Mobilization of pathogenicity islands by *Staphylococcus aureus* strain Newman bacteriophages

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*Staphylococcus aureus* pathogenicity islands (SaPIs) are mobile genetic elements that encode virulence factors and depend on helper phages for their mobilization. Such mobilization is specific and depends on the ability of a phage protein to inactivate the SaPI repressor Stl. Phage 80α can mobilize several SaPIs, including SaPI1 and SaPIbov1, via its Sri and Dut proteins, respectively. In many cases, the capsids formed in the presence of the SaPI are smaller than those normally produced by the phage. Two SaPI-encoded proteins, CpmA and CpmB, are involved in this size determination process. *S. aureus* strain Newman contains four prophages, named δNM1 through δNM4. Phages δNM1 and δNM2 are very similar to phage 80α in the structural genes, and encode almost identical Sri proteins, while their Dut proteins are highly divergent. We show that δNM1 and δNM2 are able to mobilize both SaPI1 and SaPIbov1 and yield infectious transducing particles. The majority of the capsids formed in all cases are small, showing that both SaPIs can redirect the capsid size of both δNM1 and δNM2.

**Introduction**

*Staphylococcus aureus* is a ubiquitous bacterium that is associated with a variety of clinical presentations. The emergence of community-acquired virulent and antibiotic resistant *S. aureus* strains is a significant public health issue. Virulence in *S. aureus* and other bacteria is often associated with mobile genetic elements, such as bacteriophages, plasmids and pathogenicity islands that are horizontally transferred within the bacterial population.

Bacteriophages are also involved in the mobilization of a family of *S. aureus* pathogenicity islands (SaPIs). SaPIs are 14–27 kb genetic elements that contain phage-like repressor, integrase and terminase genes, as well as genes encoding superantigen toxins and other virulence and antibiotic resistance factors. Two of the best characterized SaPIs are SaPI1 and SaPIbov1, found in *S. aureus* strains RN4282 and RF122, respectively. SaPI1 carries genes for the toxic shock syndrome toxin 1 (tst) and enterotoxins C and L, respectively, and is associated with bovine pathogenic *S. aureus*.

While normally repressed and stably integrated in the host genome, SaPIs become derepressed and mobilized by specific “helper” bacteriophages. Upon mobilization, the SaPI genomes are packaged into transducing particles formed by structural proteins encoded by the helper phage.

The first step of the mobilization process is the inactivation of the SaPI Stl repressor, which is dependent on specific interactions between Stl and a phage-encoded derepressor. Bacteriophage 80α, a transducing phage that is closely related to staphylococcal typing phage 53, encodes a protein, Sri, gene product (gp) of open reading frame (orf) 22, that binds to and deactivates SaPI1 Stl. In contrast, the SaPIbov1 Stl protein, which only shares 16% sequence identity with SaPI1 Stl, does not respond to Sri. Instead, SaPIbov1 Stl is inactivated by binding of 80α gp32 (Table 1), which encodes a dUTPase (Dut). A third mechanism is found in SaPIbov2, in which Stl is derepressed by the 80α gp15 protein. 80α has also been shown to derepress SaPI2 and SaPI1m1, by an unknown mechanism. Derepression leads to excision and replication of the SaPI genome.

Capsid assembly in 80α, as in other bacteriophages, starts with the formation of a precursor procapsid from the major capsid protein (CP; gp47 in 80α) and a scaffolding protein (SP; gp46) that binds to and incorporates into the procapsid.

The DNA is packaged through the portal in a process that requires the terminase complex, consisting of the small (TerS; gp40) and large (TerL; gp41) terminase subunits. During DNA packaging, the capsid changes from a round, thick-walled procapsid to an angular, thin-walled mature capsid.

A unique aspect of the phage-induced SaPI mobilization is that in many cases, the capsids formed are smaller (having \( T = 4 \))
isocehedral symmetry (T = 7) than those normally formed by the phage alone23 (T = 7). The smaller capsid can only accommodate the smaller SaPI genome and thus interferes with phage multiplication.8 We previously showed that SaPI1 proteins gp6 (CpmB, Capsid morphogenesis protein B) and gp7 (CpmA) (corresponding to SaPIbov1 gp8 and gp9, respectively; Table 2) are involved in this size determination,15,16,18 and that CpmB acts as an internal scaffold in the T = 4 SaPI1 procapsids.14 CpmA and CpmB are conserved, and always appear together, in the majority of SaPIs sequenced to date.5 Other factors, including the SaPI-encoded TerS subunit, also contribute to phage interference.21,22

Table 1. Genes and gene products in 80Sa, SaPI1 and SaPI2

| 80Sa ORF | Protein name | GenBank identifiers | Function |
|---------|--------------|---------------------|----------|
| int     |              | ABF71572 / ABF73033 | Integrase |
| cl      |              | ABF71577 / ABF73035 | CII-like repressor |
| sr      |              | ABF71593             | * Anti-repressor of SaPI Std |
| dut     |              | ABF71603 / ABF73036 | dUTPase, anti-repressor of SaPIbov1 Std |
| ter5    |              | ABF71611 / ABF73037 | Small terminase subunit |
| terL    |              | ABF71612 / ABF73038 | Large terminase subunit |
| pp      |              | ABF71613 / ABF73039 | Portal protein |
| gpp44   |              | ABF71615 / ABF73040 | Minor capsid protein |
| sp      |              | ABF71616 / ABF73041 | Scaffolding protein |
| cp      |              | ABF71617 / ABF73042 | Capsid protein |
| tp      |              | ABF71624 / ABF73043 | Major tail protein |
| hol     |              | ABF71641 / ABF73045 | Holin, cell lysis |
| lty     |              | ABF71642 / ABF73046 | Lysozyme, amidase, cell lysis |

*The corresponding ORF is not annotated in the SaPI1 sequence (DQ530553). It lies between ABF73049 and ABF73050.

S. aureus strain Newman (NCTC 8178) was originally isolated from a case of ‘secondarily infected tubercular osteomyelitis’.17 It produces more coagulase than other strains23 and is currently used in animal infection models.24-27 The strain Newman genome28 contains four prophages, named ΔSaPI1, ΔSaPI2, ΔSaPI3 and ΔSaPI4, which carry several virulence genes, but the other prophages also appear to contribute to the parental strain’s virulence.25

Results and Discussion

Sequences of Newman phages ΔSaPI1 and ΔSaPI2. S. aureus strain Newman phages ΔSaPI1 and ΔSaPI229 were identified in a global BLAST search for bacteriophage sequences related to the 80Sa CP and SP. The other two Newman phages, ΔSaPI3 and ΔSaPI4, are more distantly related to 80Sa and were not picked up by a BLAST search. The ΔSaPI4 capsid protein is about 37% identical to those of 80Sa, ΔSaPI1 and ΔSaPI2, while ΔSaPI3 is not homologous to the other three phages; it is non-inducible by mitomycin C and appears to be defective in excision and lysis.29 ΔSaPI3 and ΔSaPI4 will not be considered further in this paper. The complete genomic sequences of ΔSaPI1 (GenBank accession number DQ530559), ΔSaPI2 (DQ530560) and 80Sa (DQ530558) were aligned using the programs Easyfig30 (Fig. 1) and VISTA31 (Fig. S1). Large tracts of the three genomes mobilize SaPI1. In addition, both phages can mobilize SaPIbov1, which was surprising, in light of the extreme differences in the Dut protein. We also find that ΔSaPI1 and ΔSaPI2 respond to capsid size determination induced by SaPI1 and SaPIbov1, although in the case of mobilization of SaPIbov1 by 80Sa, only about 65% of the capsids formed are small. Our results suggest that prophages found in clinical isolates of S. aureus may play a role in mobilization, spread and establishment of pathogenicity islands in the bacterial population, and underscore the importance of prophages in the evolution of bacterial pathogenicity.

Table 2. Genes and gene products in SaPI1 (GenBank ID U93688) and SaPIbov1 (AF217213)

| Protein name | SaPI1 GenBank ID | SaPIbov1 GenBank ID | Function |
|---------------|------------------|---------------------|----------|
| Stl           | AAC28967         | AAG29617            | Repressor |
| CpmA          | AAC28958         | AAG29606            | Small capsid size formation |
| CpmB          | AAC28957         | AAG29605            | Small capsid size, internal scaffold |

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Comparison of the sequences, including numerous open reading frames (ORFs) that are comparable in size and location to genes in 80α, is thought to be responsible for CP binding,18 is conserved. Other genes were also conserved (Fig. 1), most notably the genes encoding the SaPI1 antirepressor (Sri) homologs, which exhibit only two amino acid substitutions at the protein level (orf22 in 80α, orf53 in 80β). Indeed, 80α and 80β Dut (ABF71603) of SaPIbov1 Stl.10 According to sequence alignment, this gene corresponds to proteins ABF73115 in 80α, and ABF73115 in 80β. The divergent regions of the sequences include numerous ORFs that are identical, in particular the structural gene cluster starting from orf69 (80α orf50), nucleotide (nt) number 11,461 (which lies between ABF73049 and ABF73050; Table 1). This similarity suggested that 80α and 80β Dut should be able to derepress SaPI1 by the same mechanism.

Differences between 80α NM1 and 80α NM2 are localized to the two ends of the genome. The integrase (ori) gene, at the leftmost end of the sequence, is similar between 80α and 80β NM2, but differs in 80α NM1 (Fig. 1). A corresponding relationship is found in the attachment (att) sites, which are recognized by the integrase.9 At the opposite end of the genome, the lysis genes (holin and lysin; 80α NM1 proteins ABF73093 and ABF73094, respectively) are similar in 80α and 80β NM1, but differ in 80β NM2 (Fig. S1).

Table 1. ORFs in 80α, 80β, 80β NM1, 80β NM2, RN450 and RN10616. RN10616 is an 80α lysogenized in the phage-cured S. aureus strain RN4509 (Table 3). RN10616 is an 80α lysogen.22 The lysogens were induced with mitomycin C, and the resulting lysates were purified by PEG precipitation and CsCl density gradient separation, as previously described.22 The purified 80α NM1 and 80β NM2 fractions were analyzed for the presence of phage particles by electron microscopy (EM). Both contained siphoviruses with 60 nm diameter icosahedral capsids and 185 nm tails (Fig. S3A,B), similar to those of phage 80α,11,17 and consistent with the previous report,7 except that we did not observe a difference in tail length between 80α NM1 and 80β NM2.

The crude lysates from the phage inductions above were filtered through a 0.22 μm filter to remove unlysed cells. The filtrates were used to infect S. aureus strain ST1, an RN4220 derivative (holin and lysin; Table 3). At 2–3 h post infection, unlysed cells were removed by centrifugation, and the resulting particles were purified as described above. EM revealed siphovirus particles similar to the phages, but having predominantly smaller diameter icosahedral capsids and 185 nm tails (Fig. S3A,B).
(~45 nm) capsids (Fig. 3 C and D). This result shows that, like 80a, wNM1 and wNM2 both respond to the previously described SaPI1-induced size determination.6

To test for the ability to transduce S. aureus, the 0.22 μm filtered lysates from the ST1 infections were mixed with S. aureus strain RN4220 and plated out on GL agar containing 5 mg/ml tetracycline. In all three cases (ST1 infected by 80a, wNM1 and wNM2), the lysates successfully conferred tetracycline resistance on RN4220, showing that the particles had been packaged with the SaPI1 tst::tetM genome (Table 4). PCR with primers specific to SaPI1 orf6 also confirmed that SaPI1 DNA was present in all three transductants (data not shown).

Figure 2. Multiple protein sequence alignments of pertinent gene products from 80a, wNM1 and wNM2, including SP (A), Sr (B), and Dut (C). (See Table 1 for protein identifiers.) In (C), corresponding proteins from phage PH15 are also included, and the conserved MNTL and GVSS motifs are indicated (arrows) above the alignment. Residues that are identical between three or more sequences are highlighted. Conservation between all six sequences is indicated underneath the alignment. The alignments were generated with Clustal Omega.34
Table 3. List of S. aureus strains used in this study and the prophages and SaPIs contained within them

| Strain     | Genotype                  | Phage | SaPI       | Reference                  |
|------------|---------------------------|-------|------------|----------------------------|
| RN450      | phage-cured version of S. aureus strain RN1 (NCTC8325) | –     | –          | Novick 1967, Novick 1991   |
| RN4220     | Transformable mutant of RN450 | –     | –          | Kreiswirth et al. 1983, Novick 1991 |
| RN10616    | RN4220(80a)               | 80a   | –          | Ubeda et al. 2009          |
| TB25       | RN450(ΔNM1)               | ΔNM1  | –          | Bae et al. 2006           |
| TB26       | RN450(ΔNM2)               | ΔNM2  | –          | Bae et al. 2006           |
| ST1*       | RN4220(ΔSaPI1)            | –     | SaPI1      | Christie et al. 2010      |
| ST137      | RN4220(SaPI1)             | –     | SaPI1      | G.E. Christie, unpublished |
| AD1        | TB25(SaPI1)               | ΔNM1  | SaPI1      | this work                 |
| AD2        | TB26(SaPI1)               | ΔNM2  | SaPI1      | this work                 |
| AD3        | TB25(SaPIbov1)            | ΔNM1  | SaPIbov1   | this work                 |
| AD4        | TB26(SaPIbov1)            | ΔNM2  | SaPIbov1   | this work                 |
| AD5        | RN10616(ΔSaPIbov1)        | 80a   | SaPIbov1   | this work                 |

*Independent isolate, equivalent to strain RN10822.11

Figure 3. Electron micrographs of negatively stained, CsCl gradient-purified formed by induction of the ΔNM1 lysogen TB25 (A) and the ΔNM2 lysogen TB26 (B). Negatively stained transducing particles formed by mobilization of SaPI1 with ΔNM1 (C) and ΔNM2 (D), and by mobilization of SaPIbov1 with ΔNM1 (E) and ΔNM2 (F). Examples of small virions (SV), large virions (LV) and large empty capsids (LE) are indicated. Scale bars equal 100 nm.
To test for mobilization of SaPIbov1, the filtered tN1M1 and tN2M2 phage lysates were used to infect ST137, which is an RN4220 derivative containing SaPIbov1 tet::tmM (Table 3) (G.E. Christie, unpublished data). As a positive control, the cells were infected with an 80ph lysate in parallel. The resulting lysates were purified and analyzed by EM. Both large and small capsids were observed (Fig 3E and F). Aliquots of 0.22 μm filtered lysates from the ST137 infections were used to infect RN4220 (Table 4). These results show that SaPIbov1 tet::tmM DNA had been successfully mobilized by both tN1M1 and tN2M2 and packaged into transducing particles, in spite of the lack of a recognizable Dut protein.

The only substantial sequence homology between 80ph Dut and the corresponding proteins in tN1M1 and tN2M2 are two short, completely conserved motifs: an N-terminal MTNTL sequence and an internal GVSS sequence (Fig 2C). These motifs are outside of the middle region that is divergent between 80ph and Dut1, and that was previously shown to affect the rate of mobilization. In S. enterica phage PHE15, which does not induce SaPIbov1, the MTNTL and GVSS motifs are not conserved, in spite of otherwise relatively high sequence conservation (40% identity). Thus, the ability to bind St and mobilize SaPIbov1 might depend on these motifs. Alternatively, another factor encoded by tN1M1 and tN2M2 could be able to carry out the SaPIbov1 derepression.

Although not yet known, there is evidence that other factors, such as the large and small capsid size, might be related to the differences in the lytic proteins, holin and lysis. To avoid the separation of different types of particles (procapsids, mature capsids, empty mature capsids) into different fractions that is inherent in the CaCl2 gradient purification, these particles were purified only by PEG precipitation and differential centrifugation, and imaged by EM (Fig 4). The samples were not as clean as the CaCl2 gradient-purified particles, but included a representative distribution of procapsids and packaged, mature transducing particles of all sizes. A minimum of 300 particles were counted and measured for each double lysogen. The capsids were divided into four groups by size (large and small) and maturity (procapsids vs. mature capsids) and plotted as a fraction of the total number of capsids (Fig 5). As observed previously many mature capsids are also empty due to abortive DNA packaging or loss of DNA during purification. These are easily distinguishable from procapsids and were grouped together with the small, empty capsids. A small number of aberrant shells that did not fit into any of the categories above were excluded from the analysis.

Capsid size determination. Double lysogens containing a tN1M1 or tN2M2 and SaPI1 or SaPI2 were made by introducing filtered lysates of SaPI1 tet::tmM or SaPIbov1 tet::tmM into TB25 and TB26, and selecting for tetracycline resistance, yielding strains AD1 (tN1M1, SaPI1), AD2 (tN2M2, SaPI1), AD3 (tN1M1, SaPIbov1) and AD4 (tN2M2, SaPIbov1) (Table 3). Each lysate was used to infect RN10616, yielding strain AD5 (80ph, SaPIbov1) (Table 3). RN10628, a double lysogen of 80ph and SaPI1, for which 90% of the resulting capsids were small (Figs 4B and 5). The samples were not as clean as the CaCl2 gradient-purified particles, but included a representative distribution of procapsids and packaged, mature transducing particles of all sizes. A minimum of 300 particles were counted and measured for each double lysogen. The capsids were divided into four groups by size (large and small) and maturity (procapsids vs. mature capsids) and plotted as a fraction of the total number of capsids (Fig 5). As observed previously many mature capsids are also empty due to abortive DNA packaging or loss of DNA during purification. These are easily distinguishable from procapsids and were grouped together with the small, empty capsids. A small number of aberrant shells that did not fit into any of the categories above were excluded from the analysis.

Table 4. Transducing titers (c.f.u./ml) for infections of SaPI-containing strains by phages 80ph, SaPI1, and SaPI2. Numbers for ST1 are an average of three technical replicates

| Recipient strain | 80ph | tN1M1 | tN2M2 |
|------------------|------|------|------|
| ST1 (SaPI1)      | 2.7 × 10^8 (1.6) | 2.0 × 10^8 (1.4) | 6.0 × 10^7 (2.2) |
| ST137 (SaPI2)    | 1.9 × 10^8 (1.3) | 3.6 × 10^7 (1.8) | 3.2 × 10^7 (1.7) |

*Numbers in parentheses were normalized to 80ph for each SaPI.
Formation of small particles is one of the mechanisms by which SaPIs interfere with phage multiplication. Some SaPIs lack homologs of the \(cpmA\) and \(cpmB\) genes\(^5\) and are thus presumed not to change the size of their helpers, although this has not been confirmed experimentally. Nevertheless, the high conservation of these genes in many SaPIs suggests that the size determination does confer an evolutionary advantage on the SaPIs under some circumstances. SaPI mobilization depends on a delicate interplay between phage and SaPI-encoded proteins, and SaPIs have evolved multiple strategies for usurping the gene products encoded by the phage for their own benefit. All these mechanisms contribute to the overall fitness of the SaPI element and its subsequent success and establishment in the \(S. \text{aureus}\) population.

**Materials and Methods**

Growth of bacteria and phages. \(S. \text{aureus}\) strains used are listed in Table 3. \(S. \text{aureus}\) lysogens were grown in CY media at 32°C as previously described.\(^3\) For induction of prophages, mitomycin C was added to 5 \(\mu\)g/ml at OD\(_{600}\) = 0.8. Cells were grown until lysis occurred, as evidenced by a drop in OD\(_{600}\), typically after 3–4 h. The clarified lysates were precipitated with 10% (w/v) PEG 6,000.

**Figure 4.** Electron micrographs of negatively stained, partially purified lysates from the double lysogens AD1 (\(\Phi\)NM1, SaPI1) (A) and AD5 (\(\Phi\)LDs, SaPIbov1) (B). Examples of particles corresponding to small procapsids (SP), small virions (SV), large procapsids (LP) and large virions (LV) are indicated in each panel. Scale bars equal 100 nm.

**Figure 5.** Particle distribution histograms for the mobilization of SaPI1 and SaPIbov1 by phages \(\Phi\)NM1 (white), \(\Phi\)NM2 (black) and \(\Phi\)LDs (hatched). Particles were scored as small procapsids (SP), small virions (SV), large procapsids (LP) and large virions (LV) and plotted as a fraction of the total number of particles scored in each sample.
in 0.5 M NaCl and pelleted at 8,600 g for 20 min. The pellets were resuspended in phage buffer (50 mM Tris- HCl pH 7.8, 100 mM NaCl, 1 mM MgSO4, 4 mM CaCl2) and centrifuged at 8,600 g for 20 min to remove insoluble material. The resulting supernatant was either centrifuged for 20 h at 70,000 rpm in a Beckman Ti70 rotor. Bands were collected and dialyzed against phage buffer.

For phage-induced SaPI mobilization, 50 ml cultures of the appropriate SaPI-containing strains (ST1 or ST137) were grown at 32°C in the presence of 0.5 g/ml tetracycline. At OD600 = 0.5, the cells were infected at an approximate m.o.i. = 1 with phage lysates that had been passed through a 0.22 µm filter to remove any intact cells. The supernatant was collected after 2-3 h of growth, and the resulting transducing particles were purified by PEG precipitation and CsCl gradient centrifugation as described above.

Transduction. For transduction assays, lysates from phage infections of equal volumes of ST1 or ST137 starting cultures were filtered through a 0.22 µm filter. Serial dilutions (100 µl volume) of the filtrates were mixed with an equal volume of RN4220 cells, incubated for 30 min at 32°C and spread on GL agar plates containing 5 µg/ml tetracycline. The plates were incubated at 32°C for 24 h and colonies were counted.

Double lysogens, containing both a SaPI and a prophage, were made similarly, by mixing the filtered lysates from infections of ST137 with the appropriate recipient phage lysogen strain (TB25, TB26 or RN40161), incubating for 30 min at 32°C and spreading on GL agar plates containing 5 µg/ml tetracycline.

After restreaking, single colonies were picked and grown in CY broth with 5 µg/ml tetracycline. For production of particles, the resulting double lysogens were grown and induced with mitomycin C as described for the phage lysogens above. Particles to be used for capsid size measurements were purified by PEG precipitation and differential centrifugation, but were not run on CsCl gradients.

Electron microscopy. The partially purified or CsCl gradient-purified material was diluted in 80 ml dialysis buffer (20 mM Tris- HCl pH 7.8, 50 mM NaCl, 1 mM MgSO4, 1 mM CaCl2), applied to glow-discharged 400 mesh carbon-only grids (Electron Microscopy Sciences), washed with two drops of dialysis buffer, and negatively stained stained with 1% uranyl acetate. The grids were observed in an FEI Tecnai F20 electron microscope operated at 200 kV and imaged with a Gatan Ultrascan 4000 CCD camera at a typical magnification of 65,000x.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here: www.landesbioscience.com/journals/bacteriophage/article/20632

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