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Bacteriophage mediated gene transfer in *Staphylococcus aureus* and genetic engineering of the bacteriophage as alternative antimicrobials

황색포도상구균의 박테리오파지 매개 유전자 전달 및 대체 항생제로의 이용

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February, 2018

Department of Veterinary Microbiology

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Bacteriophage mediated gene transfer in *Staphylococcus aureus* and genetic engineering of the bacteriophage for alternative antimicrobials

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Abstract

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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. Most *S. aureus* strains carry a variety of MGEs, including three genomic islands (νSaα, νSaβ, νSaγ) that are diverse in virulence gene content but conserved within strain lineages. 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. We also demonstrate that ϕSaBov mediates the mobilization of νSaα and νSaγ, which are located remotely from ϕSaBov, mostly to recipient strains belonging to ST151. Phage DNA sequence analysis revealed that chromosomal DNA excision events from RF122 were highly specific to MGEs, suggesting sequence-specific DNA excision and packaging events rather than generalized transduction by a temperate phage. Disruption of the int gene in ϕSaBov did not affect phage DNA excision, packaging, and integration events. However, disruption of the *terL* gene completely abolished phage DNA packing events, suggesting that the primary function of temperate phage in the transfer of genomic islands is to allow for phage DNA packaging by TerL and that transducing phage particles are the actual vehicle for transfer. These results extend our understanding of the important role of bacteriophage in the horizontal transfer and evolution of genomic islands in *S. aureus*, highlighting the central role of bacteriophages in the pathogenic evolution of *S. aureus*.

Discovery of clustered, regularly interspaced, short palindromic repeats and the Cas9 RNA-guided nuclease (CRISPR/Cas9) system provides a new opportunity to create programmable gene-specific antimicrobials that are far less likely to drive resistance than conventional antibiotics. However, the practical therapeutic use of CRISPR/Cas9 is still questionable due to current shortcomings in phage-based delivery systems such as inefficient delivery, narrow host range, and potential transfer of virulence genes by generalized transduction. In this study, we demonstrate genetic engineering strategies to overcome these shortcomings by integrating CRISPR/Cas9 system into a temperate phage genome, removing major virulence genes from the host chromosome, and expanding host specificity of the phage by complementing tail fiber protein. This significantly improved the efficacy and safety of CRISPR/Cas9 antimicrobials to therapeutic levels in both in vitro and in vivo assays. The genetic engineering tools and resources established in this study are expected to provide an

efficacious and safe CRISPR/Cas9 antimicrobial, broadly applicable to *S. aureus*.

Keywords: Bacteriophage, Genomic island transfer, Genetic engineering, CRISPR/Cas9, Alternative antimicrobial

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Literature Review

I. Bacteriophage

1. Bacteriophage and the life cycles

Bacteriophages (phages) are viruses that infect bacteria. Phages were discovered in 1915 by a British pathologist, Frederick Twort and in 1917 a French–Canadian microbiologist, Félix d'Hérelle. Twort reported the "glassy transformation" observed in micrococci colonies, and d'Hérelle isolated an "anti-microbe" of *Shigella* and devised the term 'bacteriophage' — literally meaning bacteria-eater [1,2]. On the other hand, as early as 1927, the contribution of phages to the pathogenicity of their bacterial hosts was revealed throughout the observation of Frobisher and Brown. They discovered that nontoxigenic streptococci exposed to filtered supernatant of toxigenic streptococcal cultures acquired the ability to produce scarlatinal toxin [3]. These supernatants contained a phage encoding the scarlatinal toxin and investigators were describing it transduction i.e. the transfer of genetic material to a bacterial cell via phage infection. In most environments, a pool of phages and bacteria is linked in continuous cycles of co-evolution. A number of toxin genes were found to be phage encoded, and consideration of the role of phages in bacterial pathogenesis emphasized the dissemination of toxin genes among bacterial strains [4].

The double sides of phage, the killing or evolution of bacteria, are associated with the life cycle of phage, lytic and lysogenic cycle. To infect a host bacterium, a phage will first interact with receptors on the host cell, adsorb and then inject its genome. The subsequent replication strategy will depend on whether the phage is virulent (lytic) or temperate (lysogenic) [5]. Virulent phages (for example, phage T4) can only replicate via the lytic cycle that process the production of new vial progeny and their release from the infected bacterial host cell. On the other hand, once temperate phages (phage λ) enter a cell, they either initiate the lytic cycle or form a stable association with the host, termed lysogeny. During lysogeny, a phage in bacterial genome is called a prophage and replicates in concert with the host DNA, either in a free, plasmid-like state (for example, phage P1) or integrated into the bacterial chromosome (for example, phage λ). Under stressful conditions, prophages can escape the lysogenic state and replicate more virions that are released from the bacterium. Typically, release of phage progeny results in bacterial death through cell lysis [6,7], although filamentous phages are released by 'secretion' through the outer membrane, thereby avoiding bacterial lysis but causing a chronic infection that slows growth of the host cell [8].

Phages are very choosy as to what bacteria they infect. This is referred to as the host range of the phage. For example, λ only infects certain *Escherichia coli*, whereas Spo1 phage infects only *Bacillus subtilis*. Every phage has a characteristic burst size. Different phage also

takes different amounts of time to go through one growth cycle. Once bound to the cell, the phage must be moved into the cytoplasm. The rate of phage DNA transport can be very rapid. It is different for different phages but can reach values as high as 3,000 base pairs per second. In contrast, two other methods for getting DNA from the outside of the cell to the cytoplasm (conjugation) transfer the DNA at a rate of approximately 100 bases per second. In many cases the details of how a phage genome gets into the cytoplasm are not known [9].

2. The structure of phage

Phages have a chromosome covered in a capsid that is composed of phage-encoded proteins. For many phage types, the capsid is attached to a tail structure that is also made from phage-encoded proteins. T4 and P1 contain a linear double-stranded DNA genome enclosed in a capsid and attached to a tail that is referred as the tailed bacteriophages. The T4 genome is 172 kb, while P1 is a smaller phage with a genome of 90 kb. The T4 capsid is an elongated icosahedron. T4 has a very elaborate tail structure including a collar at the base of the head and a rigid tail core surrounded by a contractile sheath. The core and sheath are attached to a hexagonal base plate. Also attached to the tail plate are tail pins and six kinked tail fibers. P1 also has an icosahedral capsid, a tail with a contractile sheath, a base plate, and tail fibers. λ contains a linear double-stranded DNA genome of 48.5 kb, a capsid, and a tail. The finished capsid is again shaped like an icosahedron whereas the tail is a thin flexible tube that ends in a small conical part and a single tail fiber. M13 contains a circular single-stranded DNA genome of 6,407 nucleotides surrounded by five phage-encoded proteins. The M13 chromosome is coated by a single layer of ~2,700 subunits of gene VIII encoded protein (gpVIII) giving it a filamentous appearance, the reason M13 is also known as a filamentous phage. At one end of the filament are bound the M13 proteins encoded by genes VII and IX (gpVII and gpIX) and at the other end are bound the M13-encoded gene III and VI proteins (gpIII and gpVI).

II. Phage-encoded virulence factors in bacterial pathogenicity

Phages serve as a driving force in bacterial pathogenesis, acting not only in the evolution of bacterial pathogens through gene transfer, but also contributing directly to bacterial pathogenesis at the time of infection. However, the impact of phages on the evolution of bacteria is underscored by the estimation that, globally, ~2 × 1016 phage-mediated gene transfer events occur every second [10]. Furthermore, in 1996, the discovery that the key virulence factor of *Vibrio cholerae* that is encoded in the genome of the transferrable filamentous phage CTXϕ highlighted the importance of phages in the evolution of bacterial pathogenicity [11]. This process, termed lysogenic conversion (or phage conversion), was first observed in the 1950s, and it describes a situation in which a prophage provides additional genes that benefit the lysogen [12].

The importance of phages for pathogenicity in bacteria was further demonstrated in the 1990s, when genome sequencing revealed the abundance of prophages and established that they account for the main genetic variability between closely related bacterial strains (for example, pathogens versus nonpathogens) [10]. For example, in *Streptococcus pyogenes*, ~10% of the genome consists of prophages, which encode multiple virulence factors, and in *E. coli* O157:H7 str. Sakai, 18 prophages constitute 16% of its genome [10]. The genome of *Staphylococcus aureus* is composed of more than 20% of mobile genetic elements (MGEs) including phages that play critical roles to the pathogenic evolution of *S. aureus*. More than 68 staphylococci phages and prophages have been found so far [13]. In some cases, the prophages define core aspects of bacterial pathogenesis. For example, in Shiga toxin-producing *E. coli*, prophage induction upregulates the toxin genes, and cell lysis is important for toxin release [10,14]. In addition, prophages carrying the regions of homology can motivate the changes of evolution through inversions or deletions and other chromosomal rearrangements. *S. pyogenes* M3 strain isolated from Japan is discriminated from the strain isolated from United States due to a chromosomal inversion between two different

prophages reshuffling of the prophage virulence genes [10]. Inversions and deletions can also modulate fitness through selection events that drive rapid evolutionary changes.

Release of bacterial DNA throughout the bacterial lysis by phage enhances horizontal gene transfer (HGT) between bacteria, which increase the chance that can be acquired by neighboring competent cells. Phages can directly inject random fragments of host or plasmid DNA into neighbouring bacteria, by generalized transduction, where bacterial DNA is accidentally packaged during phage replication and then delivered into neighboring cells [15]. Phage-inducible chromosomal islands (PICIs) can hijack phages to assist in their transfer, giving rise to high-efficiency transduction, where the islands are transferred to neighbouring bacteria. For example, the *S. aureus* pathogenicity islands (SaPIs) encode superantigens and 'parasitize' phages for high frequency transduction. As identification of PICIs and GTAs is challenging, their general contribution to gene transfer is probably underestimated [16,17]. Specialized transduction is that DNA located adjacent to the integrated prophage after imprecise excision is packaged and transferred between bacteria. The temperate phage P22 mediates both generalized and specialized transduction in *Salmonella typhimurium*. Aberrant excision events of P22 generate the substantial numbers of specialized transducing genomes that are heterogeneous but only some of which habe terminally redundant ends [18].

Transduction facilitates the mobilization of antibiotic resistance and virulence genes, and antibiotic exposure can promote these processes [19,20]. A form of 'constitutive generalized transduction' is promoted by gene transfer agents (GTAs), which have a significant role in HGT in bacteria [21]. GTAs are prophage-like elements encoded in bacterial genomes that package random host DNA but cannot package enough to enable the transmission of their own genes [16]. GTAs might have evolved from mutant prophages that became defective and subsequently decayed.

Phage-encoded genes are not always transmissible, however, either due to technical limitations in detecting transduction or due to the fact that integrated prophages frequently become defective. Analysis of the genome sequences of bacterial pathogens

can expeditiously reveal whether virulence factors are associated with phage-like DNA sequences regardless of whether they are transmissible. For example, the nontransmissible *stx* genes in *Shigella dysenteriae* are adjacent to lambdoid phage-like sequences interrupted by numerous insertion sequences, suggesting that the toxin genes lie in a prophage that has been rendered defective by the insertion sequences [22].

Moreover, transduction of a virulence gene is, in and of itself, insufficient evidence that the gene resides in a phage genome. Generalized transducing phages can package and transmit any chromosomal locus, and specific interactions between generalized transducing phages and chromosomal virulence genes have been described. For example, the transmission of the *V. cholerae* pathogenicity island (VPI), which was reported to correspond to the genome of a phage has not been ruled out [23]. Similarly, the *S. aureus* pathogenicity island SaPI1, which contains the gene for toxic shock syndrome toxin (*tst*), is also mobilized at high frequency by the generalized staphylococcal transducing helper phage ϕ80α [24].

Currently, analysis of sequences surrounding virulence factor genes offers the most direct and sensitive method of determining whether virulence genes are associated with phage-like sequences. While this approach can reveal whether a virulence gene is associated with phage sequences, it cannot reveal whether the gene is part of a prophage capable of transducing it or of influencing its expression. Mutational analyses can complement sequence analysis in this respect, revealing, for example, the necessity of phage morphogenesis genes for transduction or the necessity of phage regulatory sequences for virulence factor production [11,25].

III. Biotechnological exploitation of phages - its impact on basic and applied biology.

Phages are natural predators of bacteria, and have also evolved along with bacteria in an ancient arms race. Each ‘phage’ targets a particular strain of a bacterial species, and binds a specific receptor on the bacterial cell surface before injecting its own DNA into the cell. In terms of therapeutics, drug developers favour lytic phages, which replicate inside the bacterial cell and cause it to rupture, releasing the new viruses, although lysogenic phages, which incorporate their genetic material into the bacterial DNA, also exist, and are a less attractive therapeutic option because they might spread resistance or virulence factors among bacteria.

Central to the appeal of phages as therapeutics is the fact that their target pathogen acts as a switch to turn on replication and to rapidly increase the level of available phage until the infection is resolved. If the phage does not meet its adversary, it is cleared harmlessly by the body. The concept of bacteriophages as therapeutics dates back almost 100 years. Canadian physician Félix d’Herelle, considered the founding father of this branch of medicine, first isolated and characterized bacteriophages in 1917, and went on to show they could be used to treat bacterial infections in humans. While phage therapy became overshadowed in the West by antibiotics, it was a picked up in the former Soviet Union and other Eastern Bloc countries such as Poland, which did not have ready access to antibiotics. Today, phage preparations continue to be available over the counter in Georgia.

1. Application of phage as a therapeutic agent

Recently, the increases of antibiotic resistance along with the paucity of new antibiotics have promoted the renaissance of phage inspired antibacterial approaches in agriculture, medicine and some food industries. There is also interest in utilizing phages for sensitive and specific detection of bacteria. Multiple detection methods are being developed, including systems based on phage-induced lysis, phage amplification, and

delivery of reporter genes, cell surface-binding proteins and biosensors. Phage therapy using natural or modified virulent phages is showing encouraging therapeutic results, with controlled clinical trials underway. A cockatiel of phages used to treat *Pseudomonas aeruginosa* ear infections showed significant efficacy and safety [26]. Currently, a Phase I/II clinical trial involving three European countries is investigating the safety and efficacy of phages for the treatment of burn wounds infected with *E. coli* and *P. aeruginosa* (http://www.phagoburn.eu). For Food safety and agriculture, there has been faster uptake of phage technologies, with several approved products commercially available. LISTEX P100 (Microbes; the Netherlands) and ListShield (Intralytix; Baltimore, Maryland, USA) are typical examples for protection of processed foods form *Listeria monocytogenes*.

Other innovative antimicrobial approaches using phage products or engineered phages are being developed. Considerable research has focused on endolysins, which are peptidoglycan hydrolases involved in cell lysis during phage replication [27]. The loss of structural integrity upon peptidoglycan degradation causes lysis through osmotic imbalance [28]. In Gram-negative bacteria, the outer membrane must also be breached through complexes (known as spanins) that fuse both membranes [27]. Endolysins are typically composed of an enzymatically active domain and a cell wall-binding domain [6]. Extensive endolysin diversity exists, and most endolysins are species specific, although some are more promiscuous. The modularity of endolysins has facilitated 'mixing and matching' of domains to increase activities and alter host ranges [6]. By fusing endolysins to other domains, it is possible to deliver endolysins across the Gram-negative outer membrane or into eukaryotic cells to target intracellular bacteria [6,29]. Phase I and Phase II clinical trials are underway for the topical or intravenous use of endolysins for the control of *S. aureus* infection in humans, and preclinical trials have been carried out in various infection models [6]. The first commercially available endolysin, Staphefekt (Micreos), is available for the treatment of human skin infections caused by *S. aureus*.

The specificity of phages for their target bacteria is often considered to be a therapeutic advantage as this limits collateral damage to beneficial microorganisms, but altering the host range can be desirable. For the EPS-degrading phage T7, utility was enhanced by introducing gene 1.2 from, enabling infection of *E. coli* containing the F plasmid [30]. Evolution can also be exploited to select for altered receptor recognition and host range. For example, in vitro evolution of phage resulted in recognition of a new porin receptor, outer membrane protein F (*ompF*) [31]. In nature, *Bordetella* phage BPP-1 accelerates its tail fibre evolution to alter its host range through diversity-generating, site-specific, error-prone reverse transcription [32]. The specificity of phages, and their efficiency of DNA delivery, has been exploited for the injection of bacteria with lethal genes, which encode restriction endonucleases, holins, toxins of toxin–antitoxin systems or proteins that condense DNA (for example, SASPject; Phico Therapeutics, Cambridge, UK).

Phages can also improve antibiotic efficacy. For example, antibiotics can be conjugated to phages, enabling delivery to specific cells and causing an increase in local drug concentration [33]. Furthermore, antibiotic resistance can be overcome by using phages to inject sensitizing alleles of the mutated genes (for example, *rpsL* and *gyrA*) to restore drug efficacy [34]. Alternatively, delivery of regulatory genes can reprogramme cells in a defined manner. For instance, the introduction of genes that inhibit the stress response (such as *lexA*), improve drug uptake (such as *ompF*) or repress biofilm production (such as *csrA*) can increase the antibiotic sensitivity of *E. coli* [35]. Therefore, phages offer a wide range of potential methods to tackle bacterial infections and antibiotic resistance. However, regulatory issues need to be addressed for engineering approaches.

2. Genetic engineering of bacteriophage

1) Application of CRISPR-Cas system on a phage therapy

In bacteria, CRISPR–Cas has been used to generate mutations in several bacterial genomes with potential use for synthetic biology and metabolic pathway engineering, and has been explored as a novel antimicrobial strategy [36]. For example, CRISPR–Cas can kill bacteria in a sequence specific manner to selectively eliminate particular strains, to select for less virulent survivors owing to the loss of pathogenicity islands, and to inhibit antibiotic-resistant bacteria by targeting resistance genes [36,37]. In addition, CRISPR–Cas9 can be used to manipulate phage genomes to study phage biology with greater ease and precision [38-41]. These studies demonstrated the potential use of CRISPR/Cas9 system as a programmable antimicrobial to selectively control the target bacteria at the DNA level without disturbing the normal microbiome. However, the efficacy of CRISPR/Cas9 antimicrobials is still far from being therapeutic, mainly due to the low efficiency in phage-based delivery systems which limited the efficacy of CRISPR/Cas9 to reduce bacterial colony forming units (CFU) by only one or two logs in in vivo and in vitro assays [39,40]. Furthermore, phage-based delivery systems may deliver not only a plasmid or phagemid harboring CRISPR/Cas system, but also host chromosomal segments by generalized and specialized transduction to target cells [19]. This is particularly important for phage-based delivery systems using *S. aureus* since many important staphylococcal virulence factors such as superantigens and cytolysins are commonly located in mobile genetic elements (MGEs) and are transferred to other *S. aureus* and *Listeria monocytogenes* by temperate phage-mediated generalized transduction, thereby raising the safety issues [42-45].

Alternatively, against the bacterial defend on the phage infection, phages often carry inhibitors of CRISPR-Cas system that enhance their ability to either degrade their host genome or integrate into its genome. The first examples of phage-encoded “anti-CRISPR” proteins have been discovered in *P. aeruginosa* and identified the class 1 type I-F and I-E systems that are associated with small proteins (50–150 amino acids) of unknown function, previously [46]. Recent investigations have demonstrated the conservation of signature anti-CRISPR-associated (*aca*) gene with a predicted helix-turn-helix (HTH) motif to identify anti-CRISPRs across proteobacteria, broadly

spanning the type I-F CRISPR-Cas phylogeny [47].

Anti-CRISPR was occasionally found in the genome of prophage, when there is the genomic co-occurrence of a spacer and its target DNA with a perfect match, called “self targeting”. In such cases, the prophage can possess a DNA target with perfect identity to a CRISPR spacer in the same cell, as the CRISPR-Cas system is inactivated. Self-targeting is a consequence of autoimmunity rather than gene regulation. Interestingly, the self-targeting can induce bacterial cell death. Therefore, use of self-targeting and regulation of anti-CRISPR can be applied to the development of antimicrobials. The first report of cell death induced by a CRISPR-Cas system targeting a sequence on the bacterial chromosome was published by Edgar and colleagues [48]. They observed that inducing the CRISPR-Cas system led to the death of 98% of the cells in the population, and already speculated at the time that cell death was the result of chromosomal DNA degradation by the Cas enzymatic machinery. It was later shown that self-targeting by Type I systems efficiently killed bacteria regardless of the target location [49] and can lead to the excision of large pieces of DNA in the target region [50]. In Type I systems, the CASCADE complex binds the target and recruits the Cas3 exonuclease leading to extensive DNA degradation [51,52]. For killing cells, Cas3 is a better option than Cas9, which is commonly used in CRISPR genome editing tasks; Cas9 creates double strand breaks that cells can repair, whereas Cas3 cleavage is irreversible. CRISPR-Cas3, owing to its ability to target many sites in the genome at the same time, can avoid potential resistance mechanisms.

2) Alteration of the tail fiber protein from phages that have broad host range

The first step of bacteriophage infection is recognition and binding to the host receptor, which is mediated by the phage receptor binding protein (RBP). To change of host range, alteration of tail fiber protein of phages that have broad host range can expect expended range. Masatoshi Yoichi et al have demonstrated that gene products (gp) 37 and 38, expressed at the tip of the long tail fiber of T2 phage, were exchanged

with those of PP01 phage, an *E. coli* O157:H7 specific phage [53]. Homologous recombination between the T2 phage genome and a plasmid encoding the region around genes 37–38 of PP01 occurred in transformant *E. coli* K12 cells. The recombinant T2 phage, named T2ppD1, carried PP01 gp37 and 38 and infected the heterogeneous host cell *E. coli* O157:H7 and related species. Shuai Le et al have demonstrated that the binding specificity of the RBP of *P. aeruginosa* phages PaP1 and JG004 [54]. These two phages share high DNA sequence homology but exhibit different host specificities. A spontaneous mutant phage was isolated and exhibited broader host range compared with the parental phage JG004. Sequencing of its putative tail fiber and baseplate region indicated a single point mutation in ORF84 (a putative tail fiber gene), which resulted in the replacement of a positively charged lysine (K) by an uncharged asparagine (N). They further demonstrated that the replacement of the tail fiber gene (ORF69) of PaP1 with the corresponding gene from phage JG004 resulted in a recombinant phage that displayed altered host specificity. These results may have potential value of hybrid-phage in phage therapy.

IV. *Staphylococcus aureus*

1. Overview

S. aureus is the normal flora of the nasopharynx of about 30% of healthy humans and are carried in wide range of animals including porcine, bovine, poultry, canine, feline and horse [55-60]. In contrast of the characteristic as commensal bacteria, the microorganism is one of pathogenic and zoonotic agent causing from mild symptoms to severe diseases opportunistically. In human, *S. aureus* leads most cutaneous infections, e.g., furunculosis, impetigo and abscesses, and organ infections, e.g., osteomyelitis, endocarditis, arthritis, and necrotizing pneumonia. In addition, it is responsible for the toxinoses such as food poisoning, septic shock, scalded skin syndrome and toxic shock syndrome (TSS) [61-64]. In animal, *S. aureus* can cause the several infections including mastitis in cattle and dairy producing animals such as sheep and goats, septicemia, "bumblefoot" and chondronecrosis in chickens and other poultry species, exudative epidermitis (EE) in pigs [65,66]. *S. aureus* does not typically colonize animal dogs or cats but may have a transient association and can occasionally cause severe infections [67].

Methicillin resistant *S. aureus* (MRSA) has since spread and is endemic in most hospitals and community worldwide. *S. aureus* is the number one cause of hospital-associated infections, and a high percentage of these are caused by MRSA [68-70]. Methicillin resistant *S. aureus* carry staphylococcal chromosome cassette *mec* (SCC*mec*) that lender the resistance of beta-lactam antibiotics. SCC*mec* is a variable genetic element that contains the methicillin resistance determinant, *mecA*, and a site-specific recombinase gene, *ccrAB* or *ccrC*, and may contain additional resistance determinants [71-74]. The mortality rate associated with invasive MRSA infections is approximately 20%, and in the United States these infections are probably the leading cause of death by any single infectious agent; fatalities resulting from these infections are estimated to surpass those caused by HIV/AIDS [75].

MRSA is able to persist not only in hospitals (with a high level of antimicrobial agent use) but also in the community (with a low level of antimicrobial agent use). The former is called hospital-acquired MRSA (HA-MRSA) and the latter community-acquired MRSA (CA-MRSA). HA-MRSA strains historically contain predominantly SCC*mec*I–III, whereas CA-MRSA typically carries SCC*mec*IV [76,77]. SCC*mec*IV, which is smaller than SCC*mec*I–III, lacks other antibiotic resistance elements and appears to impart little or no fitness cost to the organism [76]. Consistent with this idea, CA-MRSA strains in general have faster growth rates than health care–associated MRSA strains [76]. In addition to SCC*mec*IV, CA-MRSA strains carry virulence genes, Panton-Valentine leukocidin (PVL) that is the toxin to cause edema, erythema, and tissue necrosis [78], arginine catabolic mobile element (ACME) that might contribute to fitness and epidemic spread [79], α-Type phenol-soluble modulins (PSMα) that lyse human neutrophils and ultimately promote *S. aureus* pathogenesis [80]. Those are often used as markers for CA-MRSA.

The economic impact on overall incidence of *S. aureus* infection has not been clearly delineated. An extensive literature search for economic studies on CA-MRSA yielded only two; one quantifying the cost impact of an epidemic on Driscoll Children's health plan81 and another focusing on just pneumonia patients [82]. Until CA-MRSA's overall economic burden is better quantified, it may be difficult for decision makers to determine where CA- MRSA should fall on public health, medical, and scientific priority lists. In South Korea, Kim et al. have reported that the estimated annual cases of nosocomial bloodstream infection (BSI) that is complication of invasive infection were 2,946 for MRSA and 986 for MSSA. The additional economic burden per case of nosocomial SA-BSI was US $20,494 for MRSA-BSI and $6,914 for MSSA-BSI. Total additional annual cost of nosocomial SA-BSI was $67,192,559 [83].

2. Mobile genetic element of *S. aureus*

S. aureus is a versatile bacterial pathogen that can survive in diverse host environments. This versatility depends on its ability to acquire and utilize nutrients from different sources, and invade and evade host immune system. The adaptation and pathogenicity of *S. aureus* in various hosts is primarily due to the production of a battery of virulence factors such as adhesion molecules promoting colonization in various tissues, cytotoxins destroying host immune cells and tissues, superantigens modulating host immune responses and antibiotic resistance leading to antibiotic treatment failure.

Introduction of microarray and whole genome sequencing revealed that *S. aureus* genome consist of core genome (~70%, shared by more than 95% of *S. aureus*), core variable (~12%, largely conserved within clonal complexes), and mobile genetic elements (MGE, ~18%) [84]. The core genes control essential metabolic pathway, information processing, and bacterial structural and regulatory components. The core variable contain regulators of virulence genes or known or predicted to be expressed on the bacterial surface and to interact with the host during colonization and infection, which may be a key motivation of the adaptation and survival of *S. aureus* [84]. The MGEs of *S. aureus* involve plasmids, transposons, *S. aureus* pathogenicity islands (SaPIs), staphylococcal chromosomal cassettes (SCC), and bacteriophages, and they mostly carry various virulence or antibiotic resistance genes [85]. These CVs and MGEs are associated with the genetic plasticity of *S. aureus* because they are diverse with numerous combinations of virulence genes in different lineages and hosts. These results postulated that those might be the major driving force for evolution of *S. aureus* by conferring the fitness under selective environments.

Mobile genetic elements (MGEs) are the DNA modules that facilitate the movement of DNA, referred as horizontal gene transfer (HGT), between prokaryotes, prokaryote to eukaryote and *vice versa*. The DNA segments contain commonly integration sequence, the related gene encoding enzymes to the own movement, such as integrase, excisionase, and packaging and variety specific virulence genes. MGEs may consist of insertion sequences, transposons, bacteriophages, plasmids, pathogenicity islands, and chromosome cassettes. Bacteria obtain genetic information from other cells or the

surrounding environment in three ways: (1) uptake of free DNA from the environment (transformation), (2) bacteriophage transduction, and (3) direct contact between bacterial cells (conjugation). In these processes, gene loss, alteration, and acquisition may occur frequently and allow the adaptive evolution of prokaryotes. Under stressful conditions, selective pressure may drive the enhancement of specific genes to prompt fitness and survival [85].

In efforts to identify virulence factors in specific diseases or host, comparisons of core variables and MGEs among the isolates from different sources such as nasal, skin, and infected site of humans and animals have studied, however, a clear linkage have yet to elucidate. Thus far, a total of 62 virulence genes, 38 antibiotic resistance genes and 72 MGEs have been characterized; twenty-six cell wall-anchored proteins classified into four distinct groups including microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), NEAT motif family, three-helical bundle family, and G5-E repeat family, twenty superantigens including staphylococcal enterotoxin (SE) A, B, C, D, E, H, and I, staphylococcal enterotoxins like (SEL) G, J, K, L, M, N, O, P, Q, R, U, X and toxic shock syndrome toxin 1, and sixteen cytotoxins including phenol soluble modulin (psm), α-hemolysin (hla), β-hemolysin (hlb), and bi component pore forming toxins including γ-hemolysin (hlg) A, B, C, leukocidin (Luk) D/E, F/S, A/B, M/F`, and P/Q (nature review, bi-component) [85-89].

MGEs is broadly classified and designated with their structure genes encoding integrase and terminase and the kinds of virulence gene. MGEs of *S. aureus* are diverse with a vast of combinations of the virulence and structural genes. These characteristics allow deducing the classification of MGEs based on the kinds of virulence genes found. For example, staphylococcal bacteriophage ϕSa3, carrying enterotoxin genes, *sea*, *sek*, and *seq*, and immune evasion gene cluster, *sak*, *chp*, and *scn* has been identified to nine types based on different combination of virulence genes (ϕSa3 type A-G, ϕSa3mw, and ϕSa3USA) and the variants [85]. In animal isolates, LukMF` that is carried with ϕPV83 and leukotoxins remarkable cellular tropism for bovine phagocytes, were mainly found in *S. aureus* isolated from bovine infection [90]. *S. aureus* pathogenicity islands, νSaβ,

carrying *spl*, *lukD/E* and enterotoxin gene cluster (egc), *seg, sei, sem, sen, seo,* and *seu* was found in a number of the variation in different CCs and host [85].

S. aureus MGEs consist of plasmid, genomic islands (GIs), *S. aureus* pathogenicity islands (SaPIs), staphylococcal chromosomal cassette (SCC), phage (prophage), which is carrying diverse virulence and resistant determinants. Table 1 presents the classification of *S. aureus* MGEs carrying virulence determinant.

1) *S. aureus* bacteriophage

Phages encode a large proportion of *S. aureus* virulence factors and provide the pathogen with a large variety of toxins, mainly allowing escaping host immune system [85,91,92]. Many factors have been described and characterized, such as the widespread immune modulator staphylokinase (*sak*) responsible for host tissue destruction, the chemotaxis inhibitory protein CHIP (*chp*), the staphylococcal inhibitor of complement SCIN (*scn*) and several superantigens (*sea, seg, sek, sek2, sep, seq*) [93]. These superantigens are enterotoxins causing food poisoning, toxic shock syndrome and necrotizing fasciitis [87]. In addition, the bi-component cytotoxin Panton-Valentine leukocidin (PVL, encoded by *lukF-PV, lukS-PV*) and related leukocidins (*lukM, lukF`*) form pores into leukocytes and cause necrotic infections [90,93]. Finally, the exfoliative toxin A (*eta*) is involved in severe skin infections [94].

In general, phages are carrying single virulence factor genes, although some exceptions have been reported. For examples, ϕSa3 phages and relatives, such as ϕN315, may encode up to five virulence factors, which form an immune escape complex (IEC) [85,95]. Virulence factor genes are not strictly associated to a specific phage and appear to be exchanged by horizontal gene transfer and recombination [10,92]. Virulence genes are often located near the attachment site (*att*) of the prophage, i.e., adjacent to the host chromosome. A possible origin for their acquisition by phages might be aberrant excision events from an ancestral bacterial chromosome [96]. They might also derive from mobile genetic elements, as suggested by the presence of transposase genes

flanking toxin genes as in ϕPV83 [97]. Thus, phages clearly impact virulence by positive lysogenic conversion, since they provide novel functions and activities to the host. Negative conversion also occurs as prophage insertion might inactivate genes [10,98,99]. In most of the cases, both phenomena occur simultaneously. As an example, ϕSa3-related phages integrate in beta-hemolysin gene, rendering lysogens defective for beta-hemolyse but effective for IEC production. The integration site relies on the specificity of the phage integrase [99,100].

Expression of phage-encoded virulence genes is maximal upon entry in the lytic cycle, since latent promoters are activated and phage genome is replicated, leading to an increase in genome copy number [95,101,102], although some expression is detected during lysogeny [96].

2) Genomic islands (GIs)

Three families of genomic islands exist among the *S.aureus* strains whose genomes have been sequenced [103-105]. These genomic islands, named νSα, νSaβ, νSaγ, are flanked by a broken transposase gene upstream and partial restriction-modification system (RM) type I downstream. Given the composition of genomic islands (remnant transposase genes and a G+C content that differs from the core genome), a current notion is that genomic islands were once mobile elements acquired by HGT [106]. A complete RM type I comprises host specificity determinant genes *hsdR*, *hsdM*, and *hsdS*, but only *hsdM* and *hsdS* are found juxtaposed to the *S. aureus* genomic islands [104,107]. Both flanking DNA segments contribute to the stability of genomic islands within the *S. aureus* chromosome. A lipoprotein gene cluster (*lpl*) and staphylococcal superantigen-like genes (*ssl*) are located on νSα [108]. νSaβ (also known as SaPIn3/m3) encodes bacteriocin, enterotoxins, hyaluronate lyase, and a serine protease gene cluster [104,109]. The third staphylococcal genomic island, νSaγ, contains genes encoding

Table 1. *Staphylococcus aureus* virulence determinant encoded on mobile genetic elements (MGEs).

	MGEs		Virulence determinant[a]	Other nomenclature	Strain
SaPIs	SaPI1		*ear, tst, sek, seq*		RN4282
	SaPI2		*tst*		RN3984
	SaPI3		*ear, seb, sek, seq*		COL
	SaPI4		None	PT1028	MRSA252
	SaPI5		*ear, sek, seq*		LAC
	SaPIbov1		*tst, sec, sel*		RF122
	SaPIbov2		*bap*		V329
	SaPIm4		*fhuD*	SaPIm4	COL, Mu50, N315
	SaPImw2		*tst, sec, sel*	SaPImw2	MW2
	SaPIn1/m1		*tst, sec, sel*	SaPIn1/m1	N315, Mu50
GIs	νSaα	Type 1	*set6-15, lpl1-9*	SaPIn2, SaPIm2	LAC, Mu50, N315, RF122
		Type 2	*set16-26, lpl10-14*		MW2
		Type 3	*set1-5, lpl2,7,8,11,13*		COL
	νSAβ	Type 1	*spl, lukD/E*		
		Type 2	*spl, lukD/E, bsa*		LAC[b], Mu50, MW2
		Type 3	*spl, lukD/E, egc*		
		Type 4	*spl, lukD/E, bsa, egc*		RF122
	νSAγ		*set, hla, psmβ*		COL, LAC, Mu50, N315, MW2, RF122
Prophage	ϕSa1		None	ϕSa1mu	Mu50
	ϕSa2		*LukF/S-pv*	ϕSLT, ϕPVL	MW2, LAC
	ϕSa3	Type A	*sea, sak, chp, scn*		
		Type B	*sak, chp, scn*	ϕSa3USA	LAC
		Type C	*chp, scn*		
		Type D	*sea, sak, scn*		
		Type E	*sak, scn*		
		Type F	*sep, sak, chp, scn*		
		Type G	*sep, sak, scn*		
		ϕSa3mw	*sak, sea, seq, sek*		MW2
		ϕSa3mu	*sak, sep*		N315, Mu50

	ϕCOL	*geh*	ϕCOL	COL
	ϕPV83	*lukM/F`*		
Plasmid	pIB485	sed, sej, ser		
SCC*mec*		arcA		LAC

a. Staphylococcal enterotoxins; *sea, seb, sec, sek, sel, sep, seq, sed, sej, ser, tst1*, Enterotoxin gene cluster (*seg, sei, sem, sen, seo, seu*); *egc*, Pathogenicity island protein; *ear*, Adhesion protein Bap; *bap*, Ferrichrome operon; *fhuD*, Staphylococcal enterotoxin-like; *set*, Lipoprotein gene cluster; *lpl*, Serine protease-like protein; *spl*, Leukotoxin D/E; *lukD/E*, α-hemolysin; *hla*, Phenol-soluble modulin β; *psmβ*, Panton-Valentine leukocidin F/S; *LukF/S-pv*, Staphylokinase; *sak*, chemotaxis inhibitory protein; *chp*, Staphylococcal inhibitor of complement; *scn*, lipase; *geh*, Leukotoxin M/F`; *lukM/F`*.

b. LAC carry epidemin gene cluster (*epi*) instead *bsa*.

β-type phenol-soluble modulins and a cluster of *ssl* genes similar to that present within νSα [105]. Genomic islands are not as competently mobile as other MGEs, due to the lack of typical genetic elements required for or indicative of mobilization such as integrases, excisionases, terminases, and associated repeat sequences [104,106].

3) Staphylococcal pathogenicity islands (SaPIs)

SaPIs carry staphylococcal enterotoxins (SEs) and have been separated into ten different SaPIs based on the specific superantigens combination and the host that they are integrated［85]. The characteristics of SEs on SaPIs contribute the pathogenicity of *S. aureus* as shown in Table 1. The mechanism of the SaPI movement has yet been clear, although the genome is organized insertion sequence (attachment site, *attS*) and functional genes related the movement, such as integrase (*int*), excisionase (*xis*), replication (*pri*, and *rep*), transcription factors (*stl*, and *str*) packaging (*terS*) [17]. Novick and colleagues have well demonstrated that SaPIs requires a resident helper phage. Depending on the induction of prophage by SOS response, SaPIs are excised from host genome. Alongside the production of phage virions, smaller SaPI capsids are produced from phage proteins. Packaging of SaPI DNA into these phage capsids is initiated by cleavage of the DNA at SaPI *pac* sites (terminase recognition sites) by *TerS*. Phage-induced lysis releases both mature phages and SaPI particles. On entry into a new host cell, SaPI DNA follows a replicative pathway, if the DNA is accompanied by an incoming phage genome and an integrative pathway if not.

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General Introduction

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. Most *S. aureus* strains carry a variety of MGEs, including three genomic islands (νSaα, νSaβ, νSaγ) that are diverse in virulence gene content but conserved within strain lineages. 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, νSaα, νSaβ, and νSaγ, encoding an array of virulence factors was transferrable in vitro to human and animal strains of multiple *S. aureus* clones via a resident prophage, ϕSaBov and investigated the mechanism of excision and integration of genomic islands and ϕSaBov.

Discovery of clustered, regularly interspaced, short palindromic repeats and the Cas9 RNA-guided nuclease (CRISPR/Cas9) system provides a new opportunity to create programmable gene-specific antimicrobials that are far less likely to drive resistance than conventional antibiotics. However, the practical therapeutic use of CRISPR/Cas9 is still questionable due to current shortcomings in phage-based delivery systems such as inefficient delivery, narrow host range, and potential transfer of virulence genes by generalized transduction. In this study, we demonstrate genetic engineering strategies using ϕSaBov to overcome these shortcomings by integrating CRISPR/Cas9 system into a temperate phage genome, removing major virulence genes from the host chromosome, and expanding host specificity of the phage by complementing tail fiber protein.

Chapter I.

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

I. Introduction

S. 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 (Fig. 1) [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 ICE6013, SCCmec and νSaα [9]. However, the mechanisms underlying the mobilization of genomic islands νSaα and νSaβ are unknown.

II. Materials and Methods

1. Bacterial strains and growth conditions.

Strains used in this study were summarized in Table 1. A collection of 207 *S. aureus* bovine mammary gland isolates (16 different farms, Washington state,

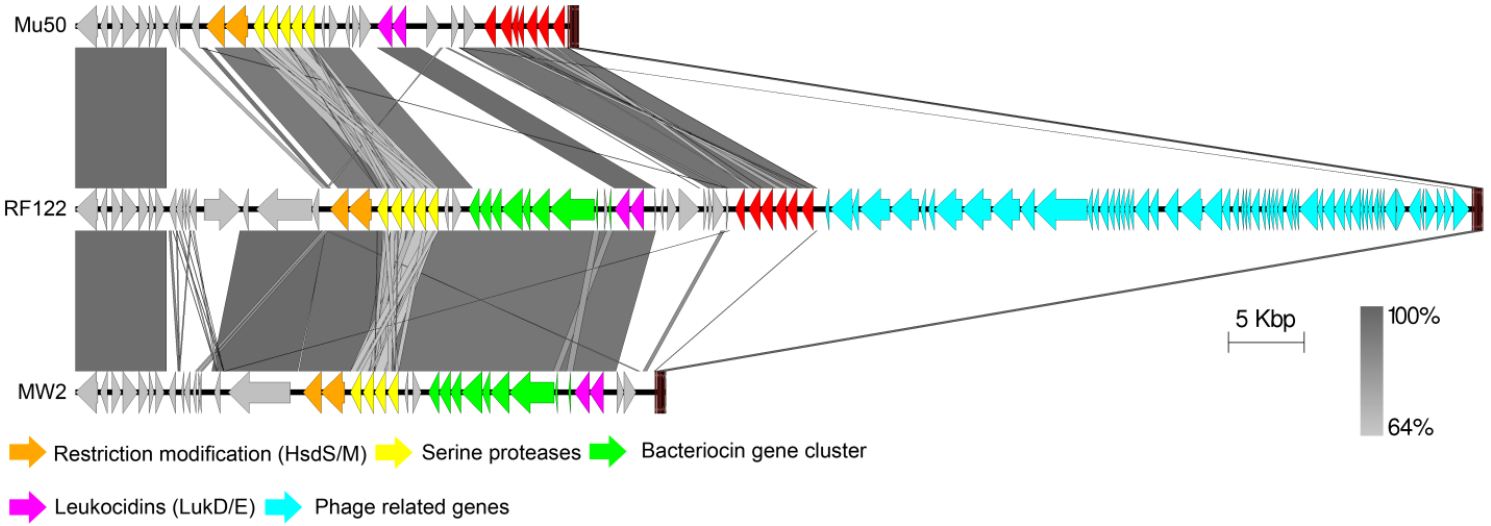

Figure 1. Sequence alignment of νSaβ in Mu50, RF122, and MW2 strains. The arrow represents annotated genes in the entries 6,10,11 and colored based on key features. Orange; restriction modification system HsdR/M, yellow; serine protease cluster (*spl*), light green; bacteriocin gene cluster (*bsa*), pink; leukocidins (*lukD/E*), red; enterotoxin gene cluster (*egc*), cyan; genes related to phage. Note that the strain Mu50 harbors the egc but lacks a bacteriocin gene cluster; the strain MW2 harbors a bacteriocin gene clusters but lacks the egc. Uniquely, the strain RF122 harbors bacteriocin gene cluster and the egc, as well as the phage (ϕSaBov) insertion at the upstream of tRNA cluster (brown bar). The shading between the entries represents the percent identity (BLASTn) from 64 % (light gray) to 100 % (dark gray).

Table 1. Bacterial strains used in this study

S. aureus strains	Description	
RF122	Bovine mastitis isolate, CC151	10, 11
RF122 *sem::tetM*	Indicative strain for transfer of ϕSaBov$_{EGC}$	This study
RF122 *SAB1737*::*tetM*	Indicative strain for transfer of ϕSaBov$_{N}$ and ϕSaBov$_{EGC}$	This study
RF122 *lukE*::*tetM*	Indicative strain for transfer of ϕSaBov$_{LUKE}$	This study
RF122 *sem::tetM, int::cat*	RF122 *sem::tetM* Int	This study
RF122 *sem*::*tetM, terL::cat*	RF122 *sem::tetM* TerL	This study
MN PE	Human MRSA USA200, ST36-SCCmecII	10, 11
MN Park	Human MRSA USA200, ST36-SCCmecII	10, 11
MN white	Human MRSA USA200, ST36-SCCmecII	10, 11
MN PAM	Human MRSA USA200, ST36-SCCmecII	10, 11
DAR1809	Human MRSA USA300, ST8-SCCmecIV	12, 13
DAR2017	Human MRSA USA300, ST8-SCCmecIV	12, 13
DAR1085	Human MRSA USA300, ST8-SCCmecIV	12, 13
DAR1964	Human MRSA USA300, ST8-SCCmecIV	12, 13
MW2	Human MRSA USA400, ST1-SCCmecIV	14, 15
MN KN	Human MRSA USA400, ST1-SCCmecIV	16, 17
MN Gary	Human MRSA USA400, ST1-SCCmecIV	14, 15
C99-193	Human MRSA USA400, ST1-SCCmecIV	14, 17
C99-529	Human MRSA USA400, ST1-SCCmecIV	14, 17
CTH96	Bovine mastitis isolate, CC151	18

USA) from 1985 to 2001, and 53 bovine mammary gland and skin isolates (8 different farms, Ohio state, USA) from 2010 to 2013 was 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 mg/mL) or chloramphenicol (10 mg/mL) when necessary.

2. Phage induction and transduction.

Cultures were grown to mid-log phase at 37°C with shaking (200 rpm), then mitomycin C (1 mg/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 mm). 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.

3. 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).

4. 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.

5. PCR and quantitative real time PCR.

All primer pairs used in PCR and outward PCR were listed in Table 2. Quantitative real time PCR was performed to estimate relative copies of $\phi SaBov_{EGC}$ to $\phi SaBov_{N}$ 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.

6. Southern blot hybridization.

Chromosomal and phage DNA were digested with *EcoRI* 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 Table 2. DNA hybridization and probe detection was performed using Chemiluminescent detection kits (Roche) according to the manufacturers' instructions.

7. Allelic exchange constructs.

All primer pairs used in allelic exchange constructs were listed in Table 2. Allelic exchange, resulting in the insertion of antibiotic markers and target gene inactivation, was done using temperature sensitive pMAD system [33] with minor modifications. The tetracycline resistance gene (*tetM*) and chloramphenicol resistance gene (*cat*) were amplified from the strain Mu50 REF23 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

Table 2. List of primers used in this study

Name	Sequences (5' to 3')	Reference
Probe synthesis for southern blot		
semf	GTGAAAAACTATTATTGTCAGGAT	This study
semr	TTGGGTTAATGGCAACCATAAAACA	
SAB1737f	GAACCAGTACGGATCACGTGAA	
SAB1737r	GTTGAATATCAGCATTACGATGATGTC	This study
Intf	CATCACTGGTGGACGCTTTG	
Intr	AATGCATCGAGCGCTTTTTC	
LukEf	CACTCGTATCACTTGAACCTTTTTCA	
LukE r	GGTGGCAATGGCTCATTTAATT	This study
pLukD	GGTGGTTTCCGCGTAAATGG	
pLukE	TCTGTGAGGTGCGTCTTGTG	
Selective marker		
tetMf	GCGCGTCGACGATCAAGAAACAAAGGCAACCCA	
tetMr	GCGCGAATTCTAGGACACAATATCCACTTGTAG	This study
Catf	GCTAGTCGACACGAAAGTCGAAGGGGGTTTTTA	
Catr	GCTAGAATTCGGCCCGGTACCCAGCTTTT	
Allelic replacement of *sem*, RF122 *sem::tetM*		
semupf	GCGCGGATCCTCTTAAGTTACCTACACC	This study
semupr	GCGCGTCGACGAAAATCATATCGCAACC	
semdnf	GCGCGAATTCTATCAAGTTCTTGTGCAG	
semdnr	GCGCAGATCTGTCTATTATCTGAGTCGC	
Allelic replacement of untranslation region of SAB1737, RF122 *SAB1737::tetM*		
1737upf	GCGCGAGCTCTTATGCTTCACTCCATTTC	This study
1737upr	GCGCGGATCCATGGGCAGTGTTGTAATTAT	
1738dnf	GCGCGAATTCTGTTGTTGCATTAAATCACT	
1738dnr	GCGCCGTCGATGATATTTAGAGGTGGCACA	
Allelic replacement of *lukE*, RF122 *lukE::tetM*		
LukDupf	GCGCGGATCCGCAGCCATCTCCAAATTC	This study
LukDupr	GCGCGTCGACCTAATCCTGGGGTATAACTG	
LukEdnf	TGATGAATTCCTATTGCCCGTTAAACGG	
LukEdnr	ATTGAGATCTCCTGTCGGTTTACTCATTG	
Generating integrase knock out strain, RF122 *sem::tetMΔInt*		
Intupf	GCGCAGATCTGTCTATTATCTGAGTCGC	This study
Intupr	GCGCGTCGACAATAAGGGTAGGCGAGCTAC	
Intdnf	GCGCGAATTCGCATATCTTGGGAACGTTTC	
Intdnr	GCGCAGATCTAACAGAGAACATGTTGCTAC	

Generating terminase knock out strain, RF122 *sem::tetMΔTerL*		
Terupf	GCGCGGATCCTGTCAACATGGCTTTTTCTG	This study
Terupr	GCGCGTCGACTTGCTGAGGGTCTTGTGTTC	
Terdnf	GCGCGAATTCCTTTCCGACCACGGGTTAA	
Terdnr	GCGCAGATCTACGAAAGTTTGCCGGAAATA	
Outward PCR and sequencing		
pInt	CGAGATTTAACGAGGGATAGG	This study
p1702	TTGACACTAGCTTTCCGTTG	
p1693	CGATGTTAATGGTAGTGATCATGC	
p1759	TTTTAGCTAGCGCGTTAGTG	
Linear phage DNA fragment characterization		
p1651	TCGGCACCACAGTTTCATTC	This study
p1654	ACCAACAGCACCAGCAATACG	
p1655	CCATTTCAGCTTGATCACTCATACC	
p1663	AACATATTTGTGGTCAGGAGCTGAA	
p1664	GTTAATGCTCTTGGCGTACCAAT	
p1975	TGAAGAAAGAAAATGTACCAGGAAATG	
p1976	TTCTAAGCAAGCACTTACATTTGTACCT	
p1691	ATCATAAGAAGAGAAGAACGAGCTAGACT	
p1694	ATGGGATCGCAATACCACC	
pseg	AGCAAGACACTGGCTCACTAA	
Identification of ϕSaBov integration		
tRNAr	GTAGCAACATGTTCTCTGTT	This study
Intf	AAAGCGCTCGATGCATTG	
LukEf	CACTCGTATCACTTGAACCTTTTTCA	
seif	TCAAGGTGATATTGGTGTAGGTAACTTAA	
seir	AAGTGGCCCCTCCATACATG	
1758f	CGAAGAATACGAATCAAAATCAGCTAA	
1692r	AGCCGTTTCAGCTTGATATAACATAT	
1760r	GAGCAATGGGTGTGTCTAATGC	
Quantitative Real time PCR		
qrt intf	CAGCGTGAAGAAGAAAAGTTTATGAG	This study
qrt intr	TAAGCGTTGTACTTCGCCAAAG	
semf	GTGAAAAACTATTATTGTCAGGAT	
semr	TTGGGTTAATGGCAACCATAAAACA	
Verification of chromosomal DNA contamination in phage DNA preparation		
LPScoreF	TAAAGGTGCGGGAACTTTCG	This study
LPScoreR	AAGCGAGATCATCTGCCGAG	

strains were cultured in 43°C (nonpermissive 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 [33].

8. Genomic DNAsequencing 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 1.23 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.

III. Result

1. Sequence analysis of νSaβ in the strain RF122.

The strain RF122 is a bovine mastitis strain which belongs to the CC151 lineage [21]. 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 43811, 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. 2A). The $attN_R$ is highly conserved in all sequenced *S. aureus* strains as it is a part of the tRNA-Ser gene [21,23]. Additionally, 33 bp imperfect direct repeats 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 [21,24]. Of note, $attEGC_R$ is highly

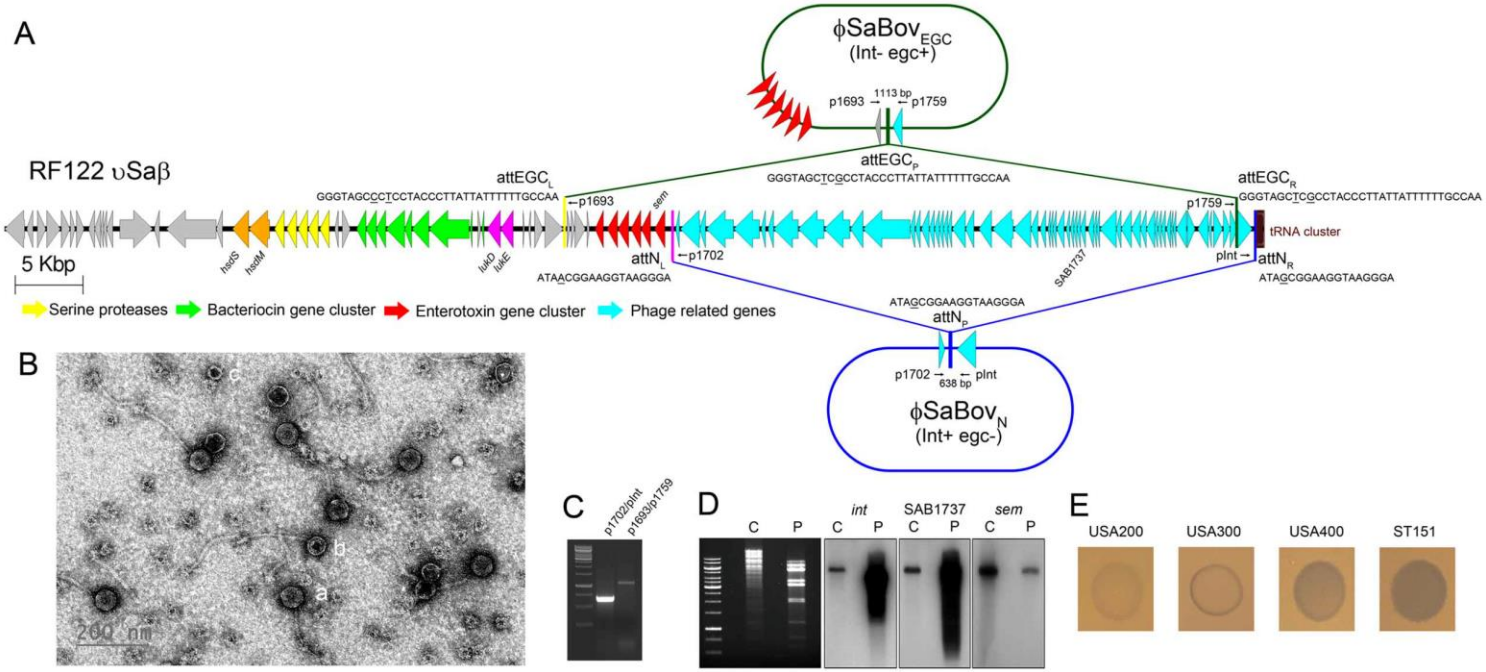

Figure 2. Heterogeneous excision products of the phage (ϕSaBov) that integrates at genomic island vSaβ. (A) A schematic map of vSaβ in the strain RF122. The arrows represent genes annotated in the GenBank entries10 and colored based on key features. Orange; restriction modification system HsdR/M, yellow; 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 *EcoRI*, separated by electrophoresis, and transferred to Nylon membrane for Southern blot analysis. Probes specific to the integrase gene (SAB1760, for wSaBovN), 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 (108 pfu/ml) was dropped onto the lawn culture of human ST36-SCCmecII (USA200), ST8-SCCmecIV (USA300), ST1-SCCmecIV (USA400), and bovine mastitis isolate (ST151).

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 [23].

2. 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 family14 with various sizes of hexagonal heads (Fig. 2B). 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[26] (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. 2A 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 ϕSaBovEGC (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. 2D). 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 (Fig. 3). 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 6 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. 2E).

3. Phage-mediated horizontal transfer of vSaβ.

Mitomycin C treatment of strain RF122 can induce heterogeneous transducing 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-SCCmecIV) strains was much higher than those to USA300 and USA200 strains (Table 3). To further confirm the transfer of the egc, a draft genome sequence of the recipients MNKN (ST1-SCCmecIV) 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 attNR 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

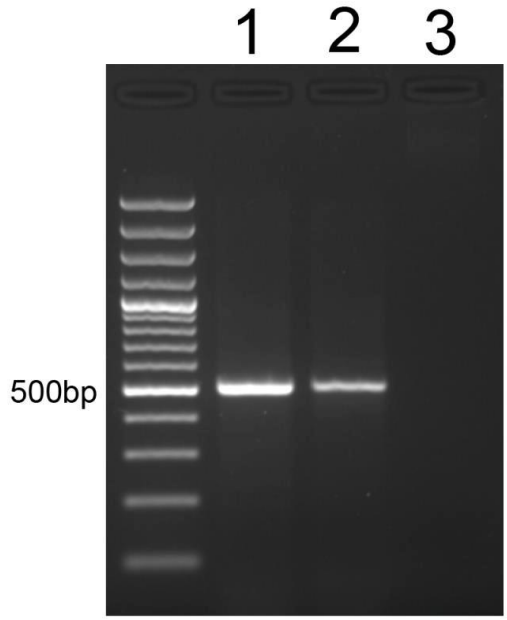

Figure 3. Test for *S. aureus* chromosomal DNA contamination in the phage DNA preparation. Exogenous chromosomal DNA (*E. coli* DH5α, 10 g) was added to the mitomycin C-induced culture lysate (10 ml) of the strain RF122, and then treated with excessive amount of RNase and DNAse I (100 unit each) for 2 hour, prior to the phage DNA extraction. PCR using primers specific to *E. coli* the *lps* gene (LPScoreF/R) was performed to check chromosomal DNA contamination in the phage DNA preparation. Results shown are PCR results with template using: Lane 1, purified *E. coli* chromosome; lane 2, *E. coli* chromosome added to mitomycin-induced culture lysate (before RNase and DNase I treatment); lane 3, phage DNA extraction after RNAse and DNAse treatments.

Table 3. Transduction frequencies of ϕSaBov$_N$, ϕSaBov$_{EGC}$, ϕSaBov$_{LUK}$

Lineage	Strain name	Transfer frequency (CFU/pfu)*		
		ϕSaBov$_N$	ϕSaBov$_{EGC}$	ϕSaBov$_{LUKE}$
ST36-SCCmecII (USA200)	MN PE	2.50×10^{-7}	5.00×10^{-8}	5.00×10^{-8}
	MN Park	None	None	None
	MN White	None	None	None
	MN PAM	None	None	None
ST8-SCCmecIV (USA300)	DAR1809	1.15×10^{-6}	3.00×10^{-7}	1.00×10^{-8}
	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-SCCmecIV (USA400)	MW2	1.85×10^{-6}	3.00×10^{-7}	1.50×10^{-8}
	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-ST151	CTH96	4.36×10^{-4}	1.22×10^{-5}	7.00×10^{-6}

(from ϕSaBov$_N$) and the egc (from ϕSaBov$_{EGC}$), but also the region upstream of the egc containing a bacteriocin gene cluster and leukotoxin D/E genes, were transferred (Fig. 4). 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. 5C). 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 pairs p1654/p1655 and p1691/p1694 but not with p1651/p1655 and p1691/pseg (Fig. 5B), suggesting a linear form of phageDNA with left flanking near SAB1654 and right flanking near SAB1694 (Fig. 5A). However, one cannot rule out the possibility that several intermediates might be detectable as a result of imperfect excision of ϕSaBov$_N$ or ϕSaBov$_{EGC}$ 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 ϕSaBov$_{LUK}$. To confirm the transduction activity of ϕSaBov$_{LUK}$, 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 3).

4. The role of integrase and terminase in the transfer of the ϕSaβ.

The phage DNA excision, package, and integration are controlled by cooperative actions of integrase, excisionase, terminase, and hostencoded DNA binding proteins[26-28]. 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

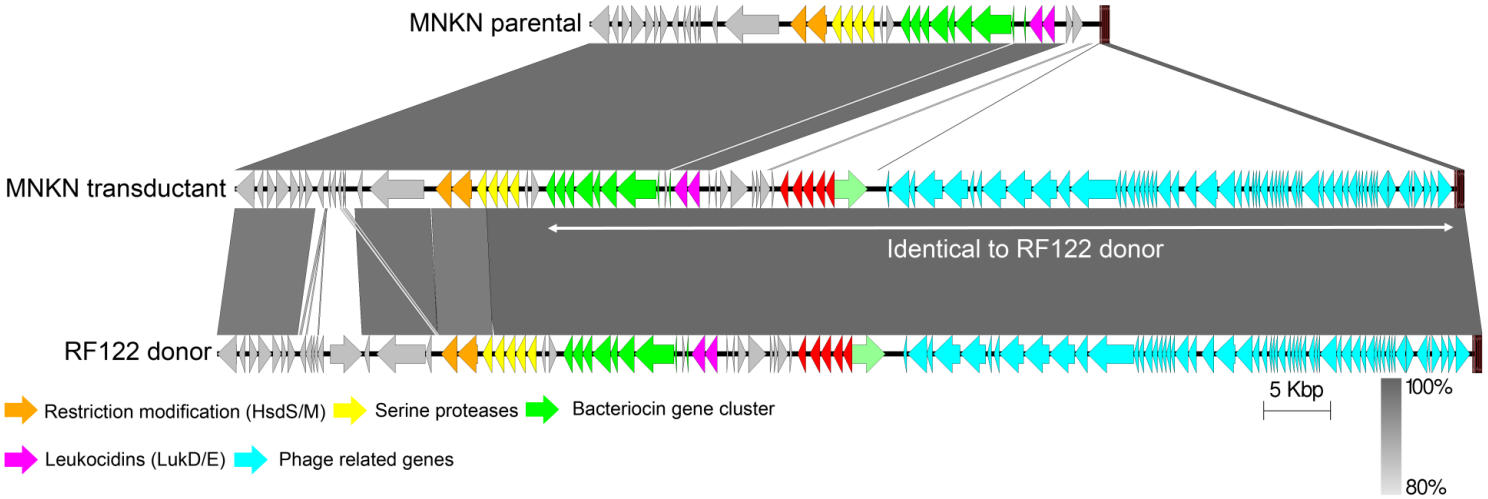

Figure 4. Sequence alignment of vSaβ in MNKN recipient and transductant with RF122 donor. White arrow indicated the sequence identical between MNKN transductant and RF122 donor. The shading between the entries represents the percent identity (BLASTn) from 80 % (light gray) to 100 % (dark gray).

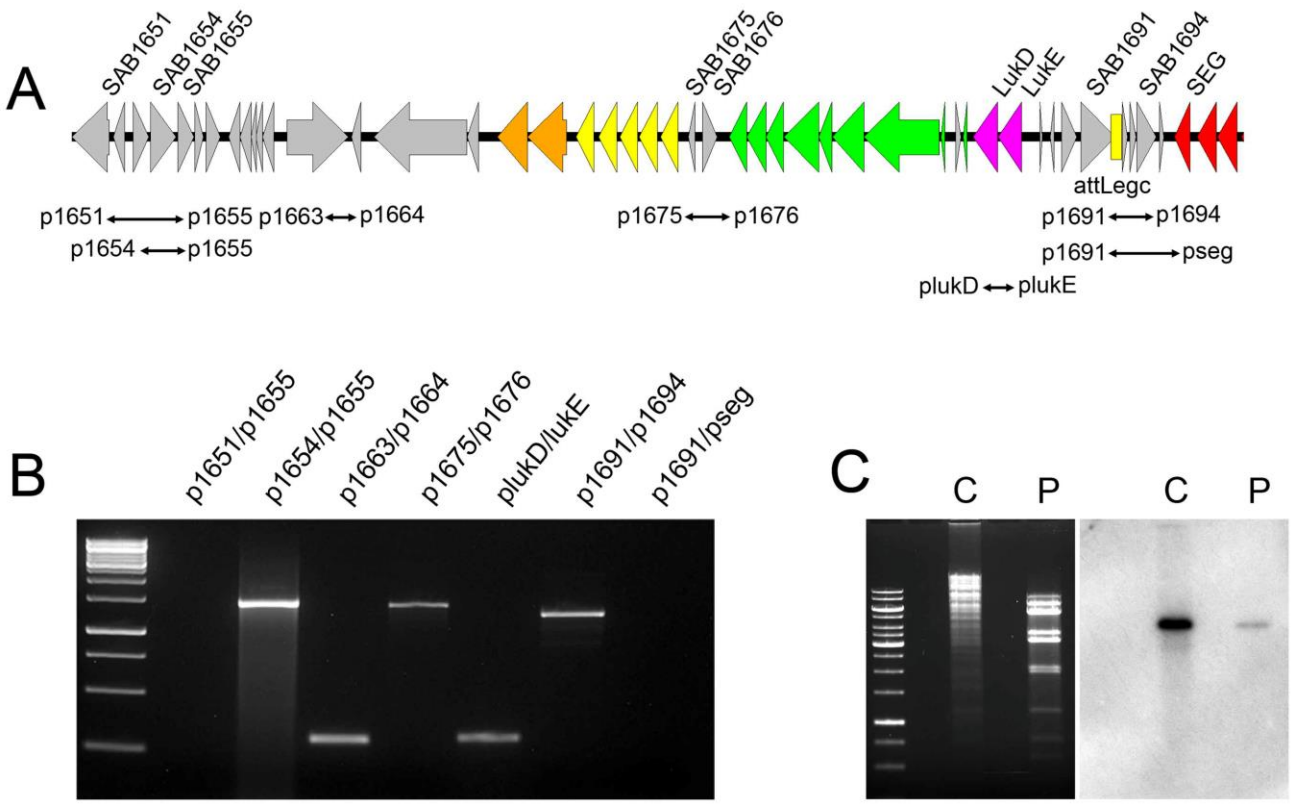
A
SAB1651
SAB1654
SAB1655
SAB1675
SAB1676
LukD
LukE
SAB1691
SAB1694
SEG
attLegc
p1651 p1655 p1663 p1664
p1654 p1655
p1675 p1676
p1691 p1694
p1691 pseg
plukD plukE
B
p1651/p1655
p1654/p1655
p1663/p1664
p1675/p1676
plukD/lukE
p1691/p1694
p1691/pseg
C
C
P
C
P

Figure 5. Identification of a transducing phage particle, ϕSaBovLUK, 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 *EcoRI* restriction enzyme using a probe specific to the *lukE* gene (the membrane used in this figure is the same as in Fig. 2).

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 (Fig. 6) and complementation of the *terL* gene restored phage DNA packaging (data not shown), suggesting phage DNAs were packaged through headful packaging mechanism by terminase [29]. In contrast, disruption of the integrase gene did not affect phage DNA excision and circularization (Fig. 6). 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 (Fig. 7), 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.

5. Postulation of the νSaβ transduction model.

Considering these data, we postulate the following νSaβ transduction model (Fig. 8). $\phi SaBov_N$ 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 att_{NL}

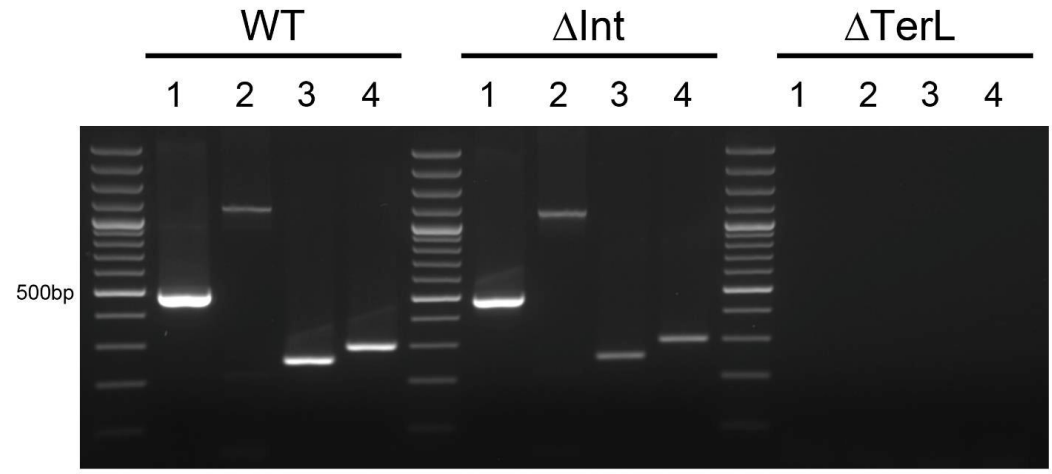

Figure 6. The role of ϕSaBov encoded integrase and terminase on phage DNA excision and packaging. The integrase (SA1760) knock out (Δ*Int*) and terminase large subunit (SA1726) knock out (Δ*TerL*) strains were generated from RF122 *sem::tetM* by allelic replacement. Phage DNAs were extracted from these strains and tested by PCR and outward PCR as described in the text. Note that the Δ*Int* strain showed identical PCR product patterns to the wild type strain, indicating a disruption of the integrase gene did not affect phage DNA excision and packaging. However, the Δ*TerL* strain did not show any PCR products, most likely due to the inability to package phage DNA. Lane 1, outward PCR using primers pInt/p1702 (a marker for circular phage DNA of ϕSaBovN); lane 2, outward PCR using primers p1693/p1759 (a marker for circular phage DNA of ϕSaBovEGC); lane 3, PCR using primers semf/semr (a marker for ϕSaBovEGC); lane 4, PCR using primers pLukD/pLukE (a marker for ϕSaBovLUK)

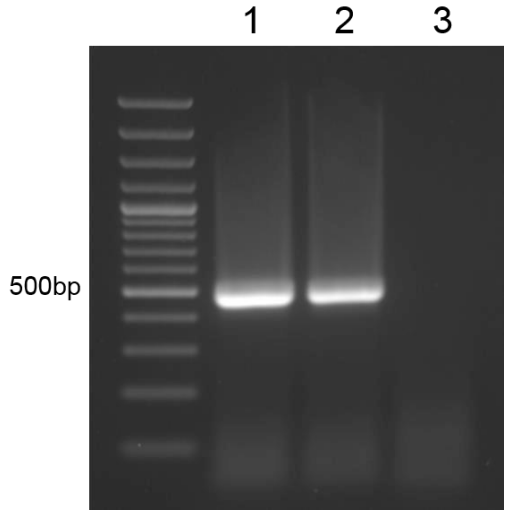

Figure 7. The excision and circularization of ϕSaBovN is dependent on host background. RF122 and RN4220 and MW2 strains carrying ϕSaBovN were treated with mitomycin C and phage DNAs were extracted. Results shown are outward PCR with pIntF/p1702R primer set (a marker for circular phage DNA of ϕSaBovN) using phage DNA template extracted from: Lane 1, RF122; lane 2, RN4220 carrying ϕSaBovN; lane 3, MW2 carrying ϕSaBovN. Note that the excision and circularization of the ϕSaBovN phage DNA was observed in RF122 and RN4220 carrying the ϕSaBovN, not in MW2 strain carrying the ϕSaBovN, indicating the excision and circularization of the ϕSaBovN phage DNA is dependent on host background.

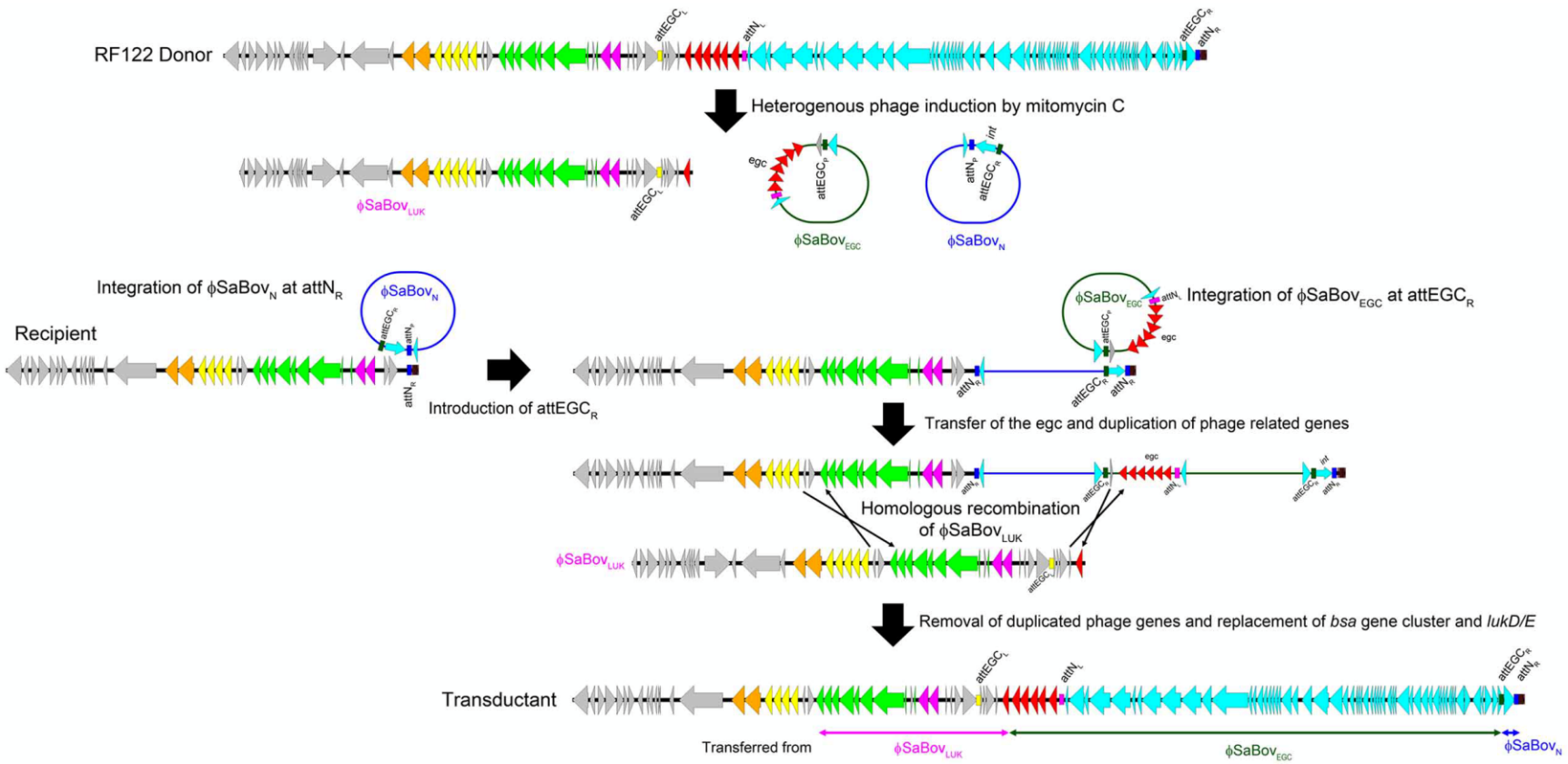
RF122 Donor
Heterogenous phage induction by mitomycin C
ϕSaBov_LUK
ϕSaBov_EGC
ϕSaBov_N
Integration of ϕSaBov_N at attN_R
Recipient
Introduction of attEGC_R
Integration of ϕSaBov_EGC at attEGC_R
Transfer of the egc and duplication of phage related genes
Homologous recombination of ϕSaBov_LUK
Removal of duplicated phage genes and replacement of bsa gene cluster and lukD/E
Transductant
Transferred from
ϕSaBov_LUK
ϕSaBov_EGC
ϕSaBov_N

Figure 8. 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 wSaBov. 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.

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 [30]. As a result, nearly all of the νSaβ (from the 141 bp upstream of the start codon of SAB1676 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 Fig. 9. 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 Table 4.

6. 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 (Fig. 10). *Spa* and MLST typing of these isolates showed that 7 and 2 isolates belong to CC97 and CC151, respectively, (Table 5) which are commonly observed clonal complexes among ruminants [31]. 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.

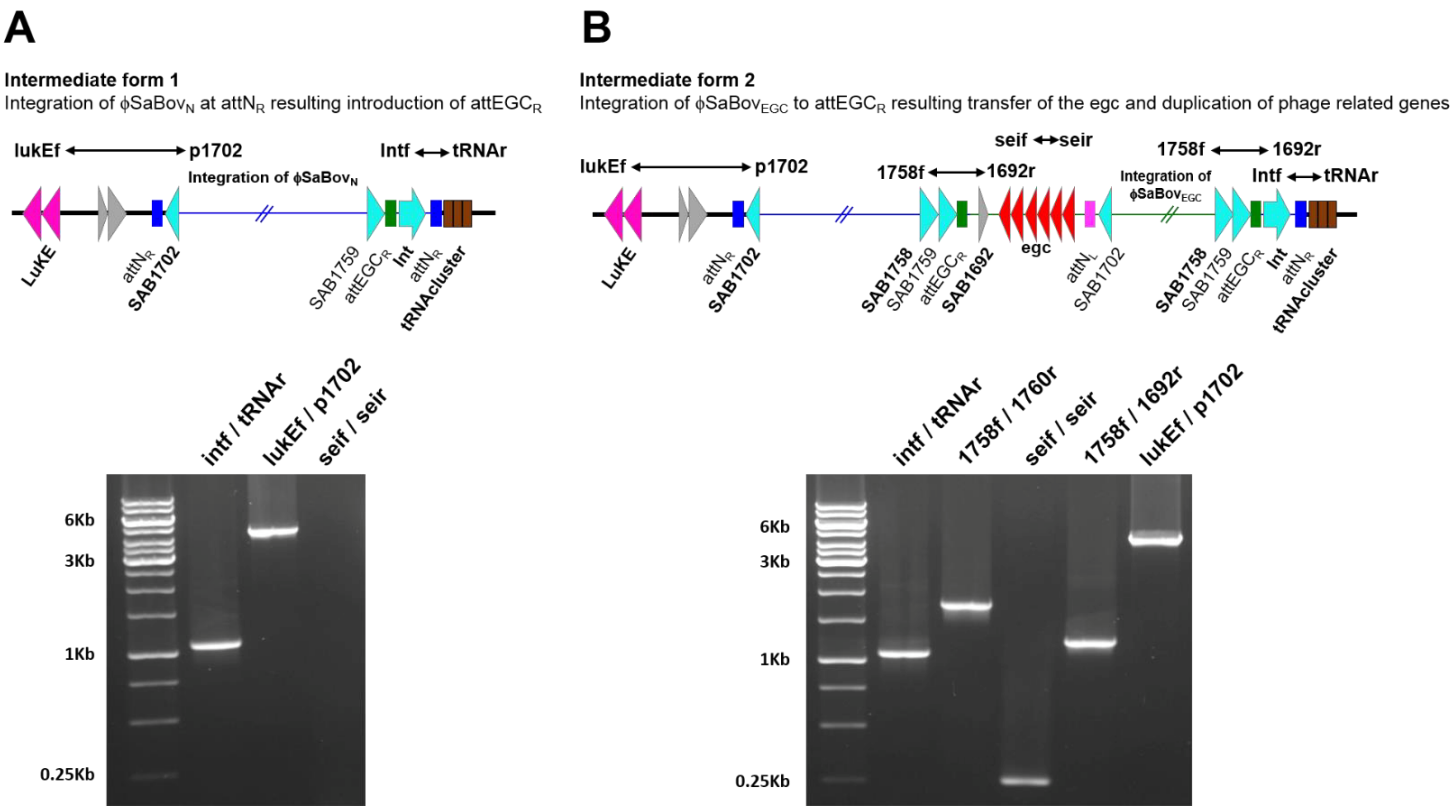
A
Intermediate form 1
Integration of ϕSaBov$_N$ at attN$_R$ resulting introduction of attEGC$_R$
lukEf
p1702
Integration of ϕSaBov$_N$
Intf
tRNAr
LuKE
attN$_R$
SAB1702
SAB1759
attEGC$_R$
Int
attN$_R$
tRNAcluster
intf / tRNAr
lukEf / p1702
seif / seir
6Kb
3Kb
1Kb
0.25Kb
B
Intermediate form 2
Integration of ϕSaBov$_{EGC}$ to attEGC$_R$ resulting transfer of the egc and duplication of phage related genes
lukEf
p1702
seif
seir
1758f
1692r
egc
attN$_L$
SAB1702
SAB1758
SAB1692
Integration of ϕSaBov$_{EGC}$
Intf
tRNAr
intf / tRNAr
1758f / 1760r
seif / seir
1758f / 1692r
lukEf / p1702
6Kb
3Kb
1Kb
0.25Kb

Figure 9. Identification of intermediate forms of transductants. The MW2 strain transduced with phage induced from RF122 was randomly selected and screened by junction PCR. (A) Top panel: A schematic map of intermediate form of transductant just carrying the ϕSaBov$_N$ at the tRNA cluster and locations of primer set for junction PCR. Bottom panel: Results of junction PCR were shown. PCR amplicons with intf/tRNAr and lukEf/p1702 primer sets and no amplicon with seif/seir primer set indicate ϕSaBov$_N$ was integrated at the attN$_R$ but not ϕSaBov$_{EGC}$. (B) Top panel: A schematic map of intermediate form of transductant harboring the integration of ϕSaBov$_{EGC}$ at the attEGC$_R$, resulting transfer of the egc and duplication of phage related genes. Bottom panel: Results of junction PCR were shown. PCR amplicons with 1758f/1692r, seif/seir, and 1758/1692r primer sets indicate the integration of the ϕSaBov$_{EGC}$ at the attEGC$_R$ and transfer of the egc. PCR amplicon with lukEf/p1702 primer set indicates the duplication of phage related genes.

Table 4. Increased transduction frequencies of ϕSaBov$_{EGC}$ and ϕSaBov$_{LUK}$ in the intermediate forms of transductants

Recipient	Transfer frequency of ϕSaBov$_{EGC}$ (CFU/pfu)*
MW2	2.5×10^{-7}
MW2 carrying ϕSaBov$_{N}$	4.6×10^{-5}
MNKN	8.5×10^{-6}
MNKN carrying ϕSaBov$_{N}$	1.4×10^{-4}
RN4220	9.0×10^{-6}
RN4220 carrying ϕSaBov$_{N}$	6.0×10^{-5}
Recipient	**Transfer frequency of ϕSaBov$_{LUKE}$ (CFU/pfu)***
MW2	1.5×10^{-8}
MW2 carrying ϕSaBov$_{N}$ and ϕSaBov$_{EGC}$	8.5×10^{-7}

*The transduction frequency of ϕSaBov$_{EGC}$ and ϕSaBov$_{LUKE}$ was measured using phages induced from RF122 *sem*::*tetM* and RF122 *lukE*::*tetM*, respectively.

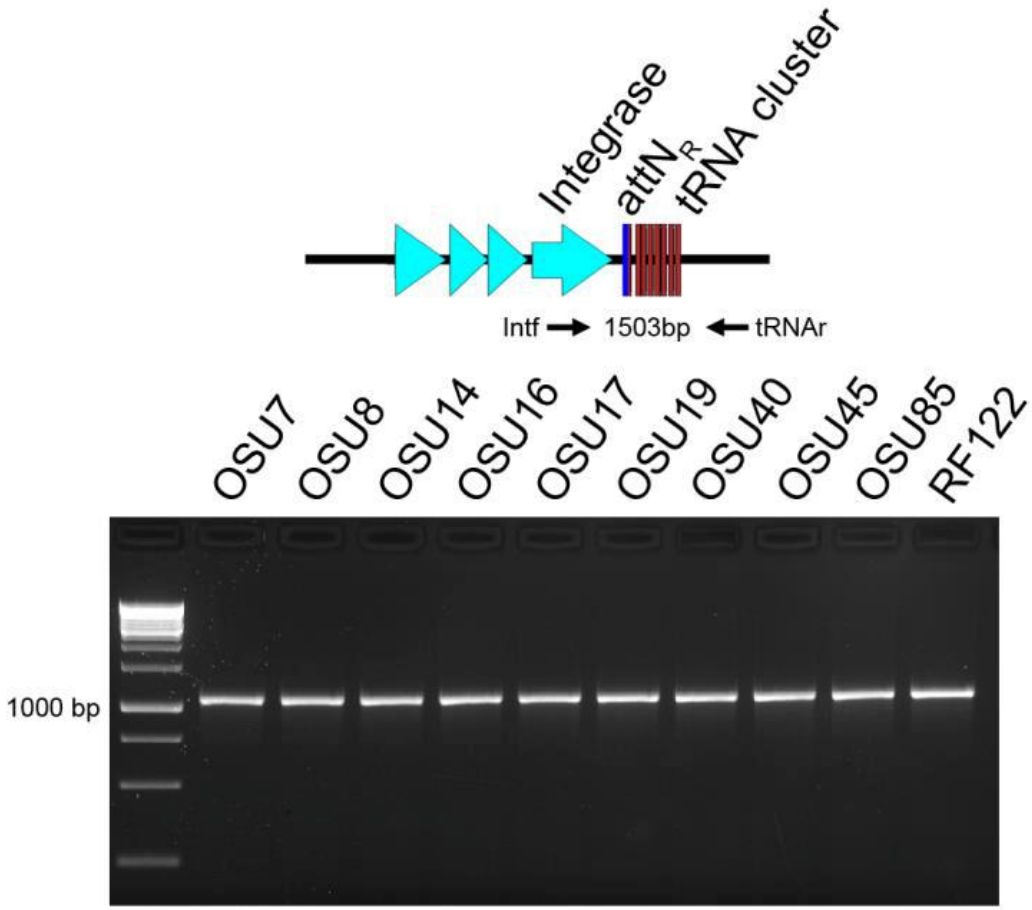

Figure 10. Screening of ϕSaBov in bovine mastitis isolates. The insertion of phage in the νSaβ was screened by a primer pair as depicted in the figure. From a collection of 53 bovine mastitis strains isolated from Ohio state, USA from 2010 to 2013, 9 strains have a phage insertion at the same location in the strain RF122 as indicated by PCR results.

Table 5. *spa* and MLST typing of bovine isolates harboring a phage insertion in the vSaβ

						Alleles at indicated locus						
ID#	Ridom *spa* type[a]	eGenomics *spa* type[a]	eGenomics *spa* repeats	Clonal complex	Sequence type[b]	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
OSU40	new1	new2	NEW-J1-G1-F1-M1-B1-B1-B1-B1-P1-B1	97	352	3	78	1	1	1	5	3
OSU8	new2	new3	T1-J1-G1-F1-B1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU7	new2	new3	T1-J1-G1-F1-B1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU17	t3992	1319	T1-J1-G1-F1-M1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU19	t2112	445	T1-J1-G1-F1-M1-B1-B1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU14	t13401	new1	U1-J1-G1-F1-G1-F1-M1-B1-B1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU16	t267	105	U1-J1-G1-F1-M1-B1-B1-B1-P1-B1	97	2187	3	317	1	1	1	5	3
OSU45	t529	102	Z1-B1	151	351	6	72	50	43	52	67	59
OSU85	t529	102	Z1-B1	151	351	6	72	50	43	52	67	59

[a] Ridom and eGenomics *spa* types determined using resources at www.spaserver.ridom.de and www.egenomics.com, respectively.

[b] Multilocus sequence types determined using resources as saureus.mlst.net.

IV. Discussion

The versatile host adaptation and successful pathogenicity of *S. 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.

V. Reference

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Chapter II.

Mobilization of Genomic Islands of *Staphylococcus aureus* by Temperate Bacteriophage

I. Introduction

Genetic variation of bacteria can be achieved through mutations, rearrangements and horizontal gene transfers and recombinations. Increasing genome sequence data have demonstrated that, besides the core genes encoding house-keeping functions such as essential metabolic activities, information processing, and bacterial structural and regulatory components, a vast number of accessory genes encoding antimicrobial resistance, toxins, and enzymes that contribute to adaptation and survival under certain environmental conditions are acquired by horizontal gene transfer of mobile genetic elements (MGEs) [1,2]. Mobile genetic elements are a heterogeneous group of molecules that include plasmids, bacteriophages, genomic islands, chromosomal cassettes, pathogenicity islands, and integrative and conjugative elements [2–4]. Genomic islands are relatively large segments of DNA ranging from 10 to 200 kb often integrated into tRNA gene clusters flanked by 16– 20 bp direct repeats [3]. They are recognized as discrete DNA segments acquired by horizontal gene transfer since they can differ from the rest of the chromosome in terms of GC content (%G+C) and codon usage [3, 5].

S. aureus is a major pathogen that colonizes the skin and mucous membranes of humans and animals, causing diseases ranging from mild skin infections to severe invasive diseases such as necrotizing pneumonia, infective endocarditis, and osteomyelitis [6–8]. The pathogenicity of this bacterium is largely influenced by the virulence genes carried on MGEs [9]. Three types of genomic islands (νSaα, νSaβ, νSaγ) are known in *S. aureus* [2]. Each type of genomic island is polymorphic in gene content but conserved within strain lineages [10–12]. Genomic islands are not as competently mobile as other MGEs, due to the lack of typical genetic elements required for or indicative of mobilization such as integrases, excisionases, terminases, and associated repeat sequences [13,14]. While efficient mobilization of SaPIs by temperate helper phages has been well documented [15,16], direct evidence indicating the mechanism of genomic island mobilization has not been well established. Previously, we reported the

transfer of νSaβ by temperate phage ϕSaBov, which integrated immediately adjacent to the νSaβ [17]. The induction of ϕSaBov by mitomycin C generated transducing particles harboring overlapping segments of νSaβ in circular and linear forms of phage DNA, which appeared to be followed by sequential integration and homologous recombination events, resulting in transfer of entire ϕSaBov and νSaβ [17]. Here we demonstrate, for the first time, phage-mediated transfer of genomic islands νSaα and νSaγ, which are remotely located from ϕSaBov. Our results also showed that the genetic background of the host and recipient strains impact the ability and efficiency of transfer of MGEs mediated by bacteriophages, suggesting the presence of MGE-specific mechanisms of excision and integration from the donor and the recipient strains, respectively, in concert with functions from bacteriophages.

II. Materials and Methods

1. Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were cultured in tryptic soy broth (TSB) or agar (TSA) plates (Difco) supplemented with tetracycline (5 μg/mL, Sigma-Aldrich) as necessary. *E. coli* were grown in Luria-Bertani (LB) broth and agar plates supplemented with ampicillin (100 μg/mL, Sigma-Aldrich) as necessary. Twenty-nine bovine mastitis isolates and 22 human isolates were kindly provided by QIA (Quarantine inspection agency, South Korea) and Patrick Schlievert (University of Iowa), respectively.

2. Phage induction and transduction

Cultures were incubated at 37°C with 200 rpm until reaching mid-exponential phase, and then mitomycin C (1 μg/mL, Sigma-Aldrich) was added. Cultures were incubated at 30°C with 80 rpm until clear lysis was observed. The lysates were sterilized with syringe filers (0.22 μm, Nalgene). A phage spot test and the plaque-forming unit (PFU) were determined by soft agar (0.5%) overlay method.

Table 1. A list of strains and plasmids used in this study

Strain	Description	Reference or source
Staphylococcus aureus		
RF122	Bovine isolate, CC151	[26]
RF122 *set::tetM*	Indicative strain for transfer of νSaα	This study
RF122 *hla::tetM*	Indicative strain for transfer of νSaγ	This study
RF122 *tst::tetM*	Indicative strain for transfer of SaPIbov	This study
RF122 *mdr::tetM*	Indicative strain for transfer of SaPI122	This study
RF122 *int::tetM*	RF122 Δ*int*	[1]
RF122 *set::tetM, int::cat*	Indicative strain for transfer of νSaα in Δ*int* background, RF122 Δ*int set::tetM*	This study
RF122 *hla::tetM, int::cat*	Indicative strain for transfer of νSaγ in Δ*int* background, RF122 Δ*int hla::tetM*	This study
RF122 *terL::tetM*	RF122 Δ*terL*	[1]
RF122 Δ*terL* pMin164 *terL*	Complementation of Δ*terL*	This study
Escherichia coli		
DH5α	Cloning host of pMAD and pMin164	Life Technologies
Top10	Cloning host of pCR4	Life Technologies
Plasmid		
pMAD-CM	Generating deletion mutants	[1]
pMAD-tetM	*tetM* insertion for screening transduction	[1]
pCR4-TOPO	TA cloning vector	Life Technologies
pMin164	High copy number vector for complementary	[2]

For transduction experiments, the recipient strains were cultured to mid-log phase and adjusted to approximately 2×10^7CFU/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 addition of sodium citrate solution (100 mM, pH 4.5). After centrifuging at 4,000 rpm at 4°C for 15min, the pellet was suspended in sodium citrate solution and plated on TSA supplemented with tetracycline (5 μ g/mL).

3. Phage DNA extraction

Phage DNA purification was performed as previously described [17]. Briefly, heterogeneous chromosomal DNA of *E. coli* K-12 was added to the culture lysates induced by mitomycin C as a control for verification of DNase treatment previously described [17]. Excessive amounts of DNase I (Sigma-Aldrich, 100 unit each) were added to remove chromosomal DNA. The 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 from the pellets using DNeasy kit (Qiagen) according to the manufacturers' instructions.

4. PCR, outward PCR, and quantitative real time PCR

All primer pairs used in PCR, outward PCR, and quantitative real time PCR are listed in Table 2. PCR was performed to determine the presence of transducing phage particles harboring MGEs using primers specific to MGEs associated with strain RF122 including: νSaα (the *set* gene), νSaβ (the *lukE* gene), and νSaγ (the *hla* gene), SaPIbov1 (the *tst* gene), SaPI122 (the *mdr* gene), and ϕSaBov (the *int* gene). To estimate the frequency of transducing phage particles harboring MGEs, the absolute copy number of each MGE per nanogram of phage DNA was determined using quantitative real time PCR. Briefly, PCR products specific to MGEs (above) were cloned into pCR™4-TOPO1TA Vector (Life Technologies). Plasmids were quantified using a Nanodrop (Thermo Scientific) and the number of DNA copies/ng was calculated as described previously [19]. Quantitative real time PCR reaction was performed using SYBR green I

master mix (Applied Biosystems) and a serial dilution of plasmid templates. Standard curves were generated by linear regression analysis calculating the slope, intercept, and correlation coefficient (R2) using Microcal OriginPro (Microcal origin, Version 7.5). Quantification of MGEs was calculated by interpolation the Ct from the standard curve.

5. Allelic exchange constructs

All PCR primer pairs used in allelic exchange constructs were listed in Table 2. The gene deletion mutants and the insertion of tetracycline resistance gene marker for screening transduction events were generated by allelic exchange using a modified pMAD-CM and pMAD-tetM temperature-sensitive shuttle vector system [17], respectively. Briefly, the gene fragments of upstream and downstream of target gene were cloned in pMAD-CM and pMAD-tetM. Resulting plasmids were electroporated to *E. coli* DH5α, and then to strain RF122. RF122 harboring the constructed plasmid were cultured at 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.

6. Phage and bacterial genomic DNA sequencing and analysis

Phage capsid DNA was isolated as described above, and bacterial genomic DNA was isolated with a DNeasy kit according to the manufacturer's instructions (Qiagen). The dsDNA was quantified with a Qubit HS Assay Kit (Invitrogen). Indexed, paired-end libraries were prepared 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). CLC Genomics Workbench v6 software was used to trim and filter reads for quality and to assemble reads de novo. Recombined regions among the RF122 donor (GenBank NC_007622), CTH96 recipient, and transductants were identified through local alignments.

Table 2. A list of primers used in this study

Name	Sequences (5' to 3')
Detection of Specific virulence gene in MGEs	
intf	CATCACTGGTGGACGCTTTG
intr	AATGCATCGAGCGCTTTTTC
set1f	GACAGTAGGCAAGCTGCGAAT
set1r	TTTCTCTGCCGTCGATTGACT
lukEf	TTTTTTTCCATCAGGCGTAACA
lukEr	ACGAATGATTTGGCCATTCC
hlaf	GCACTTACTGACAATAGTGCC
hlar	TCGCCACCTATATATACCGTTTC
tstf	TGAATTTTTTTATCGTAAGCCCTTTG
tstr	GGAAATGGATATAAGTTCCTTCGCT
mdrf	CTTTTCCTAGAAGATACCGCAATGT
mdrr	CCCATCCTTCGTGCGTTAGT
qRT-PCR	
qintf	CATCACTGGTGGACGCTTTG
qintr	AATGCATCGAGCGCTTTTTC
qsetf	AGACAAGAACGCACGCCTAAA
qsetr	TTATGGTTGGAGATTGTGGTGTGT
qlukEf	AGGTGGCAATGGCTCATTTA
qlukEr	TTGCTGAACCTGACGGACC
qhlaf	GGCCAGGCTAAACCACTTTTG
qhlar	GCTAATGCCGCAGATTCTGA
qtstf	TGAATTTTTTTATCGTAAGCCCTTTG
qtstr	GGAAATGGATATAAGTTCCTTCGCT
qmdrf	CTTTTCCTAGAAGATACCGCAATGT
qmdrr	CCCATCCTTCGTGCGTTAGT
Selective marker	
tetMf	GCGCGTCGACGATCAAGAAACAAAGGCAACCCA

tetMr	GCGCGAATTCTAGGACACAATATCCACTTGTAG
Allelic replacement of *set*, RF122 *set::tetM*	
setupf	GCGCGGATCCACGCCGAAAACTAAAGTGACA
setupr	GCGCGTCGACTGCTAA ACTTGCTTTCGCAAT
setdnf	GCGCGAATTCTTGAGTCTCTAAGAACGCCGA
setdnr	GCGCAGATCTAAAGACATCAAGGCCATGTGT
dsetr	TGCGTATAAACACCTGCGTCT
Allelic replacement of *hla*, RF122 *hla::tetM*	
hlaupf	GCGCGAATCCTTACCTCATATAGTGTCATG
hlaupr	GCGCGGTCGACGAAAGGTACCATTGCTGGTC
hladnf	GCGCGGAATTCGTCAAATTAGAATATTGCAG
hladnr	GCGCGAGATCTAATGCCTATAACTAA AAACC
dhlar	AATGAATCCTGTCGCTAATGCC
Allelic replacement of *tst*, RF122 *tst::tetM*	
tstupf	GCGCGTCGACACCAATGCGGCAGTCGGTGAT
tstupr	GCGCACGCGTATTGGAAAATAACAATGAATGACGGA
tstdnf	GCGCGAATTCCACTACTATACCAGTCTAGCAAAT
tstdnr	GCGCCCCGGGGTGTACCAACATCTTTAATTTCTTCA
dtstr	AGTTCTATTGGAGTAGGTAATTTTTCAG
Allelic replacement of *mdr*, RF122 *mdr::tetM*	
mdrupf	GCGCGTCGACTAAACCTTAAACCCTCTAATTCAGT
mdrupr	GCGCACGCGTAGGAGTACTCATAACAGGTGTCGTTA
mdrdnf	GCGCGAATTCTCTTAGATACTCCTCTTTGGTT
mdrdnr	GCGCCCCGGGAATATTCGGAATAGGCTCGCAG
dmdrr	TGGCCATAATCGCGCCAACGA
Generating integrase knock-out strain, RF122 Δ*int*	
Intupf	GCGCGGATCCGCTCCTTTACGGAGCTTTAA
Intupr	GCGCGTCGACAATAAGGGTAGGCGAGCTAC
Intdnf	GCGCGAATTCGCATATCTTGGGAACGTTTC
Intdnr	GCGCAGATCTAACAGAGAACATGTTGCTAC

Generating terminaseL knock-out strain, RF122 Δ*terL*	
Terupf	GCGCGGATCCTGTCAACATGGCTTTTTCTG
Terupr	GCGCGTCGACTTGCTGAGGGTCTTGTGTTC
Terdnf	GCGCGAATTCCTTTCCGACCACGGGTTAA
Terdnr	GCGCAGATCTACGAAAGTTTGCCGGAAATA
Outwarding PCR	
pself	AGCGGTGTGATTCTGGTGAAT
p0342r	TGGCGCACTCATCAAAGAGT
pSAB1912f	TGG AAG AGA TTT TAT AAC TAA TTT TG
pSaPI122r	CAG TGG GGA CAC CTG TGT AA
pIntf	CGAGATTTAACGAGGGATAGG
p1702r	TTGACACTAGCTTTCCGTTG
Verification of chromosomal DNA contamination in phage DNA preparation	
LPScoref	TAAAGGTGCGGGAACTTTCG
LPScorer	AAGCGAGATCATCTGCCGAG
Complementation of *terL*	
tercompf	GCTAGGATCCATCGGACTCCGTCCCGTCAT
tercompr	GCTAGAATTCAGACTACAAAGAGAATCCCG

III. Results

1. Transducing phage particles induced from RF122 harbor mobile genetic elements (MGEs)

To investigate the possibility that transducing phage particles induced from strain RF122 harbor genes uniquely associated with MGEs, PCR was performed using phage DNA extracted from RF122 following mitomycin C treatment. To ensure the removal of RF122 chromosomal DNA, exogenous *E. coli* chromosomal DNA was added to the phage-induced lysates and treated with an excessive amount of DNaseI. A PCR targeting the *waa*Q gene of *E. coli* was performed to confirm the removal of chromosomal DNA in the phage DNA preparation (Fig 1A). Results of PCR revealed that transducing phage particles induced from RF122 harbor genes associated with MGEs including genomic islands νSaα (the *set* gene), νSaβ (the *lukE* gene), and νSaγ (the *hla* gene), SaPIbov1 (the *tst* gene), SaPI122 (the *mdr* gene), and ϕSaBov (the *int* gene) (Fig 1B). The genes not belonging to MGE (*hlgB* and *rnaIII*) were not detected (Fig 1C). The SaPIbov1and ϕSaBov bordered with direct repeat sequences generated amplification products in outward PCR indicating a circular form of phage DNA. Sequencing of amplification products further confirmed these findings (data not shown). The SaPI122, not bordered with direct repeat sequence did not generate an amplification product suggesting a linear form of phage DNA, as expected for cos-type of sticky-end linear molecules (Fig 1D).

To estimate the frequency of transducing phage particles harboring MGEs, the absolute copy number of each MGE per nanogram of the phage DNA was interpolated from a standard curve generated using quantitative real time PCR (data not shown). The copy number of the ϕSaBov was the highest (8.51 Log10 copies/ng phage DNA), followed by νSaβ (5.25 Log10 copies/ ng phage DNA). The copy numbers of νSaα, νSaγ, SaPIbov1, and SaPI122 were lower than the ϕSaBov and νSaβ, and ranged between 1.64–3.00 Log10 copies/ng phage DNA (Fig 2).

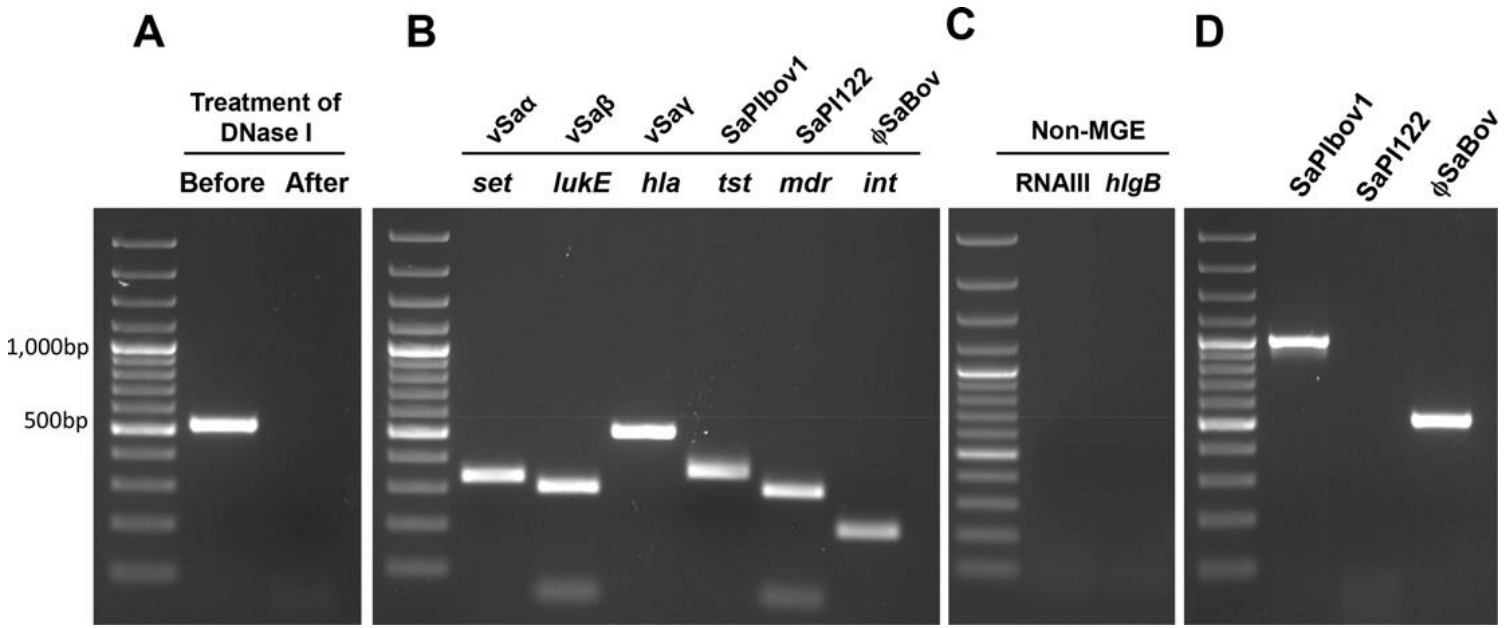


Figure 1. The presence of MGEs in transducing particles induced from the RF122 strain. (A) Verification of chromosomal DNA removal in preparation of phage DNA by adding exogenous chromosomal DNA of *E. coli*, followed by the treatment with DNase. The presence of MGEs in the phage DNA from transducing particles induced from the RF122 strain was analyzed by PCR amplification using primers specific to (B) MGEs; νSa (*set*), νSaβ (*lukE*), νSaγ (*hla*), SaPIbov1 (*tst*), SaPI122 (*mdr*), ϕSaBov (*int*) and (C) non-MGE (*rnaIII*, *hlgB*). (D) Outward PCR analysis of circularization of MGEs flanked with the direct repeat sequence; SaPIbov1, SaPI122, and ϕSaBov.

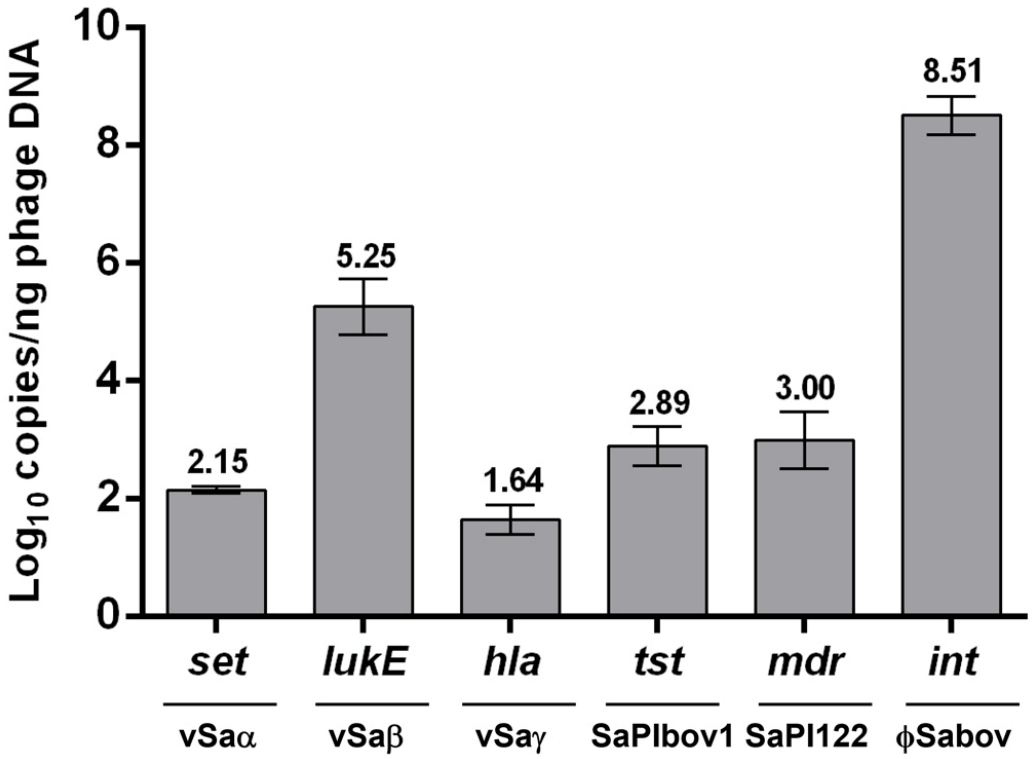


Figure 2. Estimation of the absolute copy number of MGEs in phage DNA using quantitative real time PCR.

2. Sequence analysis of phage DNA

The DNA sequences from phage capsids were determined in order to identify and map the location of the RF122 chromosomal DNA packed into transducing phage particles. A total of 47 contigs (>500 bp) were assembled which clustered in MGEs including νSaα, νSaβ, SaPIbov1, SaPI122, and ϕSaBov, except for a single short contig at SAB1107-8 not known to be located on MGEs (Fig 3). The ϕSaBov was assembled in a single contig, while other MGEs contained multiple contigs ranging 6–23 contigs. A contig corresponding to νSaγ was not found in phage DNA sequencing, which might be due to the low copy number of the νSaγ regions as shown in quantitative real time PCR. These results suggest that the mechanism of excision and packaging of phage DNA is specific to MGEs, rather than a generalized transduction mechanism, which would result in random excision and packaging of host chromosomal DNA.

3. Transfer of MGEs by transducing phage particles induced from RF122

Results of PCR and phage DNA sequencing demonstrated the presence of phage particles harboring MGEs in the RF122 strain. To test the transfer of MGEs by these transducing phage particles to other *S. aureus*, the *tetM* gene, conferring tetracycline resistance, was introduced into the MGEs as depicted in Fig 4A by homologous recombination using a modified pMAD system. The insertion of the *tetM* gene was confirmed by PCR (Fig 4B). The transfer of MGEs was successful mostly in the ST151 lineage (Table 3). Other than ST151 strains, νSaα and SaPIbov1 were transferred only to ST1-SCCmecIV and ST398, respectively. Interestingly, among the ST151 strains, SaPIbov1 was only transferred to recipients (RF114, 38963, CI2135) which do not have SaPIbov1 in the genome. SaPIbov1 was not transferred to the recipients (CTH96, RF113, DS102) which have pre-existing SaPIbov1 in the genome. We also inserted the *tetM* gene into the non-MGE gene (*hlgB*) to test random excision and packaging of phage DNA as in true generalized transduction, but transduction was not observed even with the recipients belonging to the ST151 lineage (data not shown). To further confirm the transfer of MGEs, a draft genome sequence of a recipient (CTH96) and

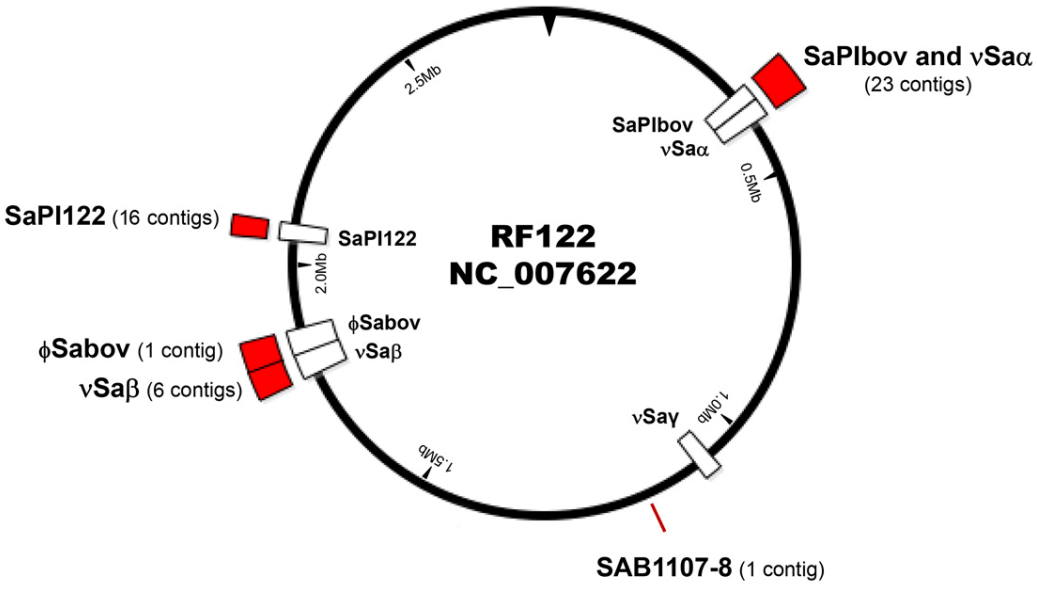


Figure 3. A schematic map of contigs determined from Illumina MiSeq analysis of phage DNA. A red box indicates the location and number of contigs detected from sequencing analysis of phage DNA. A white box indicates the location of MGEs (Sa,Sa, Saγ, SaPIbov1, SaPI122, ϕSaBov) present in the RF122 chromosome.

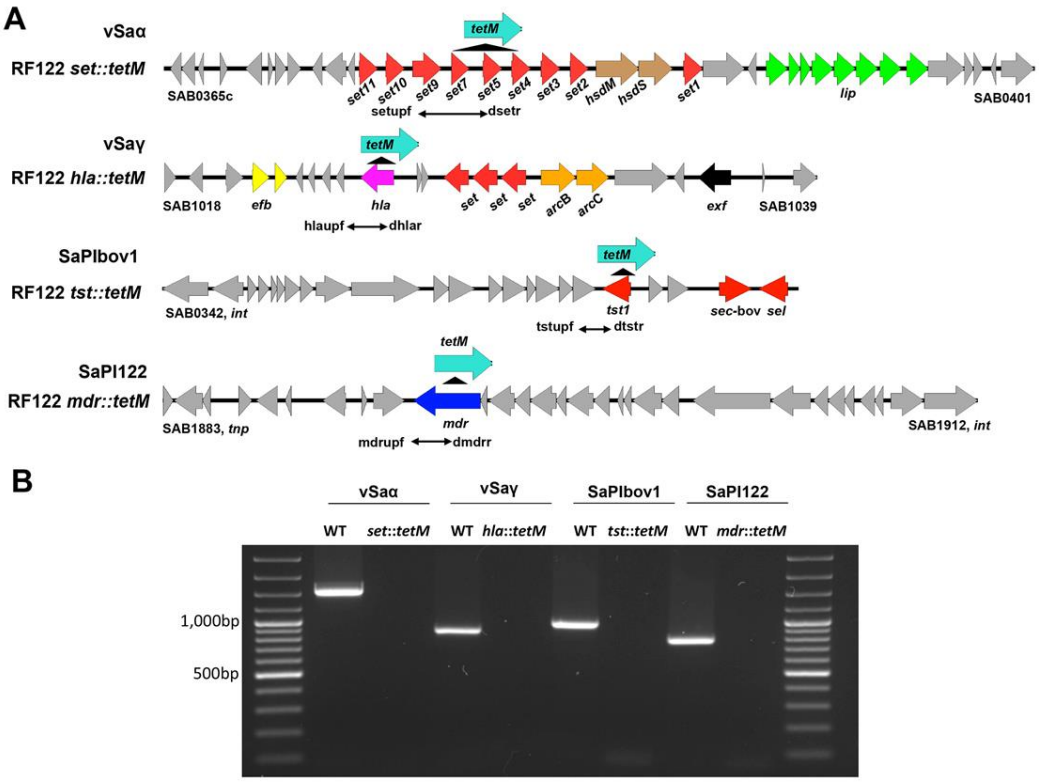


Figure 4. Schematic maps of the *tetM* gene insertion in the MGEs present in the RF122 strain. (A) The *tetM* gene was inserted into the MGEs present in the RF122 strain, including vSaα, vSaγ, SaPIbov1 and SaPI122, resulting RF122 *set::tetM*, RF122 *hla::tetM*, RF122 *tst::tetM*, and RF122 *mdr::tetM*, respectively. (B) The insertion of *tetM* was confirmed by PCR using primer sets designed to present a negative result in the insertional mutants, compared to the RF122 wild type strain (WT).

Table 3. Transduction frequencies of mobile genetic elements

Recipient Origin	Recipient Genotypes	Strain	MGE			
			νSaα	νSaγ	SaPIbov	SaPI122
[a]Bovine (29)	ST151 (6)	CTH96	[b]1.30×10^{-6}	8.00×10^{-7}	None	None
		RF113	1.65×10^{-5}	7.00×10^{-7}	None	2.43×10^{-6}
		RF114	1.10×10^{-6}	1.40×10^{-6}	3.60×10^{-7}	None
		38963	2.70×10^{-6}	1.00×10^{-6}	3.10×10^{-7}	None
		CI2135	2.70×10^{-6}	1.50×10^{-6}	4.00×10^{-8}	None
		DS102	3.50×10^{-6}	None	None	None
	ST1 (3)		None	None	None	None
	ST188 (8)		None	None	None	None
	ST20 (5)		None	None	None	None
	ST72 (4)		None	None	None	None
	ST398 (3)	K31	None	None	4.5×10^{-7}	None
Human (22)	ST1-SCCmecIV (7)	MW2	2.00×10^{-8}	None	None	None
		MN KN	3.10×10^{-7}	None	None	None
		C99-529	1.00×10^{-8}	None	None	None
		C99-193	None	None	None	None
		MN Gary	None	None	None	None
		MN Ask	None	None	None	None
		MN MA	None	None	None	None
	ST8-SCCmecIV (7)		None	None	None	None
	ST36-SCCmecII (8)		None	None	None	None

[a]The number of tested strains is in parenthesis.

[b]Transduction frequencies (CFU/pfu)

transductants (CTH96 νSaα transductant and CTH96 νSaγ transductant) was determined. Based on single nucleotide polymorphisms (SNPs) and the *tetM* gene found in the CTH96 νSaα transductant, approximately 27,162 bp of νSaα was transferred from donor to recipient, ranging from SAB0378 to SAB0403 and including a part of the set gene locus, hsdM , hsdS , and the *lip* gene locus (Fig 5A). The sequence comparison of the CTH96 νSaγ transductant suggested that approximately 28,000bp of νSaγ was transferred from donor to recipient, ranging from SAB1002 to SAB1029 and including phenylalanyl-tRNA synthetase (*pheT*), excinuclease ABC subunit C (*uvrC*), recombination and DNA strand exchange inhibitor protein (*mutS2*), ribonuclease III (*rnc*), succinate dehydrogenase (*sdh*), extracellular fibrinogen binding protein (*efb*), and the *hla* gene (Fig 5B).

4. The role of integrase and terminase on ϕSaBov in the transfer of MGEs

The integrase and terminase encoded in MGEs, including bacteriophages and SaPIs, are important for phage DNA excision, packaging, and integration, in concert with other factors derived from the donor or recipient [15]. However, the integrase and terminase were not present in the genomic islands νSaα and νSaγ. To test the involvement of the integrase (*int*) and terminase large subunit (*terL*) encoded by ϕSaBov in the transfer of these genomic islands, the *int* or *terL* gene was deleted in the RF122 strain. PCR analysis of phage DNA extracted from these strains showed that disruption of the *terL* gene completely abrogated phage DNA packaging, which could be restored by complementation of *terL* (Fig 6A). In contrast, disruption of the *int* gene did not affect DNA packaging (Fig 6B) nor the transfer of νSaα and νSaγ (Table 4). These results indicate that packaging of genomic islands in ϕSaBov is dependent on the terminase, but does not require integrase. In *pac*-type phages, the terminase small subunit (*TerS*) recognizes and binds to the phage specific packaging (*pac*) site typically located in or near to the *terL* gene that initiates a heterooligomer complex with *TerL* resulting in headful phage DNA packaging [15, 20, 21]. Analysis of phage DNA sequence showed an 11 bp consensus sequence present in ϕSaBov, νSaα and νSaγ (Fig 7), suggesting

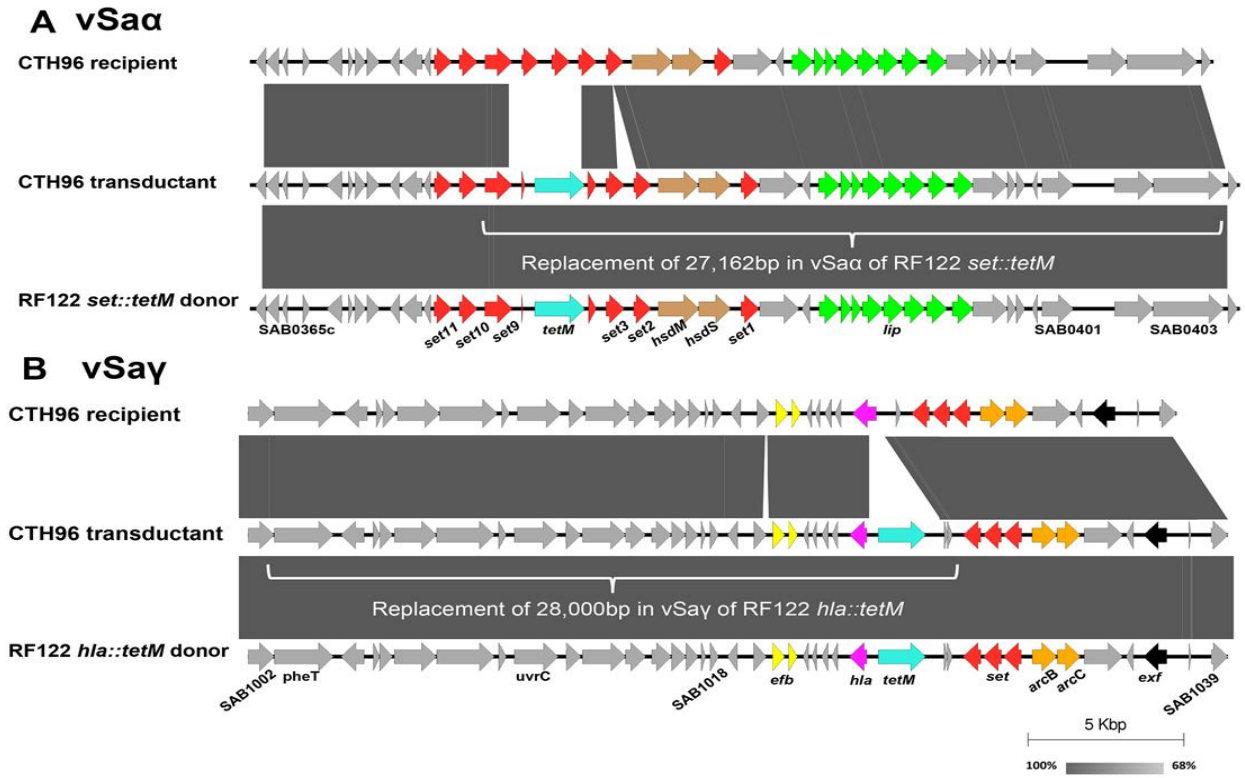


Figure 5. A schematic map of sequence alignments among RF122 (donor), CTH96 (recipient), and CTH96 transductants of phage induced from RF122 *set*::*tetM* (A) and RF122 *hla*::*tetM* (B). White brackets indicated the identical sequence between CTH96 transductant and RF122 donor, suggesting gene transferfrom RF122 to CTH96. The shading between the entries represents the percent identity (BLASTn) from 68% (light gray) to 100% (dark gray) using Easyfig.

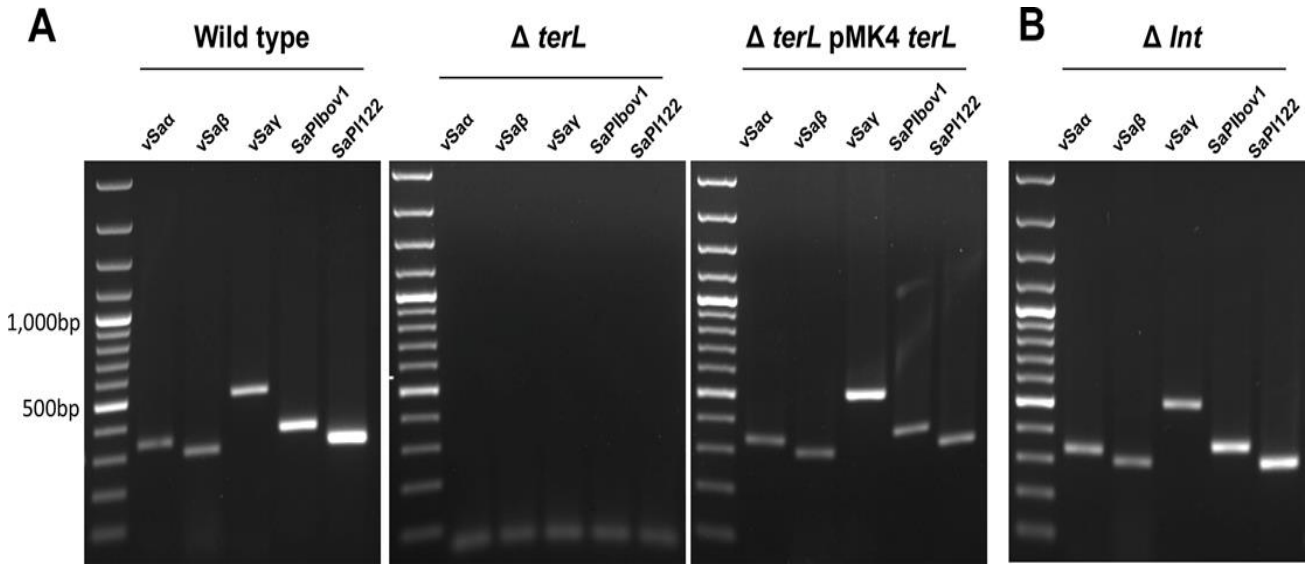
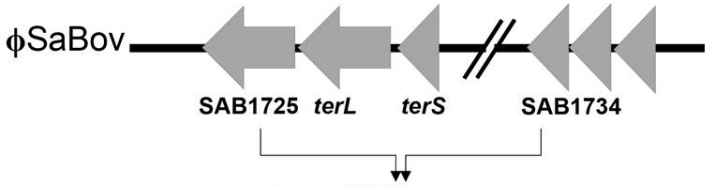


Figure 6. The roles of terminase large subunit (*TerL*) and intergrase (*Int*) on phage DNA excision and packaging. The terminase large subunit knock-out and integrase knock-out strains were generated from the RF122 strain by allelic replacement. The presence of MGEs in the phage DNA was analyzed by PCR with primers specific to each MGEs; νSaα (*set*), νSaβ (*lukD*), νSaγ (*hla*), SaPIbov1 (*tst*), and SaPI122 (*mdr*). A disruption of the *TerL* completely abolished the phage DNA packaging events. A complementation of *TerL* by pMin164 terL restored the phage DNA packaging events. (B) A disruption of the *Int* did not affect the phage DNA packaging events.

Table 4. The role of the integrase encoded in the ϕSaBov on transduction frequencies of MGEs

	RF122		RF122Δ*int*	
Recipient strains	νSaα	νSaγ	νSaα	νSaγ
CTH96	[a]1.45×10^{-6}	7.60×10^{-7}	1.50×10^{-6}	2.00×10^{-7}
RF113	1.85×10^{-5}	7.20×10^{-7}	1.70×10^{-6}	1.30×10^{-7}
RF114	1.30×10^{-6}	1.50×10^{-6}	1.00×10^{-6}	2.10×10^{-7}
38963	2.50×10^{-6}	1.20×10^{-6}	2.00×10^{-6}	1.50×10^{-7}
CI2135	2.90×10^{-6}	1.40×10^{-6}	2.50×10^{-6}	2.00×10^{-7}
DS102	3.90×10^{-6}	None	4.00×10^{-6}	None

[a]Transduction frequencies (CFU/pfu)



Locus tag	Putative *pac* site	Location
SAB1734	.....TGGAGAAGAAAGCGAACG.....	ϕSaBov
SAB1725	.....GAAAGAAGAAAAGGAGAT.....	ϕSaBov
SAB1008	.....ATGAGATGAAAAAGTTGA.....	close to vSaγ
SAB0264	.....CAAAGAAGAAGATGAAAA.....	phage related protein
*sef*9	.....ATTATACGAAACCGAGTT.....	vSaα
SAB0741c	.....TCAAGATGATAAAAATAC.....	close to *emp* and *nuc*

Figure 7. An 11 bp consensus sequence identified near to the terL gene using MEME (http://meme-suite.org/index.html) was conserved in MGEs packaged in the transducing phage particles. Putative *pac* site in MGEs. Sequence logos separated with colors indicated the frequencies scaled relative to the conservation at each position.

that MGE-specific phage DNA packaging by ϕSaBov might result from the recognition of *pac* sites in the MGE by *TerS* that induce the headful packaging mechanism in concert with TerL and other factors in the ϕSaBov.

V. Discussion

A transfer of MGEs by temperate phages requires a series of successful sequential events including excision from the host genome, packaging into transducing phage particles, transfer to a recipient and integration into the recipient genome. To investigate phage DNA excision events, phage DNA analysis using PCR and sequencing was performed. These results revealed that phage DNA excision events by ϕSaBov are highly specific to chromosomal locations of MGEs, suggesting a mechanism of sequence-specific excision events. It has been shown elsewhere that a typical *pac*-type phage DNA excision event is highly specific to the direct repeat sequence recognized by the integrase, excisionase, and terminase small unit [15, 21]. For MGEs, such as ϕSaBov and SaPIbov1, that are flanked by direct repeat sequences, phage DNA excision events occurred at the direct repeat sequence and formed a circular form of phage DNA. This process was likely controlled by their own integrase, excisionase, and terminases.

For MGEs not flanked by direct repeat sequences, including νSaα and νSaγ, phage DNA excision events occurred at various locations within these genomic islands. νSaα and νSaγ do not possess annotated genes for excision events such as integrase, excisionase, and terminase, so phage DNA excision events in these MGEs might be controlled by other elements associated with SaPIs, transposases, integrative and conjugative elements in the host background. To support this possibility, the RN4220 harboring ϕSaBov, which has a different background of integrase, transposases, and integrative and conjugative elements than RF122 and lacks MGEs such as SaPIbov1, SaPI122, did not generate transducing phage particles harboring MGEs (data not shown). As determined by PCR and DNA sequence analysis of phage DNA, transduction events are specific to MGEs rather than the random events that are expected from generalized transduction. A recent study demonstrated that the *TerS* encoded by SaPIs induced

sequence-specific excision and packaging of phage DNA at unlinked chromosomal segments [15]. In our current study, sequence analysis of MGEs present in transducing particles revealed potential consensus sequences in these MGEs which might mediate DNA excision/packaging events by *TerS* encoded in the ϕ SaBov, SaPIbov1 or SaPI122. Currently, the role of *TerS* and these potential consensus sequences on horizontal gene transfer is under investigation. A higher frequency of MGE transfer by ϕSaBov was observed with recipient strains belonging to ST151 than with other strain lineages. These results suggest that the host specificity of phage might be a primary barrier in the transfer of MGE by phage. The host specificity of phage is expected to be determined by interactions between the tail proteins of phage and the target molecules in recipient cells such as proteins and wall teichoic acid (WTA) which were generally conserved in the core genome of the same or related lineages of strain [22–25]. As such, strains belonging to ST151 lineage commonly express receptors to ϕSaBov and thereby become more susceptible to transduction. Interestingly, SaPIbov1 was not transferred to ST151 recipients that already possess SaPIbov1 in the genome. This observation is not likely superinfection immunity due to lysogenic phages since phage was not induced by mitomycin C treatment in these recipient strains [26]. It is possible that the conserved transcriptional regulators in the SaPIs, such as *stl* and *str* [27, 28], might negatively regulate transcription of elements required for the integration of the SaPIbov1. To confirm the transfer of MGEs by ϕ SaBov, a draft genome sequence of the transductants for ν Saα and νSaγ was determined. The extent of the transfer of MGEs was estimated by comparing single nucleotide polymorphisms and the presence of the *tetM* gene among donors, recipients, and transductants. Based on these comparisons, it was hypothesized that linear phage DNA segments harboring parts of νSaα and ν Saγ were transferred, rather than a transfer of a single contig harboring entire νSaα or νSaγ. This result supports findings that the overall structure of genomic islands can vary as a result of "plug and play" type of recombinational replacements [29]. To our knowledge, this is the first report to demonstrate a mobilization of νSaα and νSaγ. Although the *hla* gene was largely thought to have evolved with the core chromosome of *S. aureus*,

recombination events are evident in some strains [30, 31]. Our results suggest one mechanism by which the *hla* gene could be horizontally transferred among *S. aureus* strains. Our previous study demonstrated that the *int* gene encoded by the ϕSaBov is required for integration of phage DNA harboring ϕSaBov and νSaβ into recipient genome, but not for phage DNA excision events of ϕSaBov and νSaβ [17]. Further, the *terL* encoded in the ϕSaBov is required for packaging phage DNA of ϕSaBov and νSaβ [17]. Similarly, in this study, the int gene encoded by ϕSaBov is not required for phage DNA excision events in νSaα and νSaγ nor for the integration into recipient genome. However, the *terL* is essential for packaging into transducing phage particles. These results suggest the major role of temperate phage in transfer of νSaα and νSaγ is to package DNA and shuttle it via transducing phage particles.

Although it has long been acknowledged that bacteriophages contribute the horizontal transfer of MGEs by generalized and specialized transduction, the causal link to the transfer of genomic islands by bacteriophages or other means has not been established. In this study, for the first time, we present experimental evidence demonstrating transfer of genomic islands by a temperate phage utilizing MGE-specific DNA excision events from the host genome and presumably recipient-derived factors for integration. These results represent the complexity of underlying mechanism of phage-bacterial interaction in horizontal gene transfer and extend our understanding of the important role of bacteriophage in the horizontal transfer and evolution of genomic islands in *S. aureus*.

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Chapter III.

Genetic engineering of a temperate phage-based delivery system for CRISPR/Cas9 antimicrobials against *S. aureus*

I. Introduction

Invasive infections with methicillin resistant *S. aureus* (MRSA) in both community and healthcare settings total approximately 80,000/year and accounted for 11,285 deaths in 2011, resulting in direct heath care costs of more than $4.5 billion in the United States alone [1,2]. Moreover, the increasing occurrence of vancomycin-intermediate *S. aureus* (reduced efficacy of vancomycin) resulted from an accumulation of single nucleotide polymorphisms in the *S. aureus* chromosome by long-term exposure to vancomycin [3–5]. The increasing frequency of this problem underlines an urgent need for new antibiotics. However, the numbers of newly developed antibiotics and commercial interest in such drugs are decreasing, due to the high costs in development and rapidly rising resistance [6]. These impediments have led to an interest in the development of alternative therapeutics such as vaccines, probiotics, and phage therapy that are less likely to drive resistance.

The CRISPR (Clustered, regularly interspaced, short palindromic repeats) and CRISPR associated (Cas) genes serve as a bacterial immune system to resist foreign DNA [7,8]. The Cas9 present in the Type II CRISPR/Cas system of *Streptococcus pyogenes* is a RNA-guided endonuclease that introduces double-stranded breaks into target genes [9]. The specificity of Cas9 is guided by a trans-activating small RNA (tracrRNA) and CRISPR RNA (crRNA) harboring a short spacer sequence recognizing the target gene [10,11]. Recent studies demonstrated that a plasmid or phagemid harboring a CRISPR/Cas9 system programmed to target an antibiotic resistance gene or a specific pathogen could be delivered by a temperate phage and could successfully control antibiotic resistant *Escherichia coli* or MRSA with minimal effects on non-targeted bacteria [12–16]. These studies demonstrated the potential use of CRISPR/Cas9 system as a programmable antimicrobial to selectively control the target bacteria at the DNA level without disturbing the normal microbiome [12–16]. However, the efficacy of CRISPR/Cas9 antimicrobials is still far from being therapeutic, mainly due to the low efficiency in phage-based delivery systems which limited the efficacy of CRISPR/Cas9

to reduce bacterial colony forming units (CFU) by only one or two logs in in vivo and in vitro assays [12,14]. Furthermore, phage-based delivery systems may deliver not only a plasmid or phagemid harboring CRISPR/Cas system, but also host chromosomal segments by generalized and specialized transduction to target cells [17]. This is particularly important for phage-based delivery systems using *S. aureus* since many important staphylococcal virulence factors such as superantigens and cytolysins are commonly located in mobile genetic elements (MGEs) and are transferred to other *S. aureus* and *Listeria monocytogenes* by temperate phage-mediated generalized transduction [18,19], thereby raising the safety issues [20–22].

In this study, we demonstrate a genetic engineering strategy to overcome shortcomings in phage-based delivery systems by integrating the CRISPR/Cas9 system into the genome of temperate phage. The modifications allow for improved efficiency of delivery to target cells, expanded host specificity by complementing the tail fiber protein of the phage, and removal of virulence factor genes from the host strain to prevent contamination of harmful bacterial products in the phage lysates and subsequent spread of virulence genes by generalized transduction.

II. Methods and Materials

1. Bacterial strains and growth conditions.

All strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were cultured in tryptic soy broth (TSB) or agar (TSA) plates (Difco) supplemented with chloramphenicol (10 μg/mL, Sigma-Aldrich) as necessary. *E. coli* were grown in Luria-Bertani (LB) broth and agar plates supplemented with ampicillin (100 μg/mL, Sigma-Aldrich) as necessary.

2. Plasmid construction.

All oligos used in this study are listed in Table 2. Synthetic oligos (CRISPR_f/

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Reference or source
Staphylococcus aureus		
RF122	Bovine isolate, CC151, Lysogenized with ϕSaBov	23
RF122Δnuc	The *nuc* gene deletion mutant of RF122	This study
RF122Δ*nuc* ϕSaBov-Cas9-*nuc*	Integration of CRISPR-Cas9 system specific to the *nuc* gene into the genome of ϕSaBov lysogenized in RF122	This study
RF122Δ*nuc* ϕSaBov-Cas9-null	Integration of CRISPR-Cas9 system without spacer sequence into the genome of ϕSaBov lysogenized in RF122	This study
RF122-19	10 cytotoxins and 11 superantigen gene deletions mutant of RF122	This study
RF122-19Δ*nuc*	The *nuc* gene deletion mutant of RF122-19	This study
RF12219Δ*nuc*ϕSaBov-Cas9-*nuc*	Integration of CRISPR-Cas9 system specific to the *nuc* gene into the genome of ϕSaBov lysogenized in RF122-19	This study
RF12219Δ*nuc*ϕSaBov-Cas9-null	Integration of CRISPR-Cas9 system without spacer sequence into the genome of ϕSaBov lysogenized in RF122-19	This study

RF12219Δ*nuc*ϕSaBovpTF11	Complementation of ϕ11 tail fiber protein gene in RF122-19Δ*nuc*	This study
RF122-19Δ*nuc*ϕSaBov-Cas9-*nuc*-pTF11	Complementation of ϕ11tail fiber protein gene in RF12219Δ*nuc* ϕSaBov-Cas9-*nuc*	This study
CTH96	Bovine isolate, CC151, susceptible to ϕSaBov	24
CTH96Δ*nuc*	The *nuc* gene deletion mutant of CTH96	This study
CTH96pGFP	Expression of green fluorescence protein on CTH96	This study
NRS382	Human MRSA USA100, ST5	25
MN PE	Human MRSA USA200, ST36	26
DAR1809	Human MRSA USA300, ST8	26
MW2	Human MRSA USA400, ST1	27
Escherichia coli		
DH5α	Cloning host of pMAD and pMK4	Life Technologies
Top10	Cloning host of pCR4	Life Technologies
Plasmid	Description	
Modified pMAD-secY	Temperature sensitive shuttle vector system	This study
pCR4-TOPO	TA cloning vector	Life Technologies
pMK4	High copy number vector for complementary	28
pKS1	Cloning of synthetic oligos containing a promoter, pre-crRNA,	This study

	and two BbsI restriction sites flanked with a direct repeat (CRISPR array) into pMK4	
pKS2	Cloning of the spacer sequence specific to the *nuc* gene into pKS1	This study
pKS3	Cloning of a tracrRNA and Cas9 into modified pMAD-secY	This study
pKS4	Cloning of PCR product containing CRISPR array with spacer sequence specific to the *nuc* gene amplified from pKS2 into pKS3	This study
pKS5	Cloning of SAB1737 and SAB1738 into pKS4	This study

Table 2. Oligonucleotides used in this study

Cloning of Cloning of CRISPR array containing a promoter, pre-crRNA, DR, and BbsI sites	
CRISPR_f	GATCCCGGCCGACGTGAACTATATGATTTTCCGCAGTATA TTTTAGATGAAGATTATTTCTTAATAACTAAAAATATGGT ATAATACTCTTAATAAATGCAGTAATACAGGGGCTTTTCA AGACTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAA ATTTTTTAGACAAAAATAGTCTACGAGGTTTTAGAGCTAT GCTGTTTTGAATGGTCCCAAAACGGGTCTTCGATCGATCG ATCGAAGACGTTTTAGAGCTATGCTGTTTTGAATGGTCCC AAAACCTTGCAGGGG
CRISPR_r	AAT TCC CCT GCA AGG TTT TGG GAC CAT TCA AAA CAG CAT AGC TCT AAA ACG TCT TCG ATC GAT CGA TCG AAG ACC CGT TTT GGG ACC ATT CAA AAC AGC ATA GCT CTA AAA CCT CGT AGA CTA TTT TTG TCT AAA AAA TTT CGT AAT CGC ACT ATT TGT CTC AGC TAG ACT TCA GTC TTG AAA AGC CCC TGT ATT ACT GCA TTT ATT AAG AGT ATT ATA CCA TAT TTT TAG TTA TTA AGA AAT AAT CTT CAT CTA AAA TAT ACT GCG GAA AAT CAT ATA GTT CAC GTC GGC CGG
Cloning of spacer sequencing into pKS1	
spacer-nucf	AAACTGCAAAGAAAATTGAAGTCGAGTTTGACAAGT
spacer-nucr	TAAAACTTGTCAAACTCGACTTCAATTTTCTTTGCA
Cloning of trac-RNA and Cas9 into modified pMAD-secY	
tracrrnaf	GATCCTTAAGTGATCCCTTGAAAGATTCTGT
cas9r	GATCCGGCCGTCAGTCACCTCCTAGCTGACTCA
Cloning of CRISPR array with a spacer sequence specific to the nuc into pKS3	
leaderf	GCAGGTCGACGGATCCCGGCCGACGTGAACTATATGATTT TCCGC
drr	AACGACGGCCAGTGAATTCCCCTGCAGGGTTTTGGGACCA TTCAAAACAGCATAGCTCTAAAACGTCTTCGATCGATCGA TCGAAGACCC
Cloning of SAB1737 and SAB1738 into pKS4	

1737upf	GATCGTCGACTTATGCTTCACTCCATTTC
1737upr	GATCCTTAAGATGGGCAGTGTTGTAATTAT
1738dnf	GATCCCTGCAGGTGTTGTTGCATTAAATCACT
1738dnr	GCGCAGATCTTGATATTTAGAGGTGGCACA
Generating the *nuc* gene deletion mutant	
nucupf	GATCGTCGACGTTAACACTTTAAGCAAACCGCATC
nucupr	GATCACGCGTAACTAACACCTCTTTCTTTTTAG
nucdnf	GATCGAATTCTGCTCATTGTAAAAGTGTCACTGCT
nucdnr	GATCCCCGGGATACGTCGCTACCATCTTCT
Generating α-hemolysin (*hla*) deletion mutant	
hlaupf	GCGCGGATCCTTACCTCATATAGTGTCATG
hlaupr	GCGCGTCGACGAAAGGTACCATTGCTGGTC
hladnf	GCGCGAATTCGTCAATTTAGAATATTGCAG
hladnr	GCGCAGATCTAATGCCTCTAACTAAAAACC
Generating β-hemolysin, leukocidin G/H deletion mutant	
1874f	GCGCGGATCCCTTAATTCCGATTACATTTG
1874r	GCGCGTCGACGTGCCTTTATTAACATTAAG
1876f	GCGCGAATTCCTTCAAGTCATTCGCAATAA
1876r	GCGCAGATCTGTATCAACGATCTTATTAAC
Generating gamma hemolysin ACB deletion mutant	
hlgaupf	TAATGGATCCACCGTTGATTCTCAATCG
hlgaupr	TGAAGTCGACCATCTTAACAACTAGGGC
hlgbdnf	GCGCGAATTCGGCTTTGTGAAACCTAATCC
hlgbdnr	GCGCAGATCTGGTCGTCACAATTACTGTTG
Generating leukocidin D/E (*lukDE*) deletion mutant	
lukdeupf	GCGCGGATCCCGAATTTGGAGATGGCTGC
lukdeupr	GCGCGTCGACCTAATCCTGGAGTATAACTG

lukdednf	TGATGAATTCCTATTGCCCGTTAAACGG
lukdenr	ATTGAGATCTCCTGTCGGTTTACTCATTG
Generating leukocidin M/F` (*lukMF`*) deletion mutant	
lukmfupf	GCGCGGATCCTTCGTATAGGCTTTATAG
lukmfupr	GCGCGTCGACCTCCAATGTTATATCCTA
lukmfdnf	GCGCGAATTCCTACTTCCTAGATACCGT
lukmfdnr	GCGCAGATCTGAATAGCTTAACAACGTA
Generating Enterotoxin gene cluster (egc) deletion mutant	
egcupf	TCTTGATACGTATTTGACACTTGC
egcupr	AGCTATACGAGTTTGATGGTTCTG
egcdnf	CAGAACCATCAAACTCGTATAGCTAACTAAGCGACTCAG ATAATAGAC
egcdnr	AGAGTTGTTACAGTCGCTACACC
Generating staphylococcal enterotoxin C deletion mutant	
secupf	ATGAATTC CTGTGGATTTAGAAATAAGG
secupr	CCAACATTCCCAAGAAGTATC
secdnf	GATACTTCTTCTTGGGAATGTTGGAAGAATGGATAATGTT AATCC
secdnr	TTATCCATGGCAAGCATCAAAC
Generating staphylococcal enterotoxin like toxin L deletion mutant	
selupf	GATATATTTGAAAGGTAAGTACTTCG
selupr	AGTGTAGTATTCCATATGAATGATGGT
seldnf	ACCATCATTCATATGGAATACTACACTATACAAAAGGTTA TAGGAAGAGTT
seldnr	CAATTTCTACAGATATGACTCCC
dself	GTCATGTTTCGGTTGATAGG
dselr	TGTACAAATGGACTTAAGATATAGCG
Generating toxic shock syndrome toxin deletion mutant	

tstupf	GCGCGGATCC ACTACATGTACTTACCAATGCG
tstupr	GCGCGTCGACGCAGAAATTAATTAATTTACCACTTTTTCT
tstdnf	GCGCGAATTCCAAAGGGCTTACGATGAAAAATTTCAT
tstdnr	GCGCAGATCTAACCAATTACCAAATTCTCCATGC
Generating staphylococcal enterotoxin like toxin X deletion mutant	
selxupf	TGTCGATGCTATGGATAGTGAGG
selxupr	TAATTACCTCCTTGATGTAAAGC
selxdnf	GCTTTACATCAAGGAGGTAATTATATCGCTAATACTTTGA AAGTTAGG
selxdnr	TCAAATGTAGCAGTATACATTAATTGCG
Complementation of the tail fiber protein of ϕ11	
synf	GCTACTGCAGATCCCATTATGCTTTGGCAG
synr	GGGTTTCACTCTCCTTCTACA
ϕ11tailf	AGGAGAGTGAAACCCATGTACAAAATAAAAGATGTTGAA AC
ϕ11autor	GCTAGGATCCCTAACTGATTTCTCCCCATAAG
Confirmation of deleted cytotoxins and superantigens	
lukdf	TTGCCAGTCAACTTCATAAGTAGATGT
lukdr	GCGCGTGGTAACTTTAACCC
lukef	TTTTTTACCATCAGGCGTAACA
luker	ACGAATGATTTGGCCATTCC
lukmf	TGGAGGTAAATTCCAGTCAGCA
lukmr	TGTCGCGATAAAAGACGGATT
lukf`f	ACTGGTGGATTGAACGGGTC
lukf`r	ATCCAGTGCAAGTTGTTCCAAA
lukgf	GACTTTGCACCAAAAAATCAGGAT
lukgr	AGGTGCATAATGTATTCCAGGTCT
lukhf	GCGTCATCATTATCATGTGCAA

lukhr	CAAGGCTCAATTCATTCAAATT
hlgaf	CCAATCAGCGCCATCAATC
hlgar	CCAGTTGGGTCTTGTGCAAAT
hlgbf	CGTTGCTACTTCTATGGCAT
hlgbr	ACATTGTATTTAGCTCCCCAA
hlgcf	ATGTTAAAGCTATGCGATGGCC
hlgcr	AAGAGGTGGTAACTCACTGTCTGGA
hlaf	TGTTTGTTGTTTGGATGCTTTTCT
hlar	GGTTTAGCCTGGCCTTCAGC
hlbf	CACCTGTACTCGGCCGTTCT
hlbr	TATACATCCCATGGCTTAGGTTTTTC
segf	GGTAACAATCGACAATAGACAATCACTT
segr	TCCAGATTCAAATGCAGAACCAT
senf	TGGACTGTATTTTGGAAATAAATGTGT
senr	GCTCCCACTGAACCTTTTACGT
seuf	AGCGAGTGAATTCTCTGGTTTAATG
seur	TTGTGCTGTTATGTTTTTCATATTGG
seif	TGGCATTGATTATAATGGTCCTTG
seir	GCCTTTACCAGTGTTATTATGACC
semf	TCAGTTTCGACAGTTTTGTTGT
semr	CAGCTCAAGAAATTGATACTAAATTAAGAAG
seof	ACTACAGATAAAAAGAAAGTTACTGCAC
seor	CATCAATATGATAGTCTGATGAATCTATTG
secf	CAACCAGACCCTACGCCAGA
secr	TGTTATAAATTAAATCATGTGCCAAAAA
self	TAATTATCAATGGCAAGCATCAAAC
selr	CACTCCCCTTATCAAAACCGC
tstf	TGAATTTTTTTATCGTAAGCCCTTTG

tstr	GGAAATGGATATAAGTTCCTTCGCT
selxf	TCAACACAAAATTCCTCAAGTGT
selxr	GCGACTCTAATGTATATTTACCGCC
qRT-PCR	
qintf	CATCACTGGTGGACGCTTTG
qintr	AATGCATCGAGCGCTTTTTC
qcas9f	CGGAAGCGACTCGTCTCAA
qcas9r	CAAATACGATTCTTCCGACGTGTAT
qϕ11intf	TCTTTCTGTTGACTATGCACGATCT
qϕ11intr	TTTTGGCGTAATTGATAACTGCTT
qϕnm1intf	TTCTGTTGGCTATGCACGATCT
qϕnm1intr	TTTTGGCGTAATTGATAACTGCTT

CRISPR_r) containing promoter, pre-crRNA, and direct repeats flanked with BbsI sites (CRISPR array) were annealed, ligated into pMK4 digested with BamHI and EcoRI, resulting pKS1 (Fig. 1). Synthetic oligos (spacer-nucf/spacer-nucr) containing a spacer sequence specific to the *nuc* gene followed by protospacer-adjacent motif (NGG) were annealed and ligated into pKS1 digested with BbsI, resulting pKS2. The tracrRNA and the cas9 genes were amplified from the genomic DNA of *Streptococcus pyogenes* SF370 (ATCC) using oligos (tracrrnaf/ cas9r), followed by digestion with AflII and EagI, and ligation into corresponding sites in modified pMAD-secY temperature sensitive shuttle vector, resulting pKS3. To program tracrRNA and the cas9 gene specific to *S. aureus*, CRISPR array specific to the nuc gene was amplified from pKS2 using oligos (leaderf/drr), digested with EagI and SbfI, and cloned into corresponding sites in pKS3, resulting pKS4 (Fig. 2).

3. Allelic exchange construct.

Integration of CRISPR-Cas9 system into the genome of ϕSaBov and marker-less deletion mutants of the *nuc* gene and 21 virulence genes were generated by allelic exchange using modified pMAD-secY temperature sensitive shuttle vector system established in this study by introducing a new multi-cloning site, a GFPuv reporter gene, a chloramphenicol resistant gene (*cat*), and an anti-sense secY gene controlled by a tetracycline inducible promoter into the pMAD system [42] to improve screening process of allelic exchange (Fig. 2). Briefly, upstream and downstream fragments of target gene were amplified and cloned in modified pMAD-secY system in *E. coli*, followed by electroporation into *S. aureus* strains. The first homologous recombination was induced by culturing at 43°C (non-permissive temperature for the replication of pMAD-secY), followed by culturing at 37 °C to promote the second recombination, resulting in allelic exchange [43]. The mutant candidates were screened by growth in TSA plate supplemented with anhydrous tetracycline (0.5 μg/ml), loss of GFP expression, and no growth in TSA plate supplemented with chloramphenicol, indicating the second recombination.

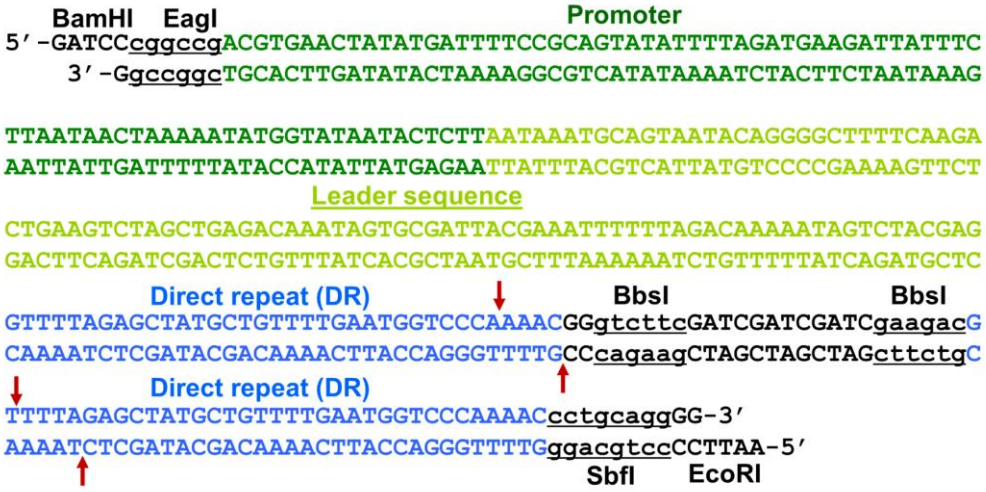

Figure 1. Synthetic oligos containing the promoter, leader, and direct repeat (DR) sequence cloned in BamHI/EcoRI sites in the pMK4 plasmid to generate pKS1. The color scheme is matched in the Figure 2. The arrow indicates the cleavage site by the BbsI restriction enzyme.

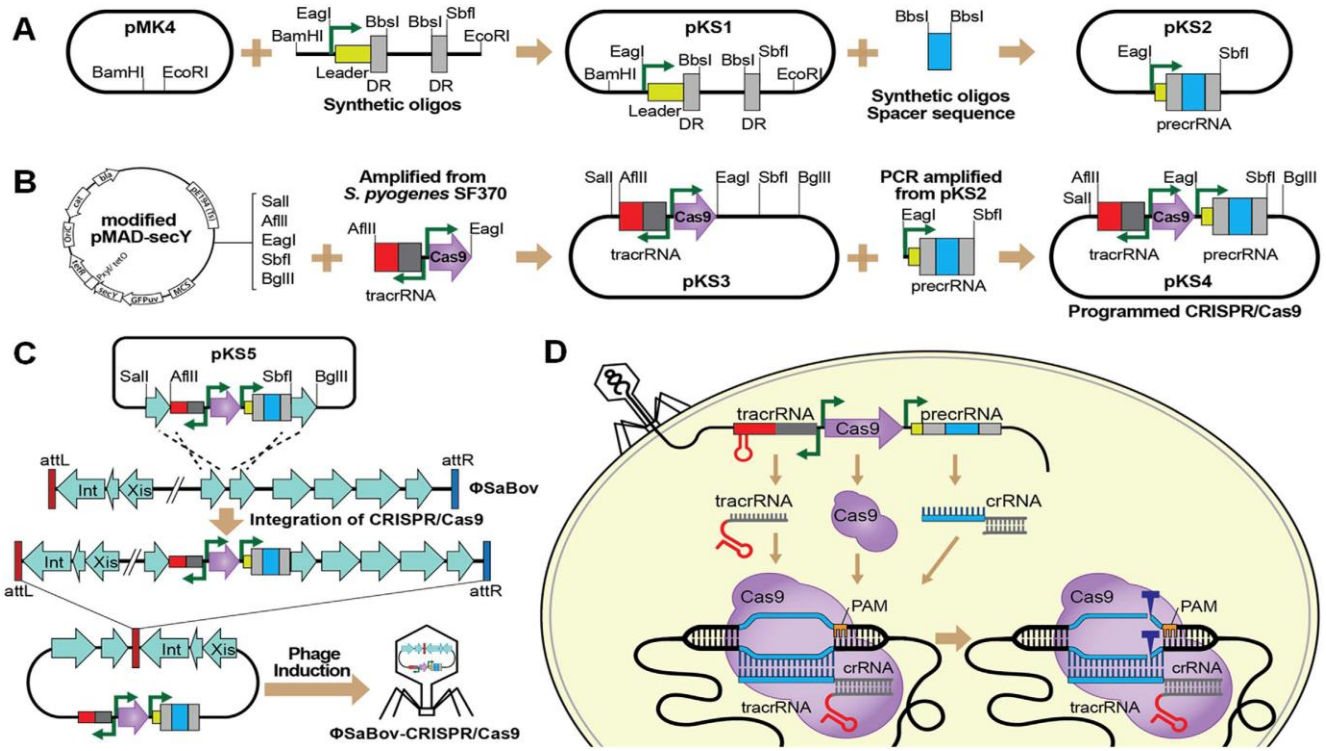
A
pMK4
BamHI
EcoRI
EagI
BamHI
BbsI
BbsI
SbfI
EcoRI
Leader
DR
DR
Synthetic oligos
pKS1
Synthetic oligos
Spacer sequence
pKS2
precrRNA
B
modified
pMAD-secY
SalI
AflII
EagI
SbfI
BglII
Amplified from
S. pyogenes SF370
Cas9
tracrRNA
pKS3
PCR amplified
from pKS2
pKS4
Programmed CRISPR/Cas9
C
pKS5
attL
attR
Int
Xis
ΦSaBov
Integration of CRISPR/Cas9
Phage
Induction
ΦSaBov-CRISPR/Cas9
D
tracrRNA
precrRNA
Cas9
crRNA
PAM

Figure 2. A schematic map of programmable and integrative CRISPR/Cas9 system (A) To generate a programmable CRISPR/Cas9 system, synthetic oligos containing a promoter, leader sequence and direct repeat (DR) sequence flanked with the BbsI restriction sites (Fig. 1) were cloned into the pMK4 shuttle vector, resulting pKS1. Synthetic oligos specific to the target gene (spacer sequence) were cloned into the BbsI site, resulting pKS2. (B) To generate integrative CRISPR/Cas9 system, genes encoding tracr-RNA and Cas9 were amplified from chromosomal DNA of *Streptococcus pyogenes* SF370 using PCR and cloned into the modified pMAD-secY temperature sensitive shuttle vector, resulting pKS3. To program CRISPR/Cas9 system specific to the target gene, the pre-crRNA (the promoter, leader sequence, DR, and spacer sequence) was amplified from pKS2 and cloned into pKS3, resulting pKS4. (C) The programmed CRISPR/Cas9 system was integrated into the non-coding region of ϕ SaBov genome by homologous recombinations. (D) The CRISPR/ Cas9 system programmed to target S. aureus is induced and delivered by ϕSaBov. The CRISPR/Cas9 system (tracrRNA, crRNA, and Cas9) is expressed and scanned the PAM sequence and recognizes the target sequence in the chromosomal DNA, leading to chromosomal DNA cleavage and bacterial death.

4. Phage lysates.

Phages were induced from the mid-exponential culture of strains by adding mitomycin C (1 μg/mL, Sigma-Aldrich) which induced clear lysis typically in 3 hours incubation at 30°C with 80 rpm. The lysates were sterilized with syringe filers (0.22 μm, Nalgene). Phage lysates were generated by propagating phage to the mid-exponential culture of the same strains from which phages were initially induced, followed by filter sterilization of lysates. The number of transducing phage particles (TP) was determined by calculating the plaque-forming unit using soft agar (0.5%) overlay method or quantitative real time PCR. Briefly, phage lysates were treated with excessive Dnase I (Sigma-Aldrich) to remove chromosomal DNA contamination, followed by DNA extraction from phage particles using DNeasy kit (Qiagen) as described previously [26,29]. Quantitative real time PCR reaction was performed using SYBR green I master mix (Applied Biosystems), primer sets specific to phages, and a serial dilution of phage DNA templates. The absolute copy number of phage DNA was calculated by interpolation of the threshold cycle from phage DNA template to the standard curves generated from cloned plasmid templates.

5. In vitro efficacy tests.

The phage stock generated from the mitomycin C induction was propagated to the RF122-19Δ*nuc*ϕSaBov-Cas9-nuc strain for 5 times to remove the mitomycin C contamination in the phage lysates. The mid exponential culture of recipient strains was harvested by centrifugation and adjusted to 1×10^6 CFU/mL in PBS. A test tube killing assay was performed in 1 mL of reaction mixtures consisting of 100 μL of recipient cell suspension, 20 μL of serially diluted phage lysates, and 880 μL TSB, and incubated at 37°C. The number of viable cells at each time point was determined by serial dilution and plating onto TSA plates. For in vitro killing under nutritionally limited conditions, an empty antibiotic disc was placed in sterile petri dish and inoculated with 100 μL of recipient cell suspension in PBS (1×10^5 CFU), followed by 20 μL of serially diluted phage lysates. After an 8 hr incubation at 37°C, the viable cells were recovered by

blotting the disk onto TSA plates.

6. In vivo efficacy tests.

All animal experiments were performed in compliance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee at the Mississippi State University (14–040). The back of C57BL/6 mice (6 to 8 week old, female, Harlan laboratory) were shaved with electric razor, depilated with Nair cream, and decontaminated with 70% ethanol swab. For intradermal infection, 100 μL of bacterial suspension in PBS containing 1×10^5 CFU was intradermally injected to the shaved skin. After 6 h, 100 μL of phage stock containing 5×10^7 transducing phage particles was intradermally injected to the infected skin. For skin surface infection, shaved skin was topically infected with 5 μL of bacterial suspension containing 2×10^4 CFU. After 1 h, phage stock containing 1×10^6 transducing phage particles/μL was applied to the infected skin in solution (10 μL) or mixing with a hydrogel (Dynarex). After 24 h, mice were euthanized with CO_2, and infected skin was excised and homogenized using Omni TH tissue homogenizer (OMNI international). Homogenates were serially diluted and plated on to BHI plate to determine the number of viable cells.

7. Phage spot and absorption assay.

For phage spot assay, recipient strains grown to the mid-log phage in BHI were harvested, adjusted to 1×10^6 CFU/ml, mixed with soft agar (0.5% w/v), and overlaid onto the BHI plate. Ten microliters of phage lysates (1×10^8 pfu/ml) was dropped onto the lawn culture. For phage absorption assay, 100 μL of the mid-exponential phase recipient *S. aureus* (5×10^8 CFU/ml) was mixed with 100 μL of phage lysates (2.5×10^6 PFU/ml) in phage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$) and incubated for 15 min at 37°C. The phages bound to the bacterial cells was removed by centrifugation at 13,000 rpm for 15 min. The PFU of the supernatant containing the unbounded phage was determined by the soft agar (0.5%) overlay method. The phage absorption (% input) was calculated by [1 − (the number PFU of unbound phage/1×10^5

input PFU)]×100. Each absorption assay was repeated at least three times.

8. Toxin detection in phage lysates.

Heparinized human venous blood was collected from healthy volunteers. All methods used in this study were carried out in accordance with the approved guidelines and all experimental protocols were approved by the Institutional Review Board for Human Subjects at the Mississippi State University (12–041). Informed written consent was obtained from all volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich). Purified PBMCs were adjusted to 2×10^6 cell per well in 96 well cell culture plate in RPMI1640 medium supplemented with 10% FBS. Phage lysates prepared from RF122Δ *nuc* or RF122-19Δ*nuc* was added to the wells. To detect superantigens in phage lysates, proliferation of T cell was measured using a [3H]-thymidine incorporation assay as described previously [44]. To detect cytotoxins in phage lysates, cytotoxicity of cells were measured using propidium iodide incorporation assay using LIVE/DEAD Cell-mediated Cytotoxicity kit (ThermoFisher).

III. Results

1. Development of programmable and integrative CRISPR/Cas9 plasmid vector systems.

The fact that bacteriophages can package their own genome more efficiently than host genetic elements, such as plasmids and phagemids, inspired us to develop a programmable and integrative vector system containing CRISPR/Cas9 integrated within the phage genome. This strategy was designed to improve packaging and delivery of the CRISPR system to target cells. To generate a programmable CRISPR/Cas system, synthetic oligonucleotides containing a CRISPR array encoding a promoter, leader sequence, and direct repeats interspaced with two BbsI restriction sites (Fig. 1) was

cloned into pMK4, resulting pKS1 (Fig. 2A). Synthetic oligos containing a spacer sequence specific to the nuc gene, uniquely present in all S. aureus, followed by a protospacer adjacent motif (PAM) NGG, was cloned into BbsI sites in pKS1, resulting in pKS2 (Fig. 2A). To generate integrative CRISPR/Cas9 system, tracrRNA and Cas9 genes were amplified from CRISPR/Cas9 system of *S. pyogenes* and cloned into the modified pMAD-secY temperature sensitive shuttle vector system developed in this study (Fig. 3), resulting in pKS3 (Fig. 2B). To program CRISPR/Cas9 system specific to *S. aureus*, CRISPR array with a spacer sequence specific to the nuc gene cloned in pKS2 was amplified by PCR and cloned allelic exchange as described below.

2. Integration of CRISPR/Cas9 system into the genome of ϕSaBov lysogenized in S. aureus strain RF122.

In order to select staphylococcal temperate phages efficiently packaging their own phage genomes, we determined the absolute copy number of phage DNA in the phage lysates using quantitative real time PCR.

(qRT-PCR) and standard curves generated from serially diluted plasmid templates (Fig. 4). Since a single transducing phage particle harbors a single copy of phage DNA, the copy number of phage DNA is equal to the number of transducing phage particles. We found that the ϕSaBov lysogenized in *S. aureus* strain RF122, a bovine mastitis isolate belonging to CC151 lineage, generated an exceptionally high number of transducing phage particles (10.31 Log copies/ml phage lysate) which was 3.44 and 3.98 Log magnitude higher than that of ϕ11 and ϕNM1, respectively (Fig. 5A). Based on these results, we selected the ϕSaBov for phage-based CRISPR/Cas9 delivery system.

To integrate the *S. aureus*-specific, programmed CRISPR/Cas9 system from pKS4 into the genome of ϕSaBov, upstream and downstream gene segments of non-coding regions between SAB1737 and SAB1738 of the ϕSaBov genome were amplified by PCR and

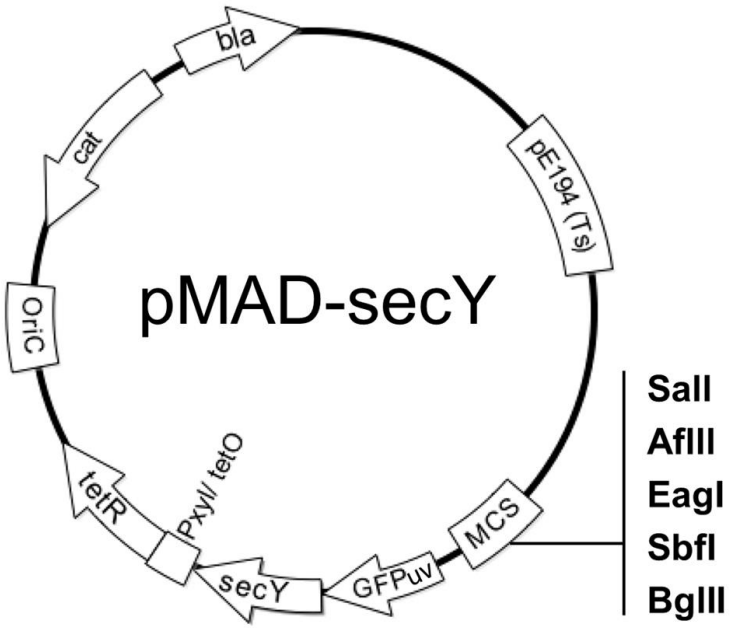

Figure 3. A schematic map of modified pMAD-secY system. For an efficient screening process of double-crossover event, genes encoding chloramphenicol resistance (*cat*), anti-sense-secY controlled by teteracycline inducible promoter (*secY*), and green fluorescent protein UV variant (GFUuv) were added to the original pMAD system.

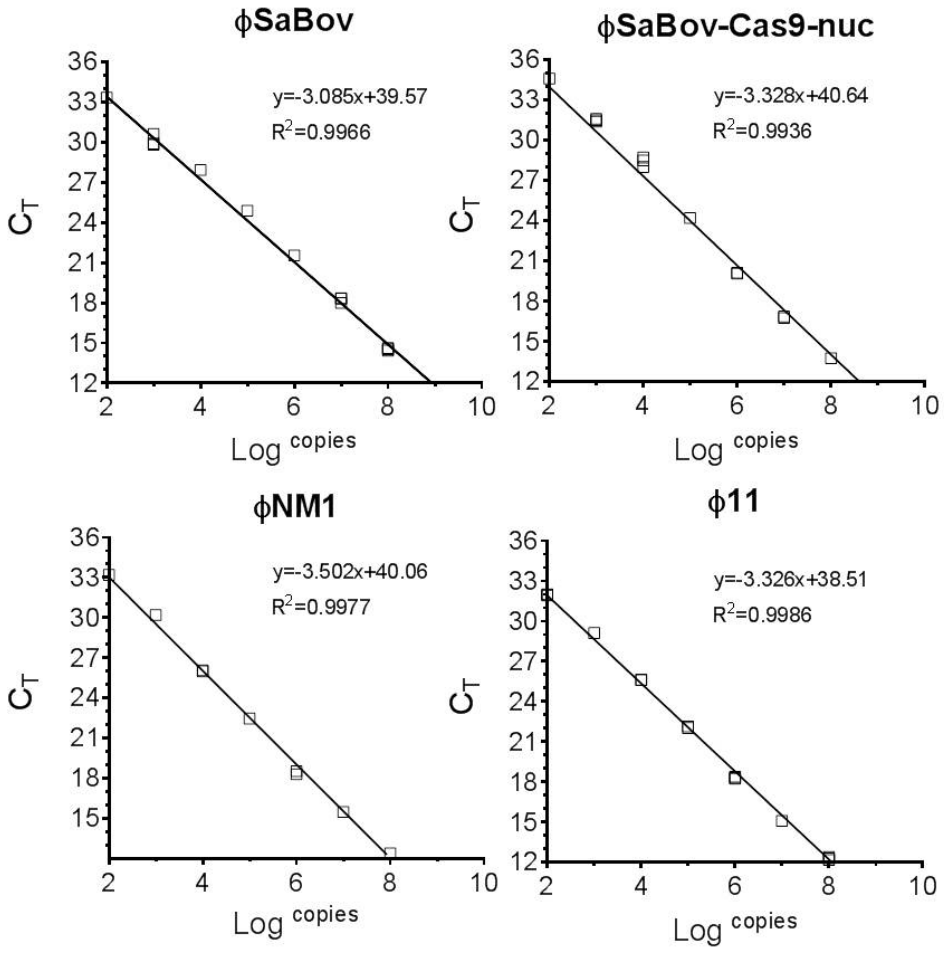

Figure 4. Standard curves to determine the absolute copy number of phage DNA. The integrase gene for each phage was amplified and cloned into pCR4-TOPO (Life technologies). A serial dilution of cloned plasmid was used in quantitative real time PCR. Standard curves were generated by linear regression analysis to calculate the slope, intercept, and correlation coefficient (R2) using Microcal OriginPro (Microcal origin, Version 7.5).

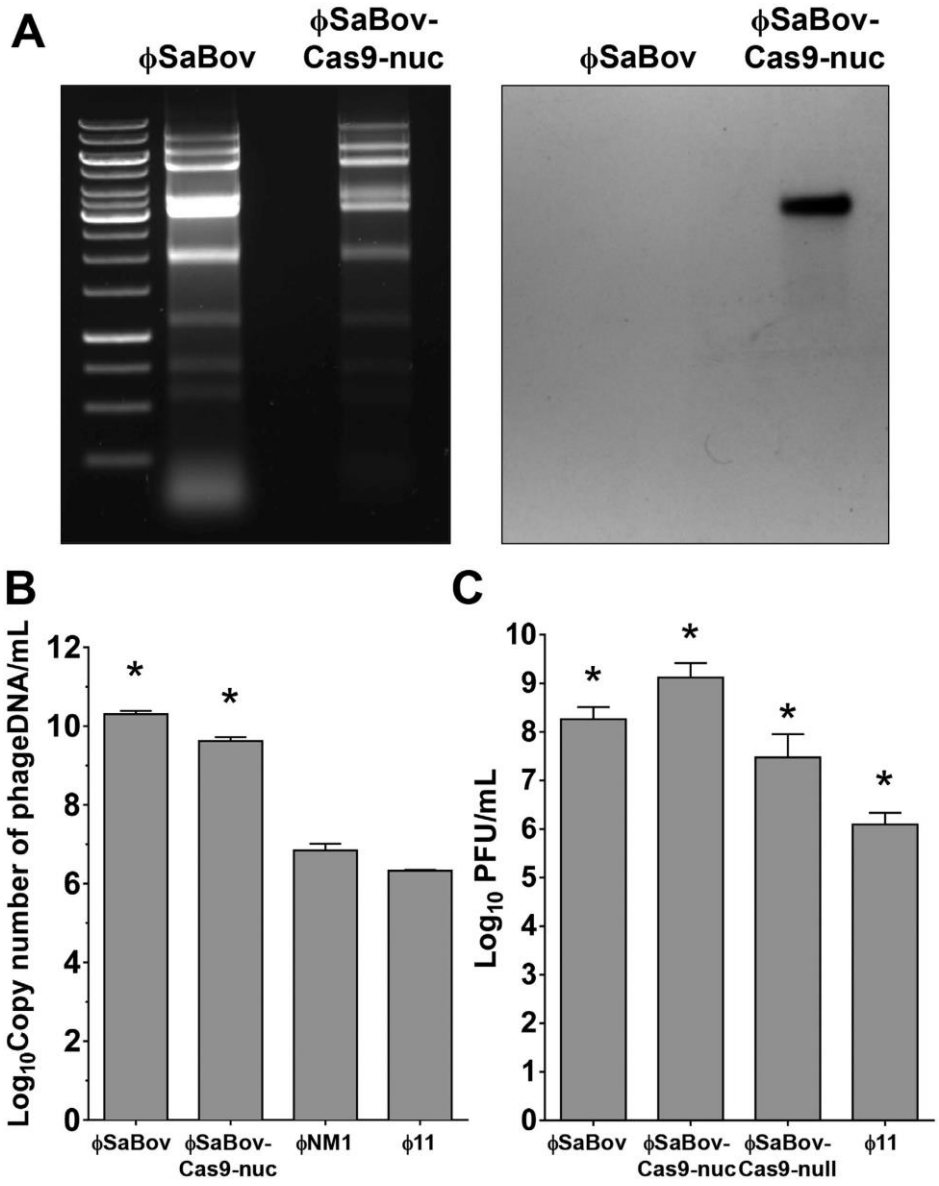

Figure 5. Characterizing the ϕSaBov integrated with CRISPR/Cas9 system. (A) Integration of CRISPR/ Cas9 system specific to *S. aureus* was verified by southern blot analysis using a probe specific to the leader sequence. (B) The copy number of phage DNA in the phage lysates was determined using quantitative real time PCR and

standard curves presented in Supplemental Figure 3. (C) The number of transducing phage particles in the phage lysates was determined by calculating the plaque forming unit (PFU) using semi-solid agar overlay method. The bar graph indicates the average and SEM combined from triple measurements of three independent experiments (n = 9). Asterisk indicates statistical significance in student t-test, compared to the results from ϕ11 ($p < 0.001$).

cloned into pKS4, resulting pKS5 (Fig. 2C). This plasmid was transformed into the strain RF122Δ*nuc*ϕSaBov in which the *nuc* gene has been removed to prevent CRISPR/Cas9 mediated-killing. The programmed CRISPR/Cas9 system was integrated into the genome of ϕSaBov by allelic exchange, resulting in RF122Δ*nuc*ϕSaBov-Cas9-*nuc* (Fig. 2C). Phages induced from this strain were referred as to ϕSaBov-Cas9-nuc. Southern blot analysis using a probe specific to the pre-crRNA gene confirmed the integration of CRISPR/Cas system into the genome of ϕSaBov (Fig. 5A). The copy number of recombinant phage genome harboring CRISPR/Cas9 system (ϕSaBov-Cas9-nuc) was determined by quantitative real time PCR (qRT-PCR) and showed a slight decrease, compared to those of wild type ϕSaBov, possibly due to the increase of genome size following integration of CRISPR/Cas9 system, but was still significantly higher than ϕ11 and ϕNM1 (Fig. 5B). The transduction efficacy of the ϕSaBov-Cas9-nuc was determined by calculating the plaque forming unit (PFU) and showed that the PFU of ϕSaBov-Cas9-nuc was higher than that of wild type ϕSaBov, presumably due to the killing effect of CRISPR/Cas9 system in ϕSaBov-Cas9-nuc which prevents lysogenic conversion events by ϕSaBov (Fig. 5C).

As a control, CRISPR/Cas9 system without a spacer sequence was integrated into the genome of ϕSaBov in RF122Δ*nuc*. Phages induced from this strain were referred as to ϕSaBov-Cas9-null.

3. The specificity and efficacy of φ SaBov-Cas9-nuc in in vitro assays.

To assess the efficacy of killing by ϕSaBov-Cas9-nuc, *S. aureus* strain CTH96, a bovine isolate susceptible to ϕSaBov, was treated with various multiplicities of infection (MOIs) of ϕSaBov-Cas9-nuc, and viable cells were recovered by plating on BHI agar. We defined the MOI as the number of transducing phage particles per recipient cell. When treated for 6 h, recipient cells were completely killed at MOI of 100 or above, and 5.01% and 0.08% of viable recipient cells were recovered at MOI of 10 and 50, respectively (Fig. 6A). In order to assess the natural occurrence of survival due to the spontaneous mutations in targets, a time-dependent killing was measured. Upon

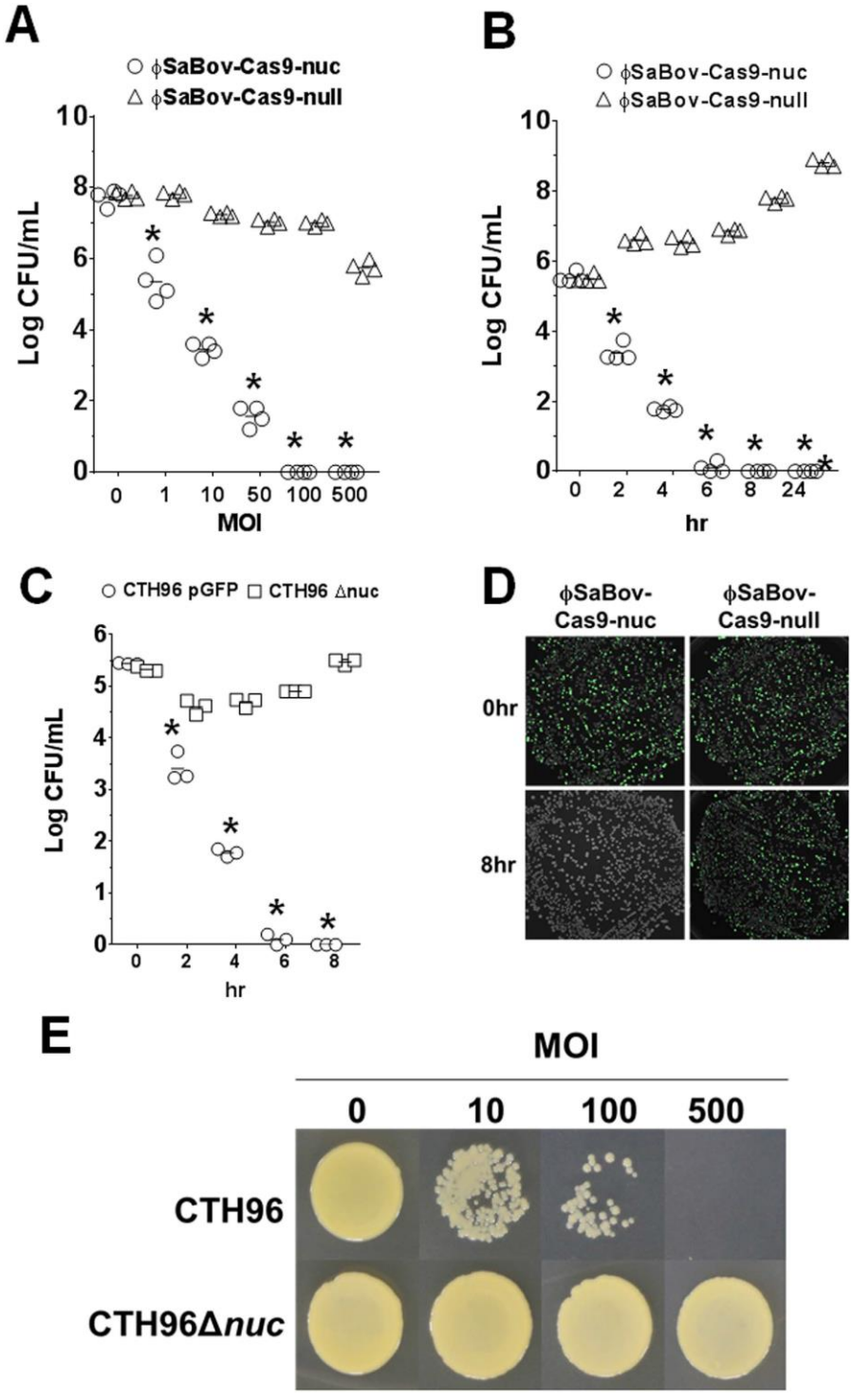
A
ϕSaBov-Cas9-nuc
ϕSaBov-Cas9-null
Log CFU/mL
MOI
0 1 10 50 100 500
B
ϕSaBov-Cas9-nuc
ϕSaBov-Cas9-null
Log CFU/mL
hr
0 2 4 6 8 24
C
CTH96 pGFP
CTH96 Δnuc
Log CFU/mL
hr
0 2 4 6 8
D
ϕSaBov-Cas9-nuc
ϕSaBov-Cas9-null
0hr
8hr
E
MOI
0 10 100 500
CTH96
CTH96Δnuc

Figure 6. The efficacy and specificity of ϕSaBov-Cas9-nuc in in vitro assays. A mid exponential culture of *S. aureus* strain CTH96 (1×10^5 CFU) was treated (A) at various MOIs of ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null for 6 h or (B) at 50 MOI of ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null up to 24 h. Viable cells were recovered on BHI plates. Data point represent the average of triple measurements which were repeated in four independent experiments. (C) A mid exponential culture of *S. aureus* strain CTH96pGFP or CTH96Δ nuc (1×10^5 CFU) was treated at 50 MOI of ϕSaBov-Cas9-nuc for 8 h. Viable cells were recovered on BHI plates. Data points indicate the average of triple measurements which were repeated in three independent experiments. Asterisk indicates statistical significance in student t-test, compared to the results from ϕSaBov-Cas9-null ($p < 0.001$). (D) A mixture of S. aureus strain CTH96pGFP and CTH96Δ nuc (1:1, each at 5×10^4 CFU) was treated with ϕSaBov-Cas9-nuc at MOI of 50 for 8 h. Viable cells were recovered from BHI plates. Pictures showing expression of green fluorescent protein were obtained under UV wave length using Gel Doc system (Bio-Rad). Results shown are a representative picture repeated in three independent experiments. (E) A sterile empty antibiotic disc was inoculated with a suspension of CTH96 or CTH96Δ int and treated with the ϕSaBov-Cas9-nuc at various MOIs for 8 h. Viable cells were recovered by blotting discs onto BHI plates. Results shown are a representative picture repeated in three independent experiments.

treatment with ϕSaBov-Cas9-nuc at MOI of 50, the number of viable recipient cells gradually decreased, and no viable cells were recovered after 8 h treatment and sustained at 24 h, suggesting rare occurrence of surviving mutants (Fig. 6B). By contrast, treatment with ϕSaBov-Cas9-null did not show any killing effect at MOI of 50 or less and minor killing effects at MOI of 100 or above, presumably due to the lytic cycle of ϕSaBov (Fig. 6A and B). To assess the *nuc* gene specific killing effect of ϕ SaBov-Cas9-nuc, we generated the nuc gene deletion mutant of CTH96 (CTH96Δ*nuc*) lacking the target gene for a spacer sequence in CRISPR/Cas9 system and CTH96 carrying a plasmid expressing green fluorescent protein (CTH96pGFPuv). When treated with ϕSaBov-Cas9-nuc at a MOI of 50, viable CTH96pGFPuv gradually decreased and completely lost viability at 8 h of treatment (Fig. 6C).

By contrast, the number of viable CTH96Δ*nuc* slightly decreased within 2 h of treatment, presumably due to the lytic cycle of ϕSaBov, and then gradually increased thereafter (Fig. 6C). Next, the mixed cultures of CTH96Δ nuc and CTH96pGFPuv were treated with ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null at MOI of 50 for 8 h, and viable cells recovered on BHI plates were analyzed under the UV lamp. When treated with the ϕSaBov-Cas9-nuc, recipient cells expressing GFP were selectively killed (Fig. 6D). By contrast, when treated with the ϕSaBov-Cas9-null, both CTH96Δ*nuc* and CTH96pGFPuv were equally recovered (Fig. 6D). The killing effect of ϕSaBov-Cas9-nuc against *S. intermedius* (coagulase positive staphylococci) and *S. epidermidis* (coagulase negative staphylococci) was also tested but was not observed (data not shown). Combined, these results clearly demonstrated the nuc gene-specific killing effect of ϕSaBov-Cas9-nuc.

The effect of CRISPR/Cas9 system requires biological activities including transcription and translation of the CRISPR/Cas9 system within the recipient cells which might limit the application of CRISPR/Cas9 antimicrobials under the nutritionally and metabolically-limited conditions. To simulate the nutritionally and metabolically-limited conditions, the empty antibiotic disc was inoculated with S. aureus in PBS (1×10^5 CFU), followed by treatment with the ϕSaBov-Cas9-nuc at MOIs of 10, 100, and 500. After 8

h treatment, viable cells were recovered by blotting the disc on to the BHI plate. Recipient cells were gradually decreased at MOIs of 10 and 100, and completely decolonized at MOIs of 500 (Fig. 6E). By contrast, the viability of recipient cells lacking the nuc gene (CTH96Δ*nuc*) was not affected (Fig. 6E). These results suggest the potential use of ϕSaBov-Cas9-nuc for sanitizing abiotic objects such as medical devices or surfaces. We chose the *nuc* gene as a target for CRISPR/Cas9 system because the *nuc* gene is uniquely present in all *S. aureus*, thus CRISRR/Cas9 system only kills *S. aureus* without affecting microbiomes. We tested the efficacy of CRISPR/Cas9 system targeting two proximate locations of the *nuc*, thereby, cleaving two chromosomal locations within the *nuc* gene which also showed a similar killing effect to CRISPR/Cas9 targeting a single location (data not shown). These results suggest that the efficacy of CRISPR/Cas9 killing effect is mainly determined by the efficiency of CRISPR/Cas9 system delivery to the target organisms.

4. The efficacy of ϕSaBov-Cas9-nuc in in vivo assays.

To test the efficacy of ϕSaBov-Cas9-nuc in vivo, the backs of C57BL/6 mice were shaved and intradermally inoculated with recipient cells (CTH96pGFP, 1×10^5 CFU). After 6 h of infection, ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null was injected into the infected skin at an MOI of 500. Following treatment for 24 h, infected skin regions were excised and homogenized to recover viable cells. The number of viable cells recovered from the skins treated with ϕSaBov-Cas9-nuc (0.647±0.128 Log CFU/g of tissue, mean ±SEM) was significantly lower than that treated with ϕSaBov-Cas9-null (3.333±0.131 Log CFU/g of tissue) (Fig. 7A). Next, to test the nuc gene-specific killing capacity of ϕSaBov-Cas9-nuc in vivo, animals were infected with a mixture of CTH96pGFP and CTH96Δ*nuc* (1:1, each at 5×104 CFU) for 6 h, followed by treatment with

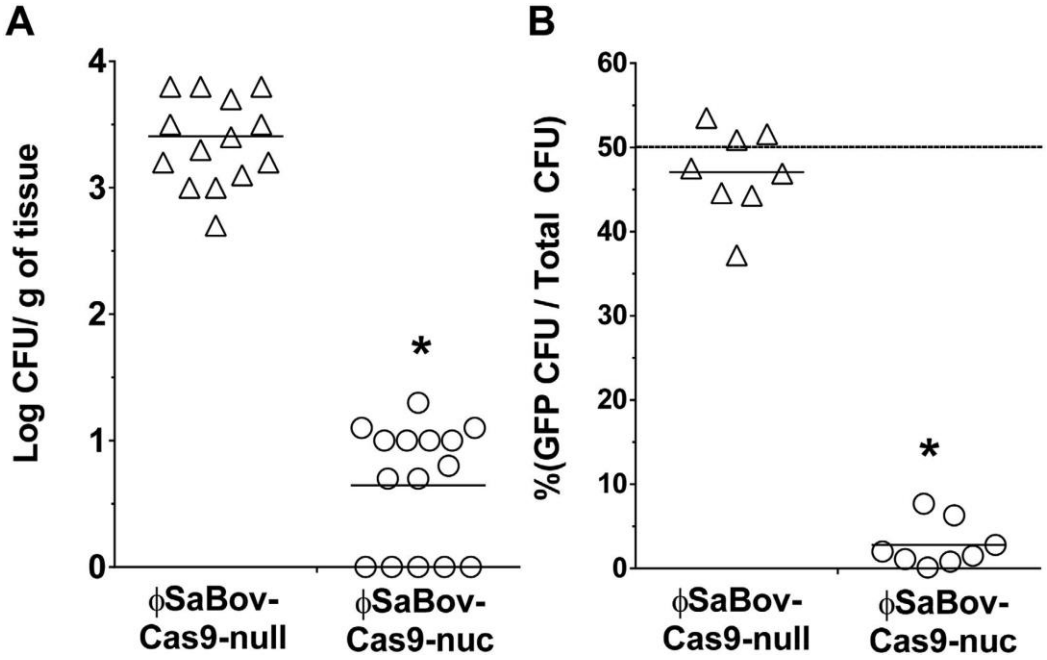

Figure 7. The efficacy of ϕSaBov-Cas9-nuc in in vivo murine skin infection. The backs of C57BL/6 mice were intradermally infected with a suspension of (A) CTH96pGFP (1 $\times 10^5$ CFU) or (B) a mixture of CTH96pGFP and CTH96Δ nuc (1:1, each at 5×10^4 CFU) for 6 h, followed by treatment with the ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null at MOI of 500. After 24 h, infected skin was excised and homogenized. Viable cells were recovered by plating serially diluted homogenates onto BHI plate. The specificity of ϕSaBov-Cas9-nuc was evaluated by the proportion of viable cells expressing green fluorescent protein in total viable cells. Data points indicate the average of triple measurements in individual mice (n = 9). Asterisk indicates statistical significance in student t-test, compared to the results from ϕSaBov-Cas9-null ($p < 0.001$).

ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null at MOI of 500 for 24 h. The proportion of viable cells expressing GFP in infected skin treated with ϕSaBov-Cas9-nuc was 2.8±2.6%, in contrast to those treated with ϕSaBov-Cas9-null showing 47.1± 4.9% (Fig. 7B), suggesting *nuc* gene-specific killing effect by ϕSaBov-Cas9-nuc in vivo.

Lastly, we tested if the ϕSaBov-Cas9-nuc was able to decolonize *S. aureus* from the surface of skin. The back of mice skin were shaved, depilated, decolonized with 70% alcohol, and colonized with CTH96pGFP (2×10^4 CFU) by cotton swab. After 6 h, ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null at MOI of 500 was topically applied by spraying. Following treatment for 24 h, infected skins were dissected and homogenized to determine the viable cell count. However, the number of viable cells recovered from infected skins treated with ϕSaBov-Cas9-nuc was not significantly different from that treated with ϕSaBov-Cas9-null (Fig. 8A). We noticed that the surface of skin was completely dried in 15 mins from the application of inoculum, or phage solution that may create an environment with limited water activity which suppress transcriptional and translational activities of *S. aureus*, thereby the machinery of CRISPR/Cas antimicrobials could not be expressed. In order to maintain water activity, ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null was mixed with a hydrogel and topically applied to the infected skin by swabbing which significantly improved the efficacy of killing effect of ϕSaBov-Cas9-nuc (Fig. 8B).

5. Prevention of toxins contaminations in phage lysates.

The strain RF122 harbors 10 superantigens (*sec, seg, sei, selm, seln, selo, selu, sell, tst1, selx*) and 11 cytolysins (*hla, hlb, hlgA, hlgB, hlgC, lukD, lukE, lukG, lukH, lukM, lukF'*) genes (Fig. 9) which could be contaminated in the phage lysates generated from the strain RF122. Furthermore, our previous studies demonstrated that induction of ϕSaBov from strain RF122 generated transducing phage particles harboring genetic segments specifically associated with MGEs such as νSaα, νSaβ, νSaγ, and SaPI1 some of which contain superantigens (*sec, seg, sei, selm, seln, selo, selu, tst*) and cytolysins

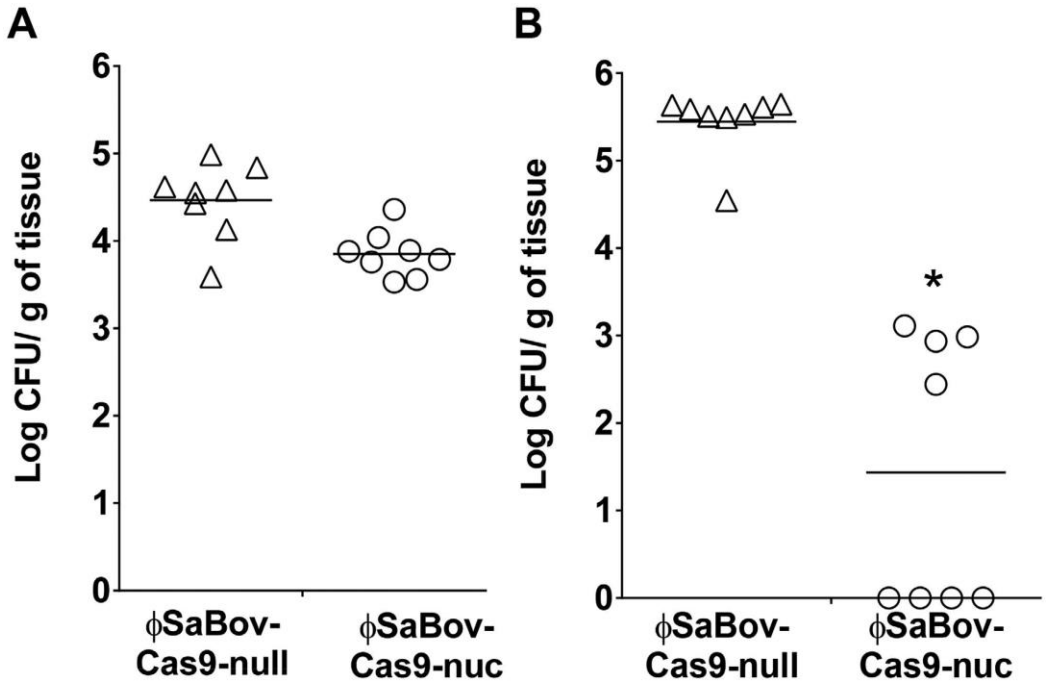

Figure 8. The efficacy of ϕSaBov-cas9-nuc in decolonization of *S. aureus* from surface of skin. The back skin of mice was shaved, depilated, and decolonized with 70% alcohol, followed by recolonization with CTH96pGFP (2×10^4 CFU). After 6 h, ϕSaBov-Cas9-nuc or ϕSaBov-Cas9-null at MOI of 500 was topically applied in solution (A) or in mixing with a hydrogel (B). Following treatment for 24 h, infected skins were dissected and homogenized to determine the viable cell count. Data points indicate the average of triple measurements in individual mice (n = 8). Asterisk indicates statistical significance in student t-test, compared to the results from ϕSaBov-Cas9-null ($p < 0.001$).

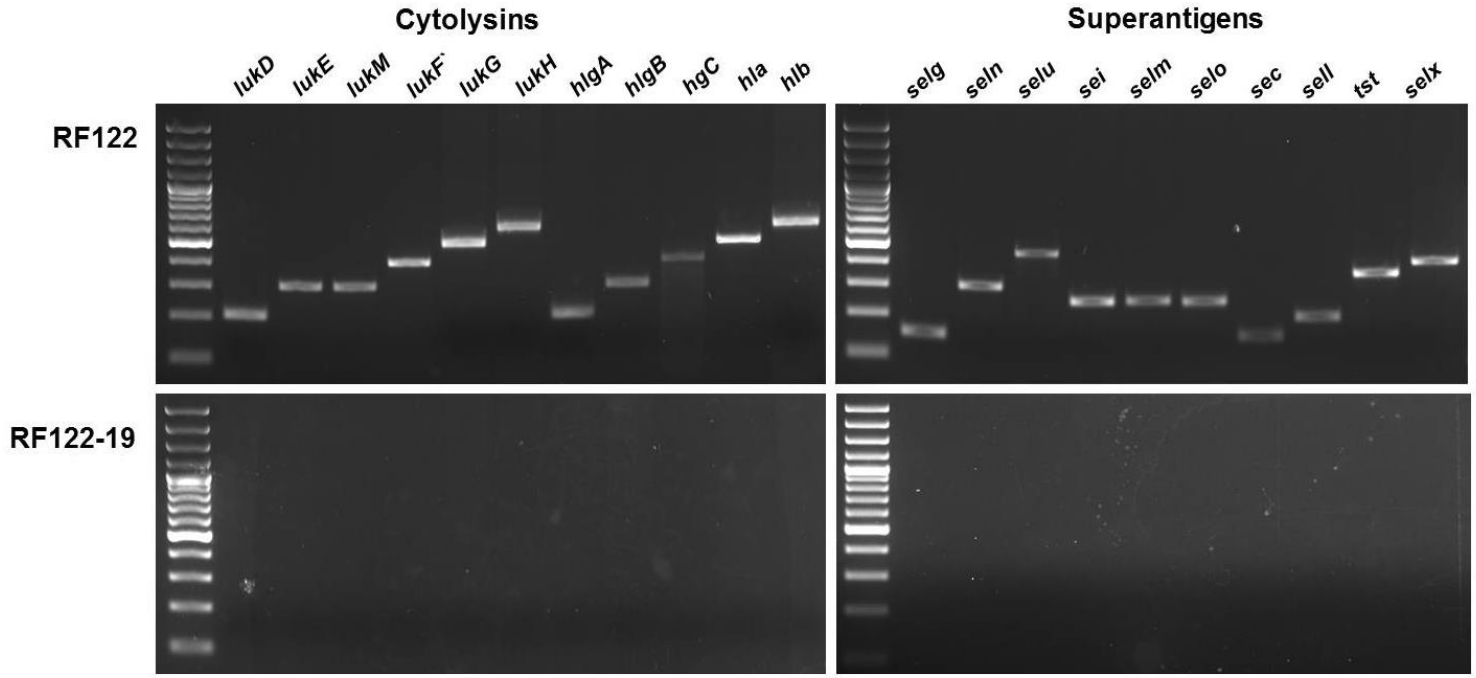

Figure 9. PCR analysis of cytolysins and superantigens genes. The strain RF122 harbors 11 cytolysins (*hla, hlb, hlgA, hlgB, hlgC, lukD, lukE, lukG, lukH, lukM, lukF'*) and 10 superantigens (*sec*, *seg*, *sei*, *selm*, *seln*, *selo*, *selu*, *sell*, *tst1*, *selx*) gene in the chromosome (RF122 panel). The 11 cytolysin and 10 superantigen genes were removed from RF122 chromosome without antibiotic selection markers by allelic exchange using modified pMAD-secY system which was confirmed by PCR analysis (RF122-19 panel).

(*hla, lukD, lukE*) and transferred these virulence genes to other *S. aureus* strains by generalized transduction [26,29].

To prevent contamination of these toxins in phage lysates and potential spread of toxin genes by the ϕSaBov-based delivery system, 10 superantigens and 11 cytotoxins present in the chromosome of RF122Δ*nuc*ϕSaBov-Cas9-nuc were removed by allelic exchange using a modified pMAD-secY system, resulting in RF122-19Δ*nuc*ϕSaBov-Cas9-nuc. The deletions were confirmed by PCR analysis (Fig. 9). As expected, phage lysates generated from the strain RF122Δ*nuc* showed superantigenicity as indicated by significantly higher count per minute (cpm) as a result of incorporation of [3H]-thymidine into dividing cellular DNA (Fig. 10A). Consistently, incubation with phage lysates generated from the strain RF122Δ*nuc* induced cytotoxicity by cytolysins in phage lysates as demonstrated by intercalation of propidium iodide into cellular DNA (Fig. 6B). By contrast, phage lysates generated from the strain RF122-19Δ*nuc* did not induce any superantigenicity or cytotoxicity (Fig. 10A and B).

6. Expansion of host specificity of ϕSaBov.

The ϕSaBov has a narrow host range highly specific to bovine CC151 lineage of *S. aureus* as shown in phage spot test (Fig. 11A, ϕSaBov panel), thereby the efficacy of ϕSaBov-Cas9-nuc to human pandemic clonal lineage of *S. aureus* (ST1, ST5, ST8 and ST36) was minimal or had no effect (Fig. 11B, black bars). Host specificity of phage is primarily determined by phage-encoded tail fiber proteins interacting with receptors on host cells such as membrane proteins or cell wall carbohydrates [30-32]. We investigated the possible expansion of host ranges of ϕSaBov by complementing the gene encoding the tail fiber protein (Tif) from ϕ11 (orf50) 28 which has a broad spectrum of host range in several pandemic clones of human S. aureus isolates in the United States (Fig. 7A, ϕ11 panel). A pMK4 shuttle vector harboring the tail fiber protein gene of ϕ11 (pTF11) was constructed and transformed into the strain RF122-19Δ*nuc*ϕSaBov and RF122-19Δ*nuc*ϕSaBov-Cas9-nuc, resulting RF122-19Δ*nuc*ϕSaBov-pTF11 and RF122-19Δ*nuc* ϕSaBov-Cas9-nuc-pTF11, respectively.

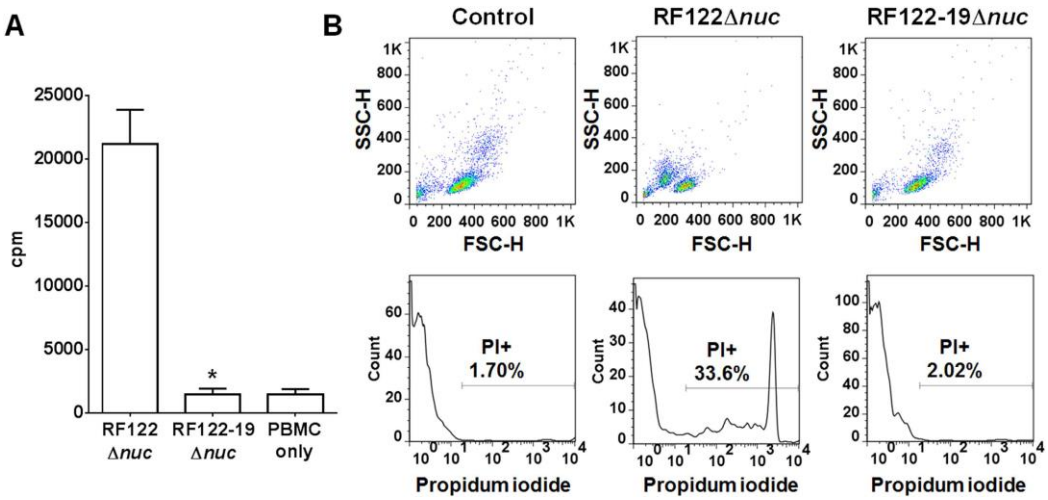

Figure 10. Prevention of toxin contamination in phage lysates. Human PBMCs were treated with phage lysates generated from RF122Δ*nuc* or RF122-19Δ*nuc*, an isogenic strain lacking 10 superantigen and 11 cytotoxin genes. (A) After 3 day incubation, proliferation of T cells caused by superantigens in phage lysages was measured by incorporation of radioactive 3H-thymidine into the cellular DNA using liquid scintillation counter. Bars indicate the count per minute (cpm) of radioactivity combined from triple measurements of three independent experiments (n = 9). Asterisk indicates statistical significance in student t-test, compared to the results from the RF122Δ*nuc* ($p < 0.001$). (B) After 3 h incubation, cytotoxicity caused by cytotoxins in phage lysates was measured by incorporation of propidium iodide into dead cells (propidium iodide positive, PI+) using flow cytometry. Data shown are representative results repeated in three independent experiments.

Phages induced from RF122Δ*nuc*ϕSaBov-pTF11 (ϕSaBov-pTF11) showed improved clear zone of lysis in phage spot tests against pandemic human clonal lineages (ST1, ST5, ST8 and ST36) of *S. aureus* strains (Fig. 11A, ϕSaBov-pTF11 panel), compared to that of ϕSaBov. Phage absorption assay showed that the complementation of the Tif from ϕ11 significantly improved the phage absorption to the pandemic human clonal lineage, albeit not as high as to the CC151 (Fig. 11C). Consistently, phages induced from RF122Δ*nuc* ϕSaBov-Cas9-nuc pTF11 significantly improved the killing effect of CRISPR/Cas9 against ST1, ST5, ST8 and ST36 lineages of S. aureus ranging from 1.51 to 3.15 order of magnitude (Fig. 11B, gray bars).

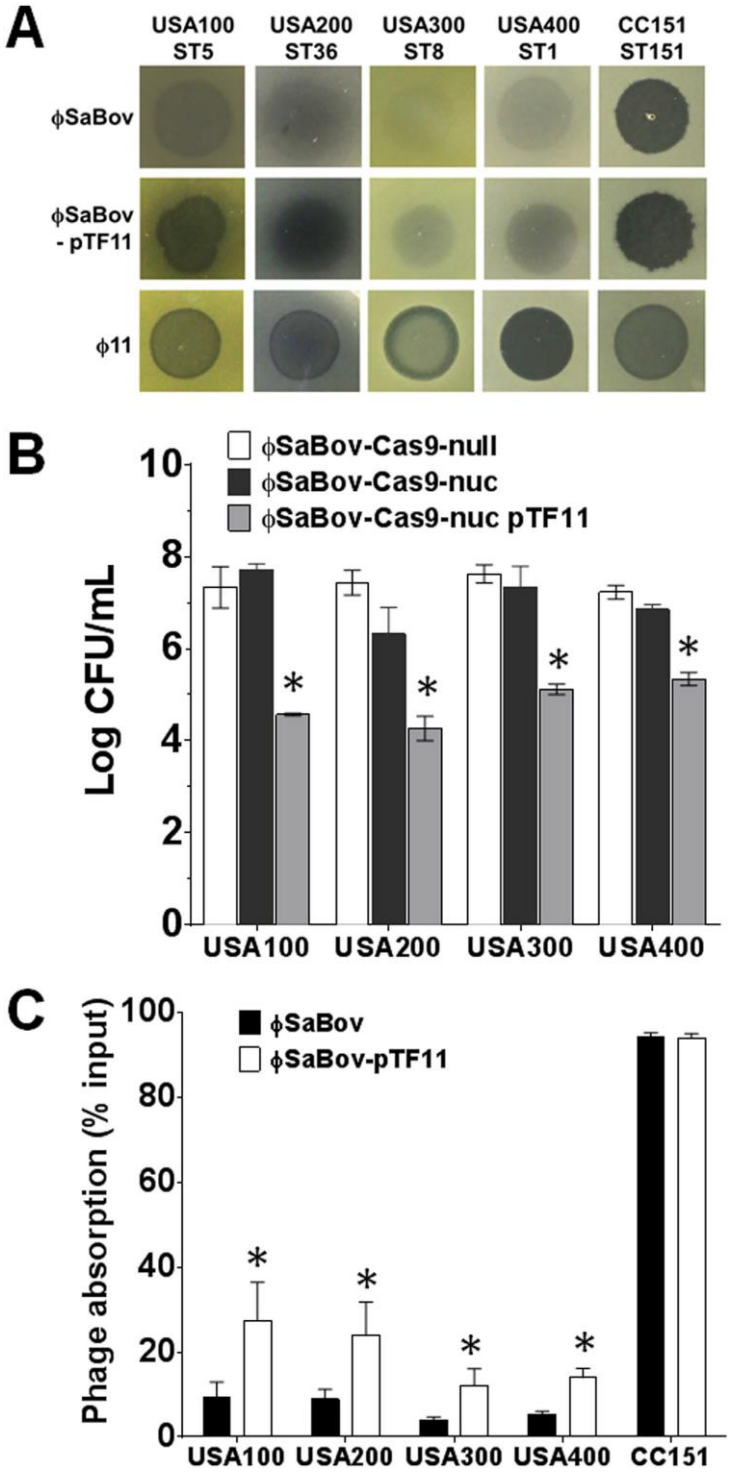
A
USA100 ST5
USA200 ST36
USA300 ST8
USA400 ST1
CC151 ST151
ϕSaBov
ϕSaBov - pTF11
ϕ11
B
ϕSaBov-Cas9-null
ϕSaBov-Cas9-nuc
ϕSaBov-Cas9-nuc pTF11
Log CFU/mL
0
2
4
6
8
10
USA100
USA200
USA300
USA400
C
ϕSaBov
ϕSaBov-pTF11
Phage absorption (% input)
0
20
40
60
80
100
USA100
USA200
USA300
USA400
CC151

Figure 11. Expansion of host specificity of ϕSaBov by complementing the tail fiber protein. (A) For phage spot test, 10 μL of phage lysates (of ϕ11, ϕSaBov, or ϕSaBov complemented with the tail fiber protein of ϕ11 (ϕSaBov-pTF11) containing 1×10^8 pfu/ml was inoculated onto the lawn culture of several pandemic human clones of *S. aureus*. (B) To measure the efficacy of CRISPR/Cas9 system delivered by ϕSaBov complemented with the tail fiber protein of ϕ11, a mid-exponential culture of pandemic human S. aureus clones (1×10^5 CFU) was treated with phage lysates of ϕSaBov-Cas9-null, ϕSaBov-Cas9-nuc, or ϕSaBov-Cas9-nuc pTF11 at MOI of 100 for 8 h. The bar graph indicates the average and SEM of Log CFU of viable cells, combined from triple measurements of three independent experiments (n = 9). (C) The phage absorption was determined by calculating the proportion of unbound phage from the total phage input (2.5×10^5 pfu) to several pandemic human clones of S. aureus (5×10^7 CFU) at the MOI of 0.005. Asterisk indicates statistical significance in student t-test, compared to the results from ϕSaBov ($p < 0.001$).

IV. Discussion

Conventional antibiotics, targeting proteins of critical bacterial cellular pathways, are often rendered ineffective due to bacteria either acquiring episomes harboring resistance genes or accruing spontaneous mutations in targets [6]. The CRISPR/Cas9 antimicrobials have become an attractive alternative due to the advantages of sequence-specific killing without disturbing the microbiome and multiplex features of spacer sequences to simultaneous target multiple genes, thereby preventing development of resistant mutants [34]. Despite promising results, a therapeutic use of CRISPR/Cas9 antimicrobials is still far from being practical due to the shortcomings in efficiency of delivery and safety aspects of phage-based delivery systems [12,14]. In this study, we demonstrated a novel genetic engineering strategy to enhance the efficacy and safety of phage-based delivery systems by integrating CRISPR/Cas9 system into the genome of a temperate phage to improve the delivery to target cells, complementing phage tail fiber protein to extend the host spectrum, and removing virulence genes from the host strain to prevent contamination by toxins and spread of virulence genes.

We chose the ϕSaBov lysogenized in *S. aureus* strain RF122 as a candidate for phage-based CRISPR/Cas9 delivery system because induction of ϕSaBov from the strain RF122 generated an exceptionally high number of transducing phage particles harboring the phage genome. Indeed, integration of CRISPR/Cas9 system into the genome of ϕSaBov significantly enhanced the efficacy of *S. aureus* specific-killing by ϕSaBov-Cas9-nuc to nearly complete decolonization in vitro under both nutritionally enriched and limited conditions and more than two orders of magnitude CFU reduction in an in vivo murine skin infection experiments. Interestingly, efficient packaging of the ϕ SaBov genome is highly specific to the RF122 background, and not reproduced in other strains such as RN4220 (Fig. 11) or MW2 [26]. These results suggest the presence of genetic elements uniquely present in the chromosome of RF122 promoting phage DNA excision and replication. Phage DNA excision, replication, and packaging are controlled by complex mechanisms involving multiple factors encoded in the phage

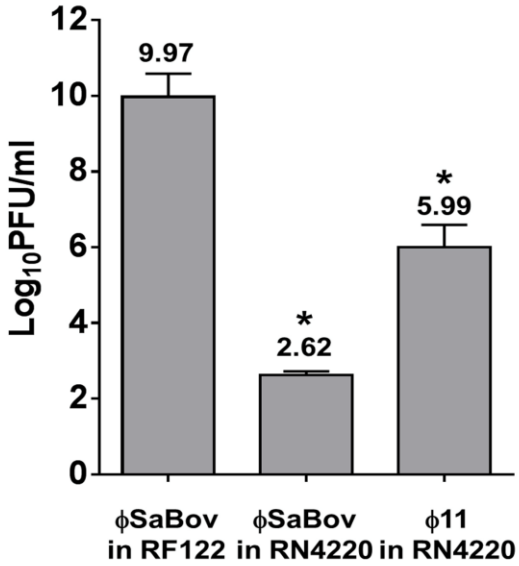

Figure 11. Packaging efficiency of the ϕSaBov and ϕ11 genome in different host. The ϕSaBov lysogenized in the RF122 and RN4220 strains were induced and the number of transducing phage particles of in the phage lysates was determined by calculating the plaque forming unit (PFU) using semi-solid agar overlay method. The bar graph indicates the average and SEM combined from triple measurements of three independent experiments (n=9). Asterisk indicates statistical significance in student t-test, compared to the results from ϕ11 ($p<0.001$).

genome and host chromosome. Upon induction of phage by SOS signals, phage-encoded rinA and rinB activate transcription of phage-encoded integrase (*Int*), excisionase (*Xis*), and unknown host encoded factors such as IHFand Fis to initiate site-specific recombination at the attachment site (*att* site) [33,35,36]. Genome sequence comparison of RF122, MW2, and RN4220 revealed several unique integrases, transposases, and integrative and conjugative elements associated with MGEs and reminiscent of inactivated phage present in the chromosome of RF122 [37]. Our results showed that the application conditions may greatly affect the efficacy of CRISPR/Cas9 mediated killing effect. When applied in the dry skin condition, the ϕSaBov-Cas9-nuc was unable to decolonize *S. aureus* from the skin surface, presumably due to the low water activity suppresses transcriptional and translational activities of *S. aureus* in which the machinery of CRISPR/Cas9 antimicrobial could not be expressed. By contrast, when mixed with a hydrogel to support water activity, the ϕSaBov-Cas9-nuc successfully decolonized *S. aureus* from the infected skin surface. It is also possible that intravenous administration of CRISPR/Cas antimicrobials delivered by phage lysates may evoke immune responses by transducing phage particles or bacterial products remaining in the phage lysates, resulting in antibody production, decreasing efficacy, and potential allergic reactions. Therefore, the most practical application of CRISPR/Cas antimicrobials delivered by phage lysates would be topical application to the condition supporting water activity such as infected tissues or the contaminated surfaces of medical and culinary devices and food products. Recently, the United States Food and Drug Administration approved phage cocktails against *Listeria monocytogenes* for use in ready to eat food as generally recognized as safe, further encouraging topical applications [38].

Phage lysates generated by induction or propagation of temperate phage to the host strain harboring a plasmid or phagemid containing CRISPR/Cas system are mixtures of bacterial components including bacterial DNA, proteins, and cell wall components, as well as transducing phage particles. As demonstrated in Fig. 8, phage lysates generated from the strain RF122Δ*nuc* contained superantigens and cytolysins expressed from the

chromosome of RF122. These aspects will clearly raise a regulatory compliance concern on pharmaceutical use of phage lysates containing CRISPR/Cas antimicrobials in Western clinical settings. To alleviate this concern, we generated RF122 containing multiple toxin gene deletions resulting in loss of 10 superantigen and 11 cytolysin genes (RF122-19Δ*nuc*) by using the modified pMAD-secY temperature sensitive shuttle vector system established in this study. Therefore, phage lysates generated from RF122-19Δ*nuc* did not show any harmful effects associated with superantigens and cytolysins. Furthermore, RF122-19Δ*nuc* can be used as a virulence factor-free host strain to propagate ϕSaBov-Cas9-nuc, without risk of spreading superantigen and cytolysin genes by temperate phage-mediated generalized transduction. One may still argue that the virulence factor-free host strain would not be an ultimate solution because phage lysates generated from RF122-19Δ*nuc* may still contain other uncharacterized virulence factors in host chromosomal segments. Our previous study showed that excision and packaging of host chromosomal segments by ϕSaBov was highly specific to MGEs within the strain RF122 background, and not in other *S. aureus* strain backgrounds such as RN4220 and MW2 [26]. A recent study demonstrated that the integrase sequence-specific excision of host chromosome unlinked to phage DNA which packaged into transducing phage particles by the terminase large subunit (*TerL*) encoded by the helper phage [39]. Genome sequence analysis of strain RF122 showed a single copy of the *terL* gene associated with the genome of ϕSaBov, and two copies of the phage absorption, an important process that determines the host specificity of phage, is mediated by the phage tail module. The phage tail module is typically composed of tape measurement protein, minor tail protein, baseplate protein, cell wall hydrolase, and tail fiber protein. The baseplate protein is linked with the tail fiber protein that can be extended out to search the host receptors such as lipopolysaccharides or the outer membrane porin protein C [30,31]. This contact triggers conformational changes in the baseplate protein of phages, causing irreversible binding of tail fibers to the outer core of lipopolysaccharides and penetration of inner tail tube to bacterial membrane allowing ejection of phage DNA [30]. A recent study in *Pseudomonas aeruginosa* Pap1 phage demonstrated that a single

nucleotide mutation in phage tail fiber protein resulted in altered host specificity [32].

These results suggest that host specificity of phage could be modulated by altering the phage tail module. The tail fiber protein of ϕ11 (orf50) contains 370 amino acid residues in which the N-terminal part of amino acid residues from 1 to 120 are highly similar to the putative tail fiber protein of ϕSaBov (orf56) with 74% identity (85/120) and 85% positivity (102/120). Recent studies demonstrated that the baseplate protein of ϕ11 (Gp45) plays an essential role in phage absorption to *S. aureus* by mediating interaction with the N-acetyl-glucosamine (GlcNAc) in the peptidoglycan [40,41]. Interestingly, the baseplate protein of ϕSaBov (orf51) showed high similarity with that of ϕ11 (Gp45) with 92% identity (587/636) and 96% positivity (615/635). These suggest a possibility that the baseplate protein is linked with the N-terminal part of tail fiber protein and the C-terminal part of tail fiber protein interact with the specific receptor in the host. This could explain the partial improvement of host specificity of ϕSaBov by complementing the plasmid harboring the gene encoding tail fiber protein of ϕ11 (pTFϕ11) because the high amino acid sequence homology in the baseplate proteins and the N-terminal part of tail fiber proteins between ϕSaBov and ϕ11 allows the interchangeable linkage of tail fiber protein from both ϕSaBov and pTFϕ11 to the baseplate protein of ϕSaBov, but causes competitions which results in partial improvement of host specificity.These results suggest that elucidating the interaction between the baseplate protein and tail fiber protein may lead to generate a chimeric tail fiber protein that bridges the C-terminal receptor recognition domain of tail fiber protein to the baseplate protein of phage for developing recombinant phage delivery system for CRISPR/Cas9.

Currently, we are pursuing either complete replacement of the tif gene within the ϕ SaBov genome with that of a broad host range phages such as ϕ11, ϕ13, and ϕNM1 by allelic exchange or integration of CRISPR/Cas9 system into the genome of broad host range phage. A phage therapy carrying CRISPR/Cas antimicrobials undoubtedly has great potential for alternative therapeutics, supplemental to conventional antibiotics, and prophylactic measurement against increasing antibiotic resistant pathogens. The genetic

engineering strategy on both phage and host genome established in this study will be useful to create an efficacious and safe CRISPR/Cas9 antimicrobials platform broadly applicable to MRSA and other important pathogens. *TerS* genes, one with ϕSaBov and the other with SaPIbov1 [37]. We are currently seeking to remove the redundant integrases and *TerS* gene to prevent excision of host chromosome mediating generalized transduction by ϕSaBov.

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General Conclusion

In this study, we demonstrated that the genomic island encoding an array of virulence factors was transferrable in vitro to human and animal strains of multiple *S. aureus* clones via a resident prophage ϕSaBov. The transfer of the νSaβ appears to have been accomplished by multiple conversions of transducing phage particles carrying overlapping segments of the νSaβ. ϕSaBov also mediates the mobilization of νSaα and νSaγ, which are located remotely from ϕSaBov, mostly to recipient strains belonging to ST151. Phage DNA sequence analysis revealed that chromosomal DNA excision events from RF122 were highly specific to MGEs, suggesting sequence-specific DNA excision and packaging events rather than generalized transduction by a temperate phage. Disruption of the *int* gene in ϕSaBov did not affect phage DNA excision, packaging, and integration events. However, disruption of the *terL* gene completely abolished phage DNA packing events, suggesting that the primary function of temperate phage in the transfer of genomic islands is to allow for phage DNA packaging by TerL and that transducing phage particles are the actual vehicle for transfer. These results extend our understanding of the important role of bacteriophage in the horizontal transfer and evolution of genomic islands in *S. aureus,* highlighting the central role of bacteriophages in the pathogenic evolution of *S. aureus*.

A phage therapy carrying CRISPR/Cas antimicrobials undoubtedly has great potential for alternative therapeutics, supplemental to conventional antibiotics and prophylactic measurement against increasing antibiotic resistant pathogens. We demonstrate genetic engineering strategies to overcome these shortcomings by integrating CRISPR/Cas9 system into a temperate phage genome ϕSaBov (ϕSaBov-Cas9-nuc), removing major virulence genes from the host chromosome, and expanding host specificity of the phage by complementing tail fiber protein. This significantly improved the efficacy and safety of CRISPR/Cas9 antimicrobials to therapeutic levels in both in vitro and in vivo assays. The genetic engineering tools and resources established in this study are expected to provide an efficacious and safe CRISPR/Cas9 antimicrobial,

broadly applicable to *S. aureus*. The genetic engineering strategy on both phage and host genome established in this study will be useful to create an efficacious and sage CRISPR/Cas9 antimicrobials platform broadly applicable to MRSA and other important pathogens.

국문초록

황색포도상구균의 박테리오파지 매개 유전자 절달 및 대체 항생제로의 이용

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문 보 연

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Bacteriophage (phage)는 세균을 감염시키는 바이러스로서 용균과 용원을 통해서 세균에 기생하여 존속하며, 병원성 유전자 전달을 통해 세균의 병원성 진화에 큰 역할을 하고 있다. 본 연구에서는 사람과 동물에서 다양한 질병을 일으키는 병원성 세균인 *Staphylococcus aureus* 에서의 phage 매개 병원성 유전자 전달 메카니즘를 규명하고, 유전적 엔지니어링을 이용하여 RNA guided sequence specific nuclease인 CRISPR(Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9을 갖는 phage를 제작하고 효율성과 안전성을 높여 항생제 대체제로서 활용 될 수 있음을 확인하고자 하였다.

다양한 숙주 및 환경에 적응하여 살아남는 *S. aureus*의 생존 능력은 다양한 병원성 유전자에 의한 것이며, 이 유전자들은 bacteriophage, genomic island, *Staphylococcus aureus* pathogenicity island (SaPI), plasmid 와 같은 mobile genetic element (MGE)와 함께 관찰되며 전달된다. Genomic island인 νSaα, νSaβ,와 νSaγ는 대부분 *S. aureus* 에서 찾을 수 있으며, 이 MGE와 함께 전달되는 병원성 유전자는 다양한 형태로 발견된다. 그러나, 현재까지 이 MGE의 전달

메카니즘과 병원성 유전자의 다양한 조합에 대한 이해는 부족한 상황이다. ϕSaBov는 *S. aureus* RF122의 prophage로 νSaβ와 매우 근접해 있어 이 MGE의 전달 및 확산에 관여할 가능성이 매우 높음을 유전자 분석을 통해 확인하였다. Phage induction 과 transduction을 통해 νSaβ의 staphylococcal superantigen, leukotocin, bacteriocin 병원성 유전자가 ϕSaBov를 통해서 사람과 동물 유래의 다양한 유전형의 *S. aureus*에 전달될 수 있음을 확인하였다. νSaβ는 이 phage의 여러번의 삽입과 전환을 통해서 새로운 *S. aureus*에 전달되었다. 반면 νSaα와 νSaγ은 ϕSaBov와 매우 떨어져 있으나 이 MGE의 전달에 있어 관여하고 있음을 확인 할 수 있었다. Phage 유전자의 NGS (next generation sequencing) 분석과 phage 유전자의 packaging에 관여하는 *terL* 유전자의 knock-out은 ϕSaBov가 MGE를 특이적으로 세균 지놈으로 부터 분리하여 phage capsid 안으로 packaging함을 보여주었다. 반면 phage 유전자의 insertion과 excision에 관여하는 *int* 유전자는 phage 및 genomic island의 insersion과 excision에 직접적으로 관여하지 않는 것으로 보이며 이것은 숙주 박테리아의 유전형에 특이적으로 나타나는 recombinase 관련 유전자에 의해 영향을 받는 것으로 보여진다. 이 연구를 통해서 phage가 *S. aureus* genomic island 다양성과 확산에 기여함을 알 수 있었으며 또한 *S. aureus* 병원성 진화에 큰 역할을 하고 있음을 확인하였다.

CRISPR-Cas9(Clustered, regularly interspaced, short palindromic repeats - Cas9 RNA-guided nuclease)은 시퀀스 특이적으로 유전자를 분해하하여 세균을 특이적으로 사멸시킬 수 있어 항생제 대체제로서의 가능성이 매우 높아 이를 전달 해 줄 수 있는 phage와 함께 많은 연구가 진행되어 왔으나 효율성과 안전성에 대한 문제가 제기되어 왔다. 이러한 문제를 극복하기 위해 high-copy를 갖으며, 유전자 전달능이 우수한 ϕSaBov지놈안에 *S. aureus* 특이적 유전자인 *nuc*를 분해할 수 있는 CRISPR-Cas9을 삽입하여 *S. aureus* 사멸효과 를 살펴본 결과 그 효과가 매우 향상됨을 확인하였다. 안전성을 높이기 위해서 ϕSaBov가 전달하는 총 19개의 병원성 유전자를 모두 knock-out 하여 cytotoxicity를 제거하였다. 또한

숙주의 적용범위를 높이기 위해서 광범위한 숙주 특이성을 갖는 ϕ11의 tail fiber 유전자를 삽입한 결과, 다양한 유전형의 *S. aureus*에서 향상된 사멸 효과를 확인할 수 있었다. 병원성 유전자를 전달하는 phage와 숙주의 유전적 엔지니어링을 통해 CRISPR-Cas9을 이용하여 항생제 대체제로서의 활용이 가능함을 확인 할 수 있었다.

주요어: 박테리오파지, 병원성 유전자 전달, CRISPR/Cas9, 유전적 엔지니어링, 항생제 대체제

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