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Phage-mediated horizontal transfer of a *Staphylococcus aureus* virulence-associated genomic island

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***Staphylococcus aureus* is a major pathogen of humans and animals. The capacity of *S. aureus* to adapt to different host species and tissue types is strongly influenced by the acquisition of mobile genetic elements encoding determinants involved in niche adaptation. The genomic islands νSaα and νSaβ are found in almost all *S. aureus* strains and are characterized by extensive variation in virulence gene content. However the basis for the diversity and the mechanism underlying mobilization of the genomic islands between strains are unexplained. Here, we demonstrated that the genomic island, νSaβ, encoding an array of virulence factors including staphylococcal superantigens, proteases, and leukotoxins, in addition to bacteriocins, was transferrable *in vitro* to human and animal strains of multiple *S. aureus* clones via a resident prophage. The transfer of the νSaβ appears to have been accomplished by multiple conversions of transducing phage particles carrying overlapping segments of the νSaβ. Our findings solve a long-standing mystery regarding the diversification and spread of the genomic island νSaβ, highlighting the central role of bacteriophages in the pathogenic evolution of *S. aureus*.**

Staphylococcus aureus is a versatile pathogen and causes a wide range of diseases in humans and animals by producing an array of factors involved in virulence and niche adaptation[1]. Genome sequencing analysis showed that *S. aureus* genomes are highly variable as only ~75% of the gene content is shared by all strains[2]. The genomic plasticity of *S. aureus* is primarily attributed to mobile genetic elements (MGEs) such as prophages, plasmids, pathogenicity islands of *S. aureus* (SaPIs), and genomic islands (νSa), which have an array of genes encoding proteins involved in antibiotic resistance, virulence, and other contingency functions[2–4]. Some MGEs are widely distributed among most *S. aureus* strains, while others are strongly associated with certain clonal complexes, presumably due to barriers such as DNA restriction-modification systems and niche separation decreasing opportunities for horizontal transfer[2,3,5].

The genomic island referred to as νSaβ (also known as SaPI3/m3) is located upstream of a tRNA gene cluster, and contains genes encoding a bacteriocin, hyaluronate lysase, serine proteases, bi-component leukotoxin D and E, and the enterotoxin gene cluster (egc)[6]. Extensive variation in virulence gene content has been observed at the νSaβ locus in different strains (Supplementary Figure S1)[2,7,8]. Moreover, recent population genetic work has identified hot spots for homologous recombination in the *S. aureus* chromosome centered on insertion sites of mobile elements, including ICE*6013*, SCC*mec* and νSaα[9]. However, the mechanisms underlying the mobilization of genomic islands νSaα and νSaβ are unknown.

Results

Sequence analysis of νSaβ in the strain RF122. The strain RF122 is a bovine mastitis strain which belongs to the CC151 lineage[10]. Genome sequence analysis of the RF122 revealed that a prophage (designated as ϕSaBov in this study), belonging to serogroup B, integrase group Sa8, and holin group 438[11], is integrated adjacent to νSaβ between an upstream tRNA cluster and downstream of the egc locus, flanked by 18 bp imperfect direct repeats, designated as $attN_R$ and $attN_L$, respectively, with a single SNP (Fig. 1A). The $attN_R$ is highly conserved in all sequenced *S. aureus* strains as it is a part of the tRNA-Ser gene[10,12]. Additionally, 33 bp imperfect direct repeats

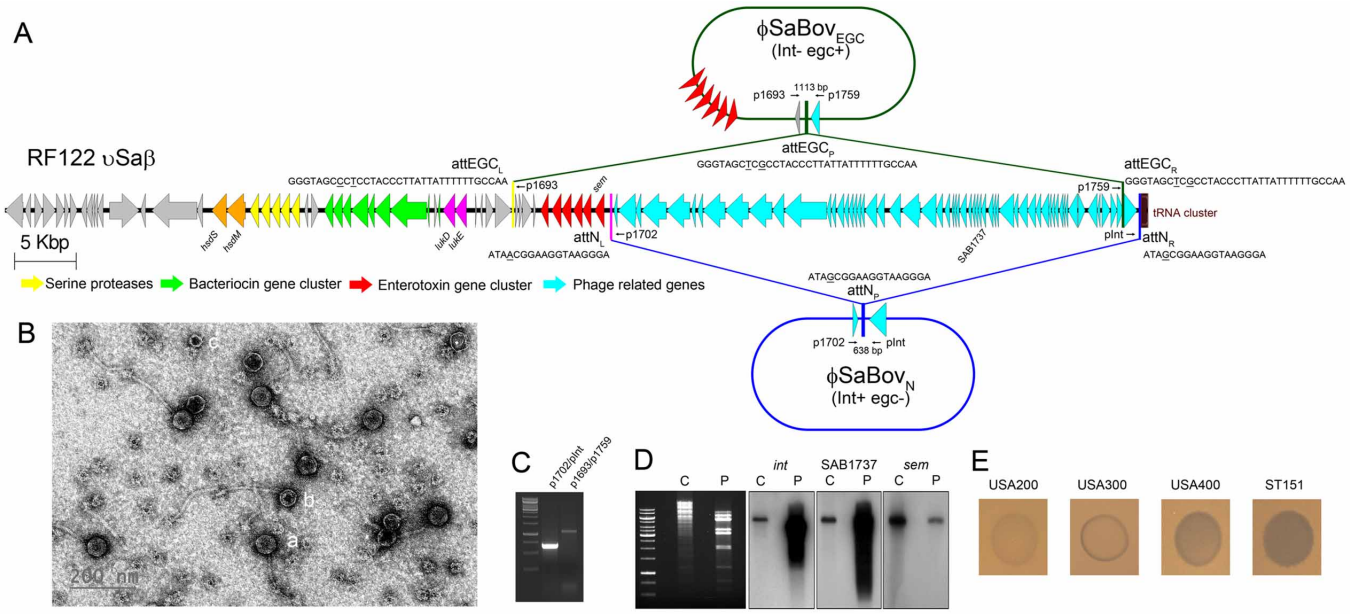

Figure 1 | Heterogeneous excision products of the phage (ϕSaBov) that integrates at genomic island νSaβ. (A) A schematic map of νSaβ in the strain RF122. The arrows represent genes annotated in the GenBank entries[10] and colored based on key features. Orange; restriction modification system HsdR/M, yellow; serine protease cluster (*spl*), light green; bacteriocin gene cluster (*bsa*), pink; leukocidins (*lukD/E*), red; enterotoxin gene cluster (*egc*), cyan; genes related to phage. Direct repeat sequences associated with phage and those associated with the egc were annotated as $attN_L$ and $attN_R$ and $attEGC_L$ and $attEGC_R$, respectively. Sequence variations in the direct repeats were underlined. Primers used for outward PCR and sequencing results of $attN_P$ and $attEGC_p$ were depicted. (B) Transmission electron microscope analysis of phage particles induced from the strain RF122. At least, three different head sizes (a, b, and c; 58, 47, 26 nm, respectively) of phages were observed. (C) Results of outward PCR using pInt/p1702 and p1693/p1759 for $\phi SaBov_N$ and $\phi SaBov_{EGC}$, respectively. (D) RF122 chromosomal DNA (C) and phage DNA (P) were digested with *Eco*RI, separated by electrophoresis, and transferred to Nylon membrane for Southern blot analysis. Probes specific to the integrase gene (SAB1760, for $\phi SaBov_N$), SAB1737 (for $\phi SaBov_N$ and $\phi SaBov_{EGC}$), and the *sem* gene (for $\phi SaBov_{EGC}$) were used. (E) Phage spot test. Mitomycin C induced culture lysate from the strain RF122 (10^8 pfu/ml) was dropped onto the lawn culture of human ST36-SCCmecII (USA200), ST8-SCCmecIV (USA300), ST1-SCCmecIV (USA400), and bovine mastitis isolate (ST151).

were found upstream of the *int* gene ($attEGC_R$) and upstream of the *seg* gene ($attEGC_L$). The $attEGC_L$ is conserved in all sequenced strains harboring the egc in the νSaβ[10,13]. Of note, $attEGC_R$ is highly conserved upstream of the *int* gene in 43 staphylococcal phage sequences available from NCBI GenBank and recognized by sigma factor H, a transcriptional regulator of phage related genes[12].

Phage induction and analysis of phage DNA. The presence of two different sets of direct repeats suggests that transducing phage particles induced from the strain RF122 may harbor heterogeneous phage DNA. To test this possibility, phage was induced by mitomycin C treatment. Electron microscopy demonstrated that induced phage has a long non-contractile tail typical of the *Siphoviridae* family[14] with various sizes of hexagonal heads (Fig. 1B). To identify circularized forms of phage DNA, outward PCR and sequencing were performed. The pIntF/p1702R primer set generated an approx. 652 bp amplicon. Sequencing of this fragment revealed that phage DNA was circularized between $attN_R$ and $attN_L$, resulting in $attN_P$, which was identical to $attN_R$, presumably using the Campbell mechanism[15] (Fig. 1A and C). This type of transducing phage particle only harbors typical genes related to phage, and referred to as $\phi SaBov_N$ (Int+, egc-). The other primer set p1759/p1693 generated an approx.1115 bp amplicon. Sequencing of this fragment showed that another phage DNA was circularized between $attEGC_R$ and $attEGC_L$, resulting in $attEGC_P$ which was identical to $attEGC_R$ (Fig. 1A and C). This type of transducing phage particles harbors the egc and typical genes related to phage except the *int* gene, and referred to as $\phi SaBov_{EGC}$ (Int-, egc+). As controls, outward PCR using chromosomal DNA as a template did not show any amplicons (data not shown). Southern blot analysis showed that probes specific to the integrase gene (a marker for $\phi SaBov_N$), SAB1737 (a marker for $\phi SaBov_N$ and $\phi SaBov_{EGC}$), and the *sem* (a marker for $\phi SaBov_{EGC}$) gene were specifically bound to their corresponding targets (Fig. 1D). The RF122 strain also harbors a truncated phage DNA represented by genes from SAB0258 (integrase gene) to SAB0266. We investigated the excision of this segment in the phage DNA using PCR but no mobilization was detected, indicating this phage is inactive (data not shown). To ensure *S. aureus* chromosomal DNA was not contaminating in the phage DNA preparation, an excessive amount of exogenous chromosomal DNA was added to phage induced lysates, followed by RNase and DNaseI treatment, prior to phage DNA extraction and tested by PCR (Supplementary Figure. S2). It is noteworthy that the band intensity of the *sem* gene was weaker than those of the integrase or SAB1737 gene, suggesting $\phi SaBov_N$ is more dominant than $\phi SaBov_{EGC}$. The relative copy number of $\phi SaBov_{EGC}$ compared to that of $\phi SaBov_N$ was determined by calculating the relative copy number of the *sem* gene (specific to $\phi SaBov_{EGC}$) to the *int* gene (specific to $\phi SaBov_N$) in the phage DNA using quantitative real-time PCR and found to be 0.06215 ± 0.001, indicating approx. 6 of 100 phages are $\phi SaBov_{EGC}$. A phage spot test was performed to evaluate the host range of transducing phage induced from the strain RF122; The test resulted in a clear zone of lysis in human isolates ST1-SCCmecIV (USA400) and a bovine mastitis isolate (CC151) and, to a lesser degree, in ST36-SCCmecII (USA200), and ST8-SCCmecIV (USA300) (Fig. 1E).

Phage-mediated horizontal transfer of νSaβ. Mitomycin C treatment of strain RF122 can induce heterogeneous transducing

Table 1 | Transduction frequencies of $\phi SaBov_N$, $\phi SaBov_{EGC}$, and $\phi SaBov_{LUK}$

Recipient lineage	Recipient strain name	Transfer frequency (CFU/pfu)*		
		$\phi SaBov_N$	$\phi SaBov_{EGC}$	$\phi SaBov_{LUKE}$
ST36-SCC*mec*II	MN PE	2.50×10^{-7}	5.00×10^{-8}	5.00×10^{-8}
(USA200)	MN Park	None	None	None
	MN White	None	None	None
	MN PAM	None	None	None
ST8-SCC*mec*IV	DAR1809	1.15×10^{-6}	3.00×10^{-7}	1.00×10^{-8}
(USA300)	DAR2017	8.00×10^{-7}	1.25×10^{-7}	1.00×10^{-8}
	DAR1085	5.00×10^{-7}	None	None
	DAR1964	4.50×10^{-7}	None	None
ST1-SCC*mec*IV	MW2	1.85×10^{-6}	3.00×10^{-7}	1.50×10^{-8}
(USA400)	MN KN	9.38×10^{-5}	4.80×10^{-6}	1.00×10^{-7}
	MN Gary	2.00×10^{-5}	4.80×10^{-6}	None
	C99-193	2.15×10^{-6}	1.00×10^{-7}	1.25×10^{-8}
	C99-529	2.05×10^{-6}	2.50×10^{-7}	1.00×10^{-8}
Bovine-CC151	CTH96	4.36×10^{-4}	1.22×10^{-5}	7.00×10^{-6}

Table S4To determine transduction frequency of $\phi SaBov_N$, transduction frequency of phages induced from RF122 *SAB1737*::*tetM* was subtracted by that of $\phi SaBov_{EGC}$.

phages harboring the egc, and these induced phages have a broad host specificity range, suggesting the egc could be transferred to other *S. aureus* by this phage. To test this possibility, the *tetM* gene, conferring tetracycline resistance, was introduced into the *sem* gene of the egc, resulting in RF122 *sem*::*tetM*. The phage induced from this strain was successfully transduced to various recipients. Similar to phage spot results, the transduction frequency to bovine (ST151) and USA400 (ST1-SCC*mec*IV) strains was much higher than those to USA300 and USA200 strains (Table 1). To further confirm the transfer of the egc, a draft genome sequence of the recipients MNKN (ST1-SCC*mec*IV) and CTH96 (CC151), and phage transduced strains (transductant) was determined. Strikingly, it was shown that both transductants have an identical sequence with the donor strain RF122 from the 141 bp downstream of the start codon of the SAB1676 gene (*bsaG*) to the $attN_R$ sequence at the tRNA-Ser, even preserving SNPs at direct repeats, totaling to 65,756 bp. This result indicates that not only the integrase gene (from $\phi SaBov_N$) and the egc (from $\phi SaBov_{EGC}$), but also the region upstream of the egc containing a bacteriocin gene cluster and leukotoxin D/E genes, were transferred (Supplementary Figure S3). Southern blot analysis using a probe specific to the *lukE* gene demonstrated the presence of the transducing phage particle harboring the region upstream of the egc containing a bacteriocin gene cluster and leukotoxin D/E genes (Fig. 2C). To test whether this type of transducing phage particle also carries a circular form of phage DNA, outward PCR using various sets of primers was attempted from freshly prepared phage DNA templates and repeated more than 10 times but failed (data not shown). We then investigated the possibility of the existence of a linear form of phage DNA. Indeed, PCR was positive with primer

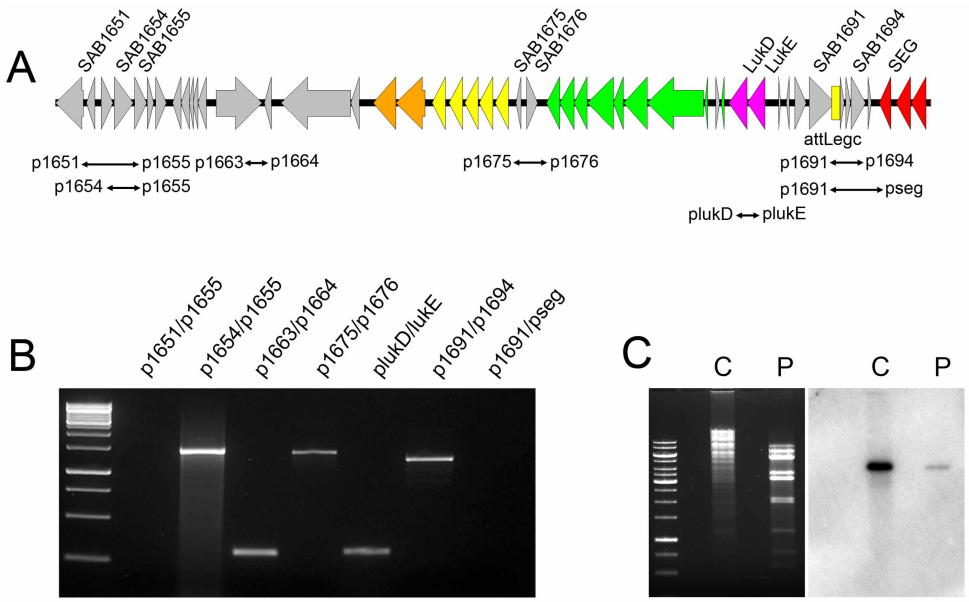

Figure 2 | Identification of a tranducing phage particle, $\phi SaBov_{LUK}$, harboring linear phage DNA. (A) A schematic map of linear phage DNA, based on PCR results (see below). Coloring of genes is as in Fig. 1. (B) Based on genome sequencing results of MNKN and CTH96 transductants, various sets of primer (see above map) were designed and tested to locate a linear form of phage DNA containing a bacteriocin gene cluster and LukD/E genes. PCR was positive with primer pairs p1654/p1655 and p1691/p1694 but not with p1651/p1655 and p1691/pseg, indicating a linear form of phage DNA with left flanking near SAB1654, and right flanking near SAB1694. (C) Southern blot analysis of RF122 chromosomal DNA (C) and phage DNA (P) digested with *Eco*RI restriction enzyme using a probe specific to the *lukE* gene (the membrane used in this figure is the same as in Fig. 1).

pairs p1654/p1655 and p1691/p1694 but not with p1651/p1655 and p1691/pseg (Fig. 2B), suggesting a linear form of phage DNA with left flanking near SAB1654 and right flanking near SAB1694 (Fig. 2A). However, one cannot rule out the possibility that several intermediates might be detectable as a result of imperfect excision of $\phi SaBov_N$ or $\phi SaBov_{EGC}$ or a stochastic event (e.g. nucleases digested at the ends of the linear DNA that was possibly fragmented by the phage). This type of transducing phage particle harboring a bacteriocin gene cluster and leukotoxin D/E genes was designated as $\phi SaBov_{LUK}$. To confirm the transduction activity of $\phi SaBov_{LUK}$, the *tetM* gene was introduced at the *lukE* gene (RF122 *lukE*::*tetM*). The phage induced from this strain was also successfully transduced the *lukE* gene to various recipients with a much lower transduction frequency (Table 1).

The role of integrase and terminase in the transfer of the νSaβ. The phage DNA excision, package, and integration are controlled by cooperative actions of integrase, excisionase, terminase, and host-encoded DNA binding proteins[15–17]. To examine the role of these genes from ϕSaBov on the transfer of the νSaβ, the *cat* gene, conferring chloramphenicol resistance, was inserted into the integrase (SAB1760) and, separately, into the terminase large subunit (TerL, SAB1726) gene in the RF122 *sem*::*tetM* strain. The mitomycin C treatment of these strains still induced a clear lysis within 3 hours, indicating that disruptions of these genes did not affect phage induction. However, outward PCR and PCR analysis showed that a disruption of the *terL* gene completely abolished the phage DNA packaging (Supplementary Figure S4) and complementation of the *terL* gene restored phage DNA packaging (data not shown), suggesting phage DNAs were packaged through headful packaging mechanism by terminase[18]. In contrast, disruption of the integrase gene did not affect phage DNA excision and circularization (Supplementary Figure S4). However, none of the transducing phage particles induced from this strain was transduced to the recipient strains and the complementation of the integrase gene restored transducibility (data not shown). These results suggest that the integrase encoded in the ϕSaBov is not required for the phage DNA excision and packaging but is required for phage DNA integration into the recipient chromosome. Furthermore, mitomycin C treatment of the RN4220 strain just carrying $\phi SaBov_N$ induced excision and circularization of $\phi SaBov_N$ phage DNA but a similar treatment of the MW2 strain did not (Supplemental Figure S5), indicating the excision and circularization of the $\phi SaBov_N$ phage DNA is dependent on host background. Strain RF122 harbors 5 alternative integrase genes associated with other MGE such as SaPIm4, SaPI122, SaPIBov1, or two inactivated phages. Currently, we are investigating the restoration of the phage DNA excision and circularization in the MW2 strain carrying the $\phi SaBov_N$ by complementation with an alternative integrase gene in the RF122.

Postulation of the νSaβ transduction model. Considering these data, we postulate the following νSaβ transduction model (Fig. 3). $\phi SaBov_N$ is firstly integrated into the $attN_R$ sequence at the tRNA-Ser which introduces the $attEGC_R$ site upstream of the int gene. Then, $\phi SaBov_{EGC}$ is integrated into the $attEGC_R$, resulting in the transfer of the egc and the duplication of the region spanning between $attN_L$ and $attEGC_R$. Homologous recombination events occur upstream of the SAB1676 gene, and downstream of $attEGC_R$ with the linear phage DNA introduced by $\phi SaBov_{LUK}$, resulting in the removal of the duplicating region spanning between $attN_L$ and $attEGC_R$ and the replacement of the region spanning the *lukE* gene, similar to Panton-Valentine leukocidin-phage mediated homologous recombination events between direct repeats of the two paralogous genes adjacent to the phage integration site[19]. As a result, nearly all of the νSaβ (from the 141 bp upstream of the start codon of SAB1676

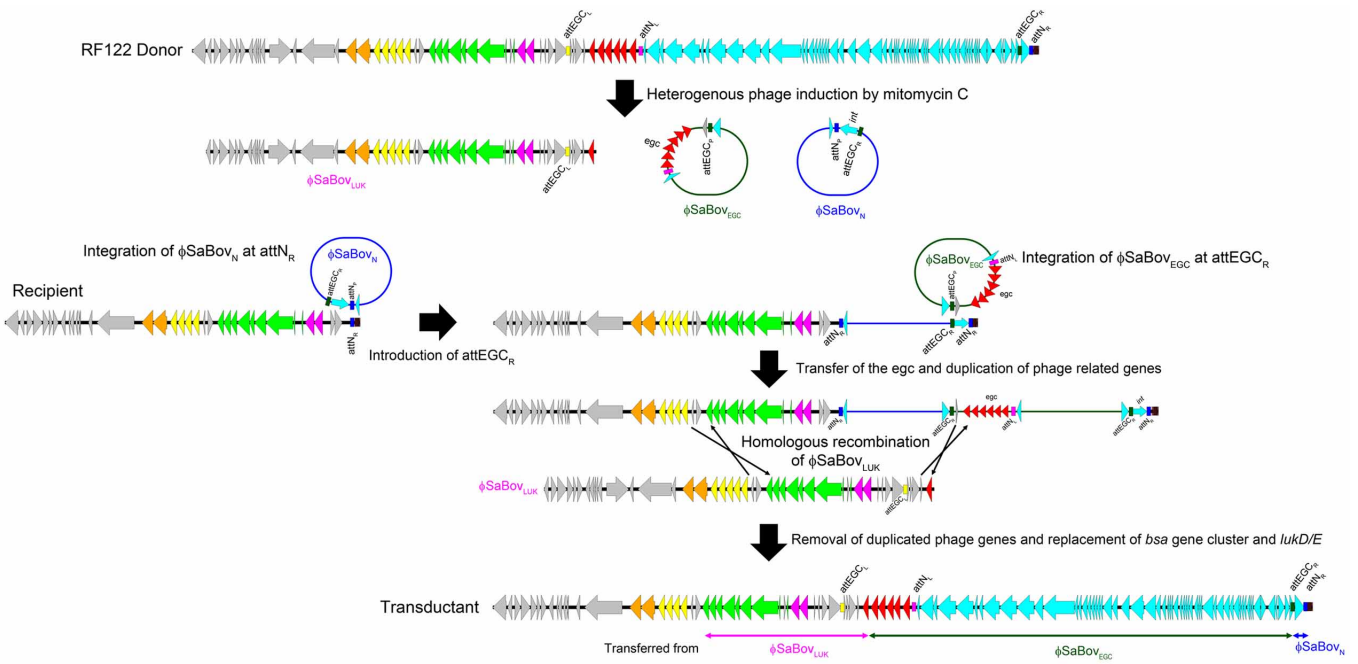

Figure 3 | Proposed model for transfer of νSaβ mediated by ϕSaBov. Upon induction by mitomycin C, phage DNA ($\phi SaBov_N$, $\phi SaBov_{EGC}$, and $\phi SaBov_{LUK}$) were excised from the RF122 chromosomal DNA and packed into phage head by terminase encoded in ϕSaBov. Upon entry to recipient strains, $\phi SaBov_N$ phage DNA is firstly integrated into recipient host chromosomal DNA through recombination between $attN_P$ (from $\phi SaBov_N$) and $attN_R$ (recipient chromosomal DNA). This event introduces the $attEGC_R$ in recipient chromosomal DNA which allows $\phi SaBov_{EGC}$ phage DNA for integrating into recipient chromosomal DNA through recombination between $attEGC_P$ (from $\phi SaBov_{EGC}$) and $attEGC_R$ (recipient chromosomal DNA). This event generates duplication of phage DNA. Homologous recombination occurs between $\phi SaBov_{LUK}$ phage DNA and integrated phage DNA, resulting removal of duplicated phage DNA. As a result of triple conversions, nearly all of the νSaβ from the donor strain is transferred to the recipient strain.

gene to the attN$_R$ sequence at the tRNA-Ser, a size of 65,767 bp) from strain RF122 was transferred to the recipient. Supporting this model, we were able to isolate transductant strains carrying intermediated forms of transduction carrying the ϕSaBov$_N$ at tRNA cluster and the ϕSaBov$_{EGC}$ at attEGC$_R$ without homologous recombination of the ϕSaBov$_{LUK}$ using a junction PCR as shown in Supplemental Figure S6. Furthermore, transductant strains carrying the ϕSaBov$_N$ or both ϕSaBov$_N$ and ϕSaBov$_{EGC}$ exhibited an increased capacity to accept the ϕSaBov$_{EGC}$ or the ϕSaBov$_{LUK}$, respectively, as shown in supplemental Table S3.

Distribution of a prophage in the νSaβ. To understand the significance of prophage in the dissemination of νSaβ, the prevalence of νSaβ and prophage in a collection of bovine isolates was investigated. From a collection of 2010–2013 bovine skin and mammary gland isolates from 8 different farms in the Ohio state, USA (n = 53), the presence of νSaβ was common (52/53, 98.1%) and 9 isolates (17.0%) have phage insertion between the egc and tRNA cluster, similar to the RF122 strain (Supplemental Figure S7). *spa* and MLST typing of these isolates showed that 7 and 2 isolates belong to CC97 and CC151, respectively, (Supplementary Table S4) which are commonly observed clonal complexes among ruminants[20]. By contrast, from another collection of bovine mammary gland isolates from 16 different farms in the Washington state, USA from 1985 to 2001 (n = 207), νSaβ was rare (102/207, 49.3%) and none of the isolates has the phage insertion at the νSaβ (data not shown). These results suggest that phage localization adjacent to νSaβ may have an important role in wide dissemination of the νSaβ in certain clonal complexes of bovine isolates.

Discussion

The versatile host adaptation and successful pathogenicity of *Staphylococcus aureus* is strongly influenced by the acquisition of virulence factors encoded in the mobile genetic elements such as prophages, plasmids, pathogenicity islands, and genomic islands. The genomic island, νSaβ, is found in almost all *S. aureus* strains and is characterized by extensive variation in virulence gene content[2,7,8]. However the basis for the diversity and the mechanism underlying mobilization of the genomic islands between strains are unexplained. This is the first experimental evidence demonstrating the transfer of the genomic island, νSaβ, by the naturally occurring staphylococcal temperate phage, ϕSaBov. Remarkable features of ϕSaBov are that it generated heterogeneous transducing phage particles harboring circular and linear forms of phage DNA containing overlapping segments of the νSaβ, totaling to 65.7 kb, and sequentially integrated into the host chromosome by specific recombination events. The exact mechanism of linear phage DNA excision and site specific homologous recombination still remain elusive. Given the high transduction frequency of ϕSaBov to the epidemic human and animal isolates and the rapid spread of the νSaβ in the isolates from bovine mastitis with concurrent existence of phage insertion at the νSaβ, our findings highlight the importance of bacteriophages in the pathogenic evolution of *S. aureus* and the need for caution in the therapeutic use of phage as it may cause undesirable consequences, such as transfer of potent toxins and other virulence factors.

Methods

Bacterial strains and growth conditions. Strains used in this study were summarized in supplementary table S1. A collection of 207 *S. aureus* bovine mammary gland isolates (16 different farms, Washington state, USA) from 1985 to 2001, and 53 bovine mammary gland and skin isolates (8 different farms, Ohio state, USA) from 2010 to 2013 were kind gifts from Drs. Fox (Washington State University) and Rajala-Schlultz (Ohio State University), respectively. Multilocus sequence typing and *spa* sequence typing was done for nine isolates from a collection of Ohio state isolates harboring phage insertion in the νSaβ, using previously described methods[21]. *S. aureus* strains were typically grown in tryptic soy broth (TSB) or agar (TSA), with the supplementation of tetracycline (5 μg/mL) or chloramphenicol (10 μg/mL) when necessary.

Phage induction and transduction. Cultures were grown to mid-log phase at 37°C with shaking (200 rpm), then mitomycin C (1 μg/mL) was added. The mixtures were incubated at 30°C with 80 rpm until complete lysis occurred (approximately 3 hours). The lysates were sterilized with syringe filers (0.22 μm). A phage spot test and the plaque forming unit (pfu) was determined by soft agar (0.5%) overlay method.

For transduction experiments, the recipient strains were cultured to mid-log phase and adjusted to approximately 2×10^7 CFU/mL. A phage solution containing approximately 10^8 PFU/mL was added to the culture, and incubated for 30 min at 30°C for the phage absorption, followed by adding sodium citrate solution (100 mM, pH 4.5). After centrifuging at 4,000 rpm, 4°C for 15 min, the pellet was resuspended in sodium citrate solution and plated on TSA supplemented with appropriate antibiotics.

Transmission electron microscope (TEM) analysis of phages. Phage particles were placed on carbon-coated copper grids and washed briefly on water droplets. After washing, grids were dried and mounted with 2% uranyl acetate for 1 min and analyzed using TEM (Philips CM200).

Phage DNA extraction and PCR. The mitomycin C treated culture lysates were treated with excessive amounts of RNase and DNase I (Sigma-Aldrich, 100 unit each), and then phage particles were precipitated with NaCl (0.5 M final concentration) and polyethylene glycol 8000 (10%, wt/vol), followed by ultracentrifugation at 100,000 × g for 1 h. Phage DNA was extracted using DNeasy kit (Qiagen) according to the manufacturers' instructions.

PCR and quantitative real time PCR. All primer pairs used in PCR and outward PCR were listed in supplementary table S2. Quantitative real time PCR was performed to estimate relative copies of ϕSaBov$_{EGC}$ to ϕSaBov$_N$ using SYBR Green master mix (Applied biosystem) by calculating ΔC_T of the *sem* gene to the integrase gene in the phage DNA, according to the manufactures' instructions.

Southern blot hybridization. Chromosomal and phage DNA were digested with *EcoR*I and resolved by electrophoresis in 0.5% agarose gels and transferred onto nylon membranes. Digoxigenin (DIG)-labeled DNA probes were synthesized using PCR-DIG DNA labeling kit (Roche) according to the manufacturers' instructions and primers listed in supplementary table S2. DNA hybridization and probe detection was performed using Chemiluminescent detection kits (Roche) according to the manufacturers' instructions.

Allelic exchange constructs. All primer pairs used in allelic exchange constructs were listed in supplementary table S2. Allelic exchange, resulting in the insertion of antibiotic markers and target gene inactivation, was done using temperature sensitive pMAD system[22] with minor modifications. The tetracycline resistance gene (*tetM*) and chloramphenicol resistance gene (*cat*) were amplified from the strain Mu50[23] and pMK4 and cloned into the pMAD, resulting in pMAD-tet and pMAD-cat, respectively. The upstream and downstream gene fragments of target genes were amplified, and cloned into pMAD-tet or pMAD-cat. Resulting plasmids were electroporated into the strain RF122. Results strains were cultured in 43°C (non-permissive temperature for the replication of pMAD) to promote the first homologous recombination, followed by culturing 37°C to promote the second recombination, resulting in allelic exchange as described previously[22].

Genomic DNA sequencing and analysis. Genomic DNA was isolated with a DNeasy Kit (Qiagen), and dsDNA was quantified with a Qubit HS Assay Kit (Invitrogen). Indexed, paired-end libraries were made from 1 ng samples of the MNKN recipient and the transductant with a Nextera XT DNA Sample Preparation Kit (Illumina). Libraries were cleaned with 1.2× AMPure XP beads (Agencourt) and sequenced using a 300 cycle MiSeq Reagent Kit v2 on an Illumina MiSeq instrument (Illumina). Using CLC Genomics Workbench v6, reads were trimmed and filtered for base quality, and assembled *de novo*. Recombined regions between the MNKN recipient and RF122 donor (GenBank NC_007622) were identified through local alignments.

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Author contributions

K.S.S designed the research. B.Y.M., J.Y.P. and S.Y.H. conducted the experiments. D.A.R. and J.C.T. provided critical sequencing information. Y.H.P., J.C.T, J.R.F., G.A.B., D.A.R. and K.S.S. analyzed data and wrote the manuscript.

Additional information

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