaPlbov1 (30 μl = 1.35 x10⁹ particles) and 80α Δ44, which cannot make functional phage particles, were boiled for 15 minutes in XT Sample buffer under reducing conditions, then vortexed for 20 minutes. The samples were run on a Criterion Bis-Tris 10% polyacrylamide gel with Precision Plus Dual Color Protein Ladder for size comparison. The gel was stained with 0.5% Coomassie Brilliant Blue dye and destained in 40% methanol, 10% acetic acid.
Fig. 21. Adsorption of noninfectious phage particles does not inhibit cell growth. RN4220 and RN10616 were grown to Klett=30, pelleted and washed to remove free phage. The pellets were resuspended in equal volume of BHI, diluted 1:1 with phage buffer and 12.5 μl of ST64 (80α Δ44) particles were added (~MOI=3). The cell-phage mix was allowed to stand 15 minutes, then incubated for 4 hours at 32°C with reduced shaking. Growth was monitored every hour. Graphs represent 3 independent replicates; error bars represent standard deviation.
minutes and run on a polyacrylamide gel, pictured in Fig. 20. This allowed us to approximate the titer of 80α Δ44 relative to SaPIbov1 so that we could test the effect of phage particles. 80α Δ44 particles, approximating an MOI=3, were added to the recipient cells and control cells were mock infected using an equal volume of phage buffer. At four hours post-infection all strains had reached an equivalent Klett OD of 300-400 (Fig. 21), demonstrating that the negative growth effect was not simply an artifact due to an excess of phage tails puncturing the cell wall.

**Incoming SaPI affects phage early gene expression**

Finally, 80α gene expression was examined directly following infection by SaPI1 in order to determine if the SaPI was indeed activating the phage and this effect was masked by the host growth defect. RNA was isolated from cells 60 minutes post-infection and prepared as described in the Methods section. Gene expression was assessed by qRT-PCR and normalized to 16S rRNA levels. The schematic in Fig. 22A depicts the locations of the genes assayed by qRT-PCR. All of the prophage genes tested showed increased levels after SaPI1 infection. The early phage genes cro, orf14 and orf20 were affected by an incoming SaPI1 to a greater extent than the terminase genes (2-fold terS increase, 2.5-fold terL increase) located in the late operon (Fig. 22B.). By 60 minutes post-infection, cro levels were increased 9 fold (p<0.05), the 5’ end of orf14 was increased 40 fold (p=0.007) and orf20 levels were up 7 fold (p<0.05). It is not entirely clear whether
Fig 22. Effect of incoming SaPI1 on $80\alpha$ gene expression. A. Map of $80\alpha$ showing genes used for qRT-PCR assessment. B. Transcript levels of selected $80\alpha$ genes. RNA was isolated from incoming SaPI1 experiments at 60 minutes post-infection and qRT-PCR was used to determine transcript titers. Fold differences: $cro$ (9x), $orf14$ (41x), $orf20$
(7x). Expression was normalized to 16S rRNA levels, the graphed results are the average of 3 independent experiments, error bars represent standard deviation. *p<0.05, **p=0.007.
cro, orf14, and orf20 are on the same transcript. There are divergent open reading frames between cro and orf14, and just upstream of orf20 suggesting the presence of multiple transcriptional units. However, tiling array data suggests that there is continuous transcription from cro to rinA in a single unit (Quiles-Puchalt et al., 2013). Either way, the differences in magnitude of expression for cro and orf14 (9 fold versus 40 fold) suggest that the increased transcript levels do not result from a simple increase in overall rightward operon expression. The 40-fold increase in transcript level seen in the phage gene orf14 indicates that incoming SaPI1 has specific effects on 80α early gene expression. The modest increase in terminase expression (2-fold in terS, 2.5-fold in terL) is hard to interpret. It may be that the incoming SaPI1 effect extends through to the late operon. Given the high rate of spontaneous release of lysogens (Fig. 13), the SaPI1-mediated effect on terminase expression is likely masked by the expression from lytic 80α present in the culture.

Discussion

Due to the prevalence of prophages in all sequenced strains of S. aureus, it is easy to presume that during the course of a staphylococcal infection, SaPI1 particles exiting from a lysed cell would enter neighboring cells containing a prophage. We speculated that SaPIs could potentially interact with 80α or other helper prophages in order to activate the prophages or their late genes for further mobilization. We were able to exploit an 80α mutant, 80α ΔterS, which is unable to package its own genome into virions in order to isolate a large, pure lysate of
SaPI1 particles. This allowed us to examine the results of SaPI1 entering a cell alone, as opposed to the usual laboratory context of a phage induction or infection.

Incoming SaPI1 appears unable to fully derepress a resident prophage or to activate the phage operon, as evidenced by only a slight increase in both TU production at 3 hours post infection (Fig. 16D) and terminase expression at 60 minutes post infection (Fig. 22B, RN10616 compared to SaPI1). It is likely that the both slight increases in TU and terminase expression levels were due SaPI1 particles entering a cell prior to spontaneous release of a resident prophage, enabling SaPI1 to replicate and propagate. Despite the evidence arguing against SaPI1 activation in whole or in part of 80α, we cannot discard the effect on incoming SaPI1 particles on early and middle gene expression (Fig. 22B). Notably, while expression levels were increased for all three genes examined, they appear either to be activated or processed independently. Levels of cro increased 9-fold and transcript levels for orf20 increased 7-fold. While these could be the result of an activation of the operon itself, however, the intervening gene, orf14, had a 40-fold increase in expression level 60 minutes post-infection relative to the no SaPI control. This represents a novel SaPI1 target, and the first instance of its identification.

The orf14 gene product is an 86 residue protein, function unknown, belonging to the DUF1108 superfamily (ABF71585.1), and conserved among staphylococcal phages. The structure has not been solved, however, QUARK ab initio modeling software predicts two anti-parallel alpha helices and four anti-
parallel beta sheets (TM=0.4898 ±0.0833). Orf14 is predicted to be 10.2 kDa, has an isoelectric point of 4.36, and is annotated as being similar to φPVL ORF39. The φ11 homolog is orf11. The two homologs have 59.3% pairwise identity at the amino acid level. Deletion of the φ11 orf11 results in loss of definitive cell lysis, zero phage titer and low SaPlbov1 transduction titer (≤ 1% of wildtype) (J.P. Penadés, unpublished data). Potentially 80α orf14 up-regulation is advantageous to an incoming SaPl1 particle, by influencing late operon expression at a low level or by some as-yet undiscovered mechanism.

From the data presented in Fig. 16-19, it is clear that incoming SaPl1 particles have a deleterious effect on recipient cell growth and, that this is not the result of simple cell wall breach by the tails from SaPl-containing particles (Fig. 21). By one hour post-infection, there is a 14-fold increase in CFUs in the no SaPl1 control relative to the +SaPl1 strain (Fig. 16B, p<1x10^-4). The effect of the incoming SaPl1 Δstr mutant on recipient cell growth was both comparable to wildtype SaPl1, and MOI dependent (Fig. 18), suggesting that Str is not responsible for the negative growth phenotype. Potentially, this deleterious growth effect is due to an uncharacterized SaPl1 gene product complexing with host RNA or, more likely, a target protein that affects an essential pathway such as replication. There is precedent in staphylococcal Siphoviridae for phage proteins to have an inhibitory effect on host growth. Multiple phages, including 80α, φ77 and 80 (all SaPl helper phages) encode the sri gene, which as a secondary function, acts as an antirepressor to SaPl1 (Harwich MD, 2009, Tallent SM, 2007, Tormo-Mas et al., 2010). The primary function of sri is to
interfere with host cell replication by binding the helicase loader protein, DnaI, thus arresting cell growth, presumably to the advantage of the phage (Liu et al., 2004).

Finally, incoming assays with SaPlbov1 compared to SaPl1 suggest that the deleterious growth defect could be a SaPl-family trait with variable strength. If that is the case, then a core SaPl gene must be the effector. A strong candidate would be the SaPl1 Ppi protein. There is about 30% homology between the SaPl1 and SaPlbov1 Ppi proteins at the amino acid level, suggesting that sequence variance could play a role in moderating the growth defect. These experiments should be repeated using the SaPl1Δppi mutant to determine if the growth defect would be reduced or eliminated.

To date, SaPls have been known to target phage capsid genes and the DNA packaging machinery, exploiting these functions for SaPl advantage. We have now demonstrated that the host chromosome or gene product thereof is also a target for SaPl-mediated interference. Elucidation of the SaPl1 host target could potentially reveal novel targets for anti-staphylococcal agents.
Chapter 6. SaPI1 can directly activate the phage late operon

Introduction

Work described in the previous chapter showed that SaPI entering into both 80α lysogens and nonlysogenic cells caused a severe growth defect, attributable to SaPI-mediated effects on the host. We were unable to show conclusively that SaPI1 can either derepress the prophage or directly activate the late operon. However, we identified a novel phage target, orf14, which is greatly up-regulated 60 minutes post-infection by SaPI1 as assessed by qRT-PCR. This encouraged us to continue and expand our hunt for regulating crosstalk between SaPIs and 80α.

Our lab has an 80α mutant, 80α ΔrinA, which is unable to activate the phage late operon encoding the structural and packaging machinery. Therefore, in an induced prophage strain, virions are not produced and host cells are not lysed. However, it had been published that in a SaPIbov1-RN450 (80α ΔrinA) strain, ample SaPIbov1 transduction was detected by transduction assay. This indicated that the presence of SaPIbov1 activated the phage late operon (Ferrer et al., 2011). SaPIbov1 contains numerous open reading frames with no determined function to date. However none of these had any significant homology to the RinA protein. Thus, it was probable that the mechanism was direct activation.
**SaPI1 directly activates 80α late operon expression**

On our hunt for crosstalk between 80α and SaPIs, we moved SaPI1 into both the RN10616 [RN4220(80α)] and the ST280 [RN4220(80α ΔrinA)] strains, then characterized each using growth curves, phage and transduction titers, and assessing expression of several phage genes following induction by Mitomycin C. As expected, both the RN4220(80α) control and the SaPI1-RN4220(80α) cultures lysed by three hours post-induction (Fig. 23A). In 80α ΔrinA strains, which cannot activate the phage late operon, the no SaPI1 control cultures never lysed. Unexpectedly, the SaPI1-RN4220(80α ΔrinA) cultures all lysed by three hours post-induction (Fig. 23B). This indicates activation of the lysis cassette, located at the 3’-end of the phage late operon. We had demonstrated SaPI1 activation of the late operon distal genes, we next asked if the entire late operon, including the structural genes, was being activated.

Lysates resulting from the growth curve experiments were titered to assess phage production and SaPI1 mobilization (Table 5). 80α titers in the induced prophage cultures were within normal levels. As expected, titers in the induced SaPI1(80α) cultures were reduced by SaPI-mediated interference with 80α. In both the 80α ΔrinA and SaPI1(80α ΔrinA) mutants, there was a lack of phage titer consistent with the mutant being unable to activate expression of the late operon. However, while assessing SaPI1 mobilization, we found that both the SaPI1(80α) and SaPI1(80α ΔrinA) had equivalent SaPI1 titers. This indicates that SaPI1 is activating the entire phage late operon and not simply the lysis cassette.
Fig. 23. In the $80\alpha\Delta rinA$ strain, SaPI1 directly activates the phage late operon resulting in cell lysis. A. Strains containing an $80\alpha$ lysogen and one containing both the prophage and SaPI1 show normal lysis kinetics. B. The $80\alpha\Delta rinA$ strain is unable to activate the phage late operon, however lysis is observed in the SaPI1-$80\alpha\Delta rinA$ cultures. Klett readings were taken post-induction at 15 minutes and every hour thereafter for 4 hours. Each graph represents $n=3-12$; error bars represent standard deviation of all the experiments.
Table 5. SaPI1 activates 80α late operon expression to produce transduction units. The deletion mutant panel was grown to Klett= 30, diluted 1:1 with phage buffer and induced with 2 μg/ml of MC. The cultures were incubated until lysis at 32°C with reduced shaking, then sterile filtered to remove any remaining bacteria. Each experiment is the average of n=3-9 independent experiments ± standard deviation.

<table>
<thead>
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<th>Strain</th>
<th>PFU/ml (x10^8)</th>
<th>TU/ml (x10^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220(80α)</td>
<td>180±49.2</td>
<td>NA</td>
</tr>
<tr>
<td>SaPI1-RN4220(80α)</td>
<td>1.3±0.5</td>
<td>4.9±0.9</td>
</tr>
<tr>
<td>80α ΔrinA</td>
<td>&lt;10</td>
<td>NA</td>
</tr>
<tr>
<td>SaPI1-80α ΔrinA</td>
<td>&lt;10</td>
<td>1.9 ± 0.5 x 10^8</td>
</tr>
</tbody>
</table>
In 80α, RinA is necessary and sufficient to activate the phage late operon (Quiles-Puchalt et al., 2013), which encodes all the structural proteins as well as the DNA packaging machinery (Christie et al., 2010). In order for transduction to occur, SaPI1 had to directly activate the phage late operon.

We knew that SaPI1 did not encode a rinA homolog, which left us with two hypotheses to explain how the SaPIs were activating the late operon. SaPI1 could produce a protein that was able to bind to the terS promoter at the 5'-end of the operon and activate transcription. Alternately, SaPI1 could somehow be influencing transcription in the phage so that late operon expression was coupled to expression of the phage middle genes. RNA was isolated from cultures at 60 minutes post-MC induction, which was a time point corroborated by expression studies previously done in our lab (Harwich MD, 2009) and a tiling array performed measuring 80α expression (Quiles-Puchalt et al., 2013). The RNA was treated to degrade any contaminating genomic DNA and cDNA synthesis was done. All methods, reagents and procedures were kept identical throughout the scope of this study. Raw expression levels were normalized to the 16S rRNA subunit.
**Fig. 24. SaPI1(80α) activates terL but not terS expression.** Gene expression in WT 80α and SaPI1(80α), and expression in 80α ΔrinA and SaPI1(80α ΔrinA) were compared by Students T-test. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. *p<0.02, **p<0.005, ***p<0.0008.
Figure 24 shows wildtype 80α and 80α ΔrinA gene expression in the presence and absence of SaPI1. In the WT 80α cultures, SaPI1 negatively affected terS and terL gene expression. A knockdown effect on terS is easily understood as SaPI1 encodes its own terS homolog, which it uses for DNA packaging. A decline in phage terS transcript levels means less message for translation and could result in a decreased pool of TerS_{80α} for phage genome packaging. The phage large subunit of terminase is absolutely required for both phage and SaPI1 DNA incorporation into capsids; however, SaPI1 has numerous methods to interfere with phage yield prior to DNA packaging, including directing the formation of small capsids, which exclude the entire phage genome based on size.

In the 80α ΔrinA strain, terS and terL expression is decreased as expected because the late operon can not be activated. We had clear indications that SaPI1 was affecting late operon expression as measured by culture lysis (Fig. 23) and production of transduction units (Table 5). Strikingly, in the SaPI1(80α ΔrinA) strain, expression of terS was decreased 5-fold (p<0.005) relative to the 80α ΔrinA control strain. The negative effect on terS was unexpected and interesting given the 1329-fold increase in terL expression (p<0.0008). Given the clear dichotomy of terminase expression in the SaPI1(80α ΔrinA), there were several possibilities. Expression of terL was activated from a new promoter within the terS gene. We think this is an unlikely possibility. Attempts to map the 5’-end of a terL transcript have failed, suggesting that the message was a result of processing and thus was degraded during the 5’ RACE protocol. Expression of
terL was the result of either transcriptional read through from an upstream transcriptional unit or from activation of the terS promoter. If the terL transcript resulted from read through or terS promoter activation, the terS transcript was preferentially degraded.

SaPI1(80α ΔrinA) directly activates the phage late operon as evidenced by culture lysis, high frequency SaPI1 mobilization and expression of terL that bypasses terS. To date, SaPI1 interference with 80α is primarily Cpm-mediated. Additionally, all SaPIs target the helper phage terminase complex during packaging. In the wildtype 80α background, SaPI1(80α) decreases expression of both terminase genes. SaPI1 terS would be expressed at normal levels, suggesting that the SaPI1-encoded TerS would outcompete the phage-encoded TerS to complex with TerL. In the 80α ΔrinA background, SaPI1-mediated effects on terminase result in decreased terS and increased terL expression. Modulation of terminase expression is a novel mechanism for SaPIs to interfere with the established target that is the phage DNA packaging process.

**SaPI1 effects expression of the 5’-end of 80α orf14**

Having demonstrated that SaPI1 had a direct effect on 80α late operon expression, we next focused on whether SaPI1 could somehow be influencing transcription in the phage so that late operon expression was coupled to expression of the phage middle genes. We generated expression profiles of several early phage genes in the wildtype SaPI1(80α) and SaPI1(80α ΔrinA) strains.
Fig. 25. Genetic map of 80α. Putative transcriptional units are represented by red arrows, genes used for expression analysis by qRT-PCR are shown colored blue.
Fig. 26. Early phage gene expression profile of SaPI1(80α) and SaPI1(80α ΔrinA). A. Expression profile of SaPI1(80α) post induction show NT orf14 expression was decreased 12.6-fold compared to 80α. B. Expression of SaPI1(80α ΔrinA) decreased 255-fold compared to 80α ΔrinA. Graphs are the average of at least 3 experiments, error bars represent standard deviation, **p<0.005.
Briefly, RNA was isolated from cultures 60 minutes post-MC induction and qRT-PCR analysis determined expression levels of the genes in question, colored blue in Fig. 26. Initially, we started with *cro*, the 5’-end of *orf14*, and *orf20*. Then added *orf13*, the 3’ end of *orf14*, and *orf15*. Due to the complexity of the strain names and orf names, the 5’ end of *orf14* hereafter will be referred to as NT *orf14*, and the 3’ end will be CT *orf14*. RNA was isolated from cultures at 60 minutes post-MC induction, which was a time point corroborated by expression studies previously done in our lab (Harwich MD, 2009) and a tiling array performed measuring 80α expression (Quiles-Puchalt *et al.*, 2013). Raw expression levels were normalized to the 16S rRNA subunit.

Comparing the wildtype 80α expression profile to the 80αΔrinA mutant, the phenotypic differences are immediately recognizable (Fig. 26). In the mutant, expression of *terS* is down 406-fold (p<0.05) and *terL* is down 939-fold relative to the wildtype control (p<0.05). Additionally, NT *orf14* is down (2.5-fold, p<0.05), which was unexpected and raises the question of whether *rinA* might regulate more than just late operon expression; perhaps it feeds back to augment the early genes as well.

Early phage gene expression was mostly unaffected by the presence of SaPI1 in either the 80α or the 80α ΔrinA strains. However, expression of the 5’-end of 80α *orf14* (NT *orf14*) was dramatically altered in both the 80α background (12.5-fold decrease) and especially in the 80α ΔrinA strains (255-fold decrease). The primer pairs for NT and CT *orf14* exactly overlap in the center of the 261 base pair gene, indicating that this activity is specific for only the 5’-end of the
gene. This is at odds with what was seen in the incoming SaPI1 experiments, where SaPI1 actually increased NT orf14 expression 40-fold compared to 80α (Fig. 22). We believe this discrepancy exists due to the state of the phage at the time of measurement. In the incoming SaPI1 experiments, an integrated 80α lysogen has expectedly low levels of NT orf14 expression. Under the current conditions, by 60 minutes the phage has excised, and been replicating, transcribing and translating its gene products. Compared to the lysogen with a single copy of orf14, there is an additive gene dosage effect in the actively induced phage. The results from both infection (Fig. 22) and induction (Fig. 27) conditions confirm that modulating expression of 80α orf14 is a novel SaPI1 target.

**Discussion**

We initiated this study to answer a simple question, does cross-talk happen between SaPI1 and 80α? Using an 80α mutant that cannot activate its late operon (80α ΔrinA) and thus cannot make virions or package DNA, we were able to show conclusively that wildtype SaPI1 can activate the late operon and transduce at levels comparable to that observed in WT 80α. Additionally, we demonstrated that in the 80α background, SaPI1 downregulated expression of both terS and terL. This seems counterintuitive, except that by decreasing the expression of both terS and terL, there is a smaller pool of terminase subunits to which SaPI1 adds its own non-limited supply of terS\textsubscript{SaPI1}. By reducing the available pool of both subunits, the odds that the terS\textsubscript{SaPI1} finds a terL subunit are
increased. Simultaneously, SaPI1 is deploying all the phage interference mechanisms in its arsenal, including capsid size redirection.

In the $80\alpha \Delta rinA$ background, large terminase expression measured by qRT-PCR and the absence of $terS$ expression indicate that SaPI1 can directly activate $terL$ and the downstream late operon genes as evidenced by culture lysis. During the packaging process, this gives SaPI1 an enormous advantage. $80\alpha$ absolutely requires $terS_{80\alpha}$ in order to incorporate into capsids. Bypassing $terS$ transcription ensures that SaPI1 has very little phage competition for capsids. Expression profiling of phage gene expression demonstrated that WT SaPI1 expressly modulates the 5’ end of $orf14$ in both backgrounds. NT $orf14$ is upregulated in the WT $80\alpha$ background, but downregulated in the $80\alpha \Delta rinA$ background. NT $orf14$ upregulation was also observed in the incoming SaPI1 experiments and represents a novel second target for SaPI1-mediated interference. Taken together, these data indicate that cross-talk, and by extension, gene regulation in both SaPI1 and $80\alpha$ are complex and layered.
Chapter 7: SaPI1 deletion mutants modulate early phage gene expression

**Characterization of a SaPI1 deletion mutant panel**

Having identified the 80α terminase and NT orf14 genes were targets for SaPI1-mediated modulation, we wanted to know which SaPI1 genes were responsible for the observed effect. We used a panel of SaPI1 deletion mutants consisting of: SaPI1Δstr, SaPI1Δppi, SaPI1Δ10, SaPI1Δ9, SaPI1Δ8, and SaPI1Δ4 (see Fig. 1 for locations). We moved the panel into both the RN10616 [RN4220(80α)] and the ST280 [RN4220(80α ΔrinA)] strains. For the remainder of the chapter, the RN420 part of the strain genotype will be dropped in order to simplify the strains. We characterized each SaPI1 mutant using growth curves, phage and transduction titers, and assessing expression of several phage genes post-induction.

In the wildtype 80α background, growth curves were performed to assess growth post-MC induction. The control 80α and SaPI1(80α) cultures lysed by three hours post-induction (Fig. 23A and 28A) and the deletion panel cultures all lysed at two hours post-induction (Fig. 28A). In 80α ΔrinA strains, which cannot activate the phage late operon, the no SaPI1 control cultures never lysed. As previously shown, the SaPI1(80α ΔrinA) cultures all lysed by three hours post-
Fig. 27. Growth curves for the SaPI1 deletion mutant panel in the $80\alpha$ and $80\alpha\Delta rinA$ backgrounds. A. Growth curves for the panel in the $80\alpha$ background. B. Growth curves for the $80\alpha\Delta rinA$ background. Cultures were grown to Klett=30, diluted 1:1 with phage buffer and induced with 2 $\mu$g/ml of MC. Graphs are the average of 3 biological replicates, error bars represent standard deviation.
Table 6. Phage titers and SaPI transduction titers from the SaPI1 deletion mutant panel in the 80α background. The deletion mutant panel was grown to Klett= 30, diluted 1:1 with phage buffer and induced with 2 μg/ml of MC. The cultures were incubated until lysis at 32°C with reduced shaking, then sterile filtered to remove any remaining bacteria. Transduction frequency is the ratio of transduction units divided by phage particles. PFU/ml and TU/ml are the average of n=3-9 independent experiments reported with standard deviation. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.005, ***p<0.0005

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<th>Strain</th>
<th>PFU/ml (x10^8)</th>
<th>TU/ml (x10^8)</th>
<th>Transduction frequency</th>
<th>TU Fold Change to WT SaPI1</th>
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<tr>
<td>80α</td>
<td>180±49.2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>SaPI1(80α)</td>
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<td>4.9±0.9</td>
<td>3.8</td>
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<tr>
<td>SaPI1Δstr(80α)</td>
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<td>0.4</td>
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<td>SaPI1Δappi(80α)</td>
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<td>SaPI1Δ4(80α)</td>
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<td>0.5±0.2**</td>
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Table 7. Phage titers and SaPI transduction titers from the SaPI1 deletion mutant panel in the 80α ΔrinA background. The deletion mutant panel was grown to Klett= 30, diluted 1:1 with phage buffer and induced with 2 μg/ml of MC. The cultures were incubated until lysis at 32°C with reduced shaking, then sterile filtered to remove any remaining bacteria. Transduction frequency is the ratio of transduction units divided by phage particles. PFU/ml and TU/ml are the average of n=3-9 independent experiments reported with standard deviation. Students T-test was run comparing the mutants to the wildtype SaPI1, **p<0.005

<table>
<thead>
<tr>
<th>Strain</th>
<th>PFU/ml</th>
<th>TU/ml</th>
<th>TU Fold change to WT SaPI1</th>
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<tr>
<td>80α ΔrinA</td>
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<td>NA</td>
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<tr>
<td>SaPI1(80α ΔrinA)</td>
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<td>1.9 ± 0.5 x 10^8**</td>
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<td>SaPI1Δstr(80α ΔrinA)</td>
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<td>7.6 ± 0.6 x10^4**</td>
<td>4.0 x10^-4</td>
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<tr>
<td>SaPI1Δppi(80α ΔrinA)</td>
<td>&lt;10</td>
<td>7.8 ± 0.6 x10^4**</td>
<td>4.1 x10^-4</td>
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<tr>
<td>SaPI1Δ10(80α ΔrinA)</td>
<td>&lt;10</td>
<td>4.5 ± 1.9 x10^3**</td>
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<td>SaPI1Δ9(80α ΔrinA)</td>
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<td>2.0 x10^-5</td>
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<td>1.6 x10^-3</td>
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<td>2.3 ± 1.2 x10^1**</td>
<td>1.2 x10^-7</td>
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</table>
induction (Fig. 23B, 28B). However, with the sole exception of SaPI\(\Delta \)8(80\(\alpha\) \(\Delta\)rinA), none of SaPI1 deletion panel ever approached lysis, (Fig. 28B).

Next we characterized the SaPI1 deletion panel in the 80\(\alpha\) and 80\(\alpha\) \(\Delta\)rinA strains, evaluating their ability to transduce SaPI and to assess phage titers. All strains were grown in liquid culture to a specific Klett reading, diluted 1:1 with phage buffer and induced with MC. Cultures that lysed were titered post-lysis. Cultures that did not lyse were allowed to incubate with reduced shaking overnight at 32\(^\circ\)C, after which the cells were pelleted, and the supernatant sterile filtered to remove contaminating bacteria. Phage titers varied for the deletion panel in the 80\(\alpha\) strains. Relative to the control SaPI1(80\(\alpha\)), the \(\Delta\)8 mutant increased phage titers by 17-fold (p<0.0005), the \(\Delta\)str mutant by 14-fold (p<0.05), and \(\Delta\)ppi by 3-fold (p<0.005), the remainder had phage titers equivalent to the control (Table 6). The 80\(\alpha\) \(\Delta\)rinA mutants also had phage titers done; as expected, they were either <10 PFU/ml or very close (<50 PFU/ml) (Table 7).

We quantified SaPI1 mobilization of the deletion panel (80\(\alpha\) background) using our standard transduction assay (Table 6). The SaPI1\(\Delta\)str(80\(\alpha\)), SaPI1\(\Delta\)ppi(80\(\alpha\)), SaPI1\(\Delta\)10(80\(\alpha\)), and SaPI1\(\Delta\)9(80\(\alpha\)) TU titers were comparable to the wildtype control. The SaPI1\(\Delta\)9(80\(\alpha\)) and SaPI1\(\Delta\)4(80\(\alpha\)) TUs were significantly smaller, both with a p-value of <0.005 compared to the wildtype SaPI1. The SaPI1 orf9, has no known function and is positioned upstream of operon 1, which encodes the phage interference functions. SaPI1 orf4 also has no defined function and is positioned as the penultimate gene in operon 1 (see Fig. 1 for gene locations).
The wildtype SaPI1(80α ΔrinA) strain and the six deletion mutants had variable TU titers. However, a significant drop in transduction occurring when any single orf was deleted, suggesting that 80α late operon activation requires the intact SaPI for full efficiency (Table 7). The SaPI1Δ8(80α ΔrinA) mutant had the highest SaPI1 titer of the panel. Compared to WT SaPI1, this represented a 1200-fold decrease in transduction (p<0.005). The SaPI1-Δstr(80α ΔrinA) and SaPI1Δppi(80α ΔrinA) had transduction titers that were comparable in scope to each other, but had a 6500–fold and 4700–fold change compared to WT (p<0.005). The SaPI1Δ10(80α ΔrinA) and SaPI1Δ9(80α ΔrinA) were further reduced in ability to mobilize SaPI1. Significantly, the SaPI1Δ4(80α ΔrinA) mutant had a severe reduction in SaPI1 transduction. Deleting this orf nearly abolished the native SaPI ability to activate phage late transcription, suggesting that further characterization of this mutant would be informative.

**Expression analysis of the SaPI1 deletion panel: phage late genes**

In the wildtype 80α background, the entire deletion panel increased terS expression, and all but SaPI1Δ8(80α) increased expression of terL relative to the SaPI1(80α) control. The SaPI1Δstr mutant increased terS expression by ~7-fold, and terL expression by 11-fold, this is consistent with the increased phage yield (14-fold) seen in Table 6. The results are intriguing given that, by sequence analysis, str appears to be a transcriptional activator. However, the Δstr mutant has no phenotype that we have discovered.
Fig. 28. Effects of SaPI1 deletions 80α terS and terL expression in the WT 80α background. A. Expression of terS. B. Expression of terL. Expression levels were compared to the WT SaPI1(80α) strain, except SaPI1(80α) which was compared to 80α alone. Transcripts were normalized to 16S rRNA. Bars represent the average of at least 3 independent experiments, error bars represent standard deviation, *p<0.05, **p<0.006, ***p<0.0008
The SaPI1Δppi mutant also increased terS and terL expression (~5-fold and ~10-fold respectively), again consistent with plating data demonstrating increased phage mobilization (34-fold). In general, ppi is a phage interference mechanism as the SaPI1bov1 and SaPI1bov2 alleles of ppi block phage DNA packaging (Ram et al., 2012). This has not been demonstrated for ppiSaPI1; however, the two alleles have ~30% identity at the amino acid level and the observed data in Table 6 and Fig. 28 are supportive of this conclusion.

The orf10 gene in SaPI1 is positioned upstream of operon 1, which contains the phage interference functions. It is unique to SaPI1, SaPI3 and SaPI4m4 and has no determined function (Novick et al., 2010). The SaPI1Δ10(80α) mutant had only a slight, but significant (terS p<0.05, terL p<0.006), increase in terminase gene expression consistent with the previous two mutants (Fig. 28). The SaPI1Δ10 mutant had phage and SaPI1 titers that were comparable to wildtype SaPI1 (Table 6). The SaPI1Δ9(80α) mutant also had a slight change in terL expression that was statistically significant but biologically, the fold difference was not significant (Fig. 28). Plating data for this mutant showed decreased phage and SaPI1 titers relative to wildtype SaPI1(80α) (0.9 and 0.8 fold respectively) (Table 6).

The SaPI1Δ8 mutant showed a ~18-fold increase in phage titers and a 4-fold decrease in SaPI1 titers. There was a slight increase in terS expression (p<0.005, only a ~2-fold difference) and no change in terL expression relative to wildtype. The plating data is indicative of a phage interference function for orf8. This supposition would need further investigation to determine the effect of
Fig. 29. Effects of SaPI1 deletions 80α terS and terL expression in the 80α ΔrinA background. A. terS expression. B. terL expression. SaPI1(80α) was compared to 80α alone. Stats were not reported for the mutants because traditionally they would be compared to the SaPI1 parent. This resulted in fold decreases in the 400-900 fold range, therefore the mutants are resemble the no SaPI1 control in expression. Bars represent n=3-9 experiments, error bars represent standard deviation. Transcripts were normalized to 16S rRNA.
overexpression on phage yield and also to determine the mechanism of interference.

The SaPI1Δ4(80α) mutant had comparable phage yields as the wildtype, however, SaPI1 transduction titers were decreased ~10 fold. Paradoxically, terL expression was slightly increased (p<0.05, but only 2-fold). The plating data suggests that orf4 might be important in SaPI1 transduction, however, we are not sure of that role yet.

Comparing the wildtype 80α expression profile (Fig. 28) to the 80αΔrinA mutant (Fig. 29), the phenotypic differences are immediately recognizable. As expected and previously demonstrated, in a mutant that cannot activate the late operon, expression of terminase genes is decreased across the entire panel. In the SaPI1(80α ΔrinA) strain, terS expression is down and terL expression is highly upregulated relative to the 80α ΔrinA strain. Terminase expression in the entire deletion panel resembles that of the 80α ΔrinA strain, not the SaPI1(80α ΔrinA). Stats are not reported for the deletion panel, since traditionally, the mutants would be compared to the wildtype SaPI1-containing strains. These were all statistically significant, but terL was downregulated orders of magnitude relative to SaPI. Consistent with previous data suggesting a phage interference function for orf8, the SaPI1Δ8(80α ΔrinA) strain terS and terL expression levels

**Expression analysis of the SaPI1 deletion panel: phage early genes**

In the 80α background, SaPI1 downregulated expression of NT orf14 12.6-fold relative to 80α by 60 minutes post-MC induction. The entire mutant
panel increased expression of NT orf14 relative to the SaPI1(80α) control strain. Fold increase of the mutant panel: ~16-fold (SaPI1Δstr), ~5-fold (SaPI1Δppi and SaPI1Δ4); the SaPI1 Δ10, Δ9, and Δ8 mutants had statistically significant changes in expression (p<0.05), however these were not more than a 4-fold change which is biologically more relevant when discussing qRT-PCR data (Fig. 30A).

In the 80α ΔrinA background, the trend for wildtype SaPI1 to decrease NT orf14 expression (255-fold) and for the mutant panel to increase the expression of the early phage gene. Fold increases for the mutant panel: 1415-fold (SaPI1Δstr), 713-fold (SaPI1Δppi), 994-fold (SaPI1Δ10), 1013-fold (SaPI1Δ9), 894-fold (SaPI1Δ8) and 1056-fold (SaPI1Δ4). These expression changes were both biologically and statistically significant (Fig. 30B).

Next we wanted to know if the SaPI1-mediated effect on the early gene, 80α NT orf14, was an operon effect or specific to the gene. As we did in the previous chapter, we analyzed expression of cro, orf13, orf15, and orf20 (see Fig. 25 for gene locations). Given the extreme variability in NT orf14 expression, we also wanted to know if the 5’-end (NT) and 3’-end (CT) of orf14 had comparable expression levels. We designed primers for the 3’-end of orf14 that exactly overlapped the primer pair for the NT end in the middle of the gene were Therefore any change observed in expression of the NT and CT ends would indicate a specific interaction with that part of the orf14 message.
Fig. 30. Effects of SaPI1 deletions on 80a NT orf14 expression. A. 80α background B. 80α ΔrinA background. Bars represent n=3-9 experiments, error bars represent standard deviation. Transcripts were normalized to 16S rRNA. Students T-test was run comparing SaPI1 to 80α and the mutants to SaPI1, *p<0.05, **p<0.006
Fig. 31. Effect of SaPl1Δstr on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPl1, *p<0.03, **p=0.005, ***p<0.0008.
Fig. 32. Effects of SaPI1 Δppi on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p=0.005.
The SaPI1Δstr(80α) mutant demonstrated a significant increase in NT orf14 (15.8-fold, p<0.05) and CT orf14 (7-fold increase, p<0.05) expression (Fig. 31). The orf14 gene is positioned in the early phage transcript among the replication genes (Fig. 25); it is possible that by affecting orf14 SaPI1 is able to modulate phage replication. The lack of increase in expression in the surrounding genes, orf13 and orf15, indicate that the orf14 upregulation is specific to that gene and is not the result of an increase in operon expression (Fig. 32). The SaPI1Δppi(80α) mutant had an ~5-fold increase in NT orf14 expression, and the lack of increase in the surrounding genes is again consistent with a SaPI1-mediated effect specific to NT orf14.

In contrast, the SaPI1Δ10 mutant had a decreased expression of cro and orf15 with a consistent increase in NT orf14 previously seen in all the mutants (Fig. 33). The SaPI1Δ9 mutant increased expression of NT orf14, simultaneously down-regulating orf15 and orf20 (Fig. 34). Again the decreases observed in orf15 expression in both mutants with the up-regulation of NT orf14 suggest that the effect observed is specific to the NT orf14 message. The SaPI1Δ8 mutant also down-regulated expression of cro, orf15 and orf20, again, while increasing expression of NT orf14 (Fig. 35). Finally, the SaPI1Δ4 mutant again decreased cro and orf15 expression, while increasing NT and CT orf14 expression (Fig. 36). 5-fold and 11-fold higher than the 80α ΔrinA strain.
Fig. 33. Effect of SaPI1Δ10 on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.006.
Fig. 34. Effect of SaPI1Δ9 on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.006.
Fig. 35. Effect of SaPI1Δ8 on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.005.
Fig. 36. Effect of SaPl1Δ4 on 80α early gene expression. Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPl1, *p<0.05, **p<0.006.
To sum up trends observed in the 80α background, all the mutants increased NT orf14 expression and this was specific to the 5'-end of the transcript. The deletion mutants in genes downstream of ppi all decreased expression of orf15, while the deletion mutants in operon 1 decreased expression of orf15 and cro.

In the 80α ΔrinA background, we had established in the previous chapter that terS and terL expression was significantly down-regulated as expected (Fig. 24, Fig. 28). We had further established that the 80α ΔrinA mutant had reduced expression of NT orf14 (Fig. 28). SaPI1 causes further depression of both NT orf14 (Fig. 26) and terS expression while significantly increasing terL expression (Fig. 24, Fig.28). From the previous figures (Fig. 30-36), we knew that the SaPI1 deletion panel affected 80α early gene expression. Now we asked, what was the effect of the deletion panel on 80α early gene expression in the 80α ΔrinA background.

Deletion of SaPI1 str amplified expression of NT orf14 and CT orf14 in the phage expression profile in wildtype 80α. In the 80αΔrinA mutant, early phage gene expression is up regulated (Fig. 26). Increased expression of orf13 (31-fold, p<0.02), NT orf14 (1415-fold, p<0.005) and CT orf14 (26.5-fold), all early genes, was detected. This is the first detection of orf13 transcript titers modulated by SaPI1. Orf13 is a small, 53 amino acid, protein in the replication region. It has no known function but does contain a domain of unknown function, DUF1270.
Fig. 37. Effect of SaPI1Δstr on 80αΔrinA early gene expression. (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.005, ***p<0.0005.
Fig. 38. Effect of SaPI1Δppi on 80α ΔrinA early gene expression. (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05.
The SaPI1Δppi(80α ΔrinA) mutant significantly increased expression of orf13 (14-fold, p<0.05), NT orf14 (713-fold, p<0.05) and CT orf14 (10-fold, p<0.05) (Fig. 38), consistent with what was seen in the SaPI1Δppi(80α) strain where NT orf14 and was also up-regulated (Fig. 32).

The SaPI1Δ10-RN4220(80α ΔrinA) mutant increases transcription of the genes in the replication module: orf13 (4.5-fold, p<0.005), NT orf14 (933-fold, p<0.0003) and CT orf14 (11-fold, p<0.005) (Fig. 39). The NT orf14 expression is consistent with what was previously seen in with this SaPI1 mutant in the wildtype phage background (Fig. 33).

The expression profile from SaPI1Δ9(80α ΔrinA) at 60 minutes post-MC induction is shown in Fig. 40. Compared to the control strain, SaPI1(80α ΔrinA), expression of NT orf14 (1012-fold, p<0.02) and CT orf14 (7-fold, p<0.0009) is increased, while cro (p<0.0009) is decreased (Fig. 40 compared to inset). These results are semi-consistent with the results in Fig. 34, in that both SaPI1Δ9 mutants up regulate NT orf14 and down regulate orf20. These results are semi-consistent with the results in Fig. 34, in that both SaPI1Δ9 mutants up regulate NT orf14 and down regulate orf20.

The expression profile of the SaPI1Δ8(80α ΔrinA) closely resembles that of the 80α ΔrinA lysogen in the absence of SaPI1 (Fig. 41, compare to inset). NT orf14 transcript titers (894-fold, p<0.05) are elevated compared to the control strain, SaPI1(80α ΔrinA). These results are consistent with the SaPI1Δ8 mutant in the wildtype phage background in that the NT orf14 expression is elevated (Fig. 35).
Fig. 39. Effect of SaPI1Δ10 on 80α ΔrinA early gene expression. (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, *p<0.05, **p<0.005, ***p<0.0009.
Fig. 40 Effect of SaPI1Δ9 on 80α ΔrinA early gene expression. (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, p<0.05, p<0.0009
Fig. 41 Effect of SaPI1Δ8 on 80α ΔrinA early gene expression. (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, p<0.05
**Fig. 42. Effect of SaPI1Δ4 on 80α ΔrinA early gene expression.** (Inset is 80αΔrinA expression profile from Fig. 26.) Each bar is the average of n=3-9 independent experiments, error bars represent standard deviation, transcript levels normalized to 16S. Students T-test was run comparing the mutants to the wildtype SaPI1, p<0.05, p<0.005.
Deletion of SaPI1Δ4 in the wildtype 80α background, NT orf14 and CT orf14 are increased at 60 minutes post-MC induction while cro and orf15 are down regulated (Fig. 42). In the SaPI1Δ4(80α ΔrinA) mutant, NT orf14 (1056-fold change, p<0.05) and CT orf14 (11-fold change, p<0.05) increased, cro was decreased (p<0.005) relative to the control strain, SaPI1(80α) consistent with previous results (Fig. 36).

Discussion

This work began with a simple question: can SaPI1 and 80α cross-talk with each other? Using the RN4220(80α) and RN4220(80α ΔrinA) strains, we moved SaPI1 and a panel of six SaPI1 deletion mutants into the two backgrounds. We chose Δstr (a potential regulator), Δppi (a phage interference protein), Δ10, Δ9, Δ8, and Δ4. Very little is known about the numbered genes in SaPI1 (Fig. 1). The orf10 gene is unique to SaPI1, SaPI3 and SaPlm4. The orf9 gene is located upstream of operon 1, while orf8 is the first gene and orf4 is the penultimate gene in the operon (Fig. 1). The SaPI2 homolog of orf4, orf17SaPl2, is a phage interference protein that targets 80 (G.E. Christie, personal communication), and whose mechanism hasn’t been elucidated to date (Ram et al., 2012). Deletion of the SaPlbov1 homologs of orf9, orf8 and orf4 have no phenotype as measured by SaPI and phage transduction assays (Ubeda et al., 2007, Ubeda et al., 2009).
Growth curves were used to begin a characterization of the mutant panel and to analyze their lysis kinetics (Fig. 27), followed by phage and SaPI titer assays to assess mobilization.

Induction of the wildtype control, SaPI1, results in comparable TU titers in both 80α and the 80α ΔrinA mutant coupled with equivalent numbers of phage particles following induction in the WT 80α strain. This indicates that intact SaPI1 has numerous ways to both propagate itself and interfere with WT phage yield and that it can directly activate the 80α ΔrinA late operon, even though the phage can not. Expression analysis of both early and late 80α genes indicated that SaPI1 increases expression

In the WT 80α background, the SaPI1Δstr, SaPI1Δppi and SaPI1Δ8 mutants had slightly higher than normal phage yield (17-fold, 14-fold and 3-fold increase respectively), while in the 80α ΔrinA background, all the mutants had an expected phage titer of <50 (Table 6, Table 7).

In the 80α background, the SaPI1Δ4(80α) (9-fold) and SaPI1Δ8(80α) (4-fold) mutants had a lower TU titer than the rest of the panel (Table 6). Unexpectedly, the control WT SaPI1(80α ΔrinA) had a TU titer that approached TU titers resulting from a WT 80α induction. Significantly, the rest of the panel had TU titers that were logs lower, suggesting that the intact SaPI1 was required for full 80α late gene activation. The mutants SaPI1Δ8(80α ΔrinA), SaPI1Δppi(80α ΔrinA), and SaPI1Δstr(80α ΔrinA), respectively, had the highest TU titers of the deletion panel (Table 7). These correspond to a fold decrease, respectively, of 1200-fold, 4700-fold, and 6500-fold. Significantly, the SaPI1Δ4
had a TU titer lower than even generalized transduction, a $2 \times 10^7$-fold decrease in SaPI1 mobilization.

Overall, in SaPI1Δstr(80α), the increased phage titers and terS and terL expression relative to the SaPI1(80α) control (6.5-fold and 11-fold) are consistent and suggest that, in this mutant, there would be an enhanced ability to package genomes (Fig. 28, Table 7). The NT end of orf14 is up-regulated ~16-fold, while the CT end only increased 7-fold in expression (Fig. 30). The orf14 gene encodes a small, conserved hypothetical protein (ABF71585.1), similar to phiPVL’s orf39 and a member of the domain of unknown function (DUF) 1108. The DUF1108 members include both S. aureus and phage proteins and a function has not yet been identified. In SaPI1Δstr(80α ΔrinA), the NT orf14 transcript is further elevated 1415-fold and CT orf14 increased 26.5 fold (Fig. 29, Fig. 37). Additionally, the transcripts for orf13 increased 31 fold, while terL was reduced 679-fold relative to SaPI1(80α ΔrinA) (Fig. 29, Fig. 37). The orf13 gene encodes a very small, 53 residue, protein in the replication module of the phage that contains a domain of unknown function, DUF1270. The actual gene function is unknown. This represents the first identified modulation of 80α orf13 expression and solidifies the gene as a SaPI1-target for modulation.

The SaPI1Δppi(80α) expression profile was strikingly similar to the 80α alone expression of the phage early genes. Both terminase genes were up-regulated to 80α-like levels (compare Fig. 26 to Fig. 32), consistent with the observed 3-fold increase in phage titers. The NT orf14 transcript was up-regulated (4.6-fold) consistent with the SaPI1Δppi(80α ΔrinA) expression profile.
in which NT \textit{orf14} was increased 713-fold; \textit{orf13} and CT \textit{orf14} were also increased (14-fold and 10-fold).

Deletion of the SaPI1 \textit{orf10} gene in the SaPI1\(\Delta\)10(80\(\alpha\)) mutant, resulted in increased levels of NT \textit{orf14}, \textit{terS} and \textit{terL} (Fig. 28, Fig. 30), however, plating data suggest that none of these expression level effects impacted either phage or SaPI titers which were comparable to wildtype SaPI1(80\(\alpha\)) (Table 6). Interestingly, this mutant negatively affected \textit{cro} and \textit{orf15} expression, now both considered novel targets for SaPI1-mediated modulation. The \textit{orf15} gene encodes the SaPIbov2 anti-repressor (Tormo-Mas \textit{et al.}, 2010), however the function is unknown and it contains a domain of unknown function (DUF2483). Both \textit{orf14} and \textit{orf15} are conserved across siphoviridae and in \textit{Staphylococcus} strains. The SaPI1\(\Delta\)10(80\(\alpha\) \(\Delta\)rin\(A\)) mutant increased expression of the replication genes \textit{orf13}, NT \textit{orf14} and CT \textit{orf14}, by 4.5-fold, 933-fold, and 11-fold respectively. This suggests a direct interaction with the 5\textquotesingle-end of \textit{orf14} that was not part of an operon-mediated effect.

The SaPI1\(\Delta\)9(80\(\alpha\)) mutant increased expression levels of NT \textit{orf14} and \textit{terL}, consistent with the plating data presented in Table 6. Decreased expression of \textit{orf15} and \textit{orf20} were observed marking the first reported impact on 80\(\alpha\) \textit{orf20} transcript levels (Fig. 34). In the 80\(\alpha\) \(\Delta\)rin\(A\) background, terminase expression levels more closely resemble that of the 80\(\alpha\) \(\Delta\)rin\(A\) control than the SaPI1(80\(\alpha\) \(\Delta\)rin\(A\)). NT \textit{orf14} transcripts were increased 1012-fold, CT \textit{orf14} increased 7-fold, however \textit{cro} levels decreased. Depending on the background, this SaPI1 mutant affected several novel early genes, \textit{cro}, \textit{orf15} and \textit{orf20}.
Fig. 35 (right panels) shows the expression profile of the SaPI1Δ8(80α) mutant. As observed across the panel, NT orf14 and terS levels are increased. Paradoxically, the SaPI1Δ8(80α) mutant failed to increase expression of terL, suggesting the terS activation might be specific. Decreased transcript titers included: cro, orf15, and orf20. The increased NT orf14 expression levels suggest that all the phage early and middle genes are depressed while all of orf14 is specifically up regulated. The expression profile of the SaPI1Δ8(80α ΔrinA) closely resembles that of the 80α ΔrinA lysogen in the absence of SaPI1 (Fig. 41). NT orf14 transcript titers are 894-fold and terS are 26-fold elevated compared to the control strain, SaPI1(80α ΔrinA). Relative to the 80α ΔrinA lysogen, the terminase expression levels are up regulated 5-fold (terS) and 11-fold (terL) (Fig. 41 inset). These results are consistent with the SaPI1Δ8 mutant in the wildtype phage background in that the NT orf14 expression is elevated (Fig. 35). Taken together these data suggest that SaPI1 or8 has a phage interference function, however the mechanism remains to be determined.

The SaPI1Δ4(80α) mutant had a similar expression pattern at 60 minutes post-MC induction. NT orf14, CT orf14 and terL were increased, while cro and orf15 were down (Fig. 36). Again this suggests a mechanism by which the phage early and middle genes are being down regulated while orf14 expression is increased. 80α replication is not coupled to late operon activation, meaning the structural and packaging genes can be activated in the absence of replication (Harwich MD, 2009). Inhibiting replication would result in reduced numbers of phage progeny relative to replicating SaPI genomes, which suggests
a scenario where SaPI1 might outcompete 80α for virions. Deletion of SaPI1Δ4 in the wildtype 80α background, NT orf14, CT orf14, and terL are increased at 60 minutes post-MC induction while cro and orf15 are down regulated (Fig. 43). In the SaPI1Δ4(80α ΔrinA) mutant, NT orf14 increased 1056-fold, while CT orf14 increased only 11-fold, again suggesting that the NT effect is specific. Expression of cro was decreased relative to the control strain, SaPI1(80α ΔrinA) consistent with previous results (Fig. 43). Taken together, these data suggest that SaPI1 orf4 is vital for activating the phage late operon in the 80α ΔrinA mutant.

Decreased transduction titers in the SaPI1Δ4(80α) strain suggest that orf4 plays a role in SaPI1 high frequency mobilization, however we currently do not understand its function.

From the SaPI1 deletion panel, a few trends emerged. In the previous chapter, we reported that in the SaPI1-80α ΔrinA mutant, expression of NT orf14 (255-fold change) and terS (5-fold change) dropped even further relative to the 80α ΔrinA control, but terL expression increased by a striking 1329-fold (Fig. 38). Thus, in the 80α ΔrinA background, SaPI1 is able to directly activate terL expression in order to activate late gene expression. This represents a novel interference mechanism.

A second novel finding is that SaPI1 appears to be modulating the expression of the 5’ end of the phage orf14 gene in both the 80α and 80α ΔrinA backgrounds. This is at odds with the results of the incoming SaPI1 experiments in Chapter 5, during which SaPI1 entering a lysogen activated NT orf14 40-fold. We believe this is because 80α is integrated at the time of measurement. In our
induction experiment, the phage has excised and is actively replicating, transcribing, and translating gene products by 60 minutes. This would result in an increase in expression from a gene dosage effect compared to a single copy present in the incoming SaPI experiments. Conversely in both the WT 80\(\alpha\) and 80\(\alpha\) \(\Delta\)rinA background, all of the SaPI1 mutants up regulated NT orf14. The results from induction conditions confirm that SaPI1 is modulating expression of 80\(\alpha\) NT orf14. The orf14 gene encodes a small, conserved hypothetical protein (ABF71585.1) similar to phiPVL’s orf39. Orf14 (ABF71585.1) is conserved across the staphylococcal siphoviridae and contains a domain of unknown function (DUF) 1108. The DUF1108 members include both S. aureus and phage proteins and a function has not yet been identified. The \(\phi\)11 homolog is orf11. The two proteins have 59.3% identity at the amino acid level. Deletion of the \(\phi\)11 orf11 results in loss of definitive cell lysis, phage titers of <10, and extremely low SaPI transduction titers (≤ 1% of wildtype) (J.P. Penadés, unpublished data).

Potentially, up-regulation of 80\(\alpha\) NT orf14 is advantageous to SaPI1 by influencing phage replication or late operon expression at a low level. Additionally, there is significant evidence to support antisense RNA transcribed in the orf14 region, although we have no indication of the significance of that finding (Quiles-Puchalt et al., 2013).

In the 80\(\alpha\) background, all the deletion mutants up regulated expression of one or both of the terminase genes, while WT SaPI1 decreased expression of both. This seems counterintuitive, except that by decreasing the expression of both \(\text{terS}\) and \(\text{terL}\), there is a smaller pool of terminase subunits to which SaPI1
adds its own non-limited supply of \textit{\textit{terS}}_{\text{SaPI1}}. By reducing the available pool of both subunits, the odds that the \textit{\textit{terS}}_{\text{SaPI1}} finds a \textit{\textit{terL}} subunit are increased. Simultaneously, SaPI1 is deploying all the phage interference mechanisms in its arsenal, including capsid size redirection.

A second pattern emerged from the deletion mutants in the 80$\alpha$ background (Table 8). The SaPI1$\Delta$10, SaPI1$\Delta$9, SaPI1$\Delta$8, and SaPI1$\Delta$4 mutants, all down regulated \textit{cro} and \textit{orf15}. Down regulating the \textit{cro} gene would have the effect of decreasing early and middle gene expression. Potentially, this down regulation is coupled to an increase in expression of one or both of the terminase genes, which was observed.

In the 80$\alpha$ \textit{\textit{rinA}} background presented in Table 9, there was only one pattern that emerged: all the strains examined modulated NT \textit{orf14} expression. Comparing 80$\alpha$ to 80$\alpha$$\Delta$\textit{rinA}, NT \textit{orf14} expression was down. Expression of NT \textit{orf14} in the SaPI1-RN4220(80$\alpha$ \textit{\textit{rinA}}) strain was down regulated 255-fold compared to the 80$\alpha$ \textit{\textit{rinA}} transcript titer. The deletion panel (SaPI1$\Delta$str, SaPI1$\Delta$ppi, SaPI1$\Delta$10, SaPI1$\Delta$9, SaPI1$\Delta$8 and SaPI1$\Delta$4) all up regulated NT \textit{orf14} from 713-1415-fold. Of this set, all except SaPI1$\Delta$8 up regulated CT \textit{orf14} from 7-26.5-fold. Of the deletion mutants up regulating both NT \textit{orf14} and CT \textit{orf14}, three (SaPI1$\Delta$str, SaPI1$\Delta$ppi and SaPI1$\Delta$10) also up regulated \textit{orf13}. The patterns emerging from this work indicate that SaPI1 gene position influences the effect on 80$\alpha$ expression. They also suggest that the effects observed in this study were not due to a overall up regulation of the
operon, since the NT \textit{orf14} expression levels were an order of magnitude or more greater than that of the surrounding genes.
Table 8. The SaPI1 panel (WT 80α background) alters 80α expression of early and late genes. Fold change above 4 and p-value were plotted to visually show patterns in gene expression. Green indicates increased expression, red indicates decreased expression, white indicates an insignificant change (less than 4-fold, p>0.05), numbers indicate fold change >4 and p-value <0.05.
Table 9. The SaPI1 panel (WT 80α ΔrinA background) alters 80α expression of early and late genes. Fold change above 4 and p-value were plotted to visually show patterns in gene expression. Green indicates increased expression, red indicates decreased expression, white indicates an insignificant change (less than 4-fold, p>0.05), numbers indicate fold change >4 and p-value <0.05.
Chapter 8. Discussion

We began this work in order to answer seemingly simple questions about 80α and SaPI interactions. Is Sri, the SaPI1 antirepressor, a bifunctional moonlighting protein like its SaPlbov1 counterpart, dUTPase? How many promoters and transcriptional units are there in SaPI1? What happens when an incoming SaPI enters a lysogen? Can it activate the late operon or derepress the prophage? What happens when SaPI enters a cell in the absence of a prophage? Is there transcriptional cross talk between 80α and SaPI1 following SOS induction? Several of these questions remain to be answered. Our studies have revealed that things are much more complicated that they initially seemed. In essence, we have reaffirmed that the relationships between the molecular pirates and their helper phages involve strikingly complex interactions.

Like its SaPlbov1 counterpart, dUTPase, the SaPI1 antirepressor, Sri, is a bifunctional moonlighting protein. The primary function of this phage middle gene is to inhibit bacterial DNA replication by binding to the host DNA helicase loader, Dnl. This halts replication of the host cell, increasing the available pool of ATP and dNTPs for phage use during 80α replication and gene expression. A secondary function for Sri is derepression of SaPI1 following infection by 80α or induction of a 80α prophage. The sri gene is expressed as an early gene, therefore tying SaPI1 derepression temporally to phage replication. This timing is critical, as SaPI1 does not encode structural genes and must pirate structural gene products from 80α. SaPI1 derepression early in the phage lifecycle results
in the expression of SaPI1’s arsenal of phage interference proteins timed precisely to redirect capsid assembly to form small capsids, and to redirect the phage packaging machinery to efficiently recognize and encapsidate SaPI1 DNA. We now speculate that perhaps bifunctionality of the SaPI antirepressors is a conserved function.

SaPI1 transcription is initiated at a small number of promoters. The stl-str genetic switch has been long recognized as the main regulatory region of the island (Ubeda et al., 2008). The stl transcription start site in SaPI1 had previously been mapped by our lab (Harwich MD, 2009). We attempted to map the 5’ end of str by 5’ RACE in this study and were unsuccessful. In our efforts to define the SaPI1 promoters, we attempted to use a panel of SaPI1 promoter fusion constructs to evaluate putative promoters for activity using GFP as a reporter. We showed that while the str promoter was active in E. coli, we were unable to detect activity in S. aureus strains following induction of a prophage. We speculated that the induction timeline is not long enough to allow for sufficient GFP accumulation for detection, however in overnight cultures of RN4220 and RN10616 containing a plasmid copy of str, GFP expression was not detected. This suggests that, at least using this construct, SaPI1 promoters are not strong enough to drive GFP expression in S. aureus.

The SaPI1 leftward and rightward transcripts have defined starting points, upstream of stl and str respectively. In the leftward transcript, autoregulation of SaPIbov1 Stl has been demonstrated (Ubeda et al., 2008) and we speculate that SaPI1 Stl is autoregulated as well. Our lab had previously established that the
*seq* and *sek* transcript is constitutively expressed regardless of prophage induction or phage infection (Harwich MD, 2009), raising questions about regulation of expression. This left the matter of integrase regulation to be determined. In several prophage, a consensus binding site for the novel sigma factor, *sigH*, had been discovered (Tao *et al.*, 2010); this site lies upstream of SaPI1 integrase. Northern blots probing for integrase demonstrated that the *int*-containing transcript likely originated with the *seq* promoter. This expression could be partially *sigH* regulated, but the conditions for *sigH* regulation are unknown. Rightward transcription has at least two points of initiation, the *str* promoter and the LexA-mediated operon 1 promoter. We were able to detect and walk down a single long transcript originating from the *str* promoter and terminating at the end of operon 1. We were unable to determine whether there are additional downstream promoters due to extensive message processing.

The temperate 80α, on entering a cell, will either integrate into the chromosome, or initiate the lytic cycle resulting in host cell destruction and the formation of hundreds of 80α progeny. There’s a distinct lack of data regarding SaPI1 entering a host cell in the absence of either a co-infecting phage or a prophage residing in the recipient cell. We generated a high concentration of pure SaPI1 particles by using an 80α mutant, 80α ΔterS, that is unable to package its own genome but will efficiently package SaPI1. When SaPI1 entered a phage-negative cell, we saw a distinct growth defect that persisted for at least four hours. This growth defect was observed in SaPI1 entering cells with an 80α prophage as well. Because of the growth defect, we were unable to conclude that
SaPI was also capable of derepressing the prophage. SaPI1 does not encode a lysin-homolog, so there was the formal possibility that phage tails, collected with the purified particles, were puncturing the cell, resulting in the observed growth defect. This was not the case. Using an $80\alpha$ $\Delta$44 mutant, which can package DNA but not eject it, we demonstrated that RN4220 and RN10616 grow as well as the uninfected cultures. This proved the cells were not harmed by tail puncture. Expression profiling of the RN10616 cultures at 60 minutes post-SaPI1 infection showed that SaPI1 was activating $orf14$ (41 fold) and $orf20$ (7 fold) expression, suggesting that SaPI1 was able to turn on $80\alpha$ early gene expression. Demonstrating that incoming SaPI1 is able to modulate prophage gene expression is completely novel. Most striking was the effect on $orf14$, a small gene with no defined function. Deletion of the $\phi$11 homolog of $orf14$ had been shown to result in phage titers $<10$ and severely impaired SaPI titers, suggesting this is an essential phage gene that impacts both phage and SaPI DNA mobilization.

Using another phage mutant, $80\alpha$ $\Delta$rinA, we asked if SaPI1 can directly activate the phage late operon for SaPI transduction. The $80\alpha$ late operon is normally activated when phage-encoded RinA binds to the $80\alpha$ terS promoter. Following induction of a SaPI1-$80\alpha$ $\Delta$rinA strain, expression of $80\alpha$ terL was increased by 1329-fold, equivalent to WT $80\alpha$ terL expression, when assessed by qRT-PCR. Remarkably, this increased $terL$ expression was direct and bypassed the upstream $terS$ gene; $terS$ expression was nearly undetectable. Experiments to map the 5’ end of the $terL$ transcript following SaPI1 activation of
80\(\alpha\) \(\Delta\)rinA are ongoing. Phage titers following culture lysis were <10, while SaPI1 TU titers were comparable to the wildtype SaPI1-80\(\alpha\) control strain. Taken together, these data proved that in order to turn on late operon expression, SaPI1 directly activates the terL gene, turning on the 80\(\alpha\) late operon. This represents both a novel SaPI1 interference mechanism and a novel class of interference mechanisms. To date, this is the only direct effect on helper phage gene expression for interference to have been found.

Efforts to determine the gene responsible using a panel of SaPI1 deletion mutants in the WT 80\(\alpha\) and 80\(\alpha\) \(\Delta\)rinA background yielded interesting and complex results. Different SaPI1 deletion mutants had variable effects on the expression of 80\(\alpha\) genes including: cro, orf13, NT orf14, orf15, orf20, terS and terL. All of the strains tested had an impact on the expression of NT orf14, terS and terL. Only the SaPI1\(\Delta\)9 and SaPI1\(\Delta\)4 mutants failed to increase expression of both terminase genes. In the WT 80\(\alpha\) strains they only activated terL, while they failed to activate either terS or terL in the 80\(\alpha\) \(\Delta\)rinA cultures. This was consistent with SaPI1 mobilization defects observed with the plating results (Table 6).

The \(\Delta\)rinA was a trifecta of decreased expression of NT orf14, terS and terL. The terminase gene defect was expected and consistent with the plating data, indicating that the mutant could not make functional plaques. The NT orf14 is inexplicable unless there is a relationship between the rinA and NT orf14 gene products we have not yet discovered. Recent data from a published titling array examining 80\(\alpha\) gene expression suggests that in the orf14 region an antisense
transcript is generated in a WT 80α prophage. Other than the general location of the antisense transcript, we have no information on exactly how long it is or what the target may be.

The SaPI1 impact on expression of 80α genes was complicated. In the WT 80α background NT orf14 and the terminase genes were negatively affected; however, in the ΔrinA mutant, both NT orf14 and terL expression increased significantly. SaPI1 modulation of the terL transcripts represents a novel interference mechanism and adds to our knowledge of the interactions between 80α and SaPI1.

Up-regulation of CT orf14 expression was the second most observed SaPI1-mediated effect on 80α gene expression. The extreme to which the NT orf14 was elevated is much more dramatic than the CT orf14 elevation observed, suggesting that the NT effect is specific to that transcript region and is not carried through transcription of the rest of the gene (compare Tables 8 and 9), or that elevated NT orf14 expression in the absence of equivalent CT orf14 expression resulted from targeted message degradation. SaPI1-mediated effects on expression of cro, orf13 and orf15 were also observed. The cro promoter activates expression of the early genes, and in all cases where cro expression was negatively impacted, a later gene or genes were activated. Expression of orf14 was most commonly associated, as all the strains that modulated cro either up or down were observed to increase orf14 expression. These data, taken together, represent the sum knowledge that exists regarding cross talk between the molecular pirates of the SaPI family and their target 80α.
In this work, we identified a novel interference mechanism, whereby SaPI1 bypasses 80α terS to activate terL expression in a mutant prophage. This guarantees, that only the SaPI1 genome and not the phage genome will not be packaged. We’ve also identified novel phage early targets of SaPI-mediated expression modulation. These appear to represent part of an arsenal of interference mechanisms that are usually masked in a wildtype 80α infection or induction scenario. In the arms race to exit a cell destined for lysis, SaPI1 seems to have multiple back up options for interference. Thus, it remains king of the molecular pirates.
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Vita

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