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Dual pathogenicity island transfer by piggybacking lateral transduction

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Abstract:	Lateral transduction (LT) is the process by which temperate phages mobilize large sections of bacterial genomes. Despite its importance, LT has only been observed during prophage induction. Here we report that superantigen-carrying staphylococcal pathogenicity islands (SaPIs) employ a related but more versatile and complex mechanism of gene transfer to drive chromosomal hypermobility while self-transferring with additional virulence genes co-opted from the chromosome. We found that after phage infection or prophage induction, activated SaPIs form concatamers in the host chromosome by switching between parallel genomic tracks in replication bubbles. This dynamic lifecycle enables SaPI _{bov1} to piggyback its LT of staphylococcal pathogenicity island <i>vSa_c</i> , which encodes an array of genes involved in host-pathogen interactions, allowing both islands to be mobilized intact and transferred in a single infective particle. Our findings highlight previously unknown roles of pathogenicity islands in bacterial virulence and show that their evolutionary impact extends beyond the genes they carry.

Dual pathogenicity island transfer by piggybacking lateral transduction

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1 **Summary**

2 Lateral transduction (LT) is the process by which temperate phages mobilize large
3 sections of bacterial genomes. Despite its importance, LT has only been observed
4 during prophage induction. Here we report that superantigen-carrying
5 staphylococcal pathogenicity islands (SaPIs) employ a related but more versatile and
6 complex mechanism of gene transfer to drive chromosomal hypermobility while self-
7 transferring with additional virulence genes from the host. We found that after phage
8 infection or prophage induction, activated SaPIs form concatamers in the bacterial
9 chromosome by switching between parallel genomic tracks in replication bubbles. This
10 dynamic life cycle enables SaPI_{bov1} to piggyback its LT of staphylococcal pathogenicity
11 island vSa α , which encodes an array of genes involved in host-pathogen interactions,
12 allowing both islands to be mobilized intact and transferred in a single infective particle.
13 Our findings highlight previously unknown roles of pathogenicity islands in bacterial
14 virulence and show that their evolutionary impact extends beyond the genes they carry.

15

16 **Keywords**

17 Phages, pathogenicity islands, lateral transduction, concatamers, SaPIs, PICIs,
18 *Staphylococcus aureus*, cotransduction

1 **Introduction**

2 Pathogenicity islands are a class of genetic elements in pathogenic bacteria that encode
3 virulence factors and accessory proteins. They are large gene clusters that are regarded
4 as mobile and their acquisition via horizontal gene transfer can transform a benign
5 bacterium into a dangerous pathogen, though mobility has only been demonstrated for a
6 small subset of these elements. The *Staphylococcus aureus* pathogenicity islands
7 (SaPIs) are a family of small (generally 15-18 kilobases) and highly mobile genetic
8 elements that carry genes for superantigens and toxins.^{1,2} They are prototypical members
9 of the phage-inducible chromosomal islands (PICIs) found in gram-positive and gram-
10 negative bacteria,³⁻⁵ and they are regarded as molecular parasites because they exploit
11 bacteriophages (phages), the viruses of bacteria, for their reproduction and
12 dissemination. Normally, SaPIs reside in the chromosomes of their *S. aureus* hosts under
13 the maintenance of their master repressor StI.⁶ Their life cycles are activated by the
14 phages that they parasitize (depicted in Figure S1B), following the formation of a complex
15 between StI and “helper” phage-encoded antirepressor proteins that lift StI repression and
16 initiate the SaPI excision-replication-packaging (ERP) program.⁷

17 In the lysogenic cycle, temperate phages reproduce as DNA (or prophages) in the
18 genomes of their lysogenic bacterial hosts during bacterial cell division. Phage maturation
19 occurs in the lytic cycle, following host cell infection or induction from the lysogenic cycle
20 (depicted in Figure S1A). In both cases, the viral genome often circularizes episomally
21 and undergoes DNA replication to form long head-to-tail concatemers.^{8,9} DNA packaging
22 begins when a phage packaging site (*pac*) is recognized by the phage small terminase
23 (TerS ϕ), which forms hetero-oligomers with the phage large terminase (TerL) to

1 translocate the viral genome into phage heads.^{10,11} When a capsid capacity or “headful”
2 has been reached, a non-specific terminal cut is made to complete DNA packaging.^{12,13}
3 This process is highly efficient and often results in high titers of infectious phage particles.
4 The SaPIs can hijack this process by employing their own small terminase (Ter_{SP}),
5 which pairs with phage TerL to form terminase enzymes that recognize SaPI *pac*
6 sites.^{14,15} SaPI DNA is then packaged into phage heads, leading to extremely high
7 frequencies of intra- and intergeneric transfer.^{16,17}

8 Phage lysates resulting from the infection of a sensitive strain, or the induction of
9 a resident prophage, are mostly comprised of infectious phage particles; but they also
10 contain transducing particles, which contain bacterial DNA that can be transferred from
11 one bacterium to another by a process known as genetic transduction. They are formed
12 in the phage lytic cycle by the mechanisms of specialized (ST), generalized (GT), and
13 lateral transduction (LT).¹⁸ Specialized-transducing particles typically contain DNA from
14 prophages that have aberrantly excised from the chromosome with adjacent host genes
15 still attached.¹⁹ Generalized-transducing particles can contain any bacterial DNA
16 (including chromosomal or plasmid DNA), and they are made when DNA packaging
17 initiates from *pac* site homologs in the host genome.²⁰ In the recently discovered LT, the
18 formation of transducing particles begins when a prophage replicates bidirectionally prior
19 to excision to create multiple integrated phage genomes.^{21,22} Some prophages excise
20 and enter the productive lytic cycle to generate infectious particles, while DNA packaging
21 can also initiate in situ from the *pac* site of integrated prophages to generate transducing
22 particles. In this scenario, the first particle is filled with a prophage-bacteria DNA molecule
23 and then the headful packaging machinery continues in the bacterial chromosome for

1 seven or more successive capsid headfuls. This mechanism only occurs during prophage
2 induction, but it can have a profound impact because it results in the transfer of large
3 spans (several hundred kb) of the bacterial genome at very high frequencies, exceeding
4 that of most mobile genetic elements transferred via conjugation or generalized
5 transduction,²³ without affecting phage production.

6 Here, we report the discovery of a previously unrecognized stage in SaPI life
7 cycles. While it was assumed that SaPIs excise from the chromosome as the first step
8 after helper phage induction (Figure S1B), we found that induced SaPIs replicate before
9 excision, creating multiple islands on parallel strands that switch genomic tracks to form
10 head-to-tail concatamers while still integrated into the host chromosome. This atypical life
11 cycle allows them to engage in SaPI LT to mobilize large sections of the bacterial
12 chromosome at high frequencies, in addition to a second more sophisticated form of LT
13 that produces transducing particles capable of delivering an intact SaPI element with
14 bacterial DNA. As a result of this second mechanism, which we term lateral
15 cotransduction (LcT), two unrelated pathogenicity islands can be packaged intact in a
16 single infective particle and cotransduced to the same host cell at high frequencies, all in
17 parallel to the normal SaPI life cycle. Moreover, unlike phage-mediated LT, which is
18 limited to prophage induction, we found that SaPI LT and LcT overcome the limitations of
19 phage-mediated LT. Thus, in addition to occurring during the lysogenic induction of
20 strains that carry a SaPI and a helper prophage, SaPI LT and LcT occur after the infection
21 of SaPI-positive strains, and even after the infection of strains that do not carry a SaPI if
22 the infecting lysate contains both phage and SaPI particles. All these features make *S.*
23 *aureus* PICIs incredibly powerful transducing agents.

1 **Results**

2 **SaPIs mediate lateral transduction**

3 SaPIs package their DNA using strategies like those employed by their helper phages
4 and are also capable of GT.¹⁴ Therefore, we initiated these studies to determine if SaPIs
5 could also engage in LT. To test this, we used the prototypical SaPIbov1,²⁴ which is
6 clinically relevant because it encodes the genes responsible for the expression of the
7 toxic shock syndrome toxin (TSST-1) and staphylococcal enterotoxins C and L (SEC and
8 SEL). Since the SaPIbov1 *pac* site is positioned in the middle of its genome and promotes
9 unidirectional packaging towards the SaPI toxin genes,¹⁴ we hypothesized that if
10 SaPIbov1 engages in LT, this process would mobilize the bacterial DNA localized
11 downstream (in the directionality of the packaging) of the SaPIbov1 attachment site (*attC*)
12 site at very high frequencies. Importantly, this region contains the vSa α genomic island
13 and phenol-soluble modulins (PSM) α group of toxins (Figure 1A). vSa α is a non-self-
14 mobilizable pathogenicity island that carries an array of 11 genes encoding toxins and
15 lipoproteins involved in host-pathogen interactions,²⁵⁻²⁷ and the PSM α secreted peptides
16 are highly cytotoxic to a wide variety of host cells.^{28,29}

17 To test for SaPI LT, we constructed a set of strains containing a detoxified
18 SaPIbov1 marked with a tetracycline resistance gene (*tetM*) in which we chromosomally
19 inserted cadmium-resistance cassettes (Cd^R) at 10 kb upstream or 10 kb and 16 kb
20 downstream of the SaPIbov1 *attC* site as selectable proxies for host gene transfer. If SaPI
21 LT occurs, our hypothesis was that headful packaging initiated by SaPI terminase from
22 the *pac* site of an integrated SaPIbov1 would reach a phage capsid capacity (~105% of
23 the genome unit length of the classical helper phages or 46 kb) at approximately 38 kb

1 downstream of the SaPI_{bov1} *attC*, which includes vSa α and PSM α . Therefore, if SaPI LT
2 occurs, we would expect high-frequency transfers of the markers localized downstream
3 of the SaPI_{bov1} *attC* site, while the transfer of the marker localized upstream of the
4 SaPI_{bov1} *attC* site would be low-frequency and indicative of GT.

5 These strains were either lysogenized with SaPI_{bov1} helper phage 80 α or with an
6 isogenic non-helper phage (80 α Δ *dut*) deleted for its antirepressor gene and unable to
7 induce SaPI_{bov1}.⁷ Next, the SaPI_{bov1}-containing lysogenic derivatives were induced
8 with mitomycin C to trigger the SOS response to activate the resident prophages. In
9 parallel, non-lysogenic SaPI_{bov1}-positive strains carrying the different Cd^R markers were
10 infected with either 80 α or 80 α Δ *dut*. In both scenarios (prophage induction and phage
11 infection), the resulting lysates were tested as donors of cadmium resistance to a non-
12 lysogenic *S. aureus* strain to determine the existence of SaPI LT.

13 The lysates resulting from prophage induction were first confirmed for SaPI
14 transduction by selection for tetracycline resistance, and as expected, high-frequency
15 SaPI_{bov1} transfer required wild-type helper phage for induction (Figures S1D and S1E).
16 Remarkably, and in support of the existence of SaPI LT, the transfers of Cd^R markers
17 localized downstream of the SaPI_{bov1} *attC* site (+10 kb or +16 kb markers), in the
18 directionality of DNA packaging, were approximately three orders of magnitude greater
19 than that observed for the marker localized upstream of the SaPI_{bov1} *attC* site (-10 kb
20 marker) that was expected to transfer at low frequency by either phage or SaPI GT
21 (Figures 1A and S2A). These results were mirrored with lysates obtained from infecting
22 the non-lysogenic SaPI_{bov1} strains with either helper phage 80 α or non-helper 80 α Δ *dut*
23 (Figure 2A), showing that the initiation of DNA packaging (in situ) occurs during the

1 infection of SaPI-containing strains, which is indicative of the existence of SaPI-LT. Note
2 that this is an important distinction from phage-mediated LT, which is only known to occur
3 during prophage induction and not after phage infection.²¹

4 Another important observation was that SaPI LT occurred with or without induction
5 of the SaPI life cycle. While the results with the 80 α Δdut (non-helper) phage were
6 predictable since we have previously demonstrated that SaPIs remain integrated into the
7 bacterial chromosome and express TerS_{SP} after non-helper prophage induction or phage
8 infection (depicted in Figure S1B),^{14,30} the results after SaPI induction with helper phages
9 were unexpected. SaPIs are thought to excise immediately after induction by helper
10 phages (Figure S1B),^{1,2,31} and this predicted that SaPI LT would not occur because the
11 mechanism requires the SaPI genomes to remain integrated into the bacterial
12 chromosome at the time of DNA packaging. These results revealed that our current
13 understanding of the SaPI life cycle is not correct and that it likely follows a different
14 sequence of events. Additionally, since clinical strains often contain SaPIs and helper and
15 non-helper phages, these results expand the relevance of SaPI LT.

16 To confirm that the increased transfer of the markers downstream of the SaPI_{bov1}
17 *attC* site occurred by SaPI LT, we repeated the induction and infection experiments using
18 SaPI_{bov1} elements with their *terS* genes deleted (SaPI_{bov1} $\Delta terS$). Our hypothesis was
19 that the SaPI TerS_{SP} is absolutely required to specifically initiate in situ packaging from
20 the SaPI *pac* site. This was supported by our results, which showed that when only the
21 phage TerS ϕ was present in the SaPI_{bov1} $\Delta terS$ lysogens, transfers of the markers
22 downstream of the SaPI_{bov1} *attC* were significantly reduced to the same levels observed
23 for the transfer of the marker located upstream of the SaPI_{bov1} *attC* site (Figure 1A).

1 These results suggested that in these mutants, all the markers were mobilized by phage
2 GT.

3 An alternative explanation for the above results was that a strong SaPI pseudo-
4 *pac* site nearby in the bacterial chromosome directed the packaging of the downstream
5 marker. To confirm that packaging of the downstream markers initiated from the
6 integrated island, *TerS_{SP}* was expressed under the control of a tetracycline-inducible
7 promoter in strains lysogenic for 80 α with its *terS* gene deleted (80 α Δ *terS*), with or without
8 SaPI_{bov1} Δ *terS*, during prophage induction. SaPI *pac* sites are not embedded in their
9 terminase genes like they are in most phage genomes,^{14,32} so a SaPI_{bov1} Δ *terS* element
10 can still direct DNA packaging. The resulting lysates were tested for transfer of the +10
11 kb Cd^R marker, and we found that when *TerS_{SP}* was expressed, the marker was
12 transferred at the low levels of GT without SaPI_{bov1} Δ *terS* but was high frequency with
13 SaPI_{bov1} Δ *terS* (Figure S3C). These results confirmed that DNA packaging for SaPI LT
14 was initiated from the SaPI_{bov1} genome.

15 **SaPI LT is widespread and promotes the high-frequency transfer of large sections** 16 **of the bacterial chromosome**

17 Because the +10 kb and +16 kb markers transferred at high frequencies, we predicted
18 that the processive packaging machinery would continue for many more headfuls before
19 the transfer frequencies dropped to levels that were indistinguishable from GT. Many
20 SaPIs encode proteins CpmA and CpmB that redirect phage capsomeres to form capsids
21 that are one-third their normal size,^{30,33} which means that successive headfuls can be any
22 combination of small and large capsids. Here we used the metric of large capsid capacity
23 to measure the maximum coverage of SaPI LT by inserting Cd^R markers into each

1 successive phage 80 α headful, for seven total downstream markers, in non-lysogenic or
2 lysogenic strains that contain SaPI_{bov1}. We found that for lysates generated by induction
3 or infection, strains that expressed TerS_{SP} transferred all seven downstream headful
4 markers at levels well above GT [Figures 1B (normalized by PFU in S3A), 2C, and S3B].
5 These results show that SaPI LT can occur during the lysogenic induction or infection of
6 SaPI-containing strains to transfer large sections of the *S. aureus* chromosome at high
7 frequency.

8 Finally, to broaden the scope of our findings, we investigated if other SaPIs also
9 engage in SaPI LT. This was confirmed, as similar observations were made for several
10 other staphylococcal pathogenicity islands (SaPI₁₁, SaPI₂, and SaPI_{bov2}) (Figures S2D
11 to S2G and S3B), showing that SaPI LT is not limited to SaPI_{bov1}.

12 **SaPI LT occurs via infection of naïve host cells**

13 Lytic events that activate SaPI life cycles result in the simultaneous release of both phage
14 and SaPI particles in the infecting lysates. Host cells can then be serially infected: first
15 with SaPI, then by phage, or vice versa. In the instances when SaPI reaches a cell first,
16 we reasoned that the island could mediate LT in a naïve cell that has never contained a
17 SaPI. To test this, we first constructed a strain with a mutated SaPI_{bov1} *attC* site to serve
18 as a control for transient SaPI integration. Non-lysogenic strains (wt and SaPI_{bov1} *attC*
19 mutant) with downstream Cd^R markers but lacking SaPIs were infected with SaPI_{bov1}
20 phage lysates, and the resulting particles were assayed for LT. We found that 80 α /
21 SaPI_{bov1} infection transferred the 4 kb Cd^R marker approximately 22-fold more than 80 α
22 alone (Figure 2B), in a manner that required an intact SaPI_{bov1} *attC* site. However, when
23 we repeated this experiment using helper phage Φ NM1 and SaPI_{bov1}, transfer of the 4

1 kb Cd^R marker by Φ NM1 / SaPIbov1 infection was 3 orders of magnitude greater than GT
2 in the first headful and remained much higher than GT up to the seventh downstream
3 marker (Figures 2B and 2D). In addition, similar observations were made for SaPI1 and
4 SaPI2 (Figures S2I and S2J). Taken together, the above results show that SaPI LT occurs
5 in the lytic events of SaPI-containing strains and even during the infection of naïve host
6 cells, making it much more versatile than phage LT which is limited to lysogenic induction.

7 **Phage and SaPI lateral transduction combine for chromosome hypermobility**

8 Phage 80 α is not just a SaPI helper, but it also mediates high-frequency LT,²¹ such that
9 phage and SaPI LT can potentially occur in the same cell. To test this, we created strains
10 carrying Cd^R markers downstream of the 80 α *attB* site and Cm^R markers downstream of
11 the SaPIbov1 *attC* site, in the directionality of the packaging for both. These 80 α (+4 kb
12 Cd^R) / SaPIbov1 (+4 kb Cm^R) lysogens were induced and the lysates were tested for the
13 transfer of each marker. We found that both Cd^R (phage LT) and Cm^R (SaPI LT) markers
14 were transferred at frequencies several orders of magnitude greater than GT (Figure 3A),
15 showing that phage and SaPI LT can combine to transfer two distinct regions of the host
16 chromosome at high frequencies. To further illustrate the potential of LT, Φ 85 / SaPIbov1
17 / SaPI3 lysogenic strains with upstream (indicative of GT) and downstream (indicative of
18 LT) Cd^R markers for each element were induced and the lysates were tested for the
19 transfer of cadmium resistance. As shown in Figure 3B, phage and SaPI LT were again
20 complementary and transferred three distinct regions of the bacterial chromosome at high
21 frequencies in a single lytic event.

22 For a more direct visualization of phage and SaPI LT, we purified the infective
23 particles resulting from SOS induction of 80 α / SaPIbov1 lysogens and extracted the DNA

1 for sequencing. The reads were then mapped to the reference *S. aureus* genome NCTC
2 8325 and quantified based on coverage. We found that most of the encapsidated DNA of
3 bacterial origin mapped to the region next to the SaPI_{bov1} and 80 α attachment sites, in
4 the directionality of packaging for SaPI or phage LT (Figure S4). Together, these results
5 confirm that phage and SaPI LT can combine to promote the massive transfer of
6 chromosomal DNA between bacterial strains.

7 **SaPI delayed excision and escape replication**

8 As previously indicated, the observation that SaPIs mediate LT with helper phages
9 suggested that the current model of SaPI excision and episomal replication early upon
10 induction is not correct; rather, delayed excision and replication in the bacterial
11 chromosome are more likely. Bidirectional replication of an integrated prophage creates
12 genomic redundancy that allows for phage maturation and phage LT to proceed in
13 parallel, and we expected the same to occur with the SaPIs. To determine if SaPIs also
14 replicate prior to excision, we checked for escape replication, which is a term normally
15 used to describe when prophages replicate while still attached to the chromosome and
16 amplify the flanking host DNA. To do this, we induced SaPI_{bov1} strains lysogenic for 80 α
17 with mitomycin C or infected non-lysogenic strains with 80 α and collected the total
18 chromosomal DNA over time for whole-genome sequencing. At each time point, we
19 quantified the reads corresponding to SaPI_{bov1} and the adjacent bacterial DNA and
20 represented them as the coverage relative to the average of the whole genome. SOS
21 induction of helper phage lysogens showed the start of SaPI_{bov1} replication and
22 amplification of the adjacent host DNA by 60 minutes, followed by robust SaPI_{bov1}
23 episomal replication and clear amplification of the chromosomal DNA by 120 minutes

1 (Figure 4A), confirming that SaPI_{bov1} replicates in the chromosome before excision. As
2 expected, in non-helper phage lysogens, SaPI_{bov1} DNA was in line with the
3 chromosomal average at all time points because SaPI was not induced (Figure 4B).
4 Furthermore, parallel results were observed with the infection of non-lysogenic strains
5 (Figures 4C and 4D). By comparison, the DNA of both helper and non-helper phages
6 showed clear escape replication by 60 to 120 minutes after induction but not by infection
7 (Figure S5). These results confirmed that following helper phage induction, SaPI_{bov1}
8 initiated bidirectional replication while still attached to the chromosome and amplified the
9 adjacent host DNA, creating the genomic redundancy needed to allow the SaPI life cycle
10 and SaPI LT to proceed in parallel.

11 **SaPI lateral cotransduction**

12 Different types of particles are generated either by the induction of a strain carrying a
13 helper prophage and a SaPI or by the infection of a SaPI-positive strain with a helper
14 phage. Most of these contain either phage or SaPI DNA, but there are also many
15 transducing particles that contain bacterial DNA packaged by phage or SaPI LT. The
16 existence of this mixed population raises the possibility that a recipient can receive DNA
17 from two different types of transducing particles. First, we tested for the frequency of a
18 host cell receiving both types of lateral-transducing particles from a lysate of an 80 α (+4
19 kb Cd^R) / SaPI_{bov1} *tsst::tetM* (+4 kb Cm^R) lysogen. This was done by selecting for
20 recipients of one marker and then scoring for the other. No transductants were obtained
21 that had acquired both chromosomal markers (Table S1), indicating that recipients of both
22 phage (Cd^R) and SaPI (Cm^R) LT events are extremely rare (at this level of detection).
23 Next, we tested for recipients of SaPI transfer and phage or SaPI LT. When we selected

1 for the tetracycline resistance (Tet^R) of SaPIbov1 *tsst::tetM*, we did not obtain any
2 transductants that also received the phage (Cd^R) or SaPI (Cm^R) LT marker. This was
3 expected, given that SaPIbov1 transfer frequencies are generally several orders of
4 magnitude greater than those of phage and SaPI LT (Figures S1D and 3A). Next, when
5 we reversed the order and selected for phage LT first, we still did not observe any
6 transductants with SaPIbov1; but when we selected for SaPI LT (markers +4 or +10 kb
7 Cm^R) first, more than 70% of the transductants had also acquired the island (Figure 5A).
8 Of note, SaPIbov1 cotransduction occurred with a Cm^R marker (+20 kb) inserted after
9 $vSa\alpha$ (Figure S6D), showing that both islands cotransduce to the same recipient cell at
10 high frequencies. Furthermore, lateral cotransduction was also observed with lysates
11 from infections of SaPIbov1-positive strains and naïve infections (Figure 5A). Parallel
12 results were also observed for SaPI1, SaPI2, SaPIbov5, and SaPI PT1028 (Figures S6A
13 and S6B).

14 Importantly, SaPI cotransduction required a helper phage (Figure 5A), which
15 indicated that induction of the SaPI life cycle was necessary; however, the frequency
16 seemed too high to be the result of two transduction events. To confirm this, we
17 deconstructed the donor lysate to require two events for cotransduction by producing
18 SaPIbov1 or SaPI lateral-transducing particles in separate strains and combining them to
19 form a lysate with both particles in the same ratios as when they are induced from a single
20 lysogenic SaPI-positive strain. The reconstituted lysate was high frequency for SaPIbov1
21 transfer and SaPI LT, but the SaPI LT transductants were no longer positive for SaPIbov1
22 (Figure S6C), showing that cotransduction did not occur by two independent events. This
23 result was reiterated when we tested for SaPI cotransduction with Cm^R markers in

1 additional SaPI LT headfuls (Figure 5A and S6C). Selection for markers in the second or
2 third SaPI LT headful did not result in cotransduction of the island, and because these
3 lysates also contained high titers of both types of particles, they also confirmed that
4 cotransduction does not occur by two events. These results showed that cotransduction
5 occurs by a single particle in the first headful. Therefore, activated SaPIs mediate a form
6 of LT with the added feature of SaPI cotransduction that we henceforth refer to as SaPI
7 lateral cotransduction (LcT).

8 **Molecular basis of SaPI lateral cotransduction**

9 We considered two possible mechanisms that could account for LcT: aberrant excision
10 or DNA packaging from tandem SaPIs. The formation of specialized-transducing particles
11 is a classic example of aberrant excision, which typically occurs at extremely low
12 frequencies and results in defective particles.^{18,19} However, SaPI cotransduction was
13 high-frequency and the islands were intact and functional (Figure S6E), so it seemed
14 unlikely that aberrant excision was responsible for LcT.

15 Phage genomes packaged by the headful mechanism are terminally redundant
16 and contain repeated sequences at each end.¹³ Upon injection into a new cell,
17 recombination between DNA containing these sequences generates the circular genome
18 that is important for DNA replication or integration. Based on the importance of terminal
19 repeats to the phage life cycle, we reasoned that LcT could occur by a mechanism in
20 which helper phage induction results in transiently tandem SaPI genomes in the host
21 chromosome (addressed below), whereby terminase initiation from the upstream SaPI
22 element would package roughly 1.5 times a SaPI genome unit length before reaching the
23 adjacent host DNA (Figure 5B). In the case of SaPI_{bov1}, this would fill a large capsid with

1 one genome unit flanked by redundant sequences (in direct repeat) attached to the entire
2 vSa α before reaching headful capacity. To test for transiently tandem SaPIs, we looked
3 for changes in the headful demarcation by a SaPI-sized insertion. We paired a
4 downstream Cm^R marker (+10 kb) with either of two downstream Cd^R markers (+20 kb
5 and +25 kb) that are well within a headful capacity when there is a single SaPI_{bov1}, but
6 would be in two different headfuls when there are tandem islands, and looked for changes
7 in the genetic linkage of the two markers by SaPI LT. To focus on large capsid headful
8 capacity by SaPI LT, we used 80 α Δ *terS* / SaPI_{bov1} Δ *cpmAB* lysogens that were not
9 capable of phage GT and small capsid formation. These strains were induced and tested
10 for cotransduction of the two markers. We found that the Cm^R marker was strongly linked
11 to both Cd^R markers with the non-helper phage 80 α Δ (*dut*, *terS*), but not with the helper
12 phage 80 α Δ *terS* (Figure 5C), indicating that the two markers were mostly in different
13 headfuls when the SaPI_{bov1} life cycle was induced. These results are consistent with an
14 LcT mechanism that involves transiently tandem SaPIs.

15 Induction of the SaPI life cycle but not the formation of classical SaPI particles was
16 required for LcT, indicating that excision and replication were involved. To confirm this,
17 we tested for LcT of the +4 kb Cm^R marker with SaPI_{bov1} deletions of primase-replicase
18 Δ (*pri-rep*), excisionase (Δ *xis*), or integrase (Δ *int*) and found that LcT required all three
19 activities (Figure 6A). Of note, LcT was only observed when integrase was supplied in
20 both donor and recipient cells, indicating that the SaPI DNA was sufficiently redundant to
21 circularize in the recipient for site-specific recombination.

22 Based on these results, we considered two ways tandem SaPI genomes could be
23 formed following escape replication: reintegration of an episomal SaPI in a place that

1 already contains a SaPI or a bridging excision reaction (illustrated in Figure 6B) in which
2 the *attR* of one SaPI recombines with the *attL* of another SaPI. In the first proposed
3 mechanism, it seemed possible that a SaPI that had excised following escape replication
4 could reintegrate next to an integrated island at an *attL* or *attR* site; although, stably
5 tandem SaPIs have never been observed in any *S. aureus* genome sequenced to date.
6 To test if SaPIs can form tandems by integration, we transduced SaPIbov1 *tsst::tetM* into
7 a strain that contains an integrated SaPIbov1 Δ *tsst::ermC*. When we selected for the
8 incoming SaPIbov1 *tsst::tetM*, only 1 out of 300 transductants was positive for both
9 islands (Table S2). However, whole genome sequencing revealed that the double-
10 positive transductant contained islands at two different locations and not in tandem
11 (Figure S6F). Therefore, SaPIbov1 does not detectably insert into SaPIbov1 *attL* and *attR*
12 sites and it seems unlikely that tandem islands are formed by SaPI reintegration.

13 The second putative mechanism for tandem SaPI formation is a bridging excision
14 reaction in which the *attL* and *attR* of two different islands in parallel chromosomal tracks
15 are paired so that recombination between the two sites joins two SaPIs rather than
16 excising one from the chromosome. To determine if intermolecular joining occurs, we
17 used polymerase chain reaction (PCR) to amplify the fusion joints of transiently formed
18 tandem SaPIbov1 islands. To distinguish the fusion joints of tandem SaPIs (formed by
19 intermolecular joining) from those of circularized genomes or concatamers generated
20 from rolling-circle replication, we used two different SaPIbov1 genomes with unique
21 primer binding sites: primer 1875 only anneals to SaPIbov1-1875 and elongates toward
22 the *attR*, and primer 1848 only anneals to SaPIbov1-1848 and elongates toward the *attL*.
23 This primer pair was designed to generate a PCR product only when SaPIbov1-1875 and

1 SaPIbov1-1848 are joined. Next, in a non-lysogenic strain with SaPIbov1-1875 in the
2 native SaPIbov1 *attC* site, we inserted a second SaPIbov1 *attC* site 4 kb downstream. We
3 then integrated SaPIbov1-1848 at the second SaPIbov1 *attC* site so that it has intact *attL*
4 and *attR* sites (Figure 6C). This strain was infected with phage for one hour, the cells
5 were lysed, and the genomic DNA was analyzed by PCR. Primers 1875 and 1848 amplify
6 a 6.2 kb product if no intermolecular joining occurs, and a 1.6 kb product if the two SaPIs
7 are joined. PCR analysis of the infected cells showed that the two SaPIs joined after
8 infection with helper phage 80 α , but not with non-helper 80 α Δdut or mock infection
9 (Figure 6C). These results confirmed that intermolecular joining occurs between SaPIs;
10 however, they did not distinguish if SaPIs from different genomic tracks were joined.

11 To determine if SaPIs are joined from different genomic tracks, we used phage LT
12 to deliver bacterial DNA that contains an integrated SaPIbov1 to emulate a parallel DNA
13 strand of an integrated SaPI undergoing escape replication. The SaPIbov1 *attC* site is in
14 the third LT headful of helper prophage $\Phi 52a$, so we constructed a strain with SaPIbov1-
15 1958 that has a unique binding site for primer 1958 but is deleted for integrase and is
16 completely unable to excise. This SaPI-positive strain was then lysogenized with a non-
17 helper phage derivative of phage $\Phi 52a$ ($\Phi 52a$ Δdut), which is not capable of inducing
18 SaPIbov1 in the donor or recipient strain, and the lysogenic derivative strain was induced
19 to produce a lysate with LT particles containing unexcised SaPIbov1-1958 still attached
20 to bacterial DNA. This lysate was used to infect lysogenic strains carrying SaPIbov1-1848
21 that were induced for 1 hour prior to infection and the genomic DNA was analyzed by
22 PCR with primers 1958 and 1848. Primers 1958 and 1848 can only make a product if
23 SaPIbov1-1958 and SaPIbov1-1848 are covalently joined. PCR analysis of induced

1 recipient cells infected with the $\Phi 52a \Delta dut$ / SaPIbov1-1958 lysate showed that the two
2 SaPIs were joined in strains lysogenic for helper phage 80 α but not with non-helper 80 α
3 Δdut (Figure 6D). No PCR product was observed with infections of phage $\Phi 52a \Delta dut$
4 alone, showing that the primers were specific for tandem genomes created by genomic
5 track switching and not rolling-circle replication of a circular SaPI genome.

6 For additional confirmation of genomic track switching, we next used Nanopore
7 long-read sequencing to capture transiently tandem SaPI genomes in the bacterial
8 chromosome. To avoid inundating the reads with helper phage DNA, we induced the life
9 cycle of SaPIbov1 in a non-lysogenic strain by simultaneously activating the SOS
10 response and expressing 80 α dUTPase (the SaPIbov1 antirepressor) from a tetracycline-
11 inducible promoter. Reads that were long enough to capture tandem SaPIs were relatively
12 rare, but we identified 17 individual reads of SaPI concatamers (14 tandem, 2 triple, and
13 1 quadruple) that were still connected to the bacterial chromosome (Figures S7A to S7C).
14 None of the reads contained host DNA repeated or interspersed between SaPI genomes,
15 indicating they were not the result of rolling-circle replication of aberrantly excised
16 genomes. Taken together, the above results are consistent with the model that bridging
17 excision reactions catalyze genomic track switching that transiently concatamerizes post-
18 replicative SaPI genomes in the bacterial chromosome.

19 **Population analysis reveals genomic signatures of virulence gene transfer by SaPI**

20 **LT**

21 Our in vitro experimental analysis showed that SaPI LT transferred markers inserted into
22 the genomic island vSa α at high frequencies. vSa α is an integral region of the *S. aureus*
23 genome found in virtually all strains examined.²⁶ It contains large clusters of genes

1 involved in innate immune evasion (staphylococcal superantigen-like proteins; SSLs),
2 immune modulation, and invasion (lipoproteins).³⁴ Of note, the SSLs comprise a large
3 family of proteins with distinct receptor tropisms and functions in evading or inhibiting the
4 innate immune response.²⁷ Our findings reveal a mechanism of transfer for this key
5 virulence-associated genetic element that was previously considered immobile. To
6 determine if this mode of gene transfer has a broader impact on natural *S. aureus*
7 populations, we looked for elevated levels of variation in gene content and synteny in the
8 regions downstream of SaPI *attC* sites that could result from recombination events
9 between the chromosome and gene fragments transferred by SaPI LT. There are 5
10 chromosomal sites of integration for SaPIs (SaPI-I to SaPI-V) that are highly conserved
11 across the *S. aureus* species diversity.³⁵ Here we examined the extent of allelic variation
12 at the three chromosomal regions flanking SaPI-II, SaPI-III/SaPI-IV, and SaPI-V. A
13 pangenome analysis of 235 *S. aureus* genome sequences representative of the species
14 diversity was carried out followed by graphical visualization of each region (Figure 7). The
15 resulting networks represent a summary of the variation in gene content and organization
16 that occurs at each region across the *S. aureus* species diversity.

17 Extensive variation in the regions downstream of each site was observed,
18 particularly in the genomic island vSa α . Notably, these regions displayed distinct
19 combinations of genes encoding proteins involved in host-pathogen interactions,
20 indicating that they represent hot spots for genomic diversification. The presence of loops
21 or reticulation in the network implies that rearrangements, insertions, or deletions have
22 occurred in regions of the genome impacted by SaPI-LT. Of note, regions downstream of
23 SaPI-III/SaPI-IV (Fig 7 C) and SaPI-V (Fig 7 D) also contain phage integration sites and

1 resulted in highly complex networks reflecting variation in gene content in integrated
2 phages. We propose that SaPI LT may promote the recombination and reassortment of
3 integrated phage sequences between strains, thereby accelerating phage diversification.
4 Taken together, these data are consistent with the idea that the observed differences in
5 gene content and synteny, as well as the reassortment of virulence gene combinations
6 among natural populations of clinical *S. aureus* isolates, can be attributed to
7 recombination events between the chromosome and DNA transferred by SaPI LT.

8 **Discussion**

9 SaPI life cycles are thought to occur above the chromosome, where rolling-circle
10 replication generates the concatemers needed to package terminally redundant genomic
11 DNA into capsids to form mature SaPI particles.³¹ Here we have discovered an
12 unrecognized stage in the SaPI lifecycle that occurs in the chromosome and is complexed
13 with a form of lateral transduction. In our model (Figure S7D), replication prior to excision
14 creates SaPI genomes on parallel tracks that are identical and thus indistinguishable to
15 SaPI excisionase proteins. The pairing of SaPI *attL* and *attR* repeats on the same strand
16 results in an excision event, while recombination between repeats on different tracks joins
17 SaPI genomes to form tandems and concatamers in the chromosome. From these
18 polymeric structures, LT initiates to mobilize large sections of the genome while producing
19 mature SaPI particles with intact genomes joined to host accessory genes – all in parallel
20 to the normal SaPI life cycle that occurs above the chromosome after late excision. For
21 LcT by SaPI_{bov1}, roughly 1.5 SaPI_{bov1} genomes connected to the vSa α island are
22 packaged and transferred together in a single particle. Upon injection of the hybrid DNA
23 into a new cell (Figure S7E), SaPI_{bov1} DNA undergoes circularization through a single

1 crossover event between repeated sequences and integrates into a SaPI_{bov1} *attC* site,
2 whereas the vSa α island can be acquired or exchanged via homologous recombination
3 with the host chromosome.

4 Recent studies have demonstrated that GT benefits temperate phages by
5 providing adaptive power to their hosts.^{36,37} However, most phages do not benefit directly
6 from their actions since their genomes are rarely delivered intact to the recipients of host
7 genes. This is because bacterial DNA carried in transducing particles usually replaces all
8 or part of the viral genome; though there are special exceptions, such as phage Mu,
9 whose genome is packaged as a monomer flanked by short bits of host DNA.^{38,39} By
10 comparison, SaPI LT drives the massive mobility of large sections of the chromosome at
11 extremely high frequencies to provide adaptive power to host cells, while LcT enables
12 SaPI elements to benefit directly from their own host gene transfer by delivering their
13 genomes intact and attached to chromosomal DNA in the same particle. Furthermore,
14 SaPIs are common in *S. aureus* genomes and most of them occupy one of five
15 chromosomal attachment sites (Table S3);^{2,40} therefore, LcT can pair many different
16 SaPIs with the same chromosomal region, creating diverse combinations of virulence and
17 accessory genes that can be mobilized as single genetic units.

18 For the SaPIs, size matters, and maintaining a small genome appears to be more
19 of a strategy than a coincidence. Many SaPIs encode proteins that divert a percentage of
20 phage virion proteins to form SaPI-sized capsids that are too small for the larger helper
21 phage genomes.^{30,33} Now with LcT, small SaPI genomes can package more than one
22 unit length in a phage capsid and still leave room for over 20 kb of bacterial accessory
23 genes; as in the case of SaPI_{bov1}, which is small enough to include the entire vSa α

1 island from the chromosome so that both elements are mobilized intact in the same
2 infective SaPI particle. Then upon injection into a new host cell, the expanded SaPI
3 genomes circularize by recombining between the repeated sequences and integrate into
4 an *attC* site, regaining their original genome size. Thus, LcT enables SaPIs to exploit the
5 genetic capacity of the host chromosome to store accessory and virulence genes so that
6 they can remain small.

7 Transduction is driven by phages, or so we thought. Here we identified and
8 characterized a powerful mechanism of transduction mediated by pathogenicity islands,
9 showing that transduction is no longer the exclusive domain of phages. We found that
10 SaPI LT and LcT occur during lysogenic induction, infection of SaPI-containing strains,
11 and the infection of strains that do not carry a SaPI, which makes them portable and more
12 versatile than phage LT that has only been observed during prophage induction. We note
13 that this study is the first report of a pathogenicity island mediating lateral transduction
14 and the first demonstration of two unrelated pathogenicity islands mobilized together in a
15 single gene transfer event to the same recipient cell, as well as the first to show a
16 functional transducing agent mobilized together with large pieces of chromosomal DNA.

17 A notable aspect of SaPI regulation is that Ter_{SP} is expressed with both helper
18 and non-helper phages. This means that any lytic interaction with a phage could
19 potentially lead to the high-frequency transmission of hundreds of kb of the host
20 chromosome downstream of a SaPI, regardless of the phage DNA-packaging specificity.
21 Such massive transfers enable core genes and chromosomal islands that are typically
22 immobile to become highly mobile. Furthermore, SaPI LT can be considered analogous
23 to other host-beneficial accessory functions, such as toxins and virulence factors

1 commonly encoded by pathogenicity islands, since TerS_{SP} expression is not tied to the
2 SaPI life cycle.

3 Our population genomic analysis provides evidence for the historical impact of
4 SaPI LT on the *S. aureus* genome. Specifically, the co-incidence of SaPI integration sites
5 with downstream chromosomal islands containing arrays of virulence-associated genes
6 has provided the opportunity for the transfer of key mechanisms of innate immune
7 evasion and cellular invasion. SaPI LT-mediated gene re-assortment could lead to the
8 generation of novel combinations of genes that are beneficial to the recipient.⁴¹
9 Furthermore, hybrid gene variants with attenuated or novel functions may be formed that
10 may confer enhanced fitness in a particular niche.⁴² In conclusion, our results show that
11 SaPIs mediate dynamic and powerful forms of gene transfer and they are one of the most
12 important drivers of pathogen evolution.

13 **Limitations of the study**

14 To our knowledge, genomic track switching is the first report of a mechanism that
15 generates DNA concatamers in the bacterial host chromosome. This finding re-writes
16 SaPI life cycles, and we predict it will also apply to the life cycles of many other
17 chromosomal genetic elements. Here we have used SaPIs as our model system, which
18 are prototypical members of the PICIs. However, we now know that PICIs and other
19 phage satellites are widespread and common in gram-positive and gram-negative
20 bacteria.³⁻⁵ It would be important to determine whether other PICIs also engage in PICI
21 lateral and lateral cotransduction. Another point that requires further investigation is to
22 determine both the advantages that SaPIs confer in promoting LT and LcT, as well as the
23 benefits for the SaPIs themselves. Additional studies are required to fully understand the

1 impact of these processes on the ecology and evolution of the clinically relevant *S.*
2 *aureus*.

3

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13

14 **AUTHOR CONTRIBUTIONS:** J.C. and J.R.P. conceived the study. M.S.J.C., E.S., and
15 Y.N.C. conducted the experiments. J.H-L., R.B., R.M., and J.R.F. performed the
16 genomic analyses. J.C. and J.R.P. wrote the manuscript.

17

18 **DECLARATION OF INTERESTS:**

19 The authors declare no competing interests.

20

21 **INCLUSION AND DIVERSITY:**

22 We support inclusive, diverse, and equitable conduct of research.

23

1 MAIN FIGURE TITLES AND LEGENDS

2 3 **Figure 1. SaPIs transfer large spans of the chromosome at high frequencies by lateral transduction.**

4
5 (A) Transfer of Cd^R markers (purple) 10 kb upstream or downstream of SaPIbov1 *attC*
6 by prophage induction. SaPIbov1 strains lysogenic for helper 80α or non-helper 80α
7 Δ*dut* phage (Φ represents either phage) were SOS induced to generate lysates (top).
8 Prediction for in situ DNA packaging initiated from the *pac* site (red) of an integrated
9 SaPIbov1 (bottom). DNA packaging is unidirectional to the right and the headful limit is
10 ~38 kb downstream of the SaPIbov1 *attC* and includes vSaα and PSMα.

11 (B) Transfer of Cd^R markers (purple) in seven successive headfuls by prophage
12 induction. SaPIbov1 strains lysogenic for 80α were SOS induced to generate lysates.
13 (A and B) Lysogenic SaPIbov1 (Sb1) strains or *terS* deletion (Δ*terS*) derivatives were
14 induced with mitomycin C and the resulting lysates were tested for transduction into *S.*
15 *aureus*. Transduction units (TrU) per milliliter (ml) are represented as the log TrU per
16 milliliter of lysate. TrU per milliliter amounts for all phage / SaPI double *terS* deletion
17 mutants were <10. Values are means (*n* = 3 independent samples). Error bars indicate
18 standard deviation.

19 20 **Figure 2. SaPI lateral transduction mobilizes the genome of non-lysogenic host strains.**

21
22 (A) Transfer of Cd^R markers 10 kb downstream of SaPIbov1 *attC* by phage infection.
23 Non-lysogenic strains without SaPI (No SaPI), carrying SaPIbov1 (Sb1), or a SaPIbov1
24 *terS* deletion (Sb1 Δ*terS*) were infected with helper 80α or non-helper 80α Δ*dut* phage
25 to generate lysates.

26 (B) Transfer of Cd^R markers 4 kb downstream of the SaPIbov1 *attC* by naïve infection.
27 Non-lysogenic strains without SaPI and intact SaPIbov1 *attC* (Cd^R₁) or mutated *attC*
28 (Cd^R₁^{*}) were infected with helper phage only (80α or ΦNM1) or helper phage /
29 SaPIbov1 (80α / Sb1 or ΦNM1 / Sb1) to generate lysates.

30 (C) Transfer of Cd^R markers (purple) in seven successive capsid headfuls by infection.
31 Non-lysogenic SaPIbov1 strains were infected with 80α phage to generate lysates.

32 (D) Transfer of Cd^R markers (purple) in seven successive capsid headfuls by naïve
33 infection. Non-lysogenic strains without SaPI were infected with ΦNM1 phage only or
34 ΦNM1 / SaPIbov1 to generate lysates.

35 (A to D) The resulting lysates were tested for transduction into *S. aureus*. Transduction
36 units (TrU) per milliliter were normalized by plaque-forming units (PFU) per milliliter and
37 represented as the log TrU of an average phage titer (1 x 10¹⁰ PFU). For all panels,
38 values are means (*n* = 3 independent samples). Error bars indicate standard deviation.

39 40 **Figure 3. SaPI lateral transduction complements phage lateral transduction.**

41 (A) Simultaneous transfer of phage 80α and SaPIbov1 LT markers. SaPIbov1 *tsst::tetM*
42 (Sb1) strains lysogenic for helper 80α phage or *terS* deletion (Δ*terS*) derivatives with
43 both 80α phage Cd^R lateral transduction markers 4 kb downstream of the 80α *attB* and
44 SaPIbov1 Cm^R lateral transduction markers 4 kb downstream of the SaPIbov1 *attC* site
45 were induced with mitomycin C.

1 (B) Simultaneous transfer of phage $\Phi 85$, SaPIbov1, and SaPI3 LT markers. Strains
2 lysogenic for $\Phi 85$ phage or $\Phi 85$ / SaPIbov1 *tsst::tetM* (Sb1) / SaPI3 *seb::ermC* (S3)
3 with Cd^R markers 10 kb upstream and 9 kb downstream of the $\Phi 85$ *attB* or 11 kb
4 upstream and 10 kb downstream of the SaPIbov1 *attC* site or 3 kb upstream and 4 kb
5 downstream of the SaPI3 *attC* site were induced with mitomycin C.
6 (A and B) The resulting lysates were tested for transduction into *S. aureus*.
7 Transduction units (TrU) per milliliter are represented as the log TrU per milliliter of
8 lysate. Values are means ($n = 3$ independent samples). Error bars indicate standard
9 deviation.

10

11 **Figure 4. Activated SaPIs replicate in the host chromosome prior to excision.**

12 SaPIbov1 escape replication during helper phage induction or infection. Relative
13 abundance of SaPIbov1 DNA and the chromosome flanking the SaPIbov1 *attC* site. The
14 chromosomal position of SaPIbov1 is indicated in grey. Samples were analyzed at 0
15 (red), 30 (light blue), 60 (green), and 120 minutes (dark blue) after the induction (Ind) of
16 lysogenic strains (A) helper 80 α / SaPIbov1 and (B) non-helper 80 α Δdut / SaPIbov1
17 with mitomycin C or the infection (Inf) of non-lysogenic SaPIbov1 strains with (C) 80 α or
18 (D) 80 α Δdut phage.

19

20 **Figure 5. SaPIs cotransduce with adjacent bacterial DNA and form transient**
21 **tandems in the host chromosome.**

22 (A) Cotransduction of SaPIbov1 with Cm^R markers 4 kb, 10 kb, or headful 2 (HF2)
23 downstream of the SaPIbov1 *attC*. SaPIbov1 *tsst::tetM* (Sb1) lysogenic strains were
24 induced with mitomycin C (Ind), non-lysogenic SaPIbov1 *tsst::tetM* strains were infected
25 with phage only (Inf), or non-lysogenic strains without SaPI were infected with helper
26 phage / SaPIbov1 *tsst::tetM* (Naïve Inf) and the resulting lysates were tested for
27 cotransduction into *S. aureus*.

28 (B) Predictions for SaPI LT with SaPIbov1 or SaPIbov1::*tetM* as singles or tandems with
29 headful (HF) limits.

30 (C) Cotransduction of two markers downstream of SaPIbov1 *attC*. SaPIbov1 *tsst::tetM*
31 $\Delta cpmAB$ strains lysogenic for 80 α $\Delta terS$ or 80 α $\Delta (dut, terS)$ phage with a Cm^R marker
32 at 10 kb and a Cd^R marker at 20 kb or 25 kb were induced with mitomycin C and the
33 lysates tested for cotransduction. Headful limits for a single [HF(Sb1)] or tandem
34 [HF(Sb1-Sb1)] island are indicated.

35 (A and C) For each replicate, 100 Cm^R transductants were tested for Tet^R or Cd^R and
36 the percent cotransduction frequency was represented as (Tet^R or Cd^R / Cm^R) x 100.
37 Values are means ($n = 3$ independent samples). Error bars indicate standard deviation.

38

39 **Figure 6. Activated SaPIs undergo genomic track switching to form concatamers**
40 **in the host chromosome.**

41 (A) Cotransduction of SaPIbov1 mutants with Cm^R markers 4 kb downstream of the
42 SaPIbov1 *attC*. SaPIbov1 *tsst::tetM* $\Delta (pri-rep)$, Δxis , and Δint mutants lysogenic for 80 α
43 were induced with mitomycin C and anhydrotetracycline (ATc) was added for
44 complementation by $P_{tet-pri-rep}^+$, $P_{tet-xis}^+$, or $P_{tet-int}^+$, respectively. The cells were
45 mechanically lysed, and the lysates were tested for cotransduction into *S. aureus* or a
46 recipient that constitutively expresses SaPIbov1 integrase ($\Delta int-c$). For each replicate,

1 100 Cm^R transductants were tested for Tet^R and the percent cotransduction frequency
2 was represented as (Tet^R / Cm^R) x 100. Values are means (*n* = 3 independent
3 samples). Error bars indicate standard deviation.
4 (B) Two types of excision reactions. The SaPIbov1 genome (light blue) replicates in the
5 host chromosome creating parallel DNA tracks. Normal excision occurs between the
6 *attL* and *attR* on the same strand (bottom). Bridging excision occurs between the *attL*
7 and *attR* on different strands, resulting in track switching that joins two SaPIs head to
8 tail (top).
9 (C and D) Genomic track switching forms tandem SaPIs. Lane 1 is the 1 kb DNA ladder
10 for both.
11 (C) Non-lysogenic strains with SaPIbov1-1875 at SaPIbov1 *attC* and SaPIbov1-1848 at
12 the engineered +4 kb SaPIbov1 *attC* site were infected with 80α, 80α Δ*dut*, or mock.
13 PCR analysis with primers 1848 and 1875 amplifies a 1.6 kb product for tandems and
14 6.2 kb for no tandems.
15 (D) SaPIbov1-1848 lysogens were induced and infected with lysates from Φ52A Δ*dut* or
16 Φ52A Δ*dut* / SaPIbov1-1958 induced lysogens. SaPIbov1-1958 is deleted for integrase.
17 PCR analysis with primers 1848 and 1958 amplifies a 2.0 kb product if track switching
18 occurs. Primers 1845 and 1848 amplifies a 1.1 kb control.

19

20 **Figure 7. Gene synteny networks demonstrating allelic variation downstream of**
21 **SaPI integration sites.**

22 (A) Schematic representation of the *S. aureus* genome indicating the SaPI (green) and
23 phage (red) integration sites analyzed in B to D. Arrows indicate the regions included in
24 the network analysis and the direction of packaging by SaPI LT.
25 (B) Gene synteny network for the region SaPI-II to the pathogenicity island vSaα.
26 (C) Gene synteny network for the region SaPI-III/SaPI-IV, encompassing phage
27 integration sites Sa1 and Sa4 (red). Note that the region start (*) is prior to SaPI-III (at
28 801,747 bp, RF122), but thresholds applied to the network have resulted in a division of
29 this section at a high multiplicity node within SaPI-III (see Methods).
30 (D) Gene synteny network for the region SaPI-V, encompassing phage integration sites
31 Sa3 and Sa5 (red).
32 (B to D) Each node represents an allelic variant as defined at the 95% identity
33 threshold, with node size representative of the number of genomes in which the allele is
34 present. Edge thickness represents the number of genomes in which a given pair of
35 alleles neighbor one another. Colors represent the key genomic features, as labelled,
36 which were identified with reference to the strain RF122. Network visualization was
37 created using Graphia v3.0. The distribution of SaPI elements at each SaPI integration
38 site can be found in Table S3.

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1 **Supplemental figure titles and legends 1 to 7**

2
3 **Figure S1. High-frequency SaPI transfer requires helper phages, related to Figure**
4 **1.**

5 (A) Prophages in lysogenic strains are maintained under repression by their CI
6 repressor proteins (top). The phage lytic cycle (bottom). Phage infection of a sensitive
7 host strain or prophage induction by the SOS response activates the lytic cycle
8 (progresses left to right). Upon induction, prophages first replicate bi-directionally in the
9 host chromosome. The phage genome circularizes episomally and undergoes DNA
10 replication to form long head-to-tail concatemers. The phage DNA-packaging machinery
11 is comprised of the small terminase (TerS), large terminase (TerL), and portal proteins.

12 (B) SaPI elements in lysogenic strains are repressed by the SaPI StI master repressor
13 and the host LexA protein (top). The current model for SaPI life cycles (progresses left
14 to right). Helper phages express antirepressor proteins in their lytic cycles (full lytic cycle
15 shown in S1A) that activate SaPI life cycles, resulting in the production of both phage
16 and SaPI particles in the same host cell (middle). The SaPI small terminase (TerS_{SP})
17 proteins complex with phage TerL proteins to form terminase enzymes that now
18 recognize SaPI *pac* sites. Non-helper phages do not encode antirepressor proteins and
19 are unable to activate the SaPI life cycle, so only phage particles are produced
20 (bottom). Of note, the SOS response removes LexA repression, and TerS_{SP} proteins
21 are expressed with helper and non-helper phages.

22 (C) Model for in situ DNA packaging initiated from the *pac* site of an integrated
23 SaPI_{bov1}. The first phage headful limit is ~38 kb downstream of the SaPI_{bov1} *attC* and
24 includes vSa α and PSM α . Chromosomal markers were inserted 4 kb, 10 kb, 20 kb, and
25 25 kb downstream of the SaPI_{bov1} *attC*.

26 (D) SaPI_{bov1} *tsst::tetM* strains lysogenic for helper 80 α or non-helper 80 α Δ *dut* phage
27 were induced with mitomycin C.

28 (E) Non-lysogenic SaPI_{bov1}::*tetM* strains were infected with 80 α or 80 α Δ *dut* phage.

29 (D and E) The resulting lysates were tested for plaque formation or SaPI transduction
30 into *S. aureus*. The *terS* genotypes are indicated as WT (+) and deletion (Δ). No SaPI
31 (NS) is indicated. The results are represented as SaPI-specific transductants (SPST)
32 per milliliter or plaque-forming units (PFU) per milliliter. PFU per milliliter for all phage
33 *terS* deletion mutants or SPST per milliliter amounts for all phage / SaPI double *terS*
34 deletion mutants were <10. Values are means ($n = 3$ independent samples). Error bars
35 indicate standard deviation.

36
37 **Figure S2. SaPIs mediate lateral transduction during prophage induction, phage**
38 **infection, and naïve infection, related to Figure 2.**

39 (A to E) SaPI lateral transduction by prophage induction.

40 (A) SaPI_{bov1} *tsst::tetM* strains lysogenic for helper 80 α or non-helper 80 α Δ *dut* phage
41 (Φ represents either phage) with Cd^R markers 16 kb downstream of the SaPI_{bov1} *attC*
42 were induced with mitomycin C.

43 (B) SaPI_{bov1} *tsst::tetM* strains lysogenic for 80 α or 80 α Δ *dut* with Cm^R markers 4 kb
44 downstream of the SaPI_{bov1} *attC* were induced with mitomycin C.

45 (C) SaPI_{bov1} Δ *tsst::tetM* Δ (*sec-seI*) strains lysogenic for 80 α or 80 α Δ *dut* with Cm^R
46 markers 20 kb downstream of the SaPI_{bov1} *attC* were induced with mitomycin C.

1 (D) SaPI1 *tsst::tetM* strains lysogenic for helper 80 α tested for the transfer of Cd^R
2 markers 3 kb upstream or 4 kb downstream of the SaPIbov1 *attC* were induced with
3 mitomycin C.
4 (E) SaPI2 *tsst::tetM* strains lysogenic for 80 α tested for the transfer of Cd^R markers 4 kb
5 downstream of the SaPIbov1 *attC* were induced with mitomycin C.
6 (F and G) SaPI lateral transduction by infection.
7 (F) SaPI1 *tsst::tetM* non-lysogenic strains with Cd^R markers 3 kb upstream or 4 kb
8 downstream of the SaPIbov1 *attC* were infected with 80 α .
9 (G) SaPI2 *tsst::tetM* non-lysogenic strains with Cd^R markers 4 kb downstream of the
10 SaPIbov1 *attC* were infected with 80 α .
11 (H to J) SaPI lateral transduction by naïve infection. Non-lysogenic strains without SaPI
12 were infected with (H) Φ NM1 or Φ NM1 / SaPIbov1, (I) 80 α or 80 α / SaPI1, and (J) 80 α
13 or 80 α / SaPI2.
14 (A and D to G) The resulting lysates were tested for transduction into *S. aureus*.
15 Transduction units (TrU) per milliliter are represented as the log TrU per milliliter of
16 lysate. TrU per milliliter amounts for all phage / SaPI double *terS* deletion mutants (gray)
17 were <10. Values are means ($n = 3$ independent samples). Error bars indicate standard
18 deviation.
19 (B, C, H to J) The resulting lysates were tested for transduction into *S. aureus*. TrU per
20 milliliter were normalized by plaque-forming units (PFU) per milliliter and represented as
21 the log TrU of an average phage titer (1×10^{10} PFU). For all panels, values are means
22 ($n = 3$ independent samples). Error bars indicate standard deviation.

23

24 **Figure S3. SaPI lateral transduction mobilizes large regions of the host**
25 **chromosome and SaPI terminase is essential for high-frequency SaPI lateral**
26 **transduction, related to Figure 3.**

27 (A and B) Strains lysogenic for 80 α tested for the transfer of Cd^R markers in seven
28 successive headfuls for (A) SaPIbov1 and (B) SaPIbov2. Strains were induced with
29 mitomycin C and the resulting lysates were tested for transduction into *S.*
30 *aureus*. Transduction units (TrU) per milliliter were normalized by plaque-forming units
31 (PFU) per milliliter and represented as the log TrU of an average phage titer (1×10^{10}
32 PFU). For all panels, values are means ($n = 3$ independent samples). Error bars
33 indicate standard deviation.

34 (C) Strains lysogenic for helper 80 α Δ *terS* or non-helper 80 α Δ (*dut*, *terS*) phage with or
35 without SaPIbov1 *tsst::tetM* Δ *terS* and Cd^R markers 10 kb downstream of the SaPIbov1
36 *attC* were induced with mitomycin C and the resulting lysates were tested for
37 transduction into *S. aureus*. Anhydrotetracycline (ATc) was added at the same time for
38 complementation by P_{tet} -*terS*_{Sb1}. The resultant lysates were tested for transduction
39 into *S. aureus*. Transduction units (TrU) per milliliter are represented as the log TrU per
40 milliliter of lysate. TrU per milliliter amounts for no complementation (-ATc) were <10.
41 Values are means ($n = 3$ independent samples). Error bars indicate standard deviation.

42

43 **Figure S4. A direct visualization of lateral transduction by analyzing the capsid**
44 **DNA extracted from purified phage and SaPI particles, related to Figure 4.**

1 (A and B) SaPIbov1 strains lysogenic for helper 80α or non-helper 80α Δdut phage
2 were induced (Ind) with mitomycin C and the resulting phage and SaPI particles were
3 purified.
4 (C and D) Non-lysogenic strains carrying SaPIbov1 were infected (Inf) with 80α or 80α
5 Δdut phage and the resulting phage and SaPI particles were purified.
6 (A to D) The DNA from the phage and SaPI particles were extracted and sequenced.
7 The coverage of chromosomal DNA is represented for (A) 80α and 80α Δdut inductions,
8 (B) SaPIbov1 with helper phage (SaPIbov1) and non-helper phage [SaPIbov1(Δdut)]
9 inductions, (C) 80α and 80α Δdut infections, and (D) SaPIbov1 with helper phage
10 (SaPIbov1) and non-helper phage [SaPIbov1(Δdut)] infections.

11
12 **Figure S5. Phages replicate in the bacterial chromosome following prophage**
13 **induction and not during infection, related to Figure 5.**

14 (A to D) Relative abundance of (A and C) helper 80α or (B and D) non-helper 80α Δdut
15 phage genomic DNA and the chromosomal regions adjacent to the 80α *attB* site for (A
16 and B) induction (Ind) or (C and D) infection (Inf) of non-lysogenic strains. Samples
17 were analyzed at 0 (red), 30 (light blue), 60 (green), and 120 min (dark blue) after
18 induction with mitomycin C or phage infection.

19
20 **Figure S6. Lateral cotransduction occurs when an intact SaPI genome and**
21 **adjacent host chromosomal DNA are packaged together in a single phage capsid,**
22 **related to Figure 6.**

23 (A and B) The SaPI family carries out lateral cotransduction.

24 (A) Cotransduction of SaPI1 *tsst::tetM* or SaPI2 *tsst::tetM* and their *cpmAB* deletion
25 ($\Delta cpmAB$) derivatives with Cm^R markers 4 kb downstream of the SaPI1 *attC* site or
26 SaPI2 *attC* site, respectively. Strains lysogenic for helper 80α phage were induced with
27 mitomycin C (Ind), non-lysogenic SaPI1 *tsst::tetM* or SaPI2 *tsst::tetM* strains were
28 infected with 80α only (Inf), and non-lysogenic strains without SaPI were infected with
29 80α / SaPI1 *tsst::tetM* or 80α / SaPI2 *tsst::tetM* for naïve infections (Naïve Inf).

30 (B) Cotransduction of SaPIbov5 *vwb::ermC* or SaPI PT1028::*ermC* with Cm^R or Cd^R
31 markers 4 kb downstream of the SaPIbov1 *attC* or SaPI4 *attC*, respectively. SaPIbov1
32 and SaPIbov5 (Sb5) share the same SaPIbov1 *attC* site and SaPI PT1028 inserts into
33 the SaPI4 *attC* site. Strains lysogenic for helper 80α phage were induced with
34 mitomycin C (Ind).

35 (C) Cotransduction of SaPIbov1 *tsst::tetM* with Cm^R markers 4 kb, 10 kb, or capsid
36 headful 3 (HF3) downstream of the SaPIbov1 *attC*. Strains lysogenic for non-helper 80α
37 Δdut (teal) or helper 80α (dark blue) phage were induced with mitomycin C. A
38 cotransduction assay with a deconstructed (DC, gray) lysate comprised of two
39 independent donor strain lysates was also tested. A SaPIbov1 *tsst::tetM* strain lysogenic
40 for 80α phage was the SaPI donor, while a SaPIbov1 $\Delta tsst::ermC$ strain lysogenic for
41 80α Δdut with a Cm^R marker 10 kb downstream of the SaPIbov1 *attC* was the lateral
42 marker donor. Both strains were induced with mitomycin C individually and the resultant
43 lysates were combined equally. Cotransduction of the SaPIbov1 *tsst::tetM* with the Cm^R
44 marker 10 kb downstream of the SaPIbov1 *attC* was tested.

1 (D) Cotransduction of SaPIbov1 $\Delta tsst::tetM \Delta(sec-seI)$ strains lysogenic for 80 α or 80 α
 2 Δdut with Cm^R markers 20 kb downstream of the SaPIbov1 *attC* site were induced with
 3 mitomycin C.
 4 (A to D) The resulting lysates were tested for cotransduction into *S. aureus*. For each
 5 replicate, 100 Cm^R or Cd^R transductants were tested for Em^R or Tet^R and the
 6 cotransduction frequency was represented as a percentage, calculated as (Em^R or Tet^R
 7 / Cm^R or Cd^R) x 100. Values are means (*n* = 3 independent samples). Error bars
 8 indicate standard deviation.
 9 (E) SaPIbov1 *tsst::tetM* cotransduction with a Cm^R marker 4 kb downstream of the
 10 SaPIbov1 *attC* into a *S. aureus* strain lysogenic for 80 α . Tet^R and Cm^R transductants
 11 and the original donor strain (80 α / SaPIbov1 *tsst::tetM* with a Cm^R marker 4 kb
 12 downstream) were induced with mitomycin C. The resulting lysates were tested for
 13 SaPIbov1 *tsst::tetM* transduction into *S. aureus*. The results are represented as SaPI-
 14 specific transductants (SPST) per milliliter. Values are means for the original donor
 15 strain (*n* = 3 independent samples). Error bars indicate standard deviation.
 16 (F) One Em^R and Tet^R isolate from Table S2 retained the recipient SaPIbov1::*ermC*
 17 after acquiring the donor SaPIbov1::*tetM*. This isolate was sequenced, and the results
 18 showed that the second SaPIbov1::*tetM* was not in tandem with SaPIbov1::*ermC* but
 19 was located at a second site. PCR analysis with primer set 1364 and 1878 confirmed
 20 the presence of the second SaPIbov1 at a secondary *attC* site. Lane 1 is the 1 kb DNA
 21 ladder and lane 2 is the 100 bp DNA ladder.

22

23 **Figure S7. SaPIs form genomic tandems and concatamers in the host**
 24 **chromosome, related to Figure 7.**

25 Non-lysogenic SaPIbov1 *tsst::tetM* strains were induced with mitomycin C and
 26 anhydrotetracycline was added at the same time for SaPIbov1 de-repression by P_{tet-dut}.
 27 The high molecular weight genomic DNA was prepared and sequenced by Nanopore
 28 long-read sequencing. From over 2 million total reads, 17 unique reads with at least
 29 tandem SaPIs attached to bacterial DNA were recovered. Reads shown here are
 30 alignments with the (A) tandem SaPIbov1 *tsst::tetM* reference genome, (B) triple
 31 SaPIbov1 *tsst::tetM* reference genome, and (C) quadruple SaPIbov1 *tsst::tetM*
 32 reference genome. The quality scores and conservation are shown. Unique reads were
 33 aligned using Qiagen CLC Workbench.

34 (D) Model for SaPI lateral cotransduction in the donor cell. The induced SaPIbov1
 35 genome (light blue) replicates in the host chromosome (1) and amplifies the adjacent
 36 bacterial chromosome including vSa α (light red) and PSM α (green). Normal excision (2)
 37 occurs between the *attL* and *attR* sites on the same strand, while bridging excision (3)
 38 between the *attL* and *attR* sites on different strands results in track switching that joins
 39 two SaPIs head to tail. SaPI terminase initiates DNA packaging (4) and a transducing
 40 particle containing intact SaPIbov1 and vSa α is formed (5). Lateral transduction then
 41 proceeds to mobilize several hundred kb of the chromosome by headful units (6).

42 (E) Model for SaPI lateral cotransduction in the recipient cell. Approximately 1.5
 43 SaPIbov1 genomes attached to the vSa α island are injected into a new cell (1).
 44 SaPIbov1 circularizes through a single crossover event between repeated sequences
 45 (2). The circular SaPIbov1 genome integrates at a SaPIbov1 *attC* site (3). The vSa α

1 island is acquired or exchanged via homologous recombination with the host
2 chromosome (4). Each "X" indicates a crossover event.

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1 **STAR METHODS**
 2 **KEY RESOURCES TABLE**
 3

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bacterial strains, see Table S4		
Phage 80 α	43	NC_009526
Phage 85	44	NC_007050.1
Phage Φ NM1	45	DQ530359
Chemicals, peptides, and recombinant proteins		
Mitomycin C	Sigma-Aldrich	Cat# M0503
Anhydrotetracycline Hydrochloride	Sigma-Aldrich	Cat# 94664
Calcium Chloride	Fisher	Cat# BP510-500
Sodium Citrate	Sigma-Aldrich	Cat# W302600
Ampicillin Sodium Salt	Sigma-Aldrich	Cat# A9518
Chloramphenicol	Fisher	Cat# BP904-100
Erythromycin	Fisher	Cat# BP920-25
Streptomycin Sulfate	Fisher	Cat# BP910-50
Cadmium Chloride	Sigma-Aldrich	Cat# 655198
Tetracycline Hydrochloride	Fisher	Cat# BP912-100
LB Broth	BD Difco	Cat# 244620
Tryptic Soy Broth	Sigma-Aldrich	Cat# 22092
Agar	Fisher	Cat# BP2641-1
Sucrose	Sigma-Aldrich	Cat# 84097
Beadbug Silica Beads	Sigma-Aldrich	Cat# Z763721
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat# M0530L
T4 Polynucleotide Kinase	New England Biolabs	Cat# M0201S
dNTPs	Promega	Cat# U1240
Rapid DNA Ligation	Roche	Cat# 11635379001
Lysostaphin Endopeptidase	AMBI Products LLC	Cat# LSPN-50
Critical commercial assays		
Epicenter Tissue & Cell Lysis Solution	Lucigen	Cat# MTC096H
Epicenter MPC Protein Precipitation Buffer	Lucigen	Cat# MMP095H
GenElute Bacterial Genomic DNA Kit	Sigma-Aldrich	Cat# NA2110
Monarch Genomic DNA purification Kit	New England Biolabs	Cat# T3010L
QIAprep Spin miniprep Kit	Qiagen	Cat# 27106
QIAquick gel extraction Kit	Qiagen	Cat# 28706
Deposited data		
Data for the main and supplemental figures and long-read sequencing.	Mendeley Data	DOI:10.17632/yxfmv6ps4c.1
Oligonucleotides		
Primers, see Table S6		
Recombinant DNA		
Plasmids, see Table S5		
Software and algorithms		
Assembly_dereplicator	46	https://github.com/rrwick/Assembly-Dereplicator

Artemis	47	https://github.com/sanger-pathogens/Artemis
Bedtools v2.30.0	48	https://github.com/arkq5x/bedtools2
Biorender	BioRender Software	https://www.biorender.com/
BLAST v.2.12.0	49	https://ncbi.nlm.nih.gov/
Burrows-Wheeler Alignment Tool v0.7.17	50	https://github.com/lh3/bwa
FastQC v0.11.8	Babraham Bioinformatics	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Chromatiblock v1	51	https://github.com/mjsull/chromatiblock/
Graphia v3.0	52	https://github.com/JDHarlingLee/GraPPLE
GraPPLE	53	https://github.com/JDHarlingLee/GraPPLE
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
ncbi-genome-download	NCBI-genome-download, GitHub Program	https://github.com/kbclin/ncbi-genome-download
Picard-tools v2.1.1	The Picard Toolkit, Broad Institute	http://broadinstitute.github.io/picard/
PIRATE v1.0.4	54	https://github.com/SionBayliss/PIRATE
Prokka v1.14.6	55	https://github.com/tseemann/prokka
SAMtools v1.11	56	https://github.com/samtools/samtools
SeqKit v2.3.1	57	https://github.com/shenwei356/seqkit
Trimmomatic v0.39	58	https://github.com/usadellab/Trimmomatic

1 **RESOURCE AVAILABILITY**

2 **Lead contact**

3 Further information and requests for resources and reagents should be directed to and
4 will be fulfilled by the lead contact, John Chen (micciv@nus.edu).

5 **Materials availability**

6 All unique/stable reagents generated in this study are available from the Lead Contact
7 with a completed Materials Transfer Agreement.

8 **Data and code availability**

9 All data reported in this paper will be shared by the lead contacts upon request or
10 through Mendeley Data (DOI: 10.17632/yxfmv6ps4c.1). This paper does not report
11 original code. Any additional information required to reanalyze the data reported in this
12 paper is available from the lead contacts upon request.

14 **Experimental Model and Study Participant Details**

15 **Phages, bacterial strains, and growth conditions**

16 The phages used in this study are listed in the key resources table. The bacterial strains
17 used in this study are listed in Table S4. *S. aureus* strains were grown in tryptic soy broth
18 (TSB) and tryptic soy agar (TSA) plates. *E. coli* strains were grown in Luria-Bertani (LB)
19 broth or on LB agar plates. Antibiotic-resistant *S. aureus* strains were selected and
20 maintained on 5 $\mu\text{g ml}^{-1}$ erythromycin, 5 $\mu\text{g ml}^{-1}$ tetracycline, 10 $\mu\text{g ml}^{-1}$ chloramphenicol,
21 300 $\mu\text{g ml}^{-1}$ streptomycin, or 0.1 mM CdCl_2 during the strain construction or testing
22 process. For inducible-promoter induction, 31.25 or 62.5 ng ml^{-1} anhydrotetracycline was
23 used. Antibiotic-resistant *E. coli* were selected and maintained on 100 $\mu\text{g ml}^{-1}$ ampicillin.

24 **METHOD DETAILS**

1 **DNA methods**

2 Plasmids and oligonucleotides used in this study are listed in Tables S5 and S6,
3 respectively. The sequences of all phages, SaPIs, and strains were previously
4 accessed from NCBI GenBank. The primers used for cloning and screening in this study
5 were obtained from Integrated DNA Technologies. Sequencing was outsourced to
6 external vendors 1st Base (Singapore) and Macrogen (Singapore). Sequence data were
7 aligned against appropriate reference DNA sequences using SnapGene (GSL Biotech
8 LLC, San Diego, CA). Phusion High-Fidelity DNA polymerase, restriction enzymes, and
9 ligase were purchased from New England Biolabs.

10 ***S. aureus* chromosomal DNA preparation**

11 Cultures of *S. aureus* were inoculated and incubated overnight. Overnight cultures were
12 centrifuged, and cell pellets were washed and resuspended in Buffer P1 (Qiagen,
13 Singapore). Cell suspensions were incubated with 5 µg lysostaphin until cell lysis, 37°C,
14 1 hour, followed by the addition of 5 µg of proteinase K, 37°C, 1 hour. Cell lysis buffer
15 (Epicenter), and MPC protein precipitation buffer (Epicenter) were added to each tube in
16 a stepwise manner and mixed gently prior to centrifugation in pre-chilled centrifuges,
17 4°C, 15000 rpm, 15 minutes. Supernatants were harvested and precipitated with
18 isopropanol. DNA pellets were then collected and washed with 70% ethanol before
19 being air-dried. Once dry, the DNA pellets were gently resuspended in water and left to
20 stand at 4°C overnight.

21 **Allelic exchange**

22 For the generation of insertions in the *S. aureus* chromosome, allelic exchange was
23 performed as previously described.^{14,21,59}

24 **Phage titers and SaPI transductions**

1 Preparations of phage lysates, transduction, and titrations were performed as previously
2 described.^{7,14,21,60} For phage only, or phage and SaPI lysates, lysogens were grown to
3 the mid-logarithmic stage in TSB, normalized to OD₆₀₀=0.5, and adjusted to 2 µg ml⁻¹
4 MC (Sigma) until complete lysis. Phage infection lysates were made by infecting the
5 same density of naive cells with an MOI = 0.1 or MOI = 1.0 until complete lysis. Lysates
6 were then adjusted to 1 µg ml⁻¹ DNase I and 1 µg ml⁻¹ RNase and filter sterilized (0.2
7 µm pore) before use. Phage titers were determined by plaque formation on bacterial
8 lawns of RN450 plated on phage agar. Phage titration results are reported as the
9 number of plaque-forming units (PFU) ml⁻¹. For transductions, RN450 cells were
10 infected for 30 minutes and then adjusted to 100 mM sodium citrate. 3 ml of top agar
11 was added to each reaction and plated by pouring the molten mixture on the
12 appropriate selective agar. The results are reported as the number of transduction units
13 (TrU) ml⁻¹.

14 **Lateral cotransduction analysis**

15 Lysates from a phage infection or lysogen induction of strains containing both a SaPI
16 *tsst::tetM* and a chromosomal marker (Cd^R or Cm^R) at varying distances from the SaPI
17 *attC* site were used to determine SaPI cotransduction frequencies. Chromosomal
18 marker transduction was first selected by plating on appropriate selective agar. The Cd^R
19 or Cm^R transductants of three independent lysates (100 transductants each) were then
20 tested for Tet^R and the frequency was represented as the (Tet^R / Cd^R or Cm^R) x 100%.

21 **Inducible complementation of SaPIbov1 mutants**

22 Lysogens of 80α or with SaPIbov1 *tsst::tetM*, SaPIbov1 *tsst::tetM* Δ(*pri-rep*), SaPIbov1
23 *tsst::tetM* Δ*int*, or SaPIbov1 *tsst::tetM* Δ*xis* were grown to mid-logarithmic phase in TSB,
24 normalized to OD₆₀₀ = 0.5, and MC-induced (T = 0 min). Anhydrotetracycline (31.25 ng

1 ml⁻¹) was added at various time points (0, 30, 60, 90, and 120 minutes or no addition)
2 after mitomycin C for complementation by $P_{Tet}-(pri-rep)/int/xis$. Complementation results
3 at 60 minutes for $P_{tet-pri-rep}^+$ or 90 minutes for $P_{tet-xis}^+$, $P_{tet-int}^+$ showed the highest
4 lateral cotransduction activity and were represented in this work. All cultures were
5 harvested and mechanically lysed 2 hours (26°C, 100 rpm) post-anhydrotetracycline
6 induction in a Precellys 24 (Bertin) with 0.1 mm Beadbug silica beads (Sigma-Aldrich).
7 All lysates were filter sterilized (0.2 µm pore) before use.

8 **Whole genome sequencing (escape replication)**

9 Samples were induced or infected as described in previous sections. At the indicated
10 time points after MC-induction or phage infection, 12 ml of sample was taken for DNA
11 extraction using GenElute Bacterial Genomic DNA kit (Sigma) following the
12 manufacturer's instructions. The DNA was precipitated by 0.3 M NaOAc and 2.25
13 volume of 100% ethanol, then pelleted at 12,000 × g for 30 min at 4 °C and washed
14 once with 1 ml of 70% ethanol. After centrifugation, the DNA pellets were air-dried for
15 30 min and resuspended in 50 µl nuclease-free water. Quality control of DNA samples
16 was tested using Agilent Bioanalyzer 2100 and whole genome sequencing (WGS) was
17 performed at the University of Glasgow Polyomics Facility using Illumina NextSeq500
18 obtaining 2 x 75 bp pair-end reads with DNA PCR free libraries. Trimmed reads were
19 mapped to the appropriate genome: 80α (NC_009526.1), SaPI_{bov1} (AF217235.1), and
20 NTCT 8325 (CP000253).

21 **Whole genome sequencing (capsid DNA)**

22 A total of 100 ml lysates from phage infections or lysogen inductions were produced as
23 described in previous sections. Capsid precipitation and capsid DNA extraction were
24 performed as previously described.²² Quality control of DNA samples was tested using

1 Agilent Bioanalyzer 2100 and WGS was performed at the University of Glasgow
2 Polyomics Facility using Illumina TruSeq DNA Nano library prep, obtaining 2 × 75 bp
3 pair-end reads with DNA PCR free libraries. A total of 3000X bacterial genome
4 coverage, 56 M reads, were generated and trimmed reads were mapped to the
5 appropriate genome: 80α (NC_009526.1), SaPIbov1 (AF217235), and NTCT 8325
6 (CP000253).

7 **Whole genome sequencing analyses**

8 We first used FastQC v0.11.8 to assess the quality of the sequencing reads and
9 Trimmomatic v0.39 to remove adapters and low-quality reads.⁵⁸ Sequencing reads from
10 each experiment were mapped to their respective reference genomes using the
11 Burrows-Wheeler Alignment Tool v0.7.17.⁵⁰ Picard-tools v2.1.1 (Broad Institute) was
12 next used to obtain the bam files, which were merged with SAMtools v1.11,⁵⁶ sorted and
13 indexed; and Bedtools v2.30.0 subcommand *bamtobed* was used to produce the bed
14 files.⁴⁸ We computed the relative coverage over 100 sliding windows along the entire
15 chromosome for each of the experiments. For this, we computed the average
16 coverages across the full genome without phages, which were removed using Bedtools
17 subcommand *subtract*. Subsequently, the coverages across the sliding windows were
18 divided by the chromosomal averages. All the bioinformatic analyses were performed
19 using the Cloud Infrastructure for Microbial Bioinformatics (CLIMB).

20 **Detection of transient SaPI concatamers in the bacterial chromosome by PCR** 21 **analysis**

22 For intermolecular joining (Figure 6C), a non-lysogenic strain with a second SaPIbov1
23 *attC* site inserted +4 kb downstream of the native SaPIbov1 *attC* was constructed. A
24 SaPIbov1 *sec-seI* deletion mutant [SaPIbov1 Δ *tsst::tetM* Δ (*sec-seI*)] or “SaPIbov1-1875”

1 was integrated at the primary SaPIbov1 *attC* site, and SaPIbov1 with synonymous
2 codon changes in the *int* gene (SaPIbov1 $\Delta tsst::cat194 int^{syn}$) or “SaPIbov1-1848” was
3 inserted at the +4 kb secondary SaPIbov1 *attC* site. Thus, both SaPIbov1-1875 and
4 SaPIbov1-1848 have intact *attL* and *attR* sites. This double SaPIbov1-positive strain
5 was infected with phage (80 α , 80 α Δdut , or mock) for one hour and then the cells were
6 harvested and mechanically lysed in a Precellys 24 (Bertin) with 0.1 mm Beadbug silica
7 beads (Sigma-Aldrich). The genomic DNA was then analyzed by PCR. Primer 1875
8 elongates toward the *attR* and only anneals to the *sec-seI* deletion joint of SaPIbov1-
9 1875, while primer 1848 elongates toward the *attL* and only binds to synonymous codon
10 changes in the *int* gene of SaPIbov1-1848. Therefore, PCR analysis with primers 1848
11 and 1875 produces a 1.6 kb product for tandems and 6.2 kb for no tandems.

12 For track switching (Figure 6D), a non-helper mutant of $\Phi 52A$ ($\Phi 52A \Delta dut$) was
13 constructed. Of note, the $\Phi 52A \Delta dut$ mutant does not induce SaPIbov1 in the donor or
14 recipient strains. A SaPIbov1 integrase deletion mutant (SaPIbov1 *tsst::tetM* Δint) or
15 “SaPIbov1-1958” that is unable to excise was integrated into the SaPIbov1 *attC* site,
16 which is located in the third lateral headful of $\Phi 52A$. This lysogenic strain was induced
17 to generate $\Phi 52A$ lateral-transducing particles that contain SaPIbov1-1958 still
18 integrated into bacterial DNA. This lysate was used to infect lysogenic strains carrying
19 SaPIbov1-1848 that were induced for 1 hour prior to infection and the genomic DNA
20 was analyzed by PCR with primers 1958 and 1848. Primer 1958 elongates toward *attR*
21 and anneals to the *seI* gene of SaPIbov1-1958 and it can only make a product with
22 primer 1848 if SaPIbov1-1958 and SaPIbov1-1848 are covalently joined. PCR analysis
23 with primers 1848 and 1958 produces a 2.0 kb product if track switching occurs.
24 Primers 1845 and 1848 produce a 1.1 kb control.

1 **Detection of transient SaPI concatamers in the bacterial chromosome by long-** 2 **read Nanopore sequencing**

3 Bacterial cultures were grown and adjusted to $OD_{600}=0.5$ before MC treatment.

4 Anhydrotetracycline was added to these mixtures after 1 hour. The cells were then
5 harvested by centrifugation 1 or 2 hours after anhydrotetracycline addition.

6 Chromosomal DNA was prepared from the bacterial pellets accordingly as described
7 above. At least six sample replicates from each time point were prepared and analyzed.

8 Microbial Genome Sequencing Centre (USA) performed long-read Nanopore
9 sequencing. The Nanopore reads were mapped to an appropriate template comprising
10 tandem SaPI_{bov1} (AF217235), flanked by +/- 10 kb of *S. aureus* NTCT 8325
11 (CP000253) chromosomal DNA at the SaPI_{bov1} *attC* site using Qiagen CLC Genomics
12 Workbench and SnapGene.

13 **Pangenome synteny network analysis**

14 All complete *S. aureus* assemblies were downloaded from RefSeq (January 2022) using
15 NCBI-genome-download v0.2.9, giving an initial dataset of 693 genomes. This was
16 dereplicated using Assembly_dereplicator v0.1.0 at a threshold of 0.001, reducing the
17 dataset to 236 genomes; 1 further genome was excluded based on mash distance. The
18 remaining 235 genomes were annotated using Prokka v1.14.6 and the pangenome was
19 established using PIRATE v1.0.4 with default thresholds.^{54,55} The synteny map of genes
20 at the 95% identity threshold was recreated as described in,⁵³ and the resulting network
21 was visualized using Graphia v3.0.⁵² Edges of weight 1 were removed from the network,
22 and the network was clustered using the Weighted Louvain Clustering algorithm
23 (granularity = 0.800) to define regions for visualization; some specific nodes were also
24 removed for visual clarity of the resulting network. This network was used to visualize

1 the synteny after the SaPI-I and SaPI-II integration sites (Figure 7B). Flanking
2 coordinates for core genes are taken from the reference genome RF122 (AJ938182).

3 Regions downstream of SaPI-III/IV and SaPI-V were investigated in this network,
4 but due to the complexity of these regions, they were extracted and analyzed
5 individually via the following method. After orientating the 235 genomes to begin at
6 *dnaA* and visualizing their structure via Chromatiblock v1 with RF122 as the reference
7 genome,⁵¹ 22 genomes were excluded from the dataset for the synteny analysis of the
8 SaPI V and SaPI III regions due to large-scale chromosomal rearrangements. Flanking
9 core genes of either end of each individual region was identified by manually inspecting
10 the Prokka-annotated GFF file of the whole RF122 genome in Artemis.⁴⁷ The positions
11 of these flanking genes in the remaining 213 genomes were obtained by BLAST
12 v.2.12.0 and then used to extract the regions within each pair of flanking genes from the
13 whole genomes via SeqKit v2.3.1.^{49,57} Reference co-ordinates from RF122 are
14 1,793,187_bp - 2,056,198_bp for SaPI V and 801,747_bp and 1,136,250_bp for SaPI III.
15 The two extracted regions were annotated with Prokka and processed by PIRATE and
16 GraPPLE as above. The resulting synteny networks were visualised in Graphia, and
17 simplified as follows: edges with weight <3 were removed, and nodes with node degree
18 >35 were removed. This greatly reduces complexity in the network, retaining only the
19 best-represented syntenic connections. For graphical presentation, only the first part of
20 each region is shown, with coordinates as noted in Figure 7.

21 **Distribution of SaPIs across the genome dataset.**

22 For each SaPI integration site, 1000 bp sequences that included integrase and
23 contiguous chromosomal flanking sequence were used to interrogate the database of *S.*
24 *aureus* genome sequences using BLAST v.2.12.0.⁴⁹

1 **Image creation**

2 Some images were created with BioRender. Agreement numbers PW23WE7MCW,
3 AB23WE7VUA, MT23WE8HRL, DW23WE9005, KC23X8TLLK, and BD25G4PYX2.

4

5 **QUANTIFICATION AND STATISTICAL ANALYSIS**

6 The statistical details for each experiment are found in the figure legends. Data are
7 presented as means \pm standard deviation. Individual data points are superimposed onto
8 bar graphs. Statistical analyses were performed with GraphPad Prism (version 9.5.1)
9 and Microsoft Excel (version 16.69.1).

10

11 **EXCEL DATA TABLES**

12

13 **Table S4. Strains used in this study, related to STAR Methods Phages, bacterial**
14 **strains, and growth conditions.**

15

16 **Table S5. Plasmids used in this study, related to STAR DNA methods.**

17

18 **Table S6. Oligonucleotides used in this study, related to STAR DNA methods.**

19

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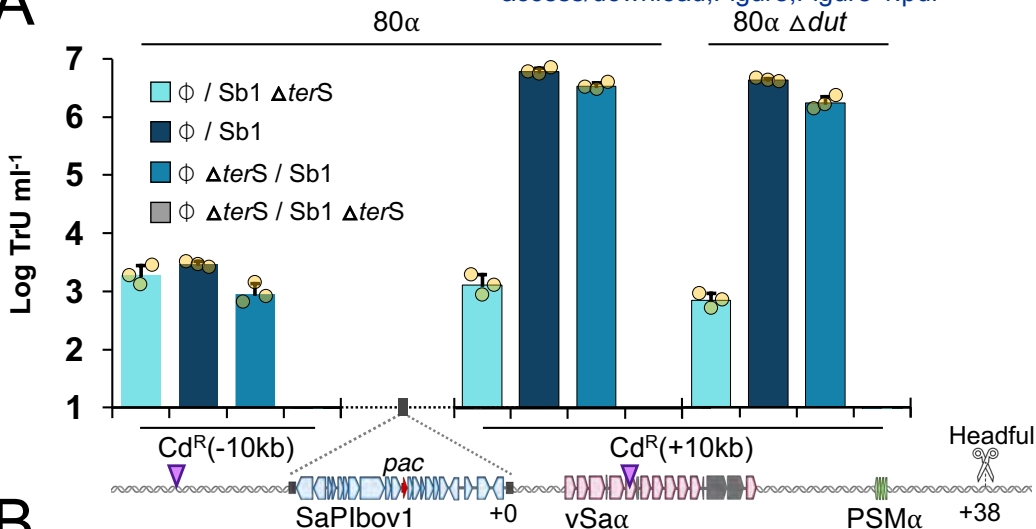
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- 22

Figure 1

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A



B

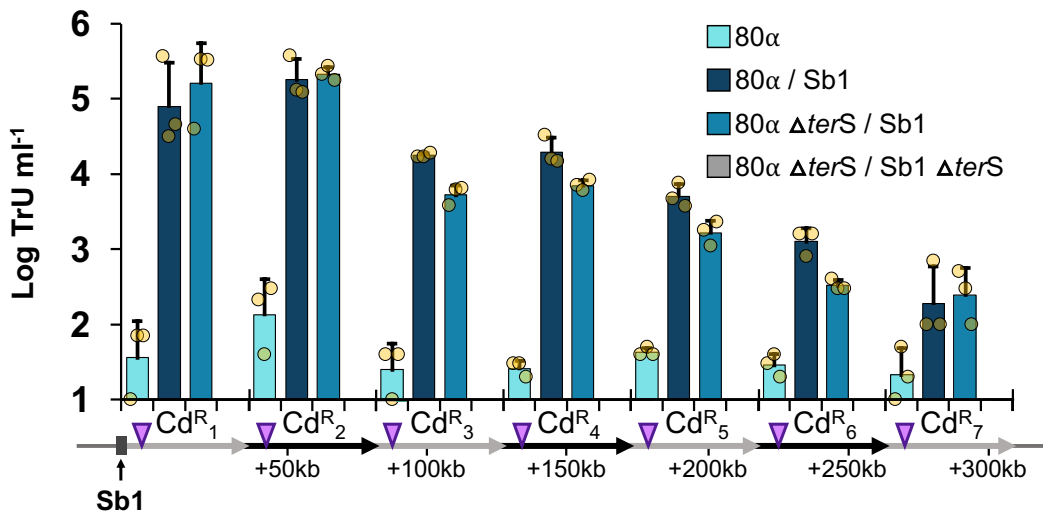
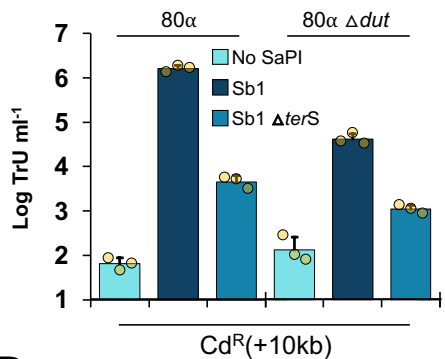
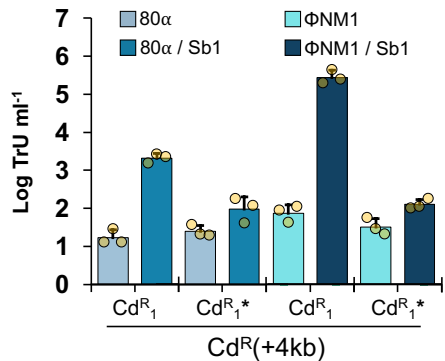


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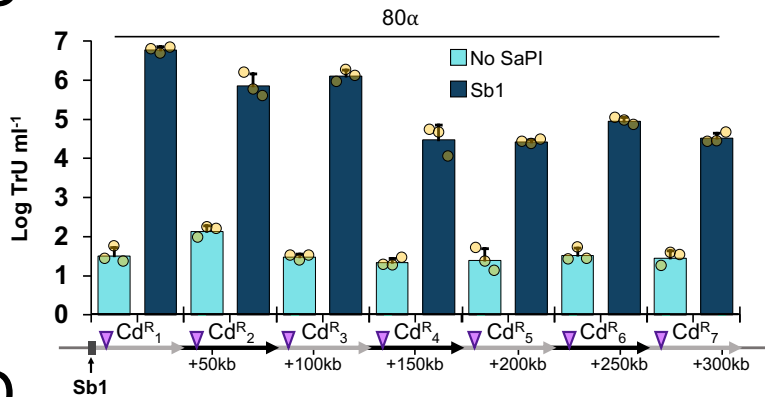
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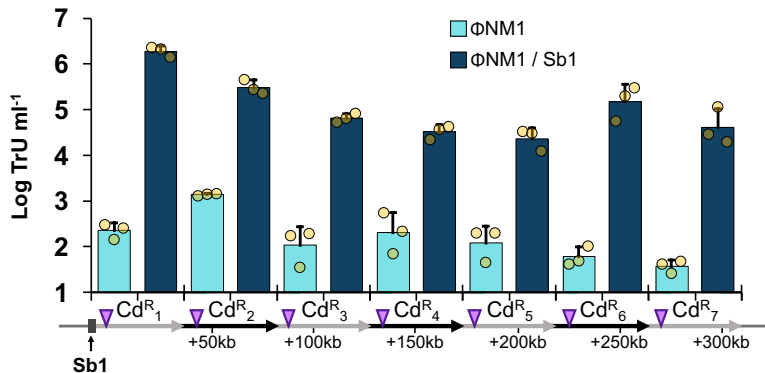
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C



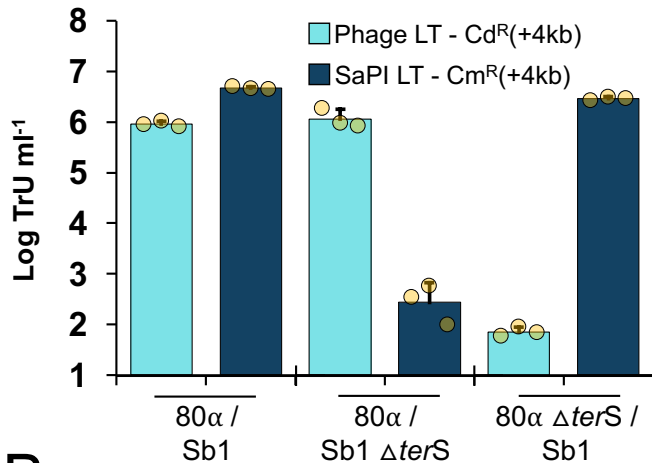
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Figure 3

A



B

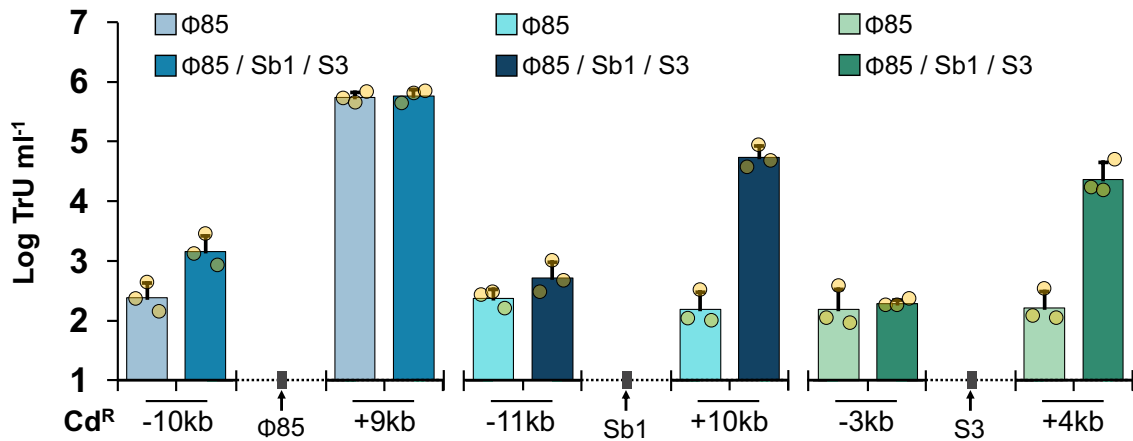
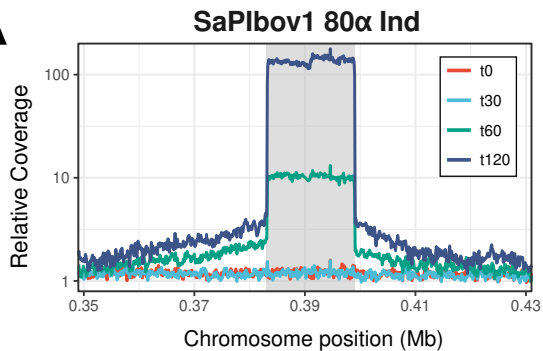
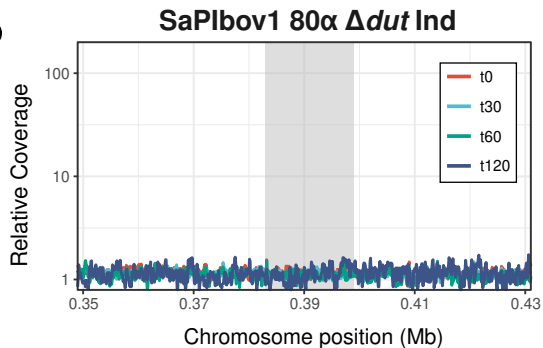


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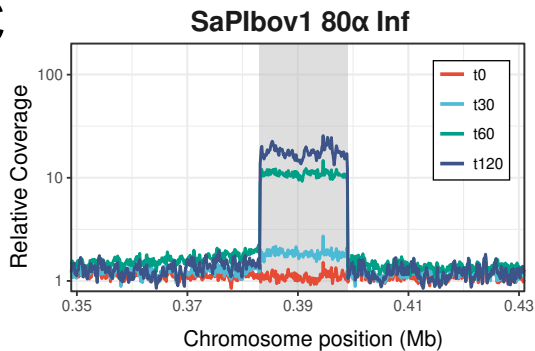
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B

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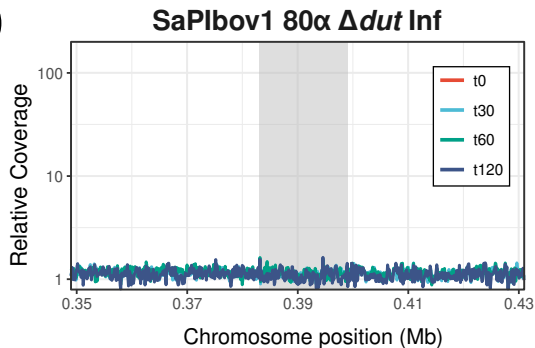


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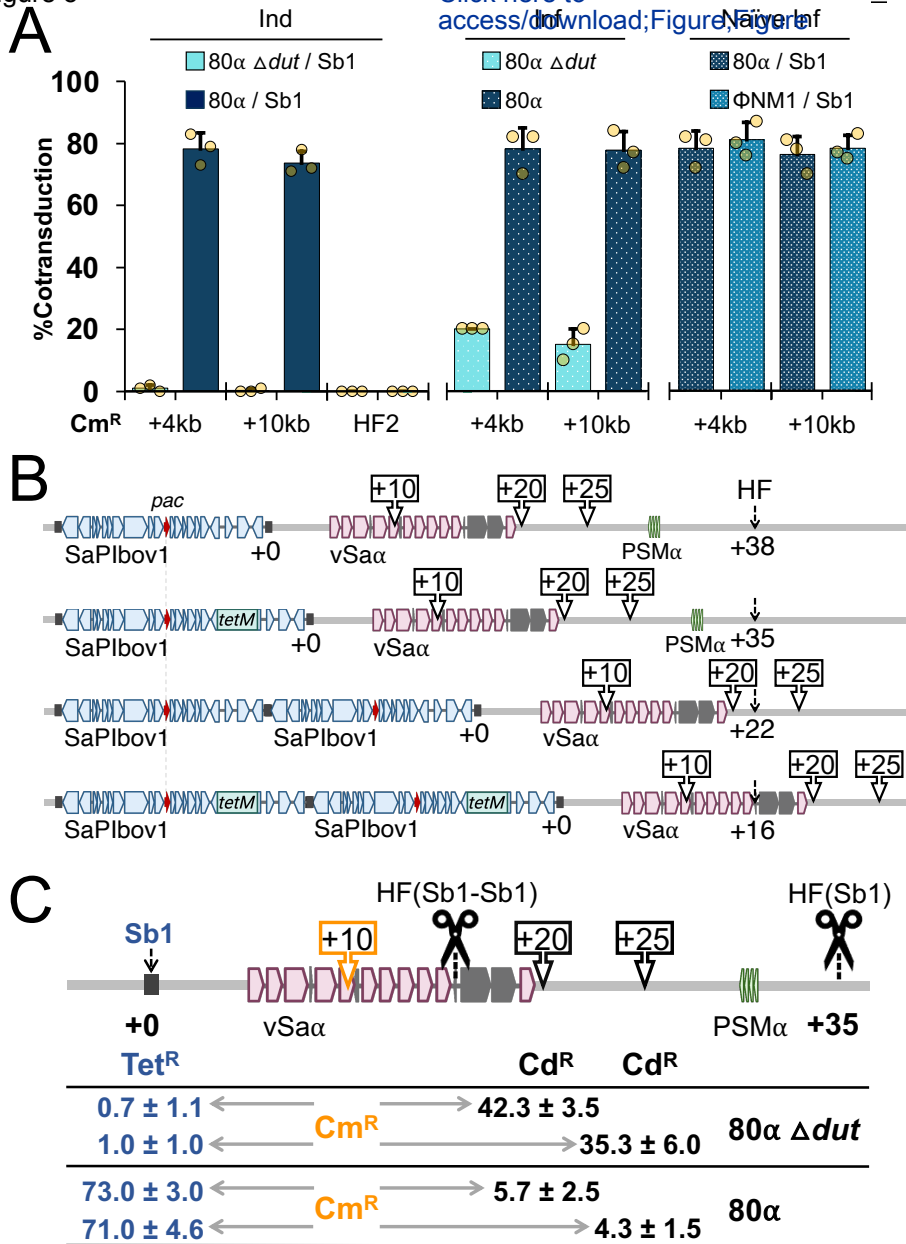
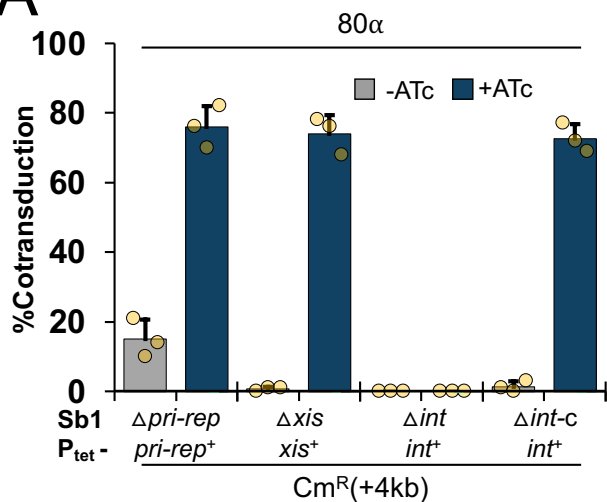
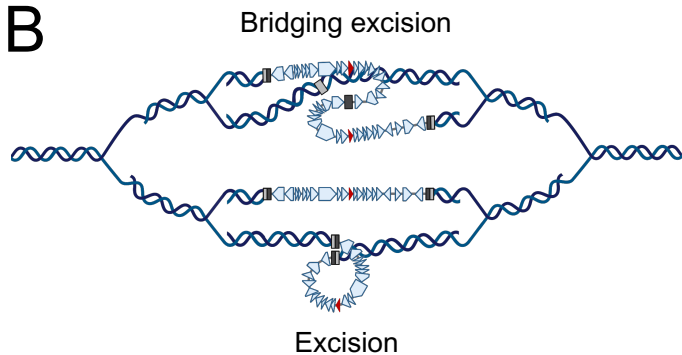
[Click here to access/download;Figure/Figure 5](#)


Figure 6

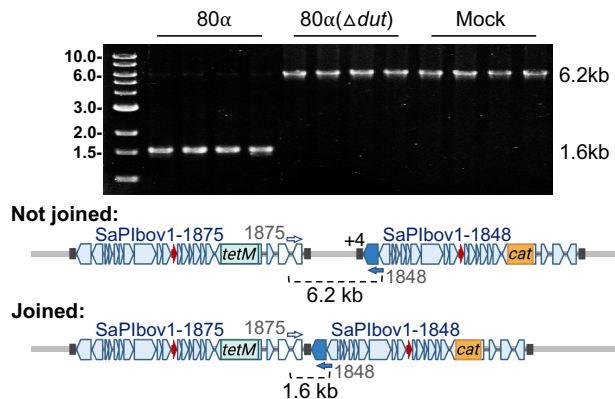
A



B



C



D

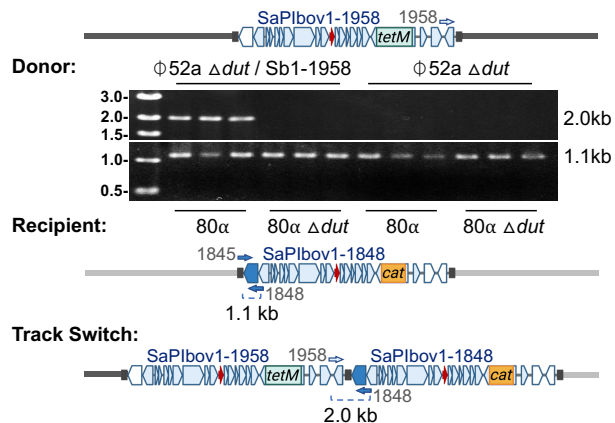


Figure 7

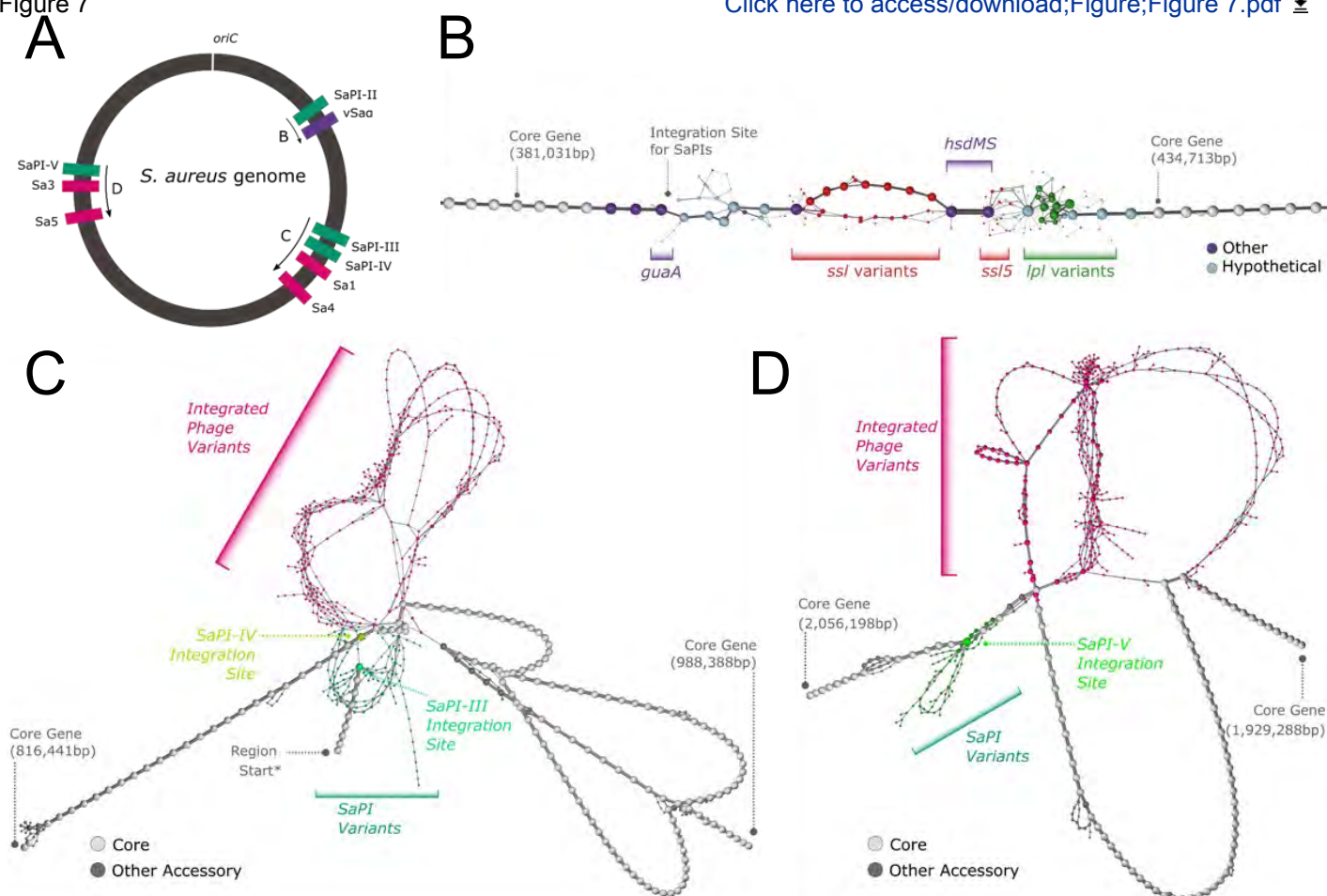
[Click here to access/download;Figure;Figure 7.pdf](#)

Table S4. Strains**Strains*****S. aureus***

RF122

RN450

RN4220

RN10616

JP12871

JP13536

JP13950

JP21092

JP21093

JP21100

JP21101

JP22013

JP22014

JP22015

JP22016

JP22017

JP22018

JP22019

JP22020

JP22278

JP22279

JP22280

JP22281

JP2392

JP22294

JP14151

JP13797

JP14153

JP22534

JP22535

JP22536

JP22537

JP22538

JP22539

JP22548

JP22549

JP22550

JP22551

JP22552

JP22553

JCSA17

JCSA438

JCSA534

JCSA631

JCSA643

JCSA651

JCSA652

JCSA653

JCSA654

JCSA657

JCSA667

JCSA668

JCSA669

JCSA671

JCSA672

JCSA673

JCSA911

JCSA913

JCSA917

JCSA918

JCSA919

JCSA920

JCSA1067

JCSA1085

JCSA1086

JCSA1087

JCSA1089

JCSA1196

JCSA1487

JCSA1488

JCSA1199

JCSA1200

JCSA1201

JCSA1202

JCSA1203

JCSA1204

JCSA1205

JCSA1206

JCSA1207

JCSA1208

JCSA1209

JCSA1210

JCSA1211

JCSA1212

JCSA1213

JCSA1214

JCSA1215

JCSA1216

JCSA1217

JCSA1218

JCSA1219

JCSA1220
JCSA1221
JCSA1222
JCSA1223
JCSA1224
JCSA1225
JCSA1226
JCSA1243
JCSA1244
JCSA1245
JCSA1246
JCSA1247
JCSA1248
JCSA1249
JCSA1250
JCSA1305
JCSA1430
JCSA1431
JCSA1487

JCSA1488

JCSA1794

JCSA1795

JCSA1796

JCSA1797

JCSA1798

JCSA1799

JCSA1800

JCSA1801

JCSA1802

JCSA1803

JCSA1816

JCSA1829

JCSA1830

JCSA1831

JCSA1832

JCSA1833

JCSA1834

JCSA1835

JCSA1836
JCSA1837
JCSA1838
JCSA1839
JCSA1840
JCSA1841
JCSA1842
JCSA1843
JCSA1844
JCSA1845
JCSA1846
JCSA1847
JCSA1848
JCSA1849
JCSA1850
JCSA1851
JCSA1852
JCSA1853
JCSA1854

JCSA1855

JCSA1856

JCSA1857

JCSA1858

JCSA1859

JCSA1860

JCSA1861

JCSA1862

JCSA1863

JCSA1913

JCSA1978

JCSA1979

JCSA1980

JCSA1981

JCSA1982

JCSA1983

JCSA1984

JCSA2013

JCSA2014

JCSA2015

JCSA2016

JCSA2018

JCSA2019

JCSA2020

JCSA2021

JCSA2034

JCSA2035

JCSA2036

JCSA2037

JCSA2038

JCSA2039

JCSA2040

JCSA2041

JCSA2042

JCSA2043

JCSA2044

JCSA2045

JCSA2046

JCSA2047
JCSA2049
JCSA2050
JCSA2051
JCSA2052
JCSA2053
JCSA2054
JCSA2055
JCSA2056
JCSA2057
JCSA2058
JCSA2059
JCSA2060
JCSA2061
JCSA2062
JCSA2068
JCSA2070
JCSA2072
JCSA2074

JCSA2076

JCSA2078

JCSA2105

JCSA2106

JCSA2109

JCSA2110

JCSA2111

JCSA2112

JCSA2113

JCSA2115

JCSA2117

JCSA2118

JCSA2119

JCSA2130

JCSA2136

JCSA2137

JCSA2140

JCSA2141

JCSA2149

JCSA2150
JCSA2202
JCSA2203
JCSA2211
JCSA2212
JCSA2215
JCSA2216
JCSA2217
JCSA2218
JCSA2219
JCSA2220
JCSA2221
JCSA2222
JCSA2223
JCSA2224
JCSA2233
JCSA2234
JCSA2235
JCSA2248

JCSA2249

JCSA2250

JCSA2251

JCSA2253

JCSA2254

JCSA2255

JCSA2287

JCSA2293

JCSA2294

JCSA2295

JCSA2316

JCSA2317

JCSA2319

JCSA2320

JCSA2321

JCSA2322

JCSA2323

JCSA2324

JCSA2325

JCSA2326

JCSA2327

JCSA2328

JCSA2329

JCSA2330

JCSA2345

JCSA2346

JCSA2347

JCSA2348

JCSA2349

JCSA2350

JCSA2351

Strains

s used in this study, related to STAR Methods Phages, bacterial strains, and growth conditions.

Description

Bovine mastitis isolate, SaPIbov1

NCTC8325 cured of Φ 11, Φ 12, and Φ 13

Restriction-defective derivative of RN450

RN4220, 80 α

RN4220 lysogenic for 80 α Δ *ter*S

RN4220 SAOUHSC_00841::*cadCA*; Cd^R marker 3 kb upstream SaPI type IV *attC*

RN4220 SAOUHSC_00848::*cadCA*; Cd^R marker 4 kb downstream SaPI type IV *attC*

JP13536 lysogenic for 80 α

JP13950 lysogenic for 80 α

JP21092, SaPI1 *tsst*::*tetM*

JP21093, SaPI1 *tsst*::*tetM*

JP21092, SaPI1 *tsst*::*tetM* Δ *ter*S

JP21093, SaPI1 *tsst*::*tetM* Δ *ter*S

JP13536 lysogenic for 80 α Δ *ter*S

JP13950 lysogenic for 80 α Δ *ter*S

JP22015, SaPI1 *tsst::tetM*

JP22016, SaPI1 *tsst::tetM*

JP22015, SaPI1 *tsst::tetM* $\Delta terS$

JP22016, SaPI1 *tsst::tetM* $\Delta terS$

JP13536, SaPI1 *tsst::tetM*

JP13950, SaPI1 *tsst::tetM*

JP13536, SaPI1 *tsst::tetM* $\Delta terS$

JP13950, SaPI1 *tsst::tetM* $\Delta terS$

RN450 lysogenic for $\Phi 85$

RN4220 SAOUHSC_00365::*cadCA*; Cd^R marker 11 kb upstream SaPI type II *attC*

RN4220 SAOUHSC_00390::*cadCA*; Cd^R marker 10 kb downstream SaPI type II *attC*

RN4220 SAOUHSC_01064::*cadCA*; Cd^R marker 10 kb upstream Sa7 *attB*

RN4220 SAOUHSC_01091::*cadCA*; Cd^R marker 9 kb downstream Sa7 *attB*

JP13536 lysogenic for $\Phi 85$

JP13950 lysogenic for $\Phi 85$

JP22294 lysogenic for $\Phi 85$

JP14151 lysogenic for $\Phi 85$

JP13797 lysogenic for $\Phi 85$

JP14153 lysogenic for $\Phi 85$

JP22534, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
JP22535, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
JP22536, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
JP22537, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
JP22538, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
JP22539, SaPIbov1 *tsst::tetM*, SaPI3 *seb::ermC*
RN4220 (*rpsL**)
RN450, 80α
JCSA17, SaPIbov1 *tsst::tetM*
RN4220, pJC1693 (SaPI4 *int+*)
JCSA438, 80α Δ*terS*
JCSA438, SaPI1 *tsst::tetM*
JCSA438, SaPI2 *tsst::tetM*
JCSA438, SaPIbov1 *tsst::tetM*
JCSA438, SaPIbov2 *bap::tetM*
JCSA438, SaPIbov1 *tsst::tetM* Δ*terS*
JCSA643, SaPI1 *tsst::tetM*
JCSA643, SaPI2 *tsst::tetM*
JCSA643, SaPIbov1 *tsst::tetM*

JCSA643, SaPI1 *tsst::tetM* Δ *terS*
JCSA643, SaPI2 *tsst::tetM* Δ *terS*
JCSA643, SaPI_{bov1} *tsst::tetM* Δ *terS*
JCSA438, 80 α Δ *dut*
JCSA911, 80 α Δ (*dut*, *terS*)
JCSA911, SaPI_{bov1} *tsst::tetM*
JCSA911, SaPI_{bov1} *tsst::tetM* Δ *terS*
JCSA913, SaPI_{bov1} *tsst::tetM*
JCSA913, SaPI_{bov1} *tsst::tetM* Δ *terS*
JCSA17, *cadCA* 4kb downstream SaPI4 *attC*
RN450, SaPI1 *tsst::tetM*
RN450, SaPI2 *tsst::tetM*
RN450, SaPI_{bov1} *tsst::tetM*
RN450, SaPI_{bov1} *tsst::tetM* Δ *terS*
RN450, Φ 52a
JCSA438, *cadCA* 4 kb downstream 80 α *attC*
JCSA643, *cadCA* 4 kb downstream 80 α *attC*
JCSA438, *cadCA* 10 kb downstream SaPI_{bov1} *attC*
RN450, *cadCA* 10 kb downstream SaPI_{bov1} *attC*

JCSA1087, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA1089, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA438, *cadCA* 16 kb downstream SaPIbov1 *attC*
RN450, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA1087, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA1089, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA653, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA657, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA669, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA673, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA653, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA657, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA669, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA673, *cadCA* 16 kb downstream SaPIbov1 *attC*
JCSA917, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA918, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA919, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA920, *cadCA* 10 kb downstream SaPIbov1 *attC*
JCSA917, *cadCA* 16 kb downstream SaPIbov1 *attC*

JCSA918, *cadCA* 16 kb downstream SaPI_{bov1} *attC*
JCSA919, *cadCA* 16 kb downstream SaPI_{bov1} *attC*
JCSA920, *cadCA* 16 kb downstream SaPI_{bov1} *attC*
JCSA643, *cadCA* 10 kb downstream SaPI_{bov1} *attC*
JCSA643, *cadCA* 16 kb downstream SaPI_{bov1} *attC*
JCSA913, *cadCA* 10 kb downstream SaPI_{bov1} *attC*
JCSA913, *cadCA* 16 kb downstream SaPI_{bov1} *attC*
JCSA1223, SaPI4 *attC*::pJC1746
JCSA1210, SaPI4 *attC*::pJC1746
JCSA1225, SaPI4 *attC*::pJC1746
JCSA1218, SaPI4 *attC*::pJC1746
JCSA1224, SaPI4 *attC*::pJC1746
JCSA1214, SaPI4 *attC*::pJC1746
JCSA1226, SaPI4 *attC*::pJC1746
JCSA1222, SaPI4 *attC*::pJC1746
RN4220, Δ *recA* pJC2094 allele exchanged
RF122, SaPI_{bov1} Δ *tsst*::*tetM* pJC2127 allele exchange
JCSA1430, SaPI_{bov1} Δ *tsst*::*tetM* Δ (*sel-sec*) pJC2126 allele exchange
JCSA438, *cadCA* 4 kb downstream 80 α *attB*

JCSA643, *cadCA* 4 kb downstream 80 α *attB*
RN450, *cadCA* 4 kb downstream SaPI1 *attC* pJC2485 allele exchange
JCSA438, *cadCA* 4 kb downstream SaPI1 *attC*
JCSA651, *cadCA* 4 kb downstream SaPI1 *attC*
JCSA667, *cadCA* 4 kb downstream SaPI1 *attC*
JCSA671, *cadCA* 4 kb downstream SaPI1 *attC*
RN450, *cadCA* 4 kb downstream SaPI2 *attC* pJC2489 allele exchange
JCSA438, *cadCA* 4 kb downstream SaPI2 *attC*
JCSA651, *cadCA* 4 kb downstream SaPI2 *attC*
JCSA667, *cadCA* 4 kb downstream SaPI2 *attC*
JCSA671, *cadCA* 4 kb downstream SaPI2 *attC*
JCSA534, SaPI_{bov1} Δ *tsst::cat194* pJC2674 allele exchange
RN450, *cadCA* 1st headful downstream of SaPI_{bov1} *attC* pJC2247 allele exchange
RN450, *cadCA* 2nd headful downstream of SaPI_{bov1} *attC* pJC2300 allele exchange
RN450, *cadCA* 3rd headful downstream of SaPI_{bov1} *attC* pJC2616 allele exchange
RN450, *cadCA* 4th headful downstream of SaPI_{bov1} *attC* pJC2617 allele exchange
RN450, *cadCA* 5th headful downstream of SaPI_{bov1} *attC* pJC2618 allele exchange
RN450, *cadCA* 6th headful downstream of SaPI_{bov1} *attC* pJC2301 allele exchange
RN450, *cadCA* 7th headful downstream of SaPI_{bov1} *attC* pJC2302 allele exchange

JCSA438, *cadCA* 1st headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 2nd headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 3rd headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 4th headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 5th headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 6th headful downstream of SaPIbov1 *attC*
JCSA438, *cadCA* 7th headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 1st headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 2nd headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 3rd headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 4th headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 5th headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 6th headful downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 7th headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 1st headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 2nd headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 3rd headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 4th headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 5th headful downstream of SaPIbov1 *attC*

JCSA669, *cadCA* 6th headful downstream of SaPIbov1 *attC*
JCSA669, *cadCA* 7th headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 1st headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 2nd headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 3rd headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 4th headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 5th headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 6th headful downstream of SaPIbov1 *attC*
JCSA673, *cadCA* 7th headful downstream of SaPIbov1 *attC*
JCSA653, SaPIbov1 *tsst::tetM* Δ (*pri-rep*) pJC2772 allele exchange
JCSA1829, SaPIbov1 *tsst::tetM*
JCSA1830, SaPIbov1 *tsst::tetM*
JCSA1831, SaPIbov1 *tsst::tetM*
JCSA1832, SaPIbov1 *tsst::tetM*
JCSA1833, SaPIbov1 *tsst::tetM*
JCSA1834, SaPIbov1 *tsst::tetM*
JCSA1835, SaPIbov1 *tsst::tetM*
JCSA1487, SaPIbov1 *tsst::tetM*, *cat194* 4 kb downstream of SaPIbov1 *attC* pJC2909 allele exchange
JCSA1487, SaPIbov1 *tsst::tetM* Δ *terS*, *cat194* 4 kb downstream of SaPIbov1 *attC* pJC2909 allele exchange

JCSA1488, SaPIbov1 *tsst::tetM*, *cat194* 4 kb downstream of SaPIbov1 *attC*, pJC2909 allele exchange
JCSA1488, SaPIbov1 *tsst::tetM* $\Delta terS$, *cat194* 4 kb downstream of SaPIbov1 *attC*, pJC2909 allele exchange
JCSA438, *cat194* 10 kb downstream of SaPIbov1 *attC* pJC2884 allele exchange
JCSA2018, SaPIbov1 *tsst::tetM*
JCSA911, *cat194* 10 kb downstream of SaPIbov1 *attC* pJC2884 allele exchange
JCSA2020, SaPIbov1 *tsst::tetM*
RN450, *cadCA* 1st headful upstream of SaPIbov1 *attC* pJC2869 allele exchange
JCSA438, *cadCA* 1st headful upstream of SaPIbov1 *attC*
JCSA643, *cadCA* 1st headful upstream of SaPIbov1 *attC*
JCSA653, *cadCA* 1st headful upstream of SaPIbov1 *attC*
JCSA657, *cadCA* 1st headful upstream of SaPIbov1 *attC*
JCSA669, *cadCA* 1st headful upstream of SaPIbov1 *attC*
JCSA673, *cadCA* 1st headful upstream of SaPIbov1 *attC*
RN450, *cadCA* 4 kb downstream of SaPIbov1 *attC* pJC2247 allele exchange
JCSA438, *cadCA* 4 kb downstream of SaPIbov1 *attC*
JCSA643, *cadCA* 4 kb downstream of SaPIbov1 *attC*
JCSA653, *cadCA* 4 kb downstream of SaPIbov1 *attC*
JCSA657, *cadCA* 4 kb downstream of SaPIbov1 *attC*
JCSA667, *cadCA* 4 kb downstream of SaPIbov1 *attC*

JCSA673, *cadCA* 4 kb downstream of SaPIbov1 *attC*
RN450, SaPIbov1 *attC** synonymous mutations pJC2626 allele exchange
JCSA2049, *cadCA* 4 kb downstream of SaPIbov1 *attC*
JCSA438, *cat194* 4 kb downstream of SaPIbov1 *attC*
JCSA911, *cat194* 4 kb downstream of SaPIbov1 *attC*
JCSA438, *cat194* 2nd headful downstream of SaPIbov1 *attC*
JCSA911, *cat194* 2nd headful downstream of SaPIbov1 *attC*
JCSA438, *cat194* 3rd headful downstream of SaPIbov1 *attC*
JCSA911, *cat194* 3rd headful downstream of SaPIbov1 *attC*
JCSA2051, SaPIbov1 *tsst::tetM*
JCSA2052, SaPIbov1 *tsst::tetM*
JCSA2053, SaPIbov1 *tsst::tetM*
JCSA2054, SaPIbov1 *tsst::tetM*
JCSA2055, SaPIbov1 *tsst::tetM*
JCSA2056, SaPIbov1 *tsst::tetM*
JCSA438, *cat194* 4 kb downstream of SaPI1 *attC*
JCSA438, *cat194* 4 kb downstream of SaPI2 *attC*
JCSA2068, SaPI1 *tsst::tetM*
JCSA2068, SaPI1 *tsst::tetM* Δ *cpmAB*

JCSA2070, SaPI2 *tsst::tetM*
JCSA2070, SaPI2 *tsst::tetM* Δ *cpmAB*
JCSA2051, SaPIbov5 *vwb::ermC*
JCSA2052, SaPIbov5 *vwb::ermC*
JCSA438, *cadCA* 4 kb downstream SaPI4 *attC*
JCSA911, *cadCA* 4 kb downstream SaPI4 *attC*
JCSA2109, SaPIpT1028::*ermC*
JCSA2110, SaPIpT1028::*ermC*
JCSA2051, SaPIbov1 *tsst::tetM* Δ *int*
JCSA2051, SaPIbov1 *tsst::tetM* Δ *xis*
JCSA1085, *cat194* 4 kb downstream of SaPI1 *attC*
JCSA1086, *cat194* 4 kb downstream of SaPI2 *attC*
JCSA1087, *cat194* 4 kb downstream of SaPIbov1 *attC*
JCSA2051, SaPIbov1 *tsst::tetM* Δ (*pri-rep*)
JCSA643, *cat194* 10 kb downstream of SaPIbov1 *attC* pJC2884 allele exchange
JCSA913, *cat194* 10 kb downstream of SaPIbov1 *attC* pJC2884 allele exchange
JCSA2136, *cadCA* 25 kb downstream of SaPIbov1 *attC* pJC2922 allele exchange
JCSA2137, *cadCA* 25 kb downstream of SaPIbov1 *attC* pJC2922 allele exchange
JCSA2040, SaPIbov1 *tsst::tetM* Δ *cpmAB*

JCSA2041, SaPIbov1 *tsst::tetM ΔcpmAB*

JCSA2136, SaPIbov1 *tsst::tetM ΔcpmAB, cadCA* 20 kb downstream of SaPIbov1 *attC* pJC2921 allele exchange

JCSA2137, SaPIbov1 *tsst::tetM ΔcpmAB, cadCA* 20 kb downstream of SaPIbov1 *attC* pJC2921 allele exchange

JCSA2051, SaPIbov1 *Δtsst::tetM Δ(sel-sec)*

JCSA2052, SaPIbov1 *Δtsst::tetM Δ(sel-sec)*

JCSA2211, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site) pJC2964 allele exchange

JCSA2212, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site) pJC2964 allele exchange

JCSA1305, SaPIbov1 *Δtsst::ermC*

RN450, *cat194* 4 kb downstream of SaPI1 *attC*

JCSA2218, SaPI1 *tsst::tetM*

RN450, *cat194* 4 kb downstream of SaPI2 *attC*

JCSA2220, SaPI2 *tsst::tetM*

RN450, *cat194* 4 kb downstream of SaPIbov1 *attC*

JCSA2222, SaPIbov1 *tsst::tetM*

JCSA1816, SaPIbov1 *Δtsst::cat194 int^{syn}* synonymous mutations pJC2984 allele exchange

JCSA2222, SaPIbov1 *Δtsst::tetM Δ(sel-sec)*

JCSA2233, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site) pJC2964 allele exchange

JCSA2234, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site)::SaPIbov1 *Δtsst::cat194 int^{syn}* synonymous mutatio

RF122, SaPIbov1 *Δtsst::cat194 int^{syn}* synonymous mutations

JCSA2248, SaPIbov1 $\Delta tsst::cat194 int^{syn}$ synonymous mutations, $\Delta(sel-sec)$ allele exchange pJC2126

JCSA2223, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site) pJC2964 allele exchange

JCSA2250, (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site)::SaPIbov1 $\Delta tsst::cat194 int^{syn}$ synonymous mutatio

JCSA17, SaPIbov1 $\Delta tsst::tetM \Delta(sel-sec)$

JCSA2253, SaPIbov1 $\Delta tsst::cat194 \Delta(sel-sec)$ pJC2847 allele exchange

JCSA2254, SaPIbov1 $\Delta tsst::cat194 int^{syn}$ synonymous mutations, $\Delta(sel-sec)$ pJC2984 allele exchange

RN450, pJC3056 (SaPIbov1 *int+*)

JCSA2130, SaPIbov1 $tsst::tetM \Delta(pri-rep)$, SaPI1 *attC*::pJC2791 (SaPIbov1 *pri-rep+*)

JCSA2115, SaPIbov1 $tsst::tetM \Delta xis$, SaPI1 *attC*::pJC2786 (SaPIbov1 *xis+*)

JCSA2130, SaPIbov1 $tsst::tetM \Delta int$, SaPI1 *attC*::pJC3038 (SaPIbov1 *int+*)

JCSA1196, $\Phi 52a \Delta dut$ pJC3067 allele exchange

JCSA2316, SaPIbov1 $tsst::tetM \Delta int$

JCSA438, SaPIbov1 $\Delta tsst::cat194 int^{syn}$ synonymous mutations, $\Delta(sel-sec)$

JCSA911, SaPIbov1 $\Delta tsst::cat194 int^{syn}$ synonymous mutations, $\Delta(sel-sec)$

RN450, *cat194* 10 kb downstream of SaPIbov1 *attC*

JCSA232, SaPIbov1 $tsst::tetM$

RN450, *cat194* 20 kb downstream of SaPIbov1 *attC* pJC2945 allele exchange

RN438, *cat194* 20 kb downstream of SaPIbov1 *attC*

RN911, *cat194* 20 kb downstream of SaPIbov1 *attC*

JCSA2323, SaPIbov1 $\Delta tsst::tetM$ $\Delta(sel-sec)$

JCSA2324, SaPIbov1 $\Delta tsst::tetM$ $\Delta(sel-sec)$

JCSA2325, SaPIbov1 $\Delta tsst::tetM$ $\Delta(sel-sec)$

JCSA2049, *cat194* 10 kb downstream of SaPIbov1 *attC*

JCSA2049, *cat194* 20 kb downstream of SaPIbov1 *attC*

JCSA1836 , SaPIbov2 *bap::tetM*

JCSA1837 , SaPIbov2 *bap::tetM*

JCSA1838 , SaPIbov2 *bap::tetM*

JCSA1839 , SaPIbov2 *bap::tetM*

JCSA1840 , SaPIbov2 *bap::tetM*

JCSA1841 , SaPIbov2 *bap::tetM*

JCSA1842 , SaPIbov2 *bap::tetM*

Description

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Table S5. Plasmids used in this study, related to STAR DNA methods.

Plasmid	Description
pBT2	Vector for allelic replacement
pUC18	<i>E. coli</i> cloning vector
pJP1872	pBT2- <i>cadCA</i>
pJP1850	pJP1872 allele exchange SAOUHSC_00841:: <i>cadCA</i> ; Cd ^R marker 3 kb upstream SaPI type IV <i>attC</i>
pJP1847	pJP1872 allele exchange SAOUHSC_00848:: <i>cadCA</i> ; Cd ^R marker 4 kb downstream SaPI type IV <i>attC</i>
pJP2803	pJP1872 allele exchange SAOUHSC_00365:: <i>cadCA</i> ; Cd ^R marker 11 kb upstream SaPI type II <i>attC</i>
pJP1853	pJP1872 allele exchange SAOUHSC_01064:: <i>cadCA</i> ; Cd ^R marker 10 kb upstream Sa7 <i>attB</i>
pJC1213	pT181 replicon, <i>cat194</i>
pJC1600	Allelic exchange vector, <i>cat194</i>
pJC1630	pUC18, PCR JCO445 + JCO446 SaPI _{bov1} <i>terS</i>
pJC1691	SaPI1 <i>attC</i> integration vector, Ptet promoter, <i>ermC</i>
pJC1673	SaPI4 <i>attC</i> integration vector, Ptet promoter, <i>ermC</i>
pJC1693	pJC1213 (SaPI4 Integrase+)
pJC1706	Allelic exchange vector, <i>ermC</i>
pJC1746	pJC1673, pJC1630 (PstI-BamHI) SaPI _{bov1} <i>terS</i>
pJC1950	Allelic exchange vector, <i>cat194</i>

pJC1992 pUC18, PCR JCO814 + JCO815 RN450, *cadCA* 10 kb downstream SaPIbov1 *attC*

pJC1993 pUC18, PCR JCO818 + JCO819 RN450, *cadCA* 16 kb downstream SaPIbov1 *attC*

pJC1994 Inverse PCR of pJC1992 JCO816 + JCO817 10 kb downstream SaPIbov1 *attC*

pJC1995 Inverse PCR of pJC1993 JCO820 + JCO821 16 kb downstream SaPIbov1 *attC*

pJC2002 pJC1706, pJC1994 (KpnI-SpHI) 10 kb downstream SaPIbov1 *attC*

pJC2003 pJC1706, pJC1995 (KpnI-SpHI) 16 kb downstream SaPIbov1 *attC*

pJC2004 pJC2002, *cadCA* (PstI-BamHI) 10 kb downstream SaPIbov1 *attC*

pJC2005 pJC2003, *cadCA* (PstI-BamHI) 16 kb downstream SaPIbov1 *attC*

pJC2094 pJC1950, $\Delta recA$

pJC2122 pUC18, PCR JCO928 + JCO930 SaPIbov1 $\Delta(sel-sec)$

pJC2123 pUC18, PCR JCO929 + JCO931 SaPIbov1 $\Delta(sel-sec)$

pJC2124 pUC18, PCR JCO932 + JCO934 SaPIbov1 $\Delta tsst::tetM$

pJC2125 pUC18, PCR JCO933 + JCO935 SaPIbov1 $\Delta tsst::tetM$

pJC2126 pJC1706, pJC2122 (SphI-XhoI) + pJC2123 (XhoI-KpnI) SaPIbov1 $\Delta(sel-sec)$

pJC2127 pJC1706, pJC2124 (SphI-PstI) + *tetM* (PstI-BamHI) + pJC2125 (BamHI-KpnI) SaPIbov1 $\Delta tsst::tetM$

pJC2244 pUC18, PCR JCO1016 + JCO1017 4 kb downstream SaPIbov1 *attC*

pJC2245 Inverse PCR of pJC2244 JCO1018 + JCO1019 4 kb downstream SaPIbov1 *attC*

pJC2246 pJC1706, pJC2245 (KpnI-SphI) 4 kb downstream SaPIbov1 *attC*

pJC2247 pJC2246, *cadCA* (PstI-BamHI) 4 kb downstream SaPIbov1 *attC*

pJC2278 pUC18, PCR JCO1112 + JCO1113 2nd headful downstream SaPIbov1 *attC*

pJC2279 pUC18, PCR JCO1114 + JCO1115 6th headful downstream SaPIbov1 *attC*

pJC2280 pUC18, PCR JCO1116 + JCO1117 7th headful downstream SaPIbov1 *attC*

pJC2281 Inverse PCR of pJC2278 JCO1120 + JCO1121 2nd headful downstream SaPIbov1 *attC*

pJC2282 Inverse PCR of pJC2279 JCO1122 + JCO1123 6th headful downstream SaPIbov1 *attC*

pJC2283 Inverse PCR of pJC2280 JCO1124 + JCO1125 7th headful downstream SaPIbov1 *attC*

pJC2284 pJC1706, pJC2281 (KpnI-SphI) 2nd headful downstream SaPIbov1 *attC*

pJC2285 pJC1706, pJC2282 (KpnI-SphI) 6th headful downstream SaPIbov1 *attC*

pJC2286 pJC1706, pJC2283 (KpnI-SphI) 7th headful downstream SaPIbov1 *attC*

pJC2300 pJC2284, *cadCA* (PstI-BamHI) 2nd headful downstream SaPIbov1 *attC*

pJC2301 pJC2285, *cadCA* (PstI-BamHI) 6th headful downstream SaPIbov1 *attC*

pJC2302 pJC2286, *cadCA* (PstI-BamHI) 7th headful downstream SaPIbov1 *attC*

pJC2311 pUC18, PCR JCO1141 + JCO1142 *sarA* P1 promoter

pJC2343 pJC1213, pJC2311 (PstI-BamHI) *sarA* P1 promoter

pJC2346 pUC18, PCR JCO1109 + JCO1170 SaPIbov1 *attC*

pJC2358 pUC18, PCR JCO1187 + JCO1188 SaPIbov1 $\Delta tsst::ermC$

pJC2359 pUC18, PCR JCO1189 + JCO1190 SaPIbov1 $\Delta tsst::ermC$

pJC2360 pJC1600, pJC2358 (KpnI-BamHI) + *ermC* (BamHI-PstI) + pJC2359 (PstI-SphI) SaPIbov1 $\Delta tsst::ermC$

pJC2482 pUC18, PCR JCO1312 + JCO1313 4 kb downstream SaPI1 *attC*

pJC2483 Inverse PCR of pJC2482 JCO1314 + JCO1315 4 kb downstream SaPI1 *attC*

pJC2484 pJC1600, pJC2483 (KpnI-SphI) 4 kb downstream SaPI1 *attC*

pJC2485 pJC2484, *cadCA* (PstI-BamHI) 4 kb downstream SaPI1 *attC*

pJC2486 pUC18, PCR JCO1316 + JCO1317 4 kb downstream SaPI2 *attC*

pJC2487 Inverse PCR of pJC2486 JCO1318 + JCO1319 4 kb downstream SaPI2 *attC*

pJC2488 pJC1600, pJC2487 (KpnI-SphI) 4 kb downstream SaPI2 *attC*

pJC2489 pJC2488, *cadCA* (PstI-BamHI) 4 kb downstream SaPI2 *attC*

pJC2602 pUC18, PCR JCO1329 + JCO1330 3rd headful downstream SaPIbov1 *attC*

pJC2603 pUC18, PCR JCO1331 + JCO1332 4th headful downstream SaPIbov1 *attC*

pJC2604 pUC18, PCR JCO1133 + JCO1134 5th headful downstream SaPIbov1 *attC*

pJC2610 Inverse PCR of pJC2602 JCO1335 + JCO1336 3rd headful downstream SaPIbov1 *attC*

pJC2611 Inverse PCR of pJC2603 JCO1337 + JCO1338 4th headful downstream SaPIbov1 *attC*

pJC2612 Inverse PCR of pJC2604 JCO1339 + JCO1340 5th headful downstream SaPIbov1 *attC*

pJC2613 pJC1706, pJC2610 (KpnI-SphI) 3rd headful downstream SaPIbov1 *attC*

pJC2614 pJC1706, pJC2611 (KpnI-SphI) 4th headful downstream SaPIbov1 *attC*

pJC2615 pJC1706, pJC2612 (KpnI-SphI) 5th headful downstream SaPIbov1 *attC*

pJC2616 pJC2613, *cadCA* (PstI-BamHI) 3rd headful downstream SaPIbov1 *attC*

pJC2617 pJC2614, *cadCA* (PstI-BamHI) 4th headful downstream SaPIbov1 *attC*

pJC2618 pJC2615, *cadCA* (PstI-BamHI) 5th headful downstream SaPIbov1 *attC*

pJC2619 Inverse PCR of pJC2482 JCO1341 + JCO1342 4 kb downstream SaPI1 *attC*

pJC2620 pJC1706, pJC2619 (KpnI-SphI) 4 kb downstream SaPI1 *attC*

pJC2625 Inverse PCR of pJC2346 JCO1214 + JCO1215 SaPIbov1 *attC** synonymous mutations

pJC2626 pJC1706, pJC2625 (KpnI-SphI) SaPIbov1 *attC** synonymous mutations

pJC2658 pUC18, PCR JCO1388 + JCO1389 SaPIbov1 Δ *tsst::cat194*

pJC2659 pUC18, PCR JCO1390 + JCO1384 SaPIbov1 Δ *tsst::cat194*

pJC2674 pJC1600, pJC2658 (SphI-AvrII) + *cat194* (AvrII-SacII) + pJC2659 (SacII-KpnI) SaPIbov1 Δ *tsst::cat194*

pJC2736 pUC18, PCR JCO1511 + JCO1513 SaPIbov1 Δ (*pri-rep*)

pJC2737 pUC18, PCR JCO1512 + JCO1514 SaPIbov1 Δ (*pri-rep*)

pJC2746 pUC18, PCR JCO1507 + JCO1508 SaPIbov1 *xis*+

pJC2786 pJC1691, pJC2746 (PstI-BamHI) SaPIbov1 *xis*+

pJC2772 pJC1706, pJC2636 (SphI-XhoI) + pJC2637 (XhoI-KpnI) SaPIbov1 Δ (*pri-rep*)

pJC2774 pUC18, PCR JCO1533 + JCO1510 SaPIbov1 *pri-rep*+

pJC2775 pUC18, PCR JCO1534 + JCO1509 SaPIbov1 *pri-rep*+

pJC2791 pJC1691, pJC2774 (BamHI-NheI) + pJC2775 (NheI-PstI) SaPIbov1 *pri-rep*+

pJC2847 pJC1600, pJC2658 (SphI-AvrII) + *cat194* (SacII-AvrII) + pJC2659 (SacII-KpnI) SaPIbov1 Δ *tsst::cat194* (reve

pJC2852 pUC18, PCR JCO1657 + JCO1660 10 kb upstream SaPIbov1 *attC*

pJC2853 pUC18, PCR JCO1658 + JCO1659 10 kb upstream SaPIbov1 *attC*

pJC2854 pJC2852, pJC2853 (EcoRI-BamHI) 10 kb upstream SaPIbov1 *attC*

pJC2868 pJC1706, pJC2854 (SphI-EcoRI) 10 kb upstream SaPIbov1 *attC*

pJC2869 pJC2868, *cadCA* (PstI-BamHI) 10 kb upstream SaPIbov1 *attC*

pJC2884 pJC2002, *cat194* (PstI-BamHI) 10 kb downstream SaPIbov1 *attC*

pJC2903 pJC2284, *cat194* (PstI-BamHI) 2nd headful downstream SaPIbov1 *attC*

pJC2904 pJC2613, *cat194* (PstI-BamHI) 3rd headful downstream SaPIbov1 *attC*

pJC2909 pJC2246, *cat194* (PstI-BamHI) 4 kb downstream SaPIbov1 *attC*

pJC2915 pUC18, PCR JCO1714 + JCO1715 20 kb downstream SaPIbov1 *attC*

pJC2916 pUC18, PCR JCO1718 + JCO1719 25 kb downstream SaPIbov1 *attC*

pJC2917 Inverse PCR of pJC2915 JCO1716 + JCO1717 20 kb downstream SaPIbov1 *attC*

pJC2918 Inverse PCR of pJC2916 JCO1720 + JCO1721 25 kb downstream SaPIbov1 *attC*

pJC2919 pJC1706, pJC2917 (KpnI-SphI) 20 kb downstream SaPIbov1 *attC*

pJC2920 pJC1706, pJC2918 (KpnI-SphI) 25 kb downstream SaPIbov1 *attC*

pJC2921 pJC2919, *cadCA* (PstI-BamHI) 20 kb downstream SaPIbov1 *attC*

pJC2922 pJC2920, *cadCA* (PstI-BamHI) 25 kb downstream SaPIbov1 *attC*

pJC2945 pJC2929, *cat194* (PstI-BamHI) 20 kb downstream SaPIbov1 *attC*

pJC2962 pUC18, PCR JCO1842 + JCO1843 (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site)

pJC2963 pUC18, PCR JCO1844 + JCO1845 SaPIbov1 *int^{syn}* synonymous mutations

pJC2964 pJC2246, pJC2962 (PstI-BamHI) (4 kb downstream of SaPIbov1 *attC*)::(SaPI Bov1 *attC* site)

pJC2983 Inverse PCR of pJC2963 JCO1846 + JCO1847 SaPIbov1 *int^{syn}* synonymous mutations

pJC2988	Allelic exchange vector, <i>ermC</i>
pJC2984	pJC1706, pJC2983 (KpnI-SphI) SaPI _{bov1} <i>int</i> ^{syn} synonymous mutations
pJC2901	pJC2620, <i>cat194</i> (PstI-BamHI) 4 kb downstream SaPI1 <i>attC</i>
pJC2902	pJC2488, <i>cat194</i> (PstI-BamHI) 4 kb downstream SaPI2 <i>attC</i>
pJC3037	pUC18, PCR JCO1944 + JCO1945 SaPI _{bov1} <i>int</i> ⁺
pJC3038	pJC1691, pJC3037 (PstI-BamHI) SaPI _{bov1} <i>int</i> ⁺
pJC3054	pT181 replicon, <i>ermC</i> , <i>sarA</i> P1 promoter
pJC3056	pJC3054, pJC3037 (PstI-BamHI) SaPI _{bov1} <i>int</i> ⁺
pJC3063	pUC18, PCR JCO1951 + JCO1952 Φ 52a Δ <i>dut</i>
pJC3066	Inverse PCR of pJC3063 JCO1953 + JCO1954 Φ 52a Δ <i>dut</i>
pJC3067	pJC2988, 3066 (PstI-BamHI) Φ 52a Δ <i>dut</i>

Plasmid	Description
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Table S6. Oligonucleotides used in this study, related to STAR DNA methods.

Oligonucleotides	Sequence (5'-3')	Reference
JCO444	<u>GGATCC</u> GCGTTCTCCCTTTTATCTTTATAACGC	14
JCO445	<u>CTGCAG</u> TTGTAGAGGTGATAGAATGAGTGAGTTAACG	14
JCO814	GCATGCGTAGAAGCACCGCAACAAACAGC	This work
JCO815	<u>GGTACCC</u> ACCAGTAGCTAACAATCCCAATACTAATGTTGCTTTAGC	This work
JCO816	<u>GAGCTGC</u> AGCTGTCAATTTTCATAGTTGTATGCTCCATTCG	This work
JCO817	<u>GAGGGATCC</u> CAATTGCGAAAGCAAGTTTAGCATTAGGTATTTTAGC	This work
JCO818	<u>GCATGCC</u> CAGAGTAACATCATCAGTTGTAGTAAACGATAATCCGG	This work
JCO819	<u>GGTACCG</u> CAGTCCATTTTCGCACTATACGGTG	This work
JCO820	<u>GAGCTGC</u> AGAGTAATAGACATGTGATTCCTCCGCC	This work
JCO821	<u>GAGGGATCC</u> GAAAAACAACGTCAGCAACAAGCTG	This work
JCO928	<u>GCATGCG</u> TCATACAACAAGTTGGTGGC	This work
JCO929	<u>GGTACCC</u> AACGCTCATGCTGAAC	This work
JCO930	<u>CTCGAG</u> CGTTAATTATGAAGTGATGTTAATTGATGTGAAG	This work
JCO931	<u>CTCGAG</u> AAGGAGAAACAGAGGATTTCTAAGCATC	This work
JCO932	<u>GCATGCG</u> TTCAGCATGAGCGTTG	This work
JCO933	<u>GGTACCC</u> CATGAGCGAACTAGAAGTGATG	This work

JCO934	<u>CTGCAGAATTAGAAAGTGTGTTTGTACATAGGGAGC</u>	This work
JCO935	<u>GGATCCGTGTTCTCCCTTTTATCTTTATAACGC</u>	This work
JCO1016	<u>GCATGCGGAAACAGAGGCAACGCTAC</u>	This work
JCO1017	<u>GGTACCCGCTTGTACTIONGATTGTACATTCGATGTAATTACACC</u>	This work
JCO1018	<u>GAGCTGCAGACTTAAACCATATTTACCAGAATTGATGAATATGC</u>	This work
JCO1019	<u>GAGGGATCCATGTACATTGCCATTCTTACATACGTATAGTC</u>	This work
JCO1109	<u>CTGCAGAAGGCGCGCCTAAACCCTCCGATCTCTATCAC</u>	This work
JCO1112	<u>GCATGCGTACGATACCTATACCACCTGTTAGTGCG</u>	This work
JCO1113	<u>GGTACCAAACACTCATTGTTGGAGGTGCCTATATCGC</u>	This work
JCO1114	<u>GCATGCGGGAGGTTGAGTAATGAATAAAGTAGAAGCG</u>	This work
JCO1115	<u>GGTACCTCGCAATAACACCAACAAACACGATAGC</u>	This work
JCO1116	<u>GCATGCTTGTTTTTGAAAGAGCGAGACGGTTCG</u>	This work
JCO1117	<u>GGTACCATTTTGCCTACTAGTGGTTCTGGCTTTAGC</u>	This work
JCO1120	<u>GAGCTGCAGCTTTGAAAATAAAAAATTAAGGGCGTATAATCACC</u>	This work
JCO1121	<u>GAGGGATCCATATTACTGCTAAGTGTAACGAAAATCATCATTGATAGC</u>	This work
JCO1122	<u>GAGCTGCAGGGATAAATATTGAGTTGCATAGAAGAATACTGC</u>	This work
JCO1123	<u>GAGGGATCCTCTCGAAAAGACAATTTACCAAGG</u>	This work
JCO1124	<u>GAGCTGCAGTACTTTTTAGTATTTTCAAATAATTTAAATGACCACATCTACAACG</u>	This work
JCO1125	<u>GAGGGATCCGTGTGTTTTCAATTGGATAGTTTAATTAAGTACTACATC</u>	This work

JCO1141	<u>CGGGCATGCGCTGATATTTTTGACTAAACCAAATGC</u>	This work
JCO1142	<u>TTCCTGCAGGATGCATCTTGCTCGATACATTTGC</u>	This work
JCO1170	<u>GGTACCACGAATCGGTGCTAAATCTAACAGC</u>	This work
JCO1187	<u>GGTACCCTCGCTATCTCCTCAGAACGTTGTG</u>	This work
JCO1188	<u>GGATCCGACCCACTACTATACCAGTCTAGCAAATCC</u>	This work
JCO1189	<u>CTGCAGAAAAACACAGATGGCAGCATCAGCC</u>	This work
JCO1190	<u>GCATGCGAAGTTGTAGTCAAGCGTGGG</u>	This work
JCO1214	GAGAATTTGAGTAGTTGGAAAATTACAATAAGGACGG	This work
JCO1215	GAGTCACTCCATTCGATAGTGCTTGGTGGTTTTGATG	This work
JCO1312	<u>GCATGCGCGTACACACAAGTTGATGATAATGC</u>	This work
JCO1313	<u>GGTACCGTCGCCACCGAACTCAATC</u>	This work
JCO1314	<u>GAGGGATCCATAGTAAAGTTGATCTGGATCAACACGACC</u>	This work
JCO1315	<u>GAGCTGCAGTTAATGAGTCGTGGTATTTCTCAAAGAGAAGC</u>	This work
JCO1316	<u>GCATGCCTGGCTTGTCCCCAGTTGATATAG</u>	This work
JCO1317	<u>GGTACCGTGCATAATGCCGGGAATGATGTAAAAGT</u>	This work
JCO1318	<u>GAGGGATCCGGGCACCACCAATAAACATAAGTAGC</u>	This work
JCO1319	<u>GAGCTGCAGCTCTCAGTGCAGCTGGAG</u>	This work
JCO1329	<u>GCATGCGGCGTTGTCGTGTTAACTGC</u>	This work
JCO1330	<u>GGTACCGTCGCATCTAACAGTGTGAAGCCATC</u>	This work

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JCO1507	<u>CTGCAGAATTAGAGATAAAATGGTAGGAGAGGAATAATATGAGCC</u>	This work
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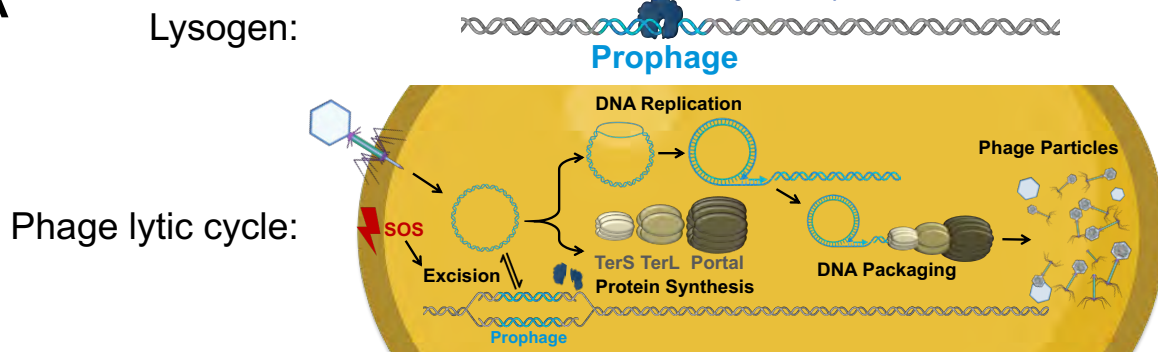
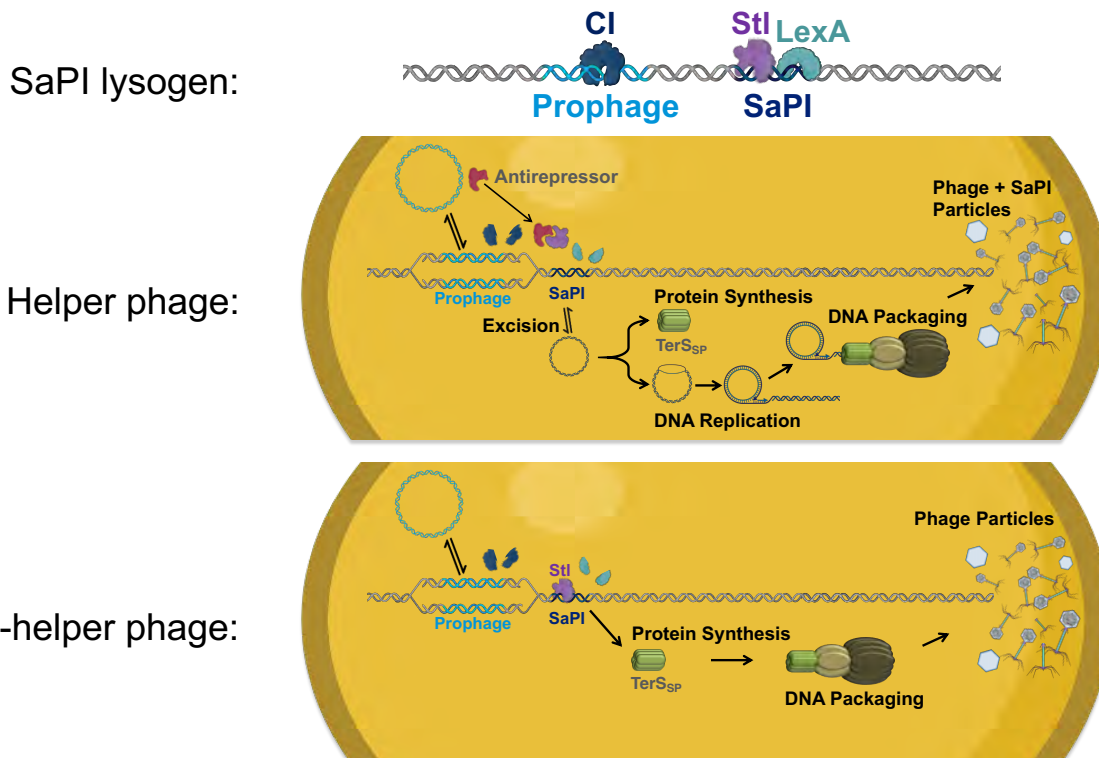
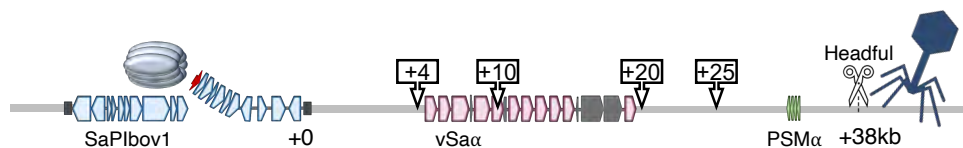
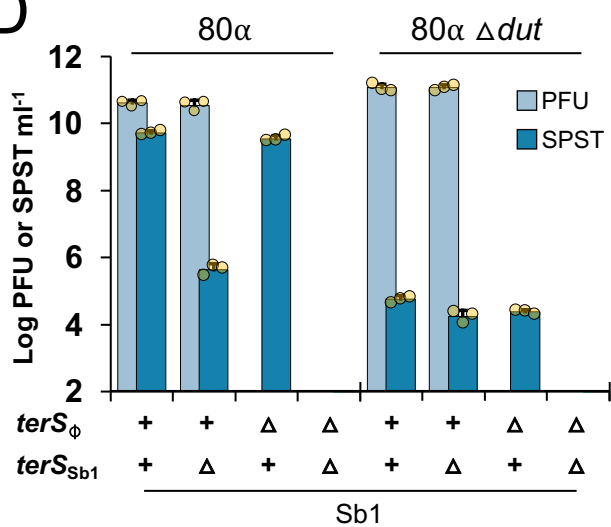
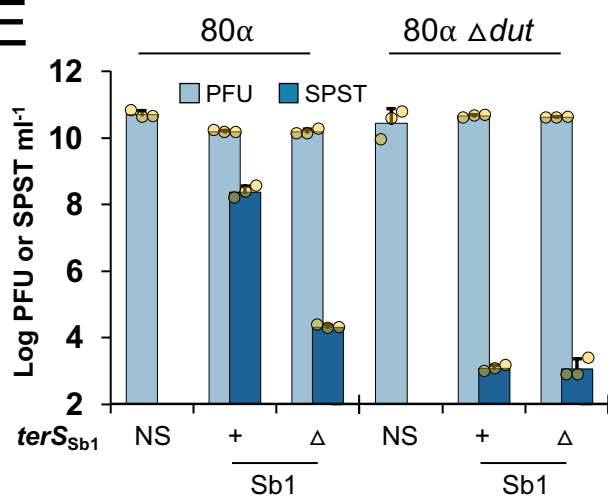
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JCO1534	GCCCAACTAAGCTAGCTAGTGCATTG	This work
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JCO1716	<u>GAGCTGC</u> AGTGCTTGTCCGTTTGTGATAATATATAACATTG	This work
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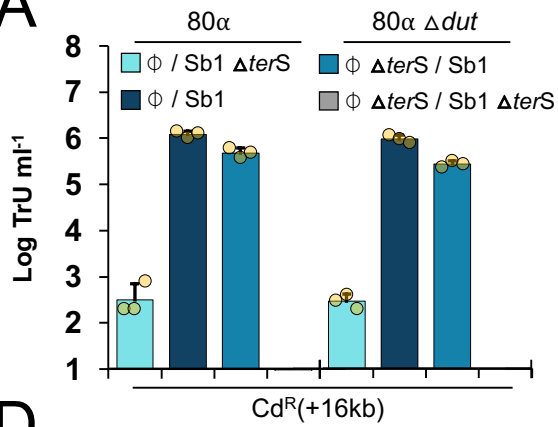
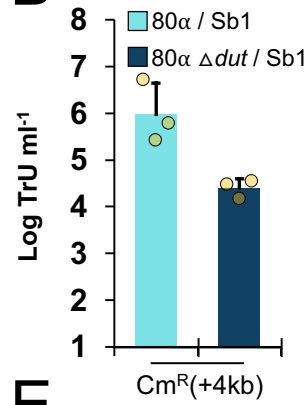
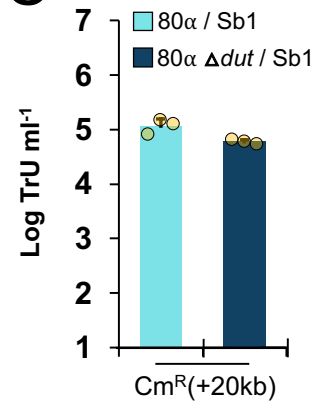
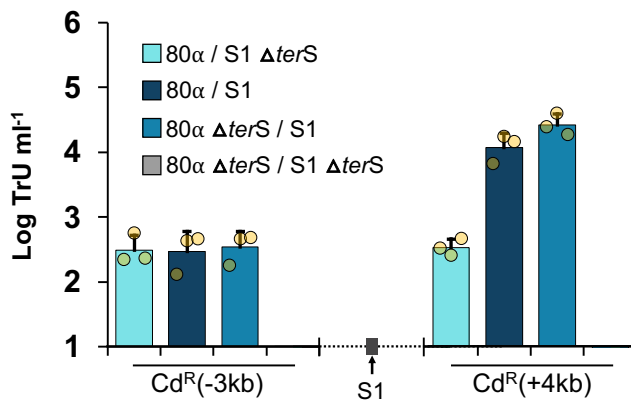
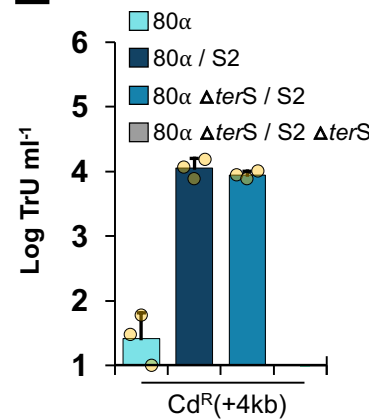
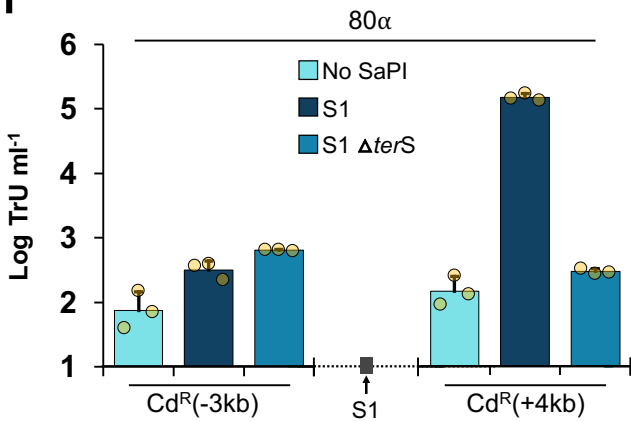
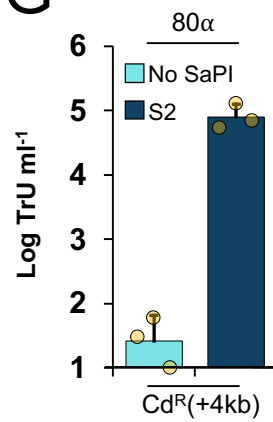
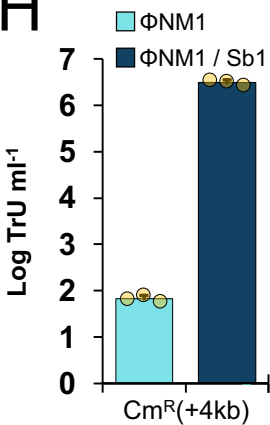
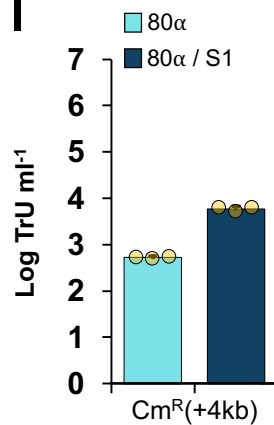
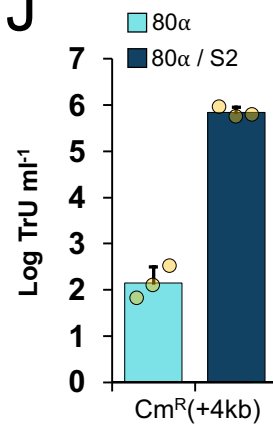
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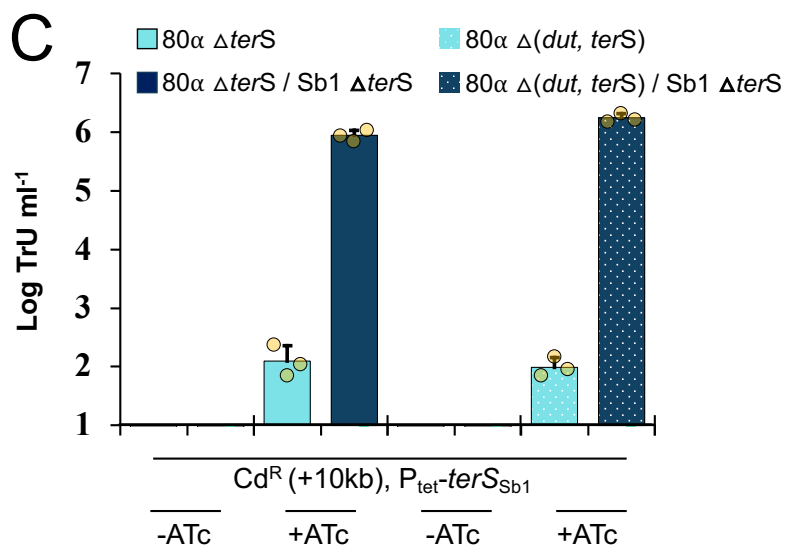
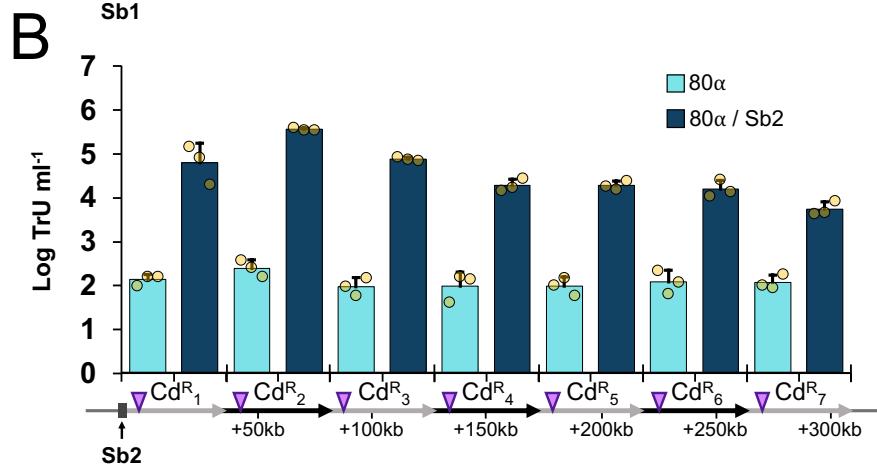
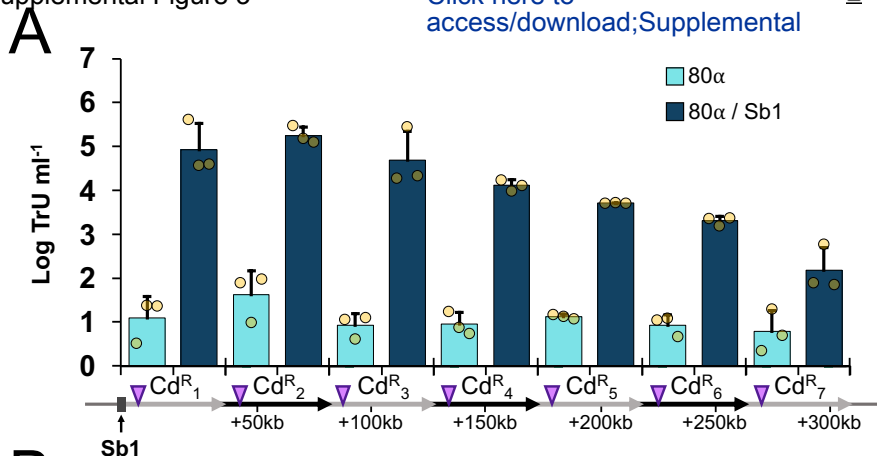
Oligonucleotides	Sequence (5'-3')	Reference
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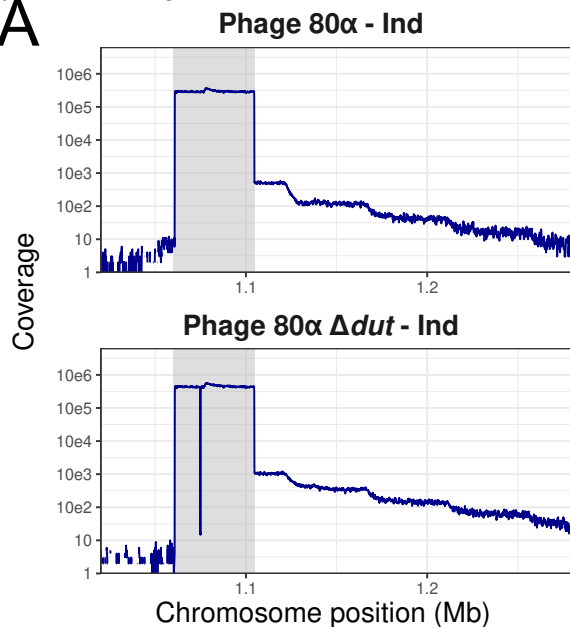
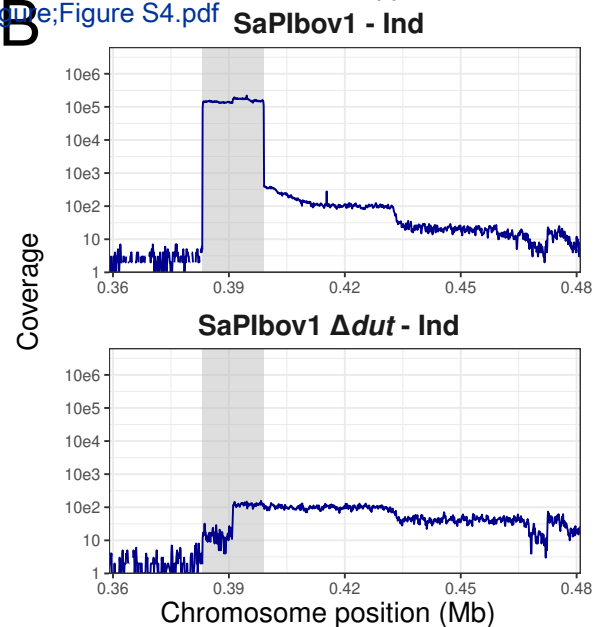
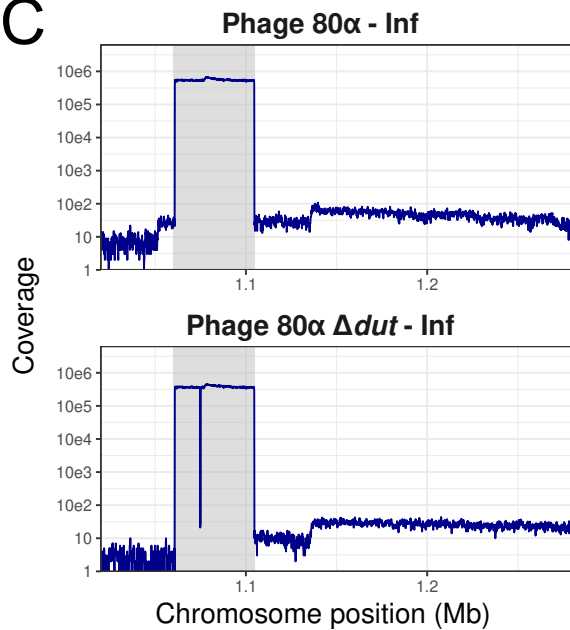
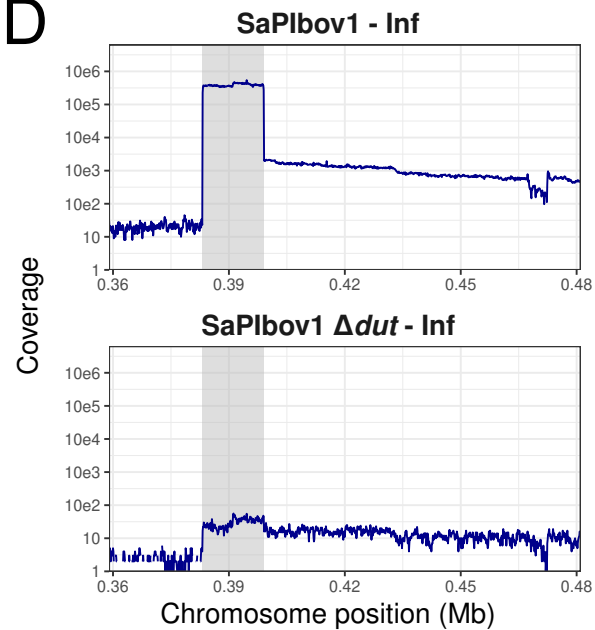
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	Cd-IV-SaPI1-2cX	GCTCTAGAGTCAATAACTATTTTAAATTTTCTAAC	This work
	Cd-IV-SaPI1-3mS	ACGCGTCGACGAAATTTTACTAAGGTGTTAGG	This work
	Cd-IV-SaPI1-4cP	AACTGCAGGAATGACATGCATTTTCATGCG	This work
pJP1847	Cd-I-SaPI1-1mB	CGCGGATCCATGTAGTTGTAGAACATCCAG	This work
	Cd-I-SaPI1-2cX	GCTCTAGAATATTATTTAGAACTTTGCGTTC	This work
	Cd-I-SaPI1-3mS	ACGCGTCGACTTGAAAATAAAAGTTTGTAATAGAT	This work
	Cd-I-SaPI1-4cP	AACTGCAGCCAAATAATACGCCAATACCTG	This work
pJP2803	Cd-XVI-1mB	CGCGGATCCGACGATTGACTGAGAACTTGG	This work
	Cd-XVI-2cX	GCTCTAGAATGTAATAATGCTAACTAAGAGATTAG	This work
	Cd-XVI-3mS	ACGCGTCGACTTTGATCCAGAATAGTCAACTGG	This work
	Cd-XVI-4cP	AACTGCAGCTACTGCCATACCAAATACCG	This work
pJP1853	Cd-IV-80alpha-1mB	CGCGGATCCTAAAGTAGTTGGTGATATGGC	This work
	Cd-IV-80alpha-2cX	GCTCTAGACATTTTAGTCAGTTGCTTTTTTC	This work
	Cd-IV-80alpha-3mS	ACGCGTCGACATTTAAATAAAACGAGATTACACAAC	This work
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Plasmid	Oligonucleotides	Sequence (5'-3')	Reference
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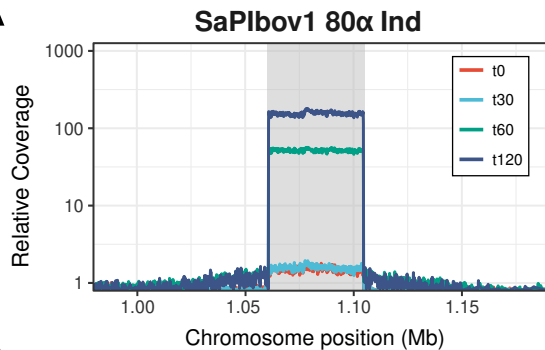
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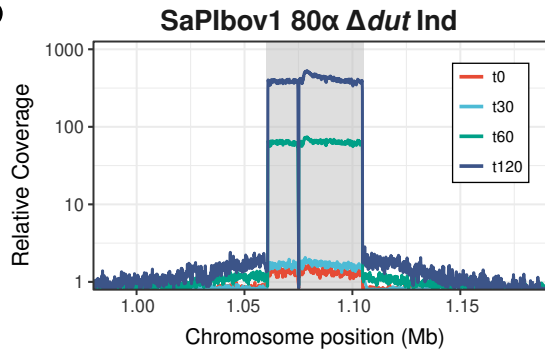


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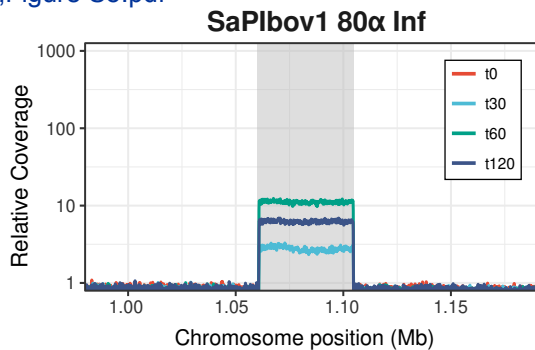
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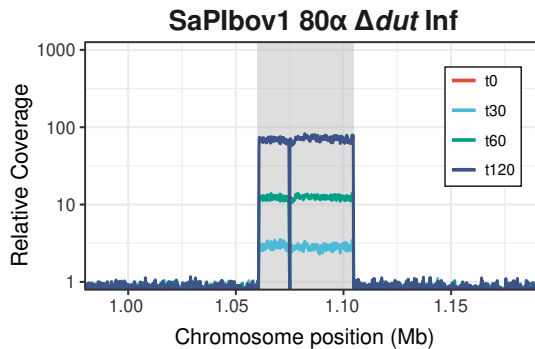
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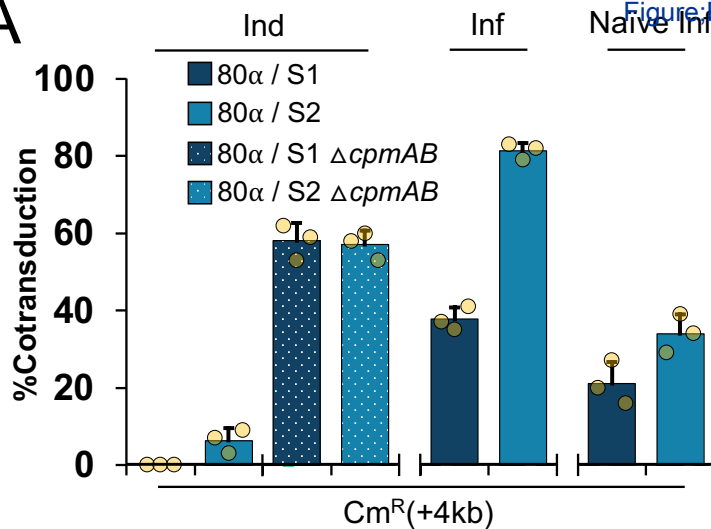
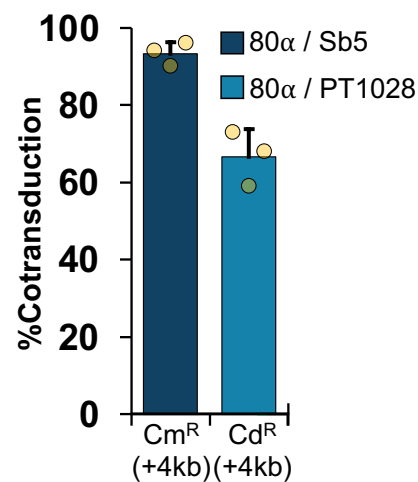
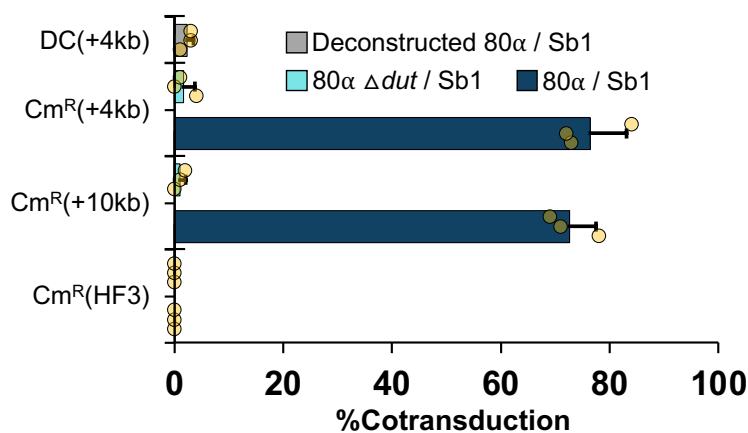
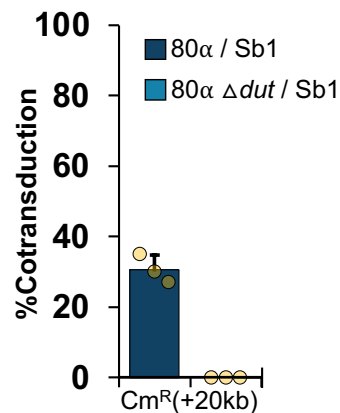
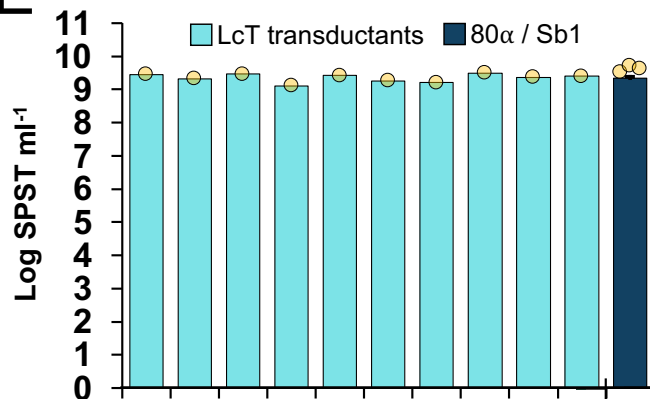
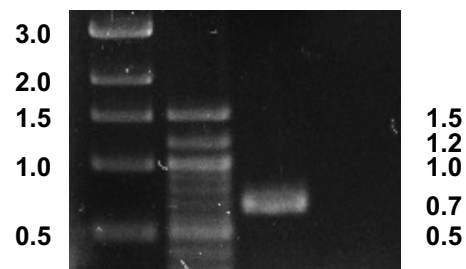


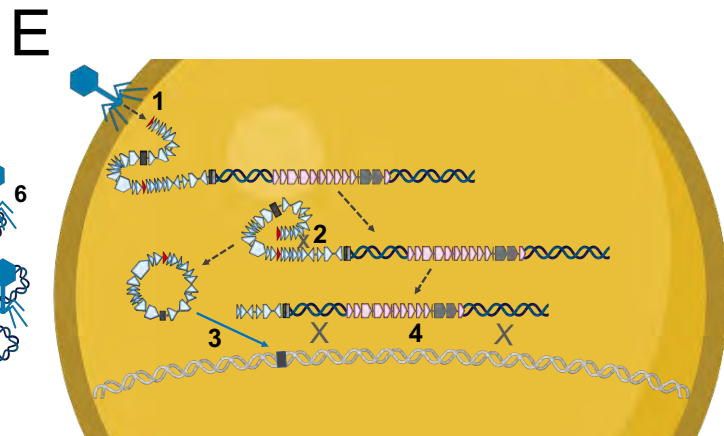
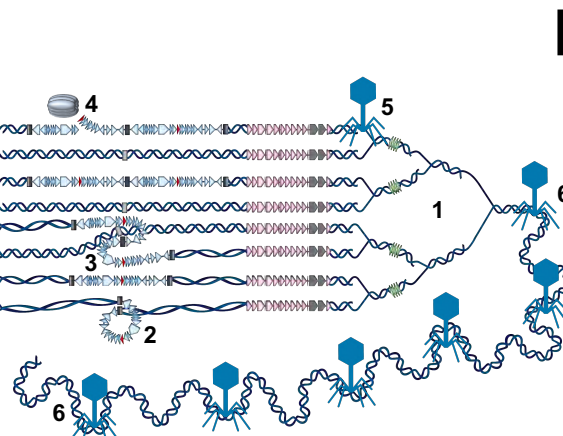
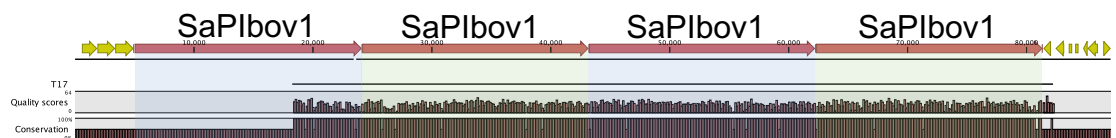
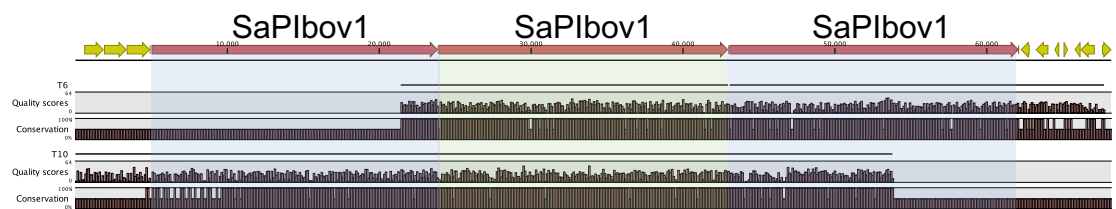
C



D



A**B****C****D****E****F**



Phage	Phage LT marker	SaPI	SaPI LT marker	Selection	(Cm ^R /Cd ^R)%	(Cd ^R /Cm ^R)%	(Cm ^R /Tet ^R)%	(Cd ^R /Tet ^R)%
80α	Cd ^R (+4 kb)	Sb1:: <i>tetM</i>	Cm ^R (+4 kb)	Cd ^R	0.0 ± 0.0	NA	NA	NA
80α	Cd ^R (+4 kb)	Sb1:: <i>tetM</i>	Cm ^R (+4 kb)	Cm ^R	NA	0.0 ± 0.0	NA	NA
80α	Cd ^R (+4 kb)	Sb1:: <i>tetM</i>	Cm ^R (+4 kb)	Tet ^R	NA	NA	0.0 ± 0.0	0.0 ± 0.0

Table S1. Cotransduction of lateral transduction markers and SaPI, related to Figure 5.

SaPIbov1 *tsst::tetM* strains lysogenic for helper 80α phage with a Cd^R marker 4 kb downstream of the 80α *attB* and Cm^R markers 4 kb downstream of the SaPIbov1 *attC* were induced with mitomycin C. The resulting lysates were tested for cotransduction into *S. aureus* by selecting for one marker (Cd^R, Cm^R, or Tet^R), followed by testing the transductants for the second marker (Cm^R or Cd^R). For each replicate, 100 transductants were tested for Cm^R or Cd^R and the cotransduction frequency was represented as a percentage, calculated as (Cm^R or Cd^R / Cm^R or Cd^R or Tet^R) x 100. Values are means ($n = 3$ independent samples) ± standard deviation.

Phage	Donor SaPI	Recipient SaPI	Selection	(Em ^R /Tet ^R)%
80α	Sb1:: <i>tetM</i>	Sb1:: <i>ermC</i>	Tet ^R	0.33 ± 0.58

Table S2. SaPIs do not form tandems by integration events, related to Figure 6.

A SaPIbov1 *tsst::tetM* strain lysogenic for 80α phage was induced with mitomycin C and the resultant lysates were tested for transduction into an *S. aureus* strain with SaPIbov1 Δ *tsst::ermC*. The recipient strain was a *recA* deletion (Δ *recA*) to focus only on integrase-mediated integration events. For each replicate, 100 Tet^R transductants were tested for Em^R and the double-positive SaPIbov1 frequency was represented as a percentage, calculated as (Em^R / Tet^R) x 100. Values are means ($n = 3$ independent samples) ± standard deviation.

SaPI Site	Representative SaPIs	# of Genomes	% of Genomes
SaPI-I	SaPI4 PT1028	39	18.3
SaPI-II	SaPIbov1 SaPIbov2	18	8.45
SaPI-III	SaPImw2 SaPIm4	33	15.5
SaPI-IV	SaPI1 SaPI3	36	16.9
SaPI-V	SaPI2 SaPI122	48	22.5

Table S3. SaPI distribution in natural populations of *S. aureus* isolates, related to Figure 7.

SaPIs are normally found at five conserved SaPI integration sites. The number or percentage of genomes indicates the number or percentage of *S. aureus* genomes (from the pangenome synteny network analysis in Figure 7) with a SaPI element present in that site. A total of 213 genomes were interrogated for this analysis.

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