

表 題 ファージミドを用いた黄色ブドウ球菌に対する抗菌カプシドシ  
ステムの確立

論 文 の 区 分 博士課程

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## 1. Introduction

Antibiotics, since their discovery, have been an important therapeutic option in overcoming bacterial infections. Nevertheless, the emergence and rapid spread of antibiotic-resistant pathogens has rendered almost all existing antibiotics ineffective and have led to bacterial infections that are now posing a severe threat to human health. Antimicrobial resistance is the ability of a particular microorganism (mainly bacteria) to resist the antimicrobial agent and make them no longer effective. As a result, standard antimicrobial treatments become ineffective and the infection persists in the patient. These antimicrobial-resistant bacteria, commonly known as "superbugs", are all not necessarily pathogens; they can be symbiotic and harmless microorganisms and so the infected individuals stay asymptomatic in related cases. Antibiotic resistance can be a natural phenomenon, in which microorganisms innately develop resistance through genetic mutations or gene transfer between microorganisms in the environment.

The treatment of bacterial infections has long relied on antibiotics since the discovery of penicillin, the world's very first antibiotic discovered by Alexander Fleming in 1928<sup>1,2</sup>. However, widespread penicillin resistance was demonstrated by 1942<sup>3</sup>. Over the next few years, the infections caused by penicillin-resistant *S. aureus* increased and spread rapidly from the hospital setting to the community. By the end of the 1960s, more than 80% of community- and hospital-acquired *S. aureus* strains were penicillin-resistant<sup>4</sup>. The resistant strains showed mutations in the penicillin target PBP2 gene as well as exhibited expression of the drug efflux pump system<sup>5</sup>. However, after the introduction of methicillin in 1959, the rapid spread of penicillin resistance temporarily stopped; yet, the emergence of methicillin-resistant strains [methicillin-resistant *Staphylococcus aureus* (MRSA)] was reported in

1961<sup>6</sup> and it has been clarified as one of the most dangerous antibiotic-resistant bacteria by WHO<sup>7</sup>.

Mankind has countered antibiotic resistance by developing new antibiotics all these years. However, bacteria counter the antibiotics by gaining functional mutations that help them in resisting the antibiotics. It is to be noted that the emergence and spread of antibiotic-resistant bacteria are faster compared to the discovery of new antibiotics in this decade<sup>8</sup>. However, the goal of winning this 'game' is not in sight, and humankind is now worn-out of new antibiotics. It has been reported that as of 2019, resistant bacterial infections have directly caused 1.27 million deaths worldwide and are also associated indirectly with 5 million more deaths<sup>9</sup>. In the same year, there were believed to be 860 thousand global deaths from AIDS<sup>10</sup> (acquired immune deficiency syndrome). A report commissioned by the British government predicted that antimicrobial resistance could directly cause about 300 million deaths and a total economic cost of \$100 trillion by 2050 if not tackled<sup>11</sup>. The emergence of AMR and lack of antibiotics to treat the emerging AMR bacterium indicates the urgent need to focus on novel or alternative strategies to combat antimicrobial resistance other than antibiotics<sup>12</sup>. In recent years, bacteriophage-based “Phage Therapy” has been attracting attention as a promising antibacterial candidate for treating bacterial infections.

Bacteriophages (in short, phages), co-discovered by Felix D'Herelle and Frederick Twort<sup>13</sup>, are viruses that infect, inject their genome and replicate within their bacterial host cells. They are ubiquitous and are the most abundant microbiological entities that exist on earth<sup>14</sup>. Phages have high specificity toward their bacterial host strains, yet they are a harmless therapeutic option as they do not infect human or animal cells. Like other viruses, a phage have a nucleocapsid comprising of genetic material wrapped in a protein shell; most phages also have a "tail" to inject the genetic material into the host. More than 95% of known phages

carry double strand DNA, generally ranging from 5,000 to 5,000,000 base pairs in length, as their genetic material; the remaining 5% of phages are RNA phages and carry RNA as their genetic material<sup>15</sup>.

Due to their bacteriolytic activity, phage therapy to treat bacterial infections has been in use since 1919<sup>16–18</sup>. In 1919, Canadian scientist Felix D'Herelle utilized phages to successfully treat four cases of bacillary dysentery. This was the first evidence that phages could be used as antibacterial therapeutic<sup>19</sup>. In 1921, Bruynoghe et al. used phage preparations to treat septic staphylococci skin infections wherein the patient recovered from the infection in 2 days<sup>20</sup>. Next, in 1931, Felix D'Herelle and Eliava utilized phages to treat cholera, a virulent infectious disease of the digestive tract during a cholera epidemic in India<sup>21</sup>. However, the uncertainty surrounding phage therapy and the efficient curative antibacterial effect of antibiotics at that time eventually led to a gradual decline in interest in phage research. Phage therapy was largely abandoned in the Western world, whereas in the former Soviet Union and a few other Eastern European countries continued the use of phage for treating bacterial infections. Nevertheless, there is a renewed interest in utilizing phages as therapeutic agents, ensuing the emergence of antibiotic-resistant bacterial strains and is now considered as one of the effective alternatives to antimicrobial agents<sup>22</sup>. Many recent pre-clinical and clinical studies have assessed the efficacy and the safety of phage therapy and manifested phage therapy as an effective alternative approach to tackle the emergence of multi-drug-resistant pathogens<sup>23</sup>.

The main limiting factor of phage therapy is that some phages may carry virulence genes. These virulent genes upon expression during the phage mediated bacterial lysis can pose adverse effects on humans<sup>24</sup>. To counteract this issue, in addition to the use of natural phages, genetically modified phages have also been developed for phage therapy applications, such

as phages that cargo exogenous antimicrobial peptides, CRISPR-Cas system, and sRNA<sup>25-29</sup>; these phages were clinically employed to treat AMR infections and were reported to be quite successful.

In many such genetic modification studies, CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 was employed as an antimicrobial agent in killing resistant bacteria<sup>27,30</sup>. The developed genetically engineered phages were designed to target resistant genes and kill the target bacteria sequence specifically. However, this strategy has a limitation as this will be ineffective when the target gene is located on a plasmid<sup>28</sup>, as in many cases the resistance genes are not located on a bacterial chromosome.

In 2015, American researchers Shmakov et al. identified a novel CRISPR-Cas system<sup>31</sup>, CRISPR-Cas13a - CRISPR-Cas type VI class 2 system, previously known as C2c2 (Class 2 CRISPR-Cas system), whose detailed molecular function was elucidated by 2016<sup>32</sup>. Cas13a uses an approximately 20- to 28-base CRISPR-RNA (crRNA) for sequence-specific interaction with its target RNA. In bacteria, once this sequence-specific binding is accomplished, RNA-guided RNA endonuclease activity of Cas13a will be enabled, resulting in the cleavage of its target RNA sequence. Post target cleavage, Cas13a functions as a non-specific ribonuclease (RNase); as a result, Cas13a will bind and cleave other RNAs regardless of homology to the crRNA. This indiscriminate degradation of RNAs in the bacterium finally leads to their cell death<sup>33,34</sup>. This distinctive bactericidal function of Cas13a prompted us to adopt CRISPR-Cas13a as a potential antibacterial system against drug-resistant pathogens. We chose to deliver CRISPR-Cas13a to the particular bacterium by phages as they are considered to be an ideal vehicle.

Phage particles that carry genetic elements other than their own genome are being used for phage therapy<sup>35,36</sup>. One such system is phage-inducible chromosomal islands, PICIs that can

hijack phage capsids to package their own genetic elements<sup>37</sup>. The advantage of PICIs is their ability to mobilize between bacterial species. Previously, we adopted SaPI (*Staphylococcus aureus* pathogenicity island) mobile genetic system to carry the CRISPR-Cas13a for packaging into a model *S. aureus* phage capsid to detect resistance gene *mecA* of MRSA with sequence specificity<sup>28</sup>. SaPI is a well-studied PICI<sup>37</sup> carried by *S. aureus*. Any foreign DNA fragment with a SaPI derived packaging signal sequence can be efficiently packaged into the phage capsids and these packaged elements could transduce other bacteria<sup>28,38</sup>. However, the genetic manipulation of SaPI is quite complex and the ability of SaPI to replicate in its host is generally considered unsafe in the perspective of using it in therapeutic applications related to cargo delivery. Probably, for this reason, a more efficient system is necessary.

Another such genetic element that was proven to exploit phage packaging to carry its own genetic cargo is phagemids, which is typically a plasmid that carries ori and phage-packaging site genes of phage<sup>39,40</sup>. The phagemid does not carry any phage protein coding gene and thus does not produce daughter progeny after transformation into a bacterium. The phage proteins coded by elements like from the chromosome of the host cell (prophage) or the helper phage can help in packaging the phagemid DNA into phage viral particles<sup>41</sup>.

A phagemid is a special type of artificially constructed vector that comprises the phage packaging sequences, the origin of replication, cloning sites, and marker genes. To simply put, it is a double-stranded plasmid containing replicons of phage origin, which can be packaged into the phage capsids (a shell of phage). This phagemid system is advantageous as it has the characteristics of both the phage and the plasmid, and can also be replicated like a plasmid. The phagemid utilizes the phage packaging sites in their genetic element to be recognized and be packaged into the hijacked phage capsids, generating non-proliferative

phage particles<sup>27</sup>. The advantage of the phagemids lies in their ease to modification, ability to carry foreign genes of interest and transduce the host bacteria yet their inability to replicate, indicating a safer mode of phage therapy<sup>42</sup>. Nevertheless, this system requires improvement concerning its practical application, such as in terms of 1. programmed designing (overcoming the complexity and flexibility in issues related to manipulation or editing), 2. host range issues (as the host range is dependent on the parental phage being exploited for phagemid packaging, indicating only those hosts that are susceptible to the phage can be targeted) and 3. the efficiency of phage capsid packaging and transduction (as low titer, impaired performance or contamination issues could impede the therapeutic application).

Here, we adopted the packaging system for *S. aureus* phage using a phagemid to address the above-mentioned issues. Considering the advantages of the phagemid system, we generated a novel sequence-specific bactericidal CRISPR-Cas13a carrying phagemid that is packaged into the phage capsids of phage vB\_SaS-JC18T2 (named phage Tan2 in this study) for targeting MRSA<sup>43</sup>. In this study, we employed a wide host range staphylococcal phage Tan2, which is a temperate phage isolated in-lab; we established an approach to package bactericidal CRISPR-Cas13a into the Tan2 phage capsid. In addition, we focused on 1. optimizing the packaging efficiency, 2. producing pure phagemid capsids without natural phage contamination, 3. evaluating their bactericidal performance and 4. using them in wide range of other practical applications. We envisage our programmable phagemid technique is feasible alternative to antibiotics in the future and has many potential applications.

**Keywords:** *Staphylococcus aureus*, CRISPR-Cas13a, bacteriophage, phagemid, capsid, antimicrobial, non-proliferation

## **2. Materials and Methods**

### **2.1 Materials and instruments**

Tryptic soy broth (TSB), tryptic soy agar (TSA), Luria-Bertani (LB) broth, LB agar (BD Difco). Mitomycin C, PEG 6000, Kanamycin, Chloramphenicol, Tetracyclin (FUJIFILM Wako Pure Chemicals, Japan). MgSO<sub>4</sub>, CaCl<sub>2</sub>, NaCl, chloroform, isoamyl alcohol, isopropyl alcohol, EDTA, ethanol, SDS (Wako Pure Chemical Industries, Japan). DNase and RNase (Sigma-Aldrich, USA). Agarose (RIKAKEN, Japan), KOD FX Neo, Ligation high ver. 2 (Toyobo, Japan). TB Green® Premix Ex Taq™ II, In-Fusion HD Cloning Kit (TaKaRa, Japan), NEBuilder® HiFi DNA Assembly (New England Biolabs, USA), Nextera mate-pair sample preparation kit and MiSeq reagent kit version 3 (Illumina, USA). Micrometer cut-off filters, 0.22  $\mu$ m filter units (Sartorius Stedim Biotech, France). ELEPO21 electroporator (Nepa Gene, Japan). MIC qPCR (Bio Molecular Systems, Australia).

### **2.2 Strains and Culture conditions**

*S. aureus* strains were grown at 37°C in TSB/TSA, when appropriate, with the antibiotic 10  $\mu$ g/mL of chloramphenicol (Cp). *E. coli* strains were grown at 37°C LB broth/LB agar when appropriate, with the following antibiotics: 30  $\mu$ g/ml for kanamycin (Km) and/or 10  $\mu$ g/ml for chloramphenicol (Cp). All *S. aureus* and *E. coli* strains used in this study were listed in Table 1.

### **2.3 Isolation of phage**

Tan2 phage was isolated from the *S. aureus* JMUB1150, methicillin-sensitive *Staphylococcus aureus* (MSSA) strain by chemical induction method, which were isolated from Jichi

Medical University, Japan, in 2015. In brief, bacterial strain JMUB1150 was treated with mitomycin C to induce the excision and production of temperate phage. Briefly, 100  $\mu$ l overnight culture of bacteria was transferred into 5 ml induction medium [TSB broth, 10 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>] and incubated at 37°C under agitation for roughly 2 hours. The cultures were grown to an optical density of 0.5 at 600 nm (OD<sub>600</sub>) and then treated with a final concentration of 2  $\mu$ g/ml mitomycin C. The induced cultures were then incubated at 30°C with agitation at 80 rpm for 4 h. Finally, cultures were filtered through 0.22  $\mu$ m filters to collect the induced phages free of cell debris. The isolated phages were stored at 4°C until use.

#### **2.4 Phage DNA extraction**

20 ml of logarithmically growing host cells *S. aureus* strain RN4220 were infected with phages of interest at a MOI of 0.1~0.01 and incubated at 37°C until the culture become comparatively clear. Residual surviving cells were lysed with 1% (v/v) chloroform to release newly assembled phage particles. Lysate was then centrifuged at 8,000 g for 5 min and the supernatant were collected into new 50 ml tubes. A final concentration of 10  $\mu$ g/ml DNase and 10  $\mu$ g/ml RNase were added to the lysate and the mixture was incubated at 37°C for 1 h with agitation. Following that, equal volume of cold PEG solution [10% (w/v) PEG 6000, 5 mM Tris-HCl (pH 7.5), 1 M NaCl, 5 mM MgSO<sub>4</sub>] was added. After overnight incubation at 4°C, precipitated phages were collected by centrifugation at 15,000 g for 30 min at 4°C. Phage pellets were resuspended in 500  $\mu$ l TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA]. Then, 5  $\mu$ l 1 M EDTA and 5  $\mu$ l 10% SDS were added to phage suspension and the mixture were incubated for 15 min at 68°C. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) was added and the solution was

mixed well by vigorously shaking the tube for 10 min. Following centrifugation, the aqueous phase was transferred into a new tube. An equal volume of chloroform: isoamyl alcohol in the ratio of 24: 1 (v/v) was then added and the mixture was again mixed well by vigorous shaking. After centrifugation, aqueous phase containing phage DNA was transferred to a new tube. DNA was precipitated by addition of an equal volume of isopropyl alcohol followed by a 1 h incubation at -20°C. Then, phage DNA was pelleted by centrifugation at 12,000 g for 10 min at 4°C. Pelleted DNA was washed with 1 ml cold 80% ethanol and centrifuged at 10,000 g for 7 min at 4°C. This washing and centrifugation step were repeated once. Finally, phage DNA was dried briefly and resuspended in an appropriate volume of TE buffer<sup>44</sup>.

## **2.5 Whole genome sequencing of phage**

Mate-pair sequencing libraries were constructed from genomic DNA of each phages using Nextera mate-pair sample preparation kit without size selection. Sequencing was performed on Illumina MiSeq platform (2 × 301 bp) using MiSeq reagent kit version 3. The generated sequence data were assembled by De Novo Assembly tool (CLC genomic work bench version 9.5.3) and automatically annotated with Microbial Genome Annotation Pipeline (MiGAP, <http://www.migap.org/>). The amino acid sequences were comparatively analyzed based on homology searches using the Basic Local Alignment Search Tool for protein (BLAST) at NCBI website (<http://www.ncbi.nlm.nih.gov/blast/>)<sup>45,46</sup>.

## **2.6 Lysogenizing RN4220 with phage Tan2**

Phage Tan2 was lysogenized into *S. aureus* strain RN4220 for the construction of host cells that is to be used for phagemid packaging. First, 100 µl of RN4220 was added to 4 ml soft

agar and overlaid on TSA bottom agar plate. Phage Tan2 suspension was then serially diluted 10-fold (to  $10^{-3}$ ) with SM buffer and 100  $\mu$ l of each dilution was spotted onto the soft agar. After drying, the plate was incubated overnight at 37°C. Surviving colonies in the spotted area were randomly selected and the lysogenization of the phage Tan2 into the host RN4220 was confirmed by PCR amplification with the primer sets Tan2 F5-s/RN4220 Tan2-as (3'-end) and RN4220 Tan2-s/Tan2 F1-as (5'-end) that amplifies the integration site.

## **2.7 Construction of *S. aureus*-*E. coli* shuttle CRISPR-Cas13a vectors**

To generate the phagemid, we constructed shuttle vector pLK628\_*mecA*/ pLK628\_ null firstly, which was a CRISPR-Cas13a plasmid that are capable to work in both *S. aureus* and *E. coli*. The fragments of CRISPR-Cas13a\_*mecA*/CRISPR-Cas13a\_null, KmR (*aphA*-3) and ori of *E. coli* were amplified with oligo pKLC21-2-F/pKLC21-2-R from pKLC21\_null or pKLC21\_*mecA*, whereas the CpR(*cat*) and ori of *S. aureus* were amplified with pKLC14-K-F/pKLC14-K-R from pKLC14. The amplified fragments were then assembled by NEBuilder HiFi DNA Assembly. All vectors (plasmid/phagemid) constructed in this study were listed in Table 2. All oligonucleotides used for amplification and confirmation in this study were listed in Table 3.

## **2.8 Construction of phagemids**

To assemble the pLK12\_*mecA*/ pLK12\_null phagemids, the phage Tan2 packaging site, *rinA-rinB-terS-terL* regions were amplified with oligos phi1150-1-FW/phi1150-1-RV; the amplified fragments were inserted into pLK628\_*mecA*/pLK628\_null. To better understand the efficiency of phagemid capsid production and their relationship with packaging site as well the natural-phages generations, we constructed the series of pLK12 phagemids by

knocking out single/sets of the packaging site genes they carry. These phagemids were pLK12\_0 (pLK12::Empty), pLK12\_1 (pLK12::terS-terL-rinA-rinB), pLK12\_2 (pLK12::terS), pLK12\_5 (pLK12::terL-terS) and pLK12\_6 (pLK12::terL-terS-rinA).

## **2.9 Generation of Phagemid-based CapsidCas13a**

The Phagemid-based SA-CapsidCas13a(s) were generated basically using the phagemid packaging system. At first, the phagemid vector and control vector were individually transformed into Tan2 prophage-integrated host cell (*S. aureus* RN4220<sup>ΦTan2 WT</sup>) or mutant cell by electroporation using ELEPO21 electroporator. The resulting transformants were then recovered at a temperature (30°C) permissive for the replicating plasmid for 5 hours and plated on TSA plates supplemented with Cp. Transformants of each phagemid are validated by colony PCR. Then, PCR-confirmed colonies were isolated and further cultured at 37°C with shaking in TSB liquid medium containing Cp along with mitomycin C for induction of prophage excision and subsequent phagemid DNA packaging. The mitomycin C was added to a final concentration of 0.2% which allowed to reach its final concentration of 2 µg/ml when the bacteria culture reached to OD<sub>600</sub> 0.5. The cultures were incubated overnight at 30°C with shaking at 80 rpm. After incubation, the supernatant (lysates) was harvested and passed through a 0.22 µm filter. Finally, the resulting positive transformants were chemically induced by mitomycin C to generate SA-CapsidCas13a(s).

Start the electroporation with the following conditions (ELEPO21): Poring Pulse (voltage: 1,800 V, pulse length: 2.5 ms, pulse interval: 50 ms, number of pulses: 1, Polarity: +); Transfer Pulse (voltage: 100 V, pulse length: 99 ms, pulse interval: 50 ms, number of pulses: 5, polarity: +/-)<sup>47</sup>.

## 2.10 Infection assay

The target *S. aureus* strains, USA300<sup>48</sup> and USA300 $\Delta$ *mecA*, were grown to an OD<sub>600</sub> of approximately 0.5. Then, 110  $\mu$ l of the culture were mixed well with 110  $\mu$ l of SA-CapsidCas13a::CpR\_*mecA* or SA-CapsidCas13a::CpR\_Nontargeting; the mixture was incubated at 37°C for 20 min. Following the incubation, 200  $\mu$ l of the mixture was added to 4 mL of soft top agar (TSB solution supplemented with 0.75% agarose) pre-warmed at 55°C and poured onto an TSB plate containing Cp. The plates were cooled at room temperature to solidify the top agar and then incubated at 37°C overnight. Post overnight incubation, the plates were scrutinized for target bacterium killing.

## 2.11 Construction of phagemids with different origin of replication

We constructed phagemid that carries different origin of replication of *S. aureus*. pLK7-623(*repC* 187C-A) was generated by insertion of origin of replication from pT181 cop623, pLK7-608(*repC*  $\Delta$ 183-362) was generated by insertion of origin of replication from pT181 cop608, whereas pLK11-KAT(*repM*) was generated by insertion of origin of replication from pKAT. The backbones of these phagemids were similar to the pLK12\_1 (pLK12::terS-terL-rinA-rinB), except the origin of replication part, *repB*.

## 2.12 Measurement of copy number

The copy number of phagemids in the *S. aureus* RN4220 <sup>$\Phi$ Tan2 WT</sup> was measured by real-time PCR. Primer sets RN4220-RT-PCR-FW and RN4220-RT-PCR-RV, pLK12-RT-PCR-FW and pLK12-RT-PCR-RV were used and TB Green® Premix Ex Taq™ II were used for real-time PCR amplification by MIC qPCR. The copy number for every sample was calculated to per chromosome.

### 2.13 Measurement of phage/capsid titers

The lysates (Tan2 phage/Phagemid-based SA-Capsid) were serially 10-fold diluted with SM buffer ranging from  $10^{-1}$  to  $10^{-7}$ . Meanwhile, overnight culture of *S. aureus* strain RN4220 diluted 1: 100 with TSB broth was incubated with agitation at 37°C until an OD<sub>600</sub> of approximately 0.5. Then, 110  $\mu$ l of each dilution of Tan2 phage/phagemid-based SA-CapsidCas13a(s) was added to the same volume of bacterial suspension and the mixture was incubated at 37°C for 20 minutes. Whereafter, 100  $\mu$ l of the culture added to 4 mL a soft top agar (TSB solution with 0.75% agarose) pre-warmed at 55°C and poured onto a TSA plate, and Cp TSA plate, respectively. The solidified plates were incubated at 37°C overnight. The colonies grown on Cp plate were counted to calculate the transduced colony-forming units, TFU/ml; whereas the plaques formed on drug-free TSA plate were counted to calculate the plaque-forming units, PFU/ml.

### 2.14 Construction of *pac* deletion mutant library

Integrated prophage Tan2 with single *TerS* deletion, or combined deletion of *TerL/TerS*, *TerL/TerS/ RinA*, or *TerL/TerS/RinA/RinB* were generated through allelic exchange using *E. coli*/staphylococcal temperature-sensitive plasmid pIMAY<sup>49</sup>/pKOR1<sup>50</sup>. For the construction of  $\Delta TerS$  mutant, 500-1000 bp upstream and downstream flanking sequences of phiTan2 *TerS* were PCR-amplified with primer sets attB2-Tan2TerS-Up-F/ sacII-Tan2TerS-Up-R and sacII-Tan2TerS-Down-F/attB1-Tan2TerS-Down-R using KOD FX Neo. The 2 amplified fragments were digested with *sacII*, ligated using Ligation high ver. 2 and purified from agarose gel. Then, PCR amplification of pKOR1 with primer set pKOR1 7190-F4/pKOR1 4549-R and ligated products with primer set Lig-F3/Lig-R4 were performed

using KOD FX Neo. The 2 DNA fragments were ligated using In-Fusion HD Cloning Kit, generating pKOR1-KO-terS. For the construction of  $\Delta TerL/TerS$ ,  $\Delta TerL/TerS/RinA$ , and  $\Delta TerL/TerS/RinA/RinB$  mutant, phiTan2 *pac* together with its 500-1000bp upstream and downstream flanking sequences were amplified with 2 different primer sets: Tan2 23519-F2/Tan2 28540-R2 (for pKOR1) and Tan2 23519-F3/Tan2 28540-R3 (for pIMAY) using KOD FX Neo. pKOR1 and pIMAY were also PCR-amplified using the same polymerase with primer set pKOR1 7280-F5/pKOR1 4509-R2 and pIMAY 2858-F/pIMAY 2769-R, respectively. The 2 phiTan2 *pac* fragments were cloned onto their corresponding plasmids using In-Fusion HD Cloning Kit to first generate pKOR1\_Tan2*pac* and pIMAY\_Tan2*pac*. Following that, inverse PCR excluding *TerL/TerS*, *TerL/TerS/RinA*, and *TerL/TerS/RinA/RinB*, respectively, were performed and the amplified fragments were self-ligated using In-Fusion HD Cloning Kit to finally generate pIMAY\_ $\Delta TerL/TerS$ , pIMAY\_ $\Delta TerL/TerS/RinA$ , or pKOR1\_ $\Delta TerL/TerS/RinA/RinB$ . All the knockout plasmids were then transformed into *E. coli* DC10B and selected on LB agar supplemented with 10  $\mu$ g/ml Cp (for pIMAY-derived plasmids) and 100  $\mu$ g/ml ampicillin (for pKOR1-derived plasmids). The plasmids were then extracted and verified by Sanger sequencing. Following that, sequence-verified knockout plasmids were electroporated into RN4220 integrated with phiTan2 as previously described<sup>47</sup>, and the transformants were grown on TSA supplemented with 10  $\mu$ g/ml Cp (for both pKOR1- and pIMAY-derived plasmids) at either 28°C (for pIMAY-derived plasmids) or 30°C (for pKOR1-derived plasmids). Single crossover was performed by growing the cells on TSA supplemented with 10  $\mu$ g/ml Cp at non-permissive temperature of 37°C (for pIMAY-derived plasmids) or 43°C (for pKOR1-derived plasmids). Finally, double crossover was performed by growing the cells on TSA supplemented with 1  $\mu$ g/mL anhydrotetracycline at 28°C (for pIMAY-derived plasmids) or 30°C (for pKOR1-derived

plasmids). The construction of  $\Delta TerS$ ,  $\Delta TerL/TerS$ ,  $\Delta TerL/TerS/RinA$ , or  $\Delta TerL/TerS/RinA/RinB$  mutants were further confirmed by PCR and Sanger sequencing.

### **2.15 Insertion of TetM gene and codon optimized Cas13a**

The KmR for *E. coli* was deleted from pLK12\_1, and swapped with CpR gene, *cat* from the plasmid pIMAY, which works both in *E. coli* and *S. aureus*, to construct pLK14. Also, we inserted tetracycline resistant (*tetR*) gene, *tetM*<sup>51</sup>, to generate pLK16. The codon-optimized Cas13a (that is smaller in length than Cas of pLK12) system that suits *S. aureus* was inserted to pLK16, to generate pLK19\_null. This was done to enable *BsaI* restriction enzyme cut that would allow us to generate various gene-targeting phagemids by inserting 25 bp spacer sequence at the cut site based on the future requirement.

### **2.16 Spacer insertion for target killing assay**

First, the CRISPR-Cas13a system of phagemid targeting 10 drug-resistant genes, *aph*(2''), *aadD*, *aph*(3'), *aac*(6'), *ermB*, *fusC*, *mphC*, *mecA*, *msrA*, *tetK* and Non-T (Non-targeting, control), were constructed on the pLK19\_null vector as follows: ten types of 85-mer oligo DNAs made of 25-mer spacer sequence (crRNA) that corresponds to the nucleotide position of each target gene flanked with 2x30-mer overlaps to the vector were designed to target several whole-genome-sequenced strains available in-house at Jichi Medical University. After that, pLK19\_null was treated with restriction enzyme *BsaI*-HF, gel electrophoresis purified and subsequently ligated with the annealed oligo DNA using NEBuilder HiFi DNA Assembly to obtain pLK19\_Cas-X. After the assembly of the phagemids, SA-CapsidCas13a::CpR;TetR\_X was generated by electroporation-induction assays.

## **2.17 Specific killing assay**

### **(Spot test assay to analyze the specific killing of clinical isolates by CapsidCas13a)**

The *S. aureus* strains to be assayed were grown to an OD<sub>600</sub> of approximately 0.5. Then, 100  $\mu$ l of the culture were added to 4 ml a soft top agar and poured onto the TSB plate containing Cp. The plates were cooled at room temperature. In the meantime, Phagemid-based SA-CapsidCas13a with known titers were adjusted to 10<sup>6</sup> TFU/ml and were then 10-fold serial diluted. Finally, 2  $\mu$ l of each dilution were spotted onto the solidified bacterial overlayer and the plates were incubated at 37°C overnight.

**Table 1 List of Bacterial strains used in this study**

Bacterial strain	Description	Origin
RN4220	Laboratory strain of <i>S. aureus</i>	Kreiswirth BN et al., 1983 (a)
RN4220 <sup>Φ</sup> Tan2 wt	RN4220 integrated with phage Tan2 genome	in this study
USA300	Methicillin-resistant <i>S. aureus</i> (MRSA), genome sequence of FPR3757	Diep BA et al., 2006 (b)
USA300Δ <i>mecA</i>	<i>mecA</i> deletion mutant of USA300	in this study
JP2025	Clinical isolate, SaPIbov2+, SaPIbov2 bap::tet, constructed by standard allelic replacement the tetM marker into the bap gene of SaPIbov2	Ubeda C et al., 2003 (c)
DC10B	<i>E. coli</i> competent cell, dam mutation in the high-efficiency <i>E. coli</i> cloning strain	Monk et al., 2012 (d)
RN4220 <sup>Φ</sup> Tan2Δ <i>TenS</i>	<i>TenS</i> deletion mutant of RN4220 <sup>Φ</sup> Tan2 WT	in this study
RN4220 <sup>Φ</sup> Tan2Δ <i>TenL</i> - <i>TenS</i>	<i>TenL</i> , <i>TenS</i> deletion mutant of RN4220 <sup>Φ</sup> Tan2 WT	in this study
RN4220 <sup>Φ</sup> Tan2Δ <i>TenL</i> - <i>TenS</i> <i>RimA</i>	<i>TenL</i> , <i>TenS</i> , <i>RimA</i> deletion mutant of RN4220 <sup>Φ</sup> Tan2 WT	in this study
RN4220 <sup>Φ</sup> Tan2Δ <i>TenL</i> - <i>TenS</i> <i>RimA</i> <i>RimB</i>	<i>TenL</i> , <i>TenS</i> , <i>RimA</i> , <i>RimB</i> deletion mutant of RN4220 <sup>Φ</sup> Tan2 WT	in this study
JMUB 1278	<i>S. aureus</i> _MSSA, isolated in Jichi Medical University, that carries resistant genes <i>aph(2'')</i> , <i>aac(6)-aph(2'')</i> , <i>mecA</i>	in this study
JMUB 4958	<i>S. aureus</i> _MSSA, isolated in Jichi Medical University, that carries resistant gene <i>fusC</i>	in this study
JMUB 3007	<i>S. aureus</i> _MRSA, isolated in Jichi Medical University, that carries resistant genes <i>aadD</i> , <i>mecA</i>	in this study
JMUB 4975	<i>S. aureus</i> _MRSA, isolated in Jichi Medical University, that carries resistant genes <i>aph(3'')</i> , <i>ernB</i> , <i>mecA</i> , <i>tetK</i>	in this study

a. Casadaban M, et al., Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol. 138(2):179-207. (1980)

b. Diep B, et al., Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. Lancet 4:367(9512):731-9. (2006)

c. Ubeda C, et al., Stp, an integrase protein with excision, circularization and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity islands. Mol Microbiol. 49(1):193-210. (2003)

d. Monk JR et al., Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. mBio. 20:3(2):d00277-11. (2012)

Table 2 List of Plasmid used in this study

Table 2. List of Plasmid used in this study					
Vector	Purpose	Description	Origin	Derived in this study as:	
pRK1C14	Construction of plasmids/phagemids	CRISPR-Cas1a expression vector in <i>S. aureus</i> CXY and rep from pC194		N/A	
pRK1C21_mecA		CRISPR-Cas1a (target mecA) expression vector in <i>E. coli</i> that also carries ColE1 ori, KanR, IT ori	Kigen et al., 2020 (a)	N/A	
pRK1C21		CRISPR-Cas1a expression vector in <i>E. coli</i> that also carries ColE1 ori, KanR, IT ori	N/A	N/A	
pIMAY		<i>E. coli</i> lambda phage-coded temperature sensitive phagemid that also carries ori for <i>E. coli</i> p15A, <i>Phage-ori</i> , <i>ori</i> and <i>ori T</i> temperature sensitive replicon for Gram positive bacteria ( <i>ori</i> pBCK20 )	Montk et al., 2012(b)	N/A	
pT1181		<i>S. aureus</i> plasmid that carries ori(pBCK20) and TetR	Novick et al., 1982 (c)	N/A	
pKAT	Construction of Mutant RM22007-Tam2	<i>E. coli</i> lambda phage-coded plasmid that carries ColE1 ori, ori <i>S. aureus</i> pB1010 ( <i>ori</i> pB), <i>lacZ</i> Q1019, cat ( <i>E. coli</i> , <i>S. aureus</i> )	Kato 2004 (d)	N/A	
pIMAY		<i>E. coli</i> lambda phage-coded temperature sensitive phagemid that also carries ori for <i>E. coli</i> p15A, <i>Phage-ori</i> , <i>ori</i> and <i>ori T</i> temperature sensitive replicon for Gram positive bacteria ( <i>ori</i> pBCK20 )	Montk et al., 2012 (b)	N/A	
pKORI		Escherichia coli <i>S. aureus</i> shuttle vector, permits rapid cloning by lambda recombination and ceph selection	Bae et al., 2006 (e)	N/A	
pLK628_mecA		Phagemid, shuttle vector for CRISPR-Cas1a (target mecA) expression in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	N/A	
pLK628_null		Phagemid, shuttle vector for CRISPR-Cas1a expression in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	N/A	
pLK12_mecA	CRISPR-Cas1a expression vector (Phagemid)	Phagemid, shuttle vector for CRISPR-Cas1a (target mecA) expression in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR_mecA	
pLK12_null		Phagemid, shuttle vector for CRISPR-Cas1a expression in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR_montargeting	
pLK7_623		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	mpC_187_C-A	
pLK11_KAK		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	mpM	
pLK7_608		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	mpC_A183:362	
pLK12_1	Phagemids for evaluating packaging efficiency	Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	pLK12::Tet <sup>r</sup> -TerS RNA:RMB	
pLK12_2		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	pLK12::Tet <sup>r</sup> -TerS	
pLK12_5		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	pLK12::Tet <sup>r</sup> -TerS	
pLK12_6		Phagemid, shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	pLK12::Tet <sup>r</sup> -TerS RNA	
pLK12_0		Shuttle vector in <i>E. coli</i> and <i>S. aureus</i> that carries ColE1 ori, IT ori, KanR ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	pLK12::Empty	
pLK16	Construction of optimized phagemid	Phagemid that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	N/A	
pLK19_null		Phagemid, shuttle vector for CRISPR-Cas1a expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_montargeting	
pLK19_aph(2?)		Phagemid, shuttle vector for CRISPR-Cas1a (target aph(2?)) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_aph12?	
pLK19_auidD		Phagemid, shuttle vector for CRISPR-Cas1a (target auidD) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_aph13?	
pLK19_aph(3?)		Phagemid, shuttle vector for CRISPR-Cas1a (target aph(3?)) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_aph12?	
pLK19_aph(2?)		Phagemid, shuttle vector for CRISPR-Cas1a (target aph(2?)) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_aph2?	
pLK19_crmB		Phagemid, shuttle vector for CRISPR-Cas1a (target crmB) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_crmB	
pLK19_fusC		Phagemid, shuttle vector for CRISPR-Cas1a (target fusC) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_fusC	
pLK19_mplC		Phagemid, shuttle vector for CRISPR-Cas1a (target mplC) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_mplC	
pLK19_mecA		Phagemid, shuttle vector for CRISPR-Cas1a (target mecA) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_mecA	
pLK19_misA		Phagemid, shuttle vector for CRISPR-Cas1a (target misA) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_misA	
pLK19_loak		Phagemid, shuttle vector for CRISPR-Cas1a (target loak) expression in <i>S. aureus</i> that carries ColE1 ori ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), <i>ori</i> ( <i>E. coli</i> ), CXY ( <i>S. aureus</i> ), rep for <i>S. aureus</i> (pC194), <i>Phage</i> Tam2 packaging site, TetR, <i>TerS</i> RNA, <i>RhoB</i>	This study	SΔCapidCas1a::CPR::TetR_loak	
a. Kigen K. et al., Development of CRISPR-Cas1a-based antimicrobials capable of sequence-specific killing of target bacteria. Nat Commun. 11(1):2934, (2020)					
b. Montk. et al., Transforming the untransformable: application of direct transformation to manipulate genetically <i>Staphylococcus aureus</i> and <i>Staphylococcus epidermidis</i> . mBio. 20(3):e00277-11, (2012)					
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d. Kato, F., PhD. Thesis, University of Tohoku, Sendai, Japan. NDL.C11751-2004-P732 (2004). ( <a href="https://www.agri.tohoku.ac.jp/intercho/ool/shuttlevector.html">https://www.agri.tohoku.ac.jp/intercho/ool/shuttlevector.html</a> )					
e. Bae T. et al., Allelic replacement in <i>Staphylococcus aureus</i> with inducible counter-selection. Plasmid. 55(1):58-63, (2006)					

**Table 3 List of primers and oligo DNA sequence for crRNA used in this study**

Table 3. List of primers and oligo DNA sequence for crRNA used in this study		
Purpose	Name of the primer	Sequence (5'-3')
Construction of cas13a shuttle vector (pLK628)	pKLC21-2-R	ggatcgtgatggcgaggggatcaag
	pKLC21-2-F	cagctgcgaattccgacgtctaag
	pKLC14-K-F	tgccgcatcagatcCAAACGAAATTTGGATAAAGTGGG
Construction of cas13a phagemids (pLK12)	pKLC14-K-R	eggaattgcagctgCGTTTGTGAACTAATGGGTGCT
	phi1150-1-FW	tctgttcaatcgtatgcttagcaatattctgtgc
	phi1150-1-RV	aatttctgttggccagctctatcagaagtaagaagg
Construction of phagemids (pLK12_1, 2, 5, 6, 0)	pLK628-3-RV	catgattgaacagatggattgc
	pLK628-3-FW	cccaacgaaattggataaagtgg
	pLK12-1-RV	agccctgcaggtacagacgtcggaattgccagctg
	pLK12-1-FW	gcaattccgacgtctgatcctgcaggccctctcc
	pLK12-2-RV	aagaaggaattgagatgattgaacaaatgattgcac
	pLK12-2-FW	tcacacgctttacgcccaaacgaaattggataaagtgg
	terS-1-FW	tcctaatctctctctctgc
	terS-1-RV	cgtaaagcggtgtgatacagtg
	terS-1-RV	cgtaaagcggtgtgatacagtg
	pLK12-2-FW	tcacacgctttacgcccaaacgaaattggataaagtgg
	pLK12-ΔrinB-F	ccaatttctgttgggaattgtatgagggatcatgac
	pLK12-ΔrinB-R	tacctccatacaaatcccaaacgaaattggataaagtgg
Construction of pLK7-608, pLK7-623 (ori)	pLK12-4-RV	caatttctgttggcatgattgaacagatgattgcac
	pLK12-4-FW	tctgttcaatcgtcccaaacgaaattggataaagtgg
	pT181-ori-F	ctgccccttttagaattgtaaat
	pT181-ori-R	cagcaacagaacacaaagaatc
	pLK12-7-RV	atttaactattctaaacggcgaggttagtgac
	pLK12-7-FW	tgttctgttgcgtgatcctgcaggccctctcc
Construction of pLK11-KAT(ori)	V-608-R	tcgtattagacnaaaacgaaattgagattaaggag
	V-608-F	atctcaatttctttttgtctaatcagatcaagctc
	pKAT(repM)-F	CGTTGAAGGTGTGGTATGTATGTG
	pKAT(repM)-R	GTGTAAACCTTCCTCCAAATCAGAC
Measurement of copy number of phagemids (qPCR)	pLK12-8-RV	TACCAACACTTCAACGaaaggcgaggttagtgac
	pLK12-8-FW	GAGGAAGGTTTACAGatcctgcaggccctctcc
	pLK12-RT-PCR-FW	cccttaccattattacagcagg
	pLK12-RT-PCR-RV	aggcctatctgacaatttctga
Construction of Phagemids (pLK16)	RN4220-RT-PCR-FW	ATCAGATCTCTTTCGTCTCTCG
	RN4220-RT-PCR-RV	TCTTACCTCGTGCAACCAAG
	pLK14-1-R	gagaatccaagcactatcatgacc
	pLK14-1-F	tgcataattcagcgtgacctc
	Tan2-pLK14-1-FW	agtgcttgatctctatgcttagcaatattctgtgc
	Tan2-pLK14-1-RV	cgcaacataaaccagctcttatcagaagtaagaagg
	pLK14-2-F	ggtttgatgtgctgattaatgc
	pIMAY-2-R	GCGTGAATTATGCAACCGCTCTAGAACTAGTGGATC
	TetM SS2	atccactagtctcagcgcgaagcaacccaatctgc
	TetM LE	taataaatcaaaagtctttgatgcatlltataatcac
Oligo DNA sequence for crRNA in <i>S. aureus</i>	pIMAY-1-R	CCGCTCTAGAACTAGTGGATC
	Vector S	aagactttttgattattataaa
	Optimized-c2c2-mecA-F	aattgacgctattgggatG
	Optimized-C2c2-mecA-R	gccattggatCTCGAGAAG
	pLK12-c2c2-vector-R	caatagcgccttaattagacgtcggaattgcagctg
	pLK12-c2c2-vector-F(2)	CGAGatcccaatggcgatcctgcaggccctctcc
	aph(2'')	gaccacccaatcgaagggaactaaacATCCATACCATAGACTATCTCAAAAGCTTCTCGAGatcccaatggcgatcctg
	aadD	gaccacccaatcgaagggaactaaacAATCTAGTAGAATCTCTCGCTATCAAGCTTCTCGAGatcccaatggcgatcctg
	aph(3')	gaccacccaatcgaagggaactaaacCTTCCAGCCATAGCATATCTGCTTAAAGCTTCTCGAGatcccaatggcgatcctg
	aac(6)-aph(2'')	gaccacccaatcgaagggaactaaacCTATAAATTGATCCATACCATAGACAAGCTTCTCGAGatcccaatggcgatcctg
Constructions of RN4220Φ Tan2 wt	ermB	gaccacccaatcgaagggaactaaacGACTGTCTAATTCAATAGACGTTACAAGCTTCTCGAGatcccaatggcgatcctg
	fusC	gaccacccaatcgaagggaactaaacTATATACCTTCGAATTTATCATCTAAAGCTTCTCGAGatcccaatggcgatcctg
	nphC	gaccacccaatcgaagggaactaaacACGTCAGGCTCTCTTGGTAGTCTCAAAAGCTTCTCGAGatcccaatggcgatcctg
	mecA	gaccacccaatcgaagggaactaaacATGCTGTTCCTGTATTTGGCCAAATCAAGCTTCTCGAGatcccaatggcgatcctg
	msrA	gaccacccaatcgaagggaactaaacGTTCATCTAATAACTACCGCTTAAAGCTTCTCGAGatcccaatggcgatcctg
	tetK	gaccacccaatcgaagggaactaaacAGTTACTATTGTAATCATAGGAAGTAAGCTTCTCGAGatcccaatggcgatcctg
	Tan2 F5-s	AGACACACGCATACATCTTTCCT
	RN4220 Tan2-as	TCGGGGACGTTTCATGGATTTC
	RN4220 Tan2-s	TTCCGCTCTCGTCAACTATTGCT
	Tan2 F1-as	GCGCGTGTCAAAATACGTGTC
Constructions of mutant RN4220Φ Tan2 wt	attB2-Tan2TerS-Up-F	ggggacacattttgacaagaagctgggtTCAGGTCGTGCAGTATCGC
	sacII-Tan2TerS-Up-R	tcctccgggggaCACGACGCAATAACGGAAGTGAT
	sacII-Tan2TerS-Down-F	tcctccgggggaTTGTTTTCCTGATATCACAGCCT
	attB1-Tan2TerS-Down-R	ggggacacattttgacaagaagcagctATGATGTAGAGGCGCCGAGT
	pKOR1 7190-F4	TAATTTCGCTTTCGCTCACTG
	pKOR1 4549-R	GTTCCGAGGCTCAACGTCAA
	Lig-F3	gttgagcctcggaactTCAGGTCGTGCAGTATCGC
	Lig-R4	agcgcaacgcaattaATGATGTAGAGGCGCCGAGT
	Tan2 23519-F2	ttctcttttctgctACAACCTCAATATGCGCCACTC
	Tan2 28540-R2	gcccaatcagcaaacTCGACCAACTCATTGACGCA
	Tan2 23519-F3	aattctctgagcccgACAACCTCAATATGCGCCACTC
	Tan2 28540-R3	ccctgttgatacggTCGACCAACTCATTGACGCA
	pKOR1 7280-F5	GTTTTCGCTATTGGGCGCTCT
	pKOR1 4509-R2	AGCGAAAAGGAGAAGTCGGTTC
	pIMAY 2858-F	CCGGTATCAACAGGGACACC
	pIMAY 2769-R	CGGCTCGAGGAATTCGAT

Detection primers for PCR	Check -1-F	agtctcattcaggcgaccggac
	Check -2-R	tctcctcgctattgtaaccag
	Check -2-F	mtctcaagcacagacaggac
	Check -1-R	cgttaatgcgcctacag
	Check -8-F	TCAATATGTATGCTTTGGTCgg
	Check -8-R	ctacagcgtgagcattgaga
	Check -5-R	caccctacaaacggagagccta
	Check -5-F	gccctcatcaatctgactg
	Check -9-R	ctggcccttgagtgactga
	Check -10-F	cagataggcctaatactggtg
	Check -9-F	ctagcctcgcggcnaatag
	Check -15-F	ttgatatgaattctgcag
	Check -15-R	attcaggattattaaagagge
	Check -13-R	CCGTCTTAATTTTCGCTTCACC
	Check -13-F	gaccaccaataatgaagg
Sequence primer for plasmids	tan2-seq-1	ttatcagcgttaattgcacg
	tan2-seq-2	tccttcaacacacnaatgctg
	tan2-seq-3	ttatctctgttcactgtcac
	tan2-seq-4	ctcgtgtaggagctacgttc
	tan2-seq-5	tgatctgttggaatccatg
	pLK12-seq-4	ggttaacaggattagcagagc
	pLK12-seq-5	tcttcgtccagatcatctg
	pLK12-seq-1	aggaggcatatcaaatgaac
	pLK12-seq-2	cagtcggtttctaattgtcac
	pLK12-seq-3	cctgaataaactaatacctg
	pT181-Seq-1	ctggttaacattacgacca
	pT181-Seq-2	gcacacagtatgtgcgtcca
	pT181-Seq-3	tccttattggcgaacctac
	repM-seq-1	GTTTCGTTGGTTGTTTCTCAC
	SaPI-tet-F	cgaacactgacatgatattagtg
	Check -15-F	ttgatatgaattctgcag
	SaPItet-seq1	catcatagacacgccaggac
	SaPItet-seq2	aacacatcgaggtcgtctg
	SaPItet-seq3	gcaaganaagtatcatgtggag
	SaPItet-seq4	acgggcatataacatgctc
	pLK16-1-F	agctggcaattccagctct
	Opt Cas Seq2	GACGAAGCAATCCGTCAAGG
	Opt Cas Seq3	GAATTCTGGAACATAACGAAGCGT
	Opt Cas Seq4	TGGTGAACGAGCAGAAGGTT
	Opt Cas Seq5	GAAACGAGATTGACAGGGAACG
	Opt Cas Seq6	AAACGCGCAGATAAGTGCATC
	Opt Cas Seq7	CGTAACGAATGCATCAGGAGA
	Opt Cas Seq8	GGAGATATACTACCCAAAGGAGCGA
	Opt Cas Seq9	CACGATTTGACGAGACATGC
	Opt Cas Seq10	GTACGTACGCATCAGTTTTCGAAG

### 3. Results

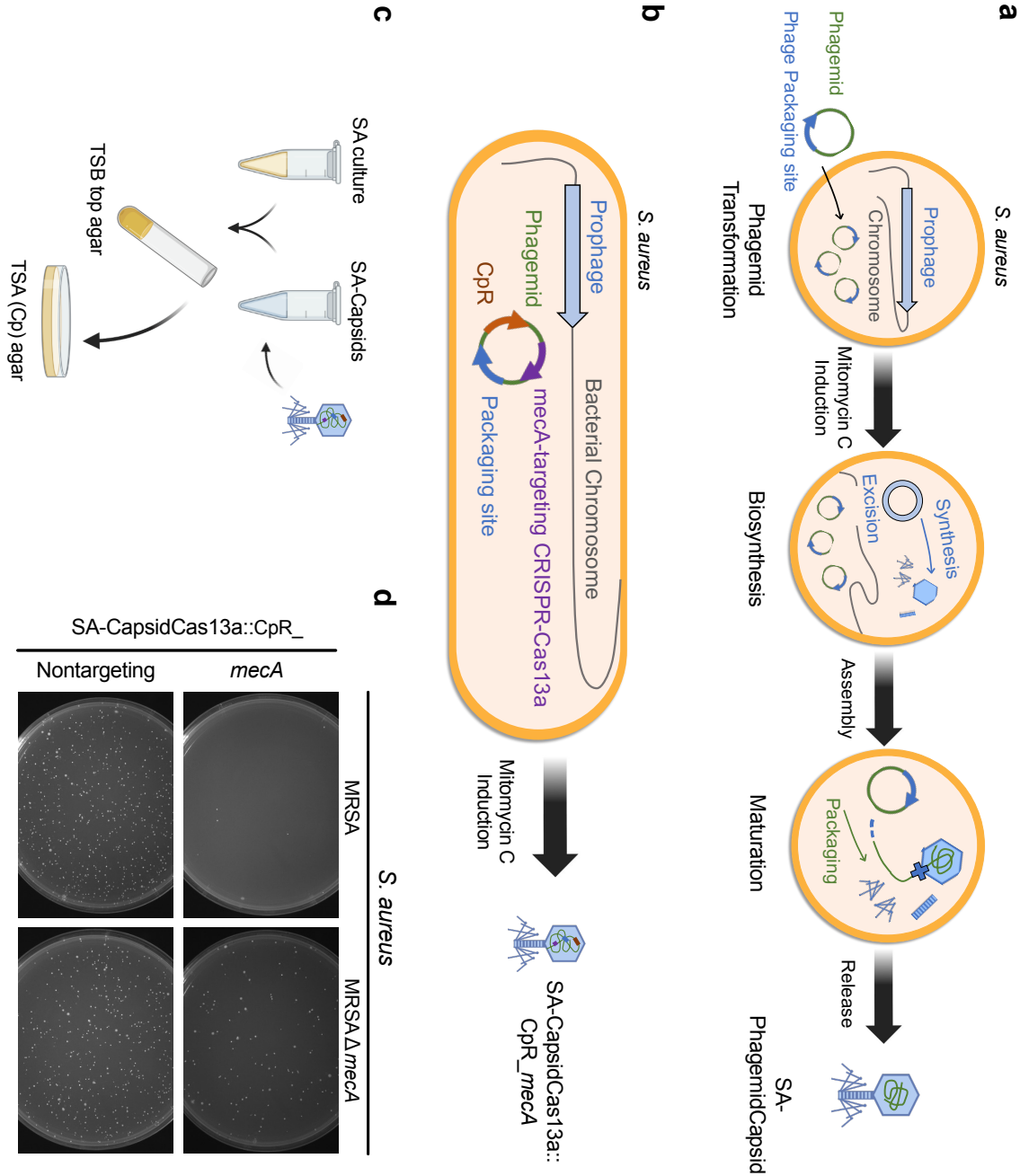
#### 3.1 Targeted Bactericidal Activity of Phagemid-based Capsid against MRSA

We utilized the capsid-packaging strategy via employing a phagemid (Fig. 1a) to prepare capsids that carry Cas13a. For this purpose, we constructed an *E. coli-S. aureus* shuttle phagemid vectors that constitute the sequence of the phage packaging sites along with LshCas13a; the phagemid was then transformed into the prophage-integrated *S. aureus* cells. The prophage-integrated *S. aureus* strain, RN4220, (RN4220<sup>Φ<sub>Tan2</sub> WT</sup>) carries the whole genome of Tan2 phage integrated into its chromosome. The phagemid when transformed into the RN4220<sup>Φ<sub>Tan2</sub> WT</sup>, would be packaged into the phage capsids due to the recognition of the phage packaging sites in their genome by taking over the capsids that are generated by excision of phage genome and translation of phage structural proteins upon mitomycin C induction of the host<sup>52</sup>, RN4220<sup>Φ<sub>Tan2</sub> WT</sup>. By this way, we successfully prepared Cas13a carrying capsids. These capsids can infect their target bacterial cells, similar to the parental phage leading to the transduction of its genetic material, herein phagemid, into the target cells. Next, we prepared methicillin resistance gene (*mecA*)\_targetable CRISPR-Cas13a system or nontargeting CRISPR-Cas13a system duly named as Phagemid-based SA-CapsidCas13a::CpR\_*mecA* (pLK12\_*mecA*), that carries a *mecA* targeting spacer and SA-CapsidCas13a::CpR\_nontargeting (pLK12\_null) as a non-targeting control. The chloramphenicol resistant (CpR) gene is used as the selection marker in this *E. coli-S. aureus* shuttle vector (Fig. 1b).

Post the construction of the phagemid system that can target *mecA*, we next examined the bactericidal ability of the pLK12\_*mecA* and pLK12\_null by utilizing two types of MRSA, one that carries *mecA* on the chromosome and other that is knocked out for *mecA*, MRSAΔ*mecA*. To test the killing effect of the pLK12\_*mecA* and pLK12\_null, an infection

assay was carried out (Fig. 1c). As expected, pLK12\_*mecA* decreased the number of bacteria by 2-3 orders of significance against the MRSA carrying *mecA*, whereas their respective non-targeting controls, pLK12\_null did not exhibit the inhibitory effect at all (Fig. 1d). On the contrary, the bacterial growth of the strain, MRSA $\Delta$ *mecA* did not show any inhibition by both the capsid types, targeting or non-targeting. These results agreed with the fact that the pLK12\_*mecA* transduced the bacteria, expressed the cargo, Cas13a, and subsequently mediated the sequence-specific killing which proved their ability as a targeted bactericidal system without inflicting any harm to cells that do not carry the target gene.

Fig.1



**Fig. 1. Phagemid-based generation of bactericidal capsids that carry *mecA* targeting CRISPR-Cas13a.**

a, Schematic representation of the generation of the phagemid-based SA-capsid. *S. aureus* cells whose chromosome carries an integrated prophage is transformed with the phagemid that carries the phage packaging site. Post transformation, mitomycin C induction of the transformed cells facilitates the excision of the phage genome and expression of phage structural proteins that assemble into capsids. These capsids then recognize the packaging signals that sit on the phagemid DNA and thereby package them yielding Phagemid-based SA-capsid.

b, The schematic representation of generation of bactericidal phagemid-based SA-Capsid Cas13a::CpR\_*mecA*. The phagemid that carries *mecA*-targeting spacer, CRISPR-Cas13a, the phage packaging site gene, and chloramphenicol resistant (CpR) gene were packaged into the capsids yielding phagemid-based SA-Capsid Cas13a::CpR\_*mecA*.

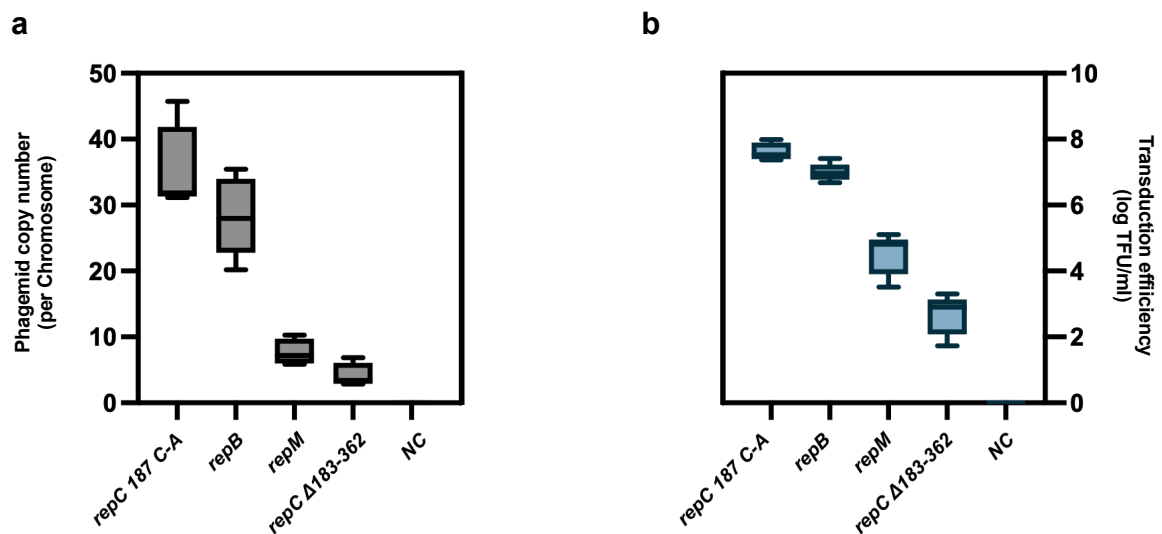
c, The experimental method is schematically depicted. The packaged capsid filtrate and the host *S. aureus* bacterial culture were mixed along with TSB soft top agar, poured on the top of chloramphenicol TSA plate, and incubated for 12 hours at 37°C to examine the transduction efficacy and sequence-specific bactericidal activity, if any.

d, MRSA or MRSA $\Delta$ *mecA* were then independently treated with *mecA*-targeting and non-targeting capsids. The *mecA*-targeting SA-CapsidCas13a selectively killed the target cells, MRSA that carries *mecA*.

### 3.2 The influence of phagemid's copy number on transduction efficacy (TFU)

After the confirmation of the targeted bactericidal activity of our capsids against MRSA, we conjecture whether the copy number of the phagemid would have any effect on packaging or transduction efficacy of capsid. In this respect, we constructed 3 new phagemids, pLK7-623, pLK11-KAT and pLK7-608 to be tested along with the previously constructed pLK12\_1. The ori of pLK7-623 and pLK7-608 were from pRN8061 and pRN8008 $\Delta$ 144 respectively<sup>53,54</sup>. These phagemids carry different the origin of replication, ori for *S. aureus* whereas same ori that works in *E. coli*; in addition, all these phagemids carried CpR marker and KmR marker for use in *S. aureus* and in *E. coli* respectively along with the phage Tan2's packaging site genes. The lengths of their genome were 7051 bp, 6412 bp, 6871 bp and 7161 bp, respectively. Post the construction of these phagemids using *E. coli* DC10B cells, we next measured the copy number of these phagemids per chromosome in the host cell RN4220 <sup>$\Phi$ Tan2 WT</sup>. All phagemids were represented by the ori name that they carry and the copy number of them ranged from 3 to 45 per chromosome (Fig. 2a). We performed infection test to measure the transduction/packaging efficacy of each of the different ori carrying phagemid-capsids; the results ranged from 1.73 to 7.99 log TFU/ml (Fig. 2, right panel). Plasmid copy-number/chromosome of *repC* 187 C-A (pLK7-623) was the highest in terms of both the copy number as well the packaging efficacy (Fig. 2). Conversely, the lowest copy number as well as the packaging efficacy was seen in *repC*  $\Delta$ 183-362 (pLK7-608) whereas no phagemids were accounted in the NC, which is the negative control strain that carries no phagemid in them when tested by qPCR.

**Fig. 2**



**Fig.2. The relationship between copy number of phagemid and transduction rate of phagemid (log TFU/ml)**

a, Optimization of the copy number of phagemid per chromosome based on different *ori*(n=3). The tested *S. aureus* *ori* were *repC* 187 C-A (pLK7-623), *repB* (pLK12\_1), *repM* (pLK11-KAT), *repC* Δ183-362 (pLK7-608) and NC indicate the negative control cells devoid of any phagemids, respectively.

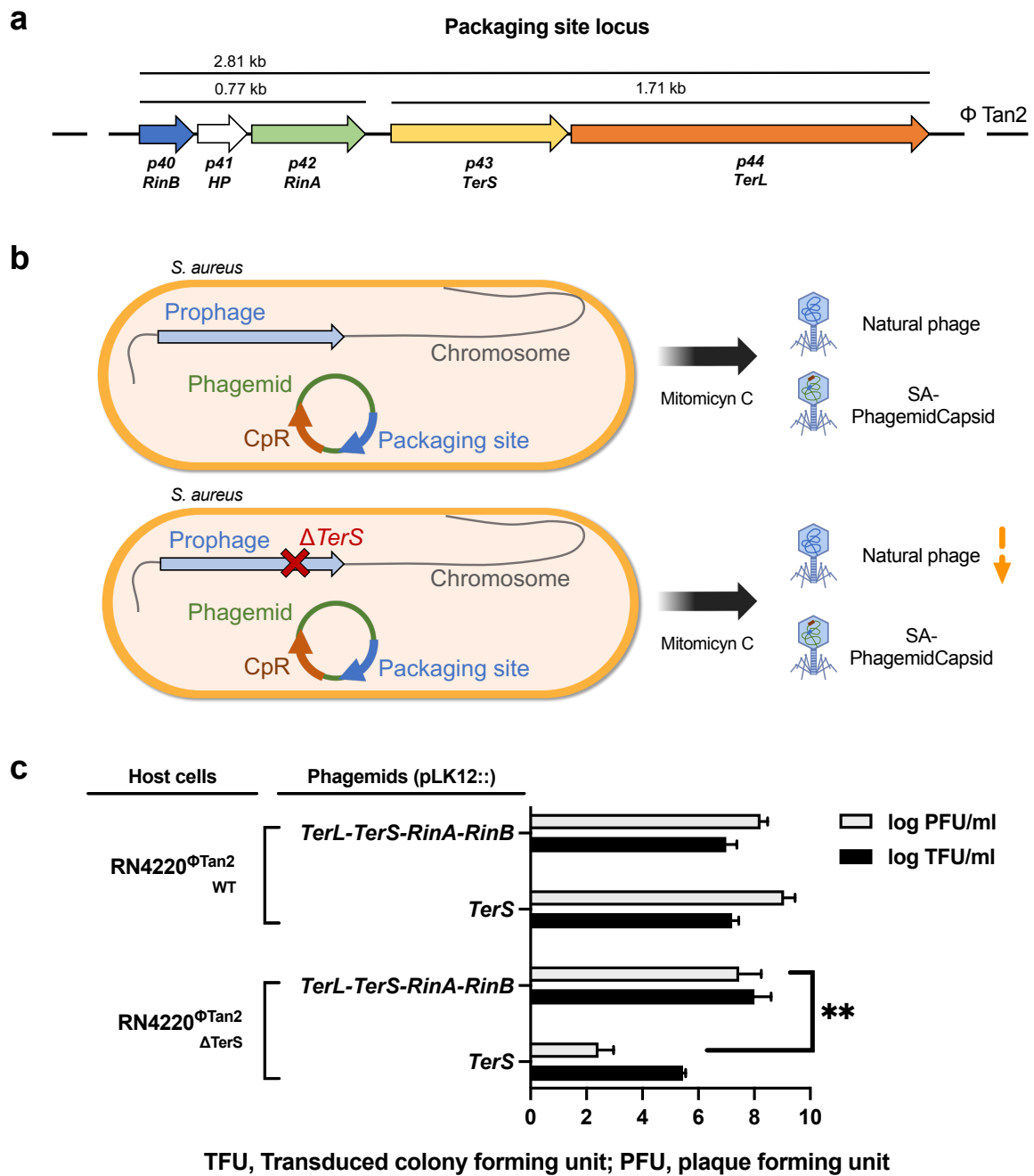
b, Exhibits the transduction rate of phagemid (log TFU/ml) vs different *ori* studied, which tested in Fig. 2a

### 3.3 The influence of *TerS*'s knockout on packaging efficacy

During the process of phagemid-capsid packaging, we did see the natural phage, Tan2 too getting packaged indicating contamination from the natural phage capsids. Therefore, next we investigated to decrease the contamination from the natural phage during the packaging of phagemid-capsids by knocking out hypothetically known packaging-correlated gene/genes from the Tan2 prophage in the host RN4220<sup>ΦTan2 WT</sup>, to understand whether these knockouts would block or decrease the self-packaging or contaminating wild-type capsids. The packaging-linked genes of Tan2 consist of *RinB*, *RinA*, *TerS* and *TerL* (Fig. 3a) which in total length is about 2.81 kb.

We first knocked out *TerS* gene from the host cell RN4220<sup>ΦTan2 WT</sup> to construct RN4220<sup>ΦTan2ΔTerS</sup>, expecting that this knock out will help in decreasing the contaminating natural Tan2 phage when the phagemid-transformed RN4220<sup>ΦTan2ΔTerS</sup> were induced (Fig. 3b). We transformed two different phagemids, pLK12::TerL-TerS-RinA-RinB (that carries the complete packaging site, length of 7161 bp) and pLK12::TerS (that carries only the *TerS*, length of 4967 bp) into the RN4220<sup>ΦTan2 WT</sup> or RN4220<sup>ΦTan2ΔTerS</sup>. Post induction, the lysates were used for infection assay against RN4220 to analyze the packaging efficacy (or transduction efficacy) by checking the transduced colony forming unit (TFU) of the phagemid and plaque forming unit (PFU) to determine the existence of contaminating natural phages (Fig. 3c) using Cp containing TSA plates. In RN4220<sup>ΦTan2ΔTerS</sup> host cells, the phagemid pLK12::TerS exhibited less plaques than that of the phagemid pLK12::TerL-TerS-RinA-RinB about 4 orders. However, this is accompanied by drop in the transduced colonies too, about 2 orders when compared with host cell RN4220<sup>ΦTan2 WT</sup>. However, the amount of contaminating Tan2 phage produced by phagemid pLK12::TerL-TerS-RinA-RinB was not changed, no matter what the host cell was.

**Fig. 3**



**Fig.3. Influence of *TerS* packaging site on TFUs and contaminating phage.**

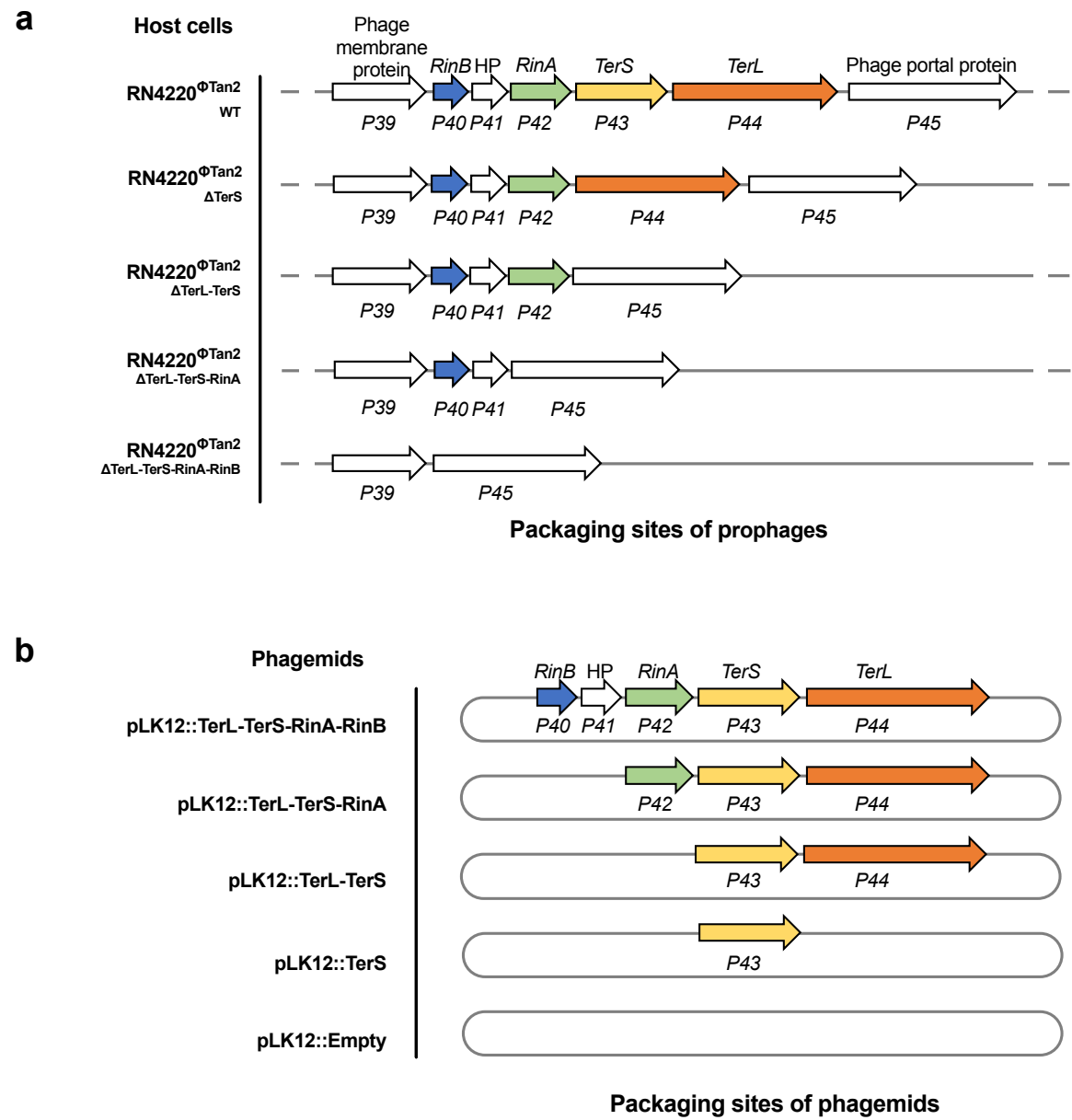
- a, The Tan2 phage map that exhibits the packaging related genes *RinB*, *RinA*, *TerS* and *TerL*.
- b, Schematic representation of how *TerS* knockout in integrated prophage in the host cells effect in reducing the natural contaminating phage capsids while packaging the phagemids.
- c, The quantification of TFUs and PFUs of the capsids packaged by the host cells, RN4220<sup>ΦTan2 WT</sup> and RN4220<sup>ΦTan2ΔTerS</sup> that were transformed with either of the two phagemids pLK12::TerS and pLK12::TerL-TerS-RinA-RinB(n=3). The bars in white color and black color represent log PFU/ml and log TFU/ml, respectively. p value is found to be 0.0062, calculated by t-test.

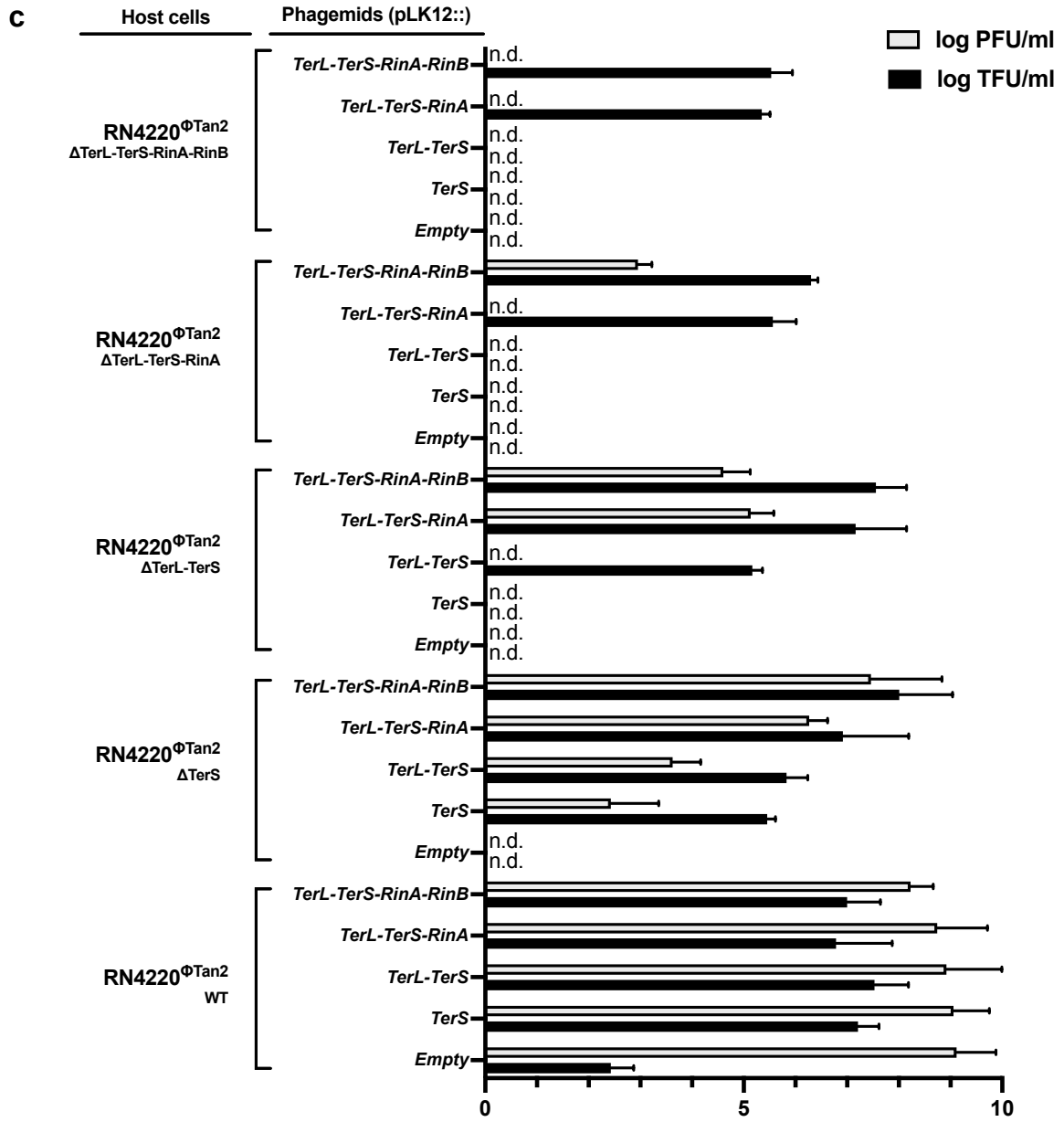
### 3.4 The influence of packaging site knockout on packaging efficacy

To understand more about the influence of packaging site knockouts on the packaging efficacy, subsequently, we knocked out different sets of packaging site genes sequentially, to construct three more different host cells RN4220<sup>ΦTan2ΔTerL-TerS</sup>, RN4220<sup>ΦTan2ΔTerL-TerS-RinA</sup>, RN4220<sup>ΦTan2ΔTerL-TerS-RinA-RinB</sup> in addition to RN4220<sup>ΦTan2 WT</sup>, RN4220<sup>ΦTan2ΔTerS</sup> (Fig. 4a). Simultaneously, we also generated four knock out phagemids: pLK12::TerL-TerS-RinA, pLK12::TerL-TerS, pLK12::TerS, pLK12::Empty (vector, without sharing packaging site) along with pLK12::TerL-TerS-RinA-RinB. All the phagemids carried specific ori for *S. aureus* and *E. coli* as well the specific resistant markers as aforementioned (Fig.4b). Post the construction of the different phagemids and host cells, we analyzed the TFU and PFU for every combination of host cells with the phagemids, 25 combinations in total.

The results showed that no plaques were formed in 4 following combinations: phagemid pLK12::TerL-TerS packaged in host cell RN4220<sup>ΦTan2ΔTerL-TerS</sup>, the phagemid pLK12::TerL-TerS-RinA packaged in host cell RN4220<sup>ΦTan2ΔTerL-TerS-RinA</sup>, and the two phagemids pLK12::TerL-TerS-RinA-RinB and pLK12::TerL-TerS-RinA packaged in host cell RN4220<sup>ΦTan2ΔTerL-TerS-RinA-RinB</sup> respectively (Fig. 4c). It is to be noted that these combinations also reported decreased TFU by about 2 orders when compared with the highest TFU seen in this assay. Also, we found that there was no plaque as well colony observed in some combinations that could be attributed to the deficient packaging genes in these combinations that are required for capsid's packaging. We managed to rule out the influence of the packaging site genes and succeeded in producing pure phagemid-loaded capsids without contaminating natural capsids.

Fig. 4





TFU, Transduced colony forming unit; PFU, plaque forming unit

**Fig.4. Removal of contaminating natural-phage during phagemid-capsid packaging.**

a, Schematic maps of wildtype or knockout in the packaging site genes of the prophages in the different constructed host cells.

b, Schematic maps of the packaging site genes retained on each constructed phagemids.

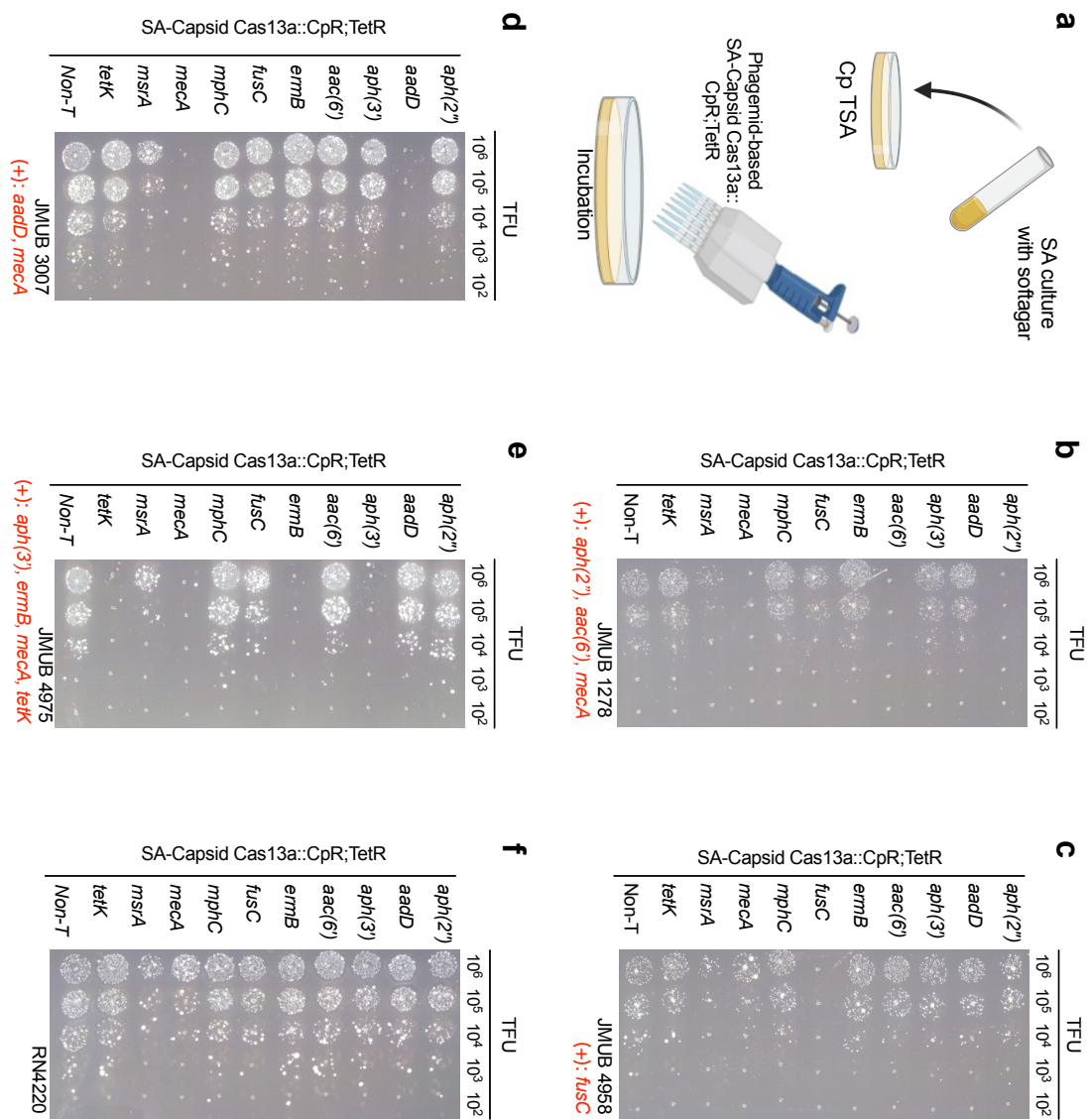
c, The PFU or TFU of SA-Capsid packaged by five phagemids pLK12::TerS, pLK12:: TerL-TerS, pLK12::TerL-TerS-RinA, pLK12::TerL-TerS-RinA-RinB and pLK12::Empty (negative control), whose packaging was compared by transforming them into five different host cells, they are RN4220<sup>ΦTan2 WT</sup>, RN4220<sup>ΦTan2ΔTerS</sup>, RN4220<sup>ΦTan2ΔTerL-TerS</sup>, RN4220<sup>ΦTan2ΔTerL-TerS-RinA</sup>, RN4220<sup>ΦTan2ΔTerL-TerS-RinA-RinB</sup>, yielding a total of twenty-five in combinations (n=3). The bars in white color and black color represent log PFU/ml and log TFU/ml, respectively.

### 3.5 Detection of various resistant genes of *S. aureus* using SA-CapsidCas13a

Finally, we wanted to test the bactericidal activity of phagemid-based SA-CapsidCas13a against other genes of *S. aureus* whereby this targeted bactericidal property against certain target genes can be visualized as resistant gene detection systems. For this purpose, we optimized pLK12\_1 by replacing the CpR and KmR markers from pLK12 by CpR from pIMAY that suitably works in both *S. aureus* and *E. coli*. In addition, we inserted tetracycline resistance (*TetM*) gene (TetR) from SaPIbov2 *bap::tet*<sup>51</sup>, as this could be possibly exploited for use in broader range of *S. aureus* clinical strains; the new phagemid that carries CpR from pIMAY and TetM from SaPIbov2 is named pLK19\_null. Next, we designed 10 different 25 bp spacer sequences that could specifically target each of the 10 different resistant genes in *S. aureus*, *aph*(2''), *aadD*, *aph*(3'), *aac*(6'), *ermB*, *fusC*, *mphC*, *mecA*, *msrA* and *tetK*. These spacers are then inserted into the phagemid, pLK19\_null yielding ten types of phagemid-based SA-CapsidCas13a::CpR;TetR; each capsid targets its own resistant gene in *S. aureus* whereas the Non-targeting capsid (Non-T) serves as negative control which do not carry any spacers. We next performed spot tests for testing the ability of sequence-specific targeted lysis of these capsids against five different *S. aureus* bacterial strains (three MRSA strains JMUB1278, JMUB4958, JMUB3007 and one MSSA JMUB4975, all of which were isolated from patients at Jichi Medical University, and RN4220 that serves as negative control) by spot assay. Comprehensive whole genome sequencing analysis of these bacterial strains detected the presence of variable resistance genes in their genome (Fig. 5a-5f). The strain JMUB 1278 carries *aph*(2''), *aac*(6') and *mecA* genes; strain JMUB4958 carries *fusC*; strain JMUB3007 carries *aadD*, and *mecA*; JMUB4975 carries *aph*(3'), *ermB*, *mecA*, and *tetK* whereas RN4220 carries none of the target gene. As expected, when the *S. aureus* clinical strains were infected with phagemid-based SA-CapsidCas13a, the growth of

only the bacteria that carried the target gene for which the spacer included in the capsids were inhibited; the growth of bacteria which were not targeted by capsid was not repressed. This showed the sequence-specific bactericidal activities of the phagemid-based SA-CapsidCas13a against various genes of *S. aureus* that could be widely applied to clinically relevant *S. aureus* gene detection or typing.

Fig. 5



**Fig.5. Methods to employ bactericidal capsid in detection of marker genes in clinical strains (sequence-specifically bacterial killing).**

a, Schematic depiction of the experimental method to detect the bactericidal activity of the capsids. Different clinical isolate strains of *S. aureus* were added to TSB top agar and poured onto the TSA-Cp plate respectively. Post the top agar solidifies, phagemid-based SA-CapsidCas13a:: CpR;TetR\_X (X stands for target genes) that is serial diluted tenfold were spotted on the different *S. aureus* clinical isolate bacterial lawns and incubated for 12 hours at 37°C to visualize the bactericidal activity.

b~e, Infection experiments were performed against clinical isolates of *S. aureus* JMUB1278, JMUB4958, JMUB3007, and JMUB4975 using antimicrobial capsids; The test results were evaluated by observing bacterial growth on TSB top agar plates supplemented with Cp. (+) indicates the presence of resistance genes in the clinical isolate bacteria. Each gene listed on the left of plate scans represents the presence of target spacers in the phagemid-based SA-CapsidCas13a:: CpR;TetR\_X against those genes. Non-T is the Non-targeting capsids that do not carry any spacers and serve as control.

f, Spot assay was performed against *S. aureus* lab strain RN4220 which does not carry any of the resistance genes that is targeted in this work. No growth arrested by antimicrobial capsid was observed in this strain.

#### 4. Discussion

Currently, phage therapy has been attempted and considered as one of the successful therapeutic options in treating infections with antimicrobial-resistant bacteria; many pre-clinical and clinical phage therapy studies were met with positive outcomes and so, has sparked worldwide interest in phage therapy<sup>55</sup>. Despite the growing attention, certain shortcoming of phage therapy remains till today and requires intervention. The primary concern is the biosafety issues associated with the use of phages<sup>56</sup>. Though phages cannot infect human cells, the ability of the phages to proliferate within their host, upon phage therapy poses an impending risk of unintentional gene transfer, as well as the emergence of new species due to the lateral spread of genes through phages between the colonized bacteria. Another limitation of the natural phage is its indiscriminate killing activity against its target bacterial strains that falls within its host range, thereby sterilizing all the bacteria within its host range, which is undesired effect<sup>57</sup>.

For these reasons, exploring the use of programmable non-replicative phages may be an ideal option. Previously, we reported the development and application of a SaPI-based novel phage-based antimicrobial agent that carried Cas13a<sup>28</sup>. However, SaPI system has many drawbacks; one of the main drawback is the designing constraints which require extensive and therefore time-consuming chromosomal gene editing, whereas the other is the limited production efficiency due to their one copy per chromosome occupancy.

In this study, we reported the phagemid-based method to deliver CRISPR-Cas13a by a cargo carrying phage capsid against *S. aureus*; this system is a programmable sequence-specific targeted bactericidal agent. We established a simple cargo packaging method that is accompanied by high efficacy and less-to-nil impurities from the natural phage capsids. We depicted the targeted antimicrobial application of our CapsidCas13a system not only against

at cellular level but also at gene level. We utilized an in-house isolated Staphylococcal phage called Tan2 for packaging purposes as this phage exhibited wide-host range against *S. aureus* (more than 99% of ~500 tested clinical strains were infected by Tan2 phage, that also comprises of considerable number of MRSA strains, Manuscript in preparation) including the antimicrobial resistant strains. Thereby, our developed capsids could have a wide range of applications against various *S. aureus* strains of clinical significance too. The developed phagemid system is optimized in detail for the design, programmable editing, flexibility, and production capability. We have attained high titers with the phagemid system out-performing our previous SaPI system. The most interesting part is that the capsid packaged by our phagemid is non-replicative that circumvents the safety concern with the phage therapy as well as are well-purified, making the analysis direct and accurate.

We utilized a phagemid system that target *mecA* and established the targeted bactericidal ability of the capsids against target MRSA that carries *mecA*, without harming the *mecA* knock-out MRSA strain. This study confirmed the ability of our phagemid-capsid system to transduce the bacteria within its host range, express the cargo and subsequently bring out sequence-specific killing. Although the copy number increased, yet not at a significant level, warranting more studies on this aspect (Fig. 2). We attribute that the not so significant increase in the capsid is due to the inherent limitation of the temperate phages. The burst size of the temperate phages from a single *S. aureus* bacterium is generally about 50 phages<sup>58</sup>, and we believe that the phagemid-derived capsids are also of copy number closer to that of this range. In addition, when the capsid is packaged with the phagemids, the *pac* sites in the prophage DNA are also recognized and packaged resulting in the production of wild type phage capsids along with the phagemid carrying capsids. We choose to knock out the *pac* site genes of the prophage, thus preventing the natural phage DNA from being recognized

and packaged, by that obtaining only the phagemid carrying pure capsids (Fig. 4c). The strategy is then expanded to closely related MRSA clinical strains to know the fine control effects of our phagemid capsids in terms of selective removal purely on their genetic sequence. Because the genomes of the MRSA clinical strains used in the study have identical cellular processes due to their sequence homology of at least 95%, selective removal of target strain based on their genetic composition is never easy. The 10 designed spacers that targets various resistant genes sitting throughout the MRSA genome exhibited targeted killing effective regardless of the gene location and or transcriptional activity (Fig. 5a-5f). For instance, targeting *mecA* killed JMUB1278, JMUB 3007 and JMUB 4975 but not JMUB 4958; targeting *fusC* killed only the JMUB 4958 but not the other strains, indicating genomic level precision targeting. This indicated that selective as well as quantitative removal of individual bacterial strains purely based on their sequence is feasible. Critically this observed programmable selective bactericidal effect is practically unfeasible with antibiotics or natural phage-based therapeutics. This antimicrobial system is undoubtedly best alternative therapeutics against the antibiotic-resistant pathogens. This genetic engineering strategy of manipulation of the phage, the host carrying prophage and of the phagemid system will be a handy, yet effectual Cas13a antimicrobial platform that is broadly expansible to MRSA or to any important pathogen. Although there are still many issues to be circumvented such as the phage resistance of bacterial cells, cross-contaminating impurities in the lysates, and concentrating method to enrich the titer, for which the studies are now underway.

In summary, we established a phagemid-based packaging system using a wide host-range phage that functions as a Cas13a delivery system to the target bacterial cells, MRSA. The method yielded high transduction efficiency by optimizing the copy number of the

phagemid; it was confirmed that the packaging efficiency positively correlated with the copy number of phagemid which is in turn dependent on the phagemid's origin of replication. Moreover, we cleared the contamination from natural phage capsids during the packaging step by knocking out the essential packaging genes from the integrated prophage in the host cells. The well-designed CapsidCas13a was able to sequence-specifically edit various genes at gene level and thus induce targeted killing against *S. aureus*. Our strategy could be extended for other varied applications, such as for clinical therapy against antibiotic resistant bacterial infections, for development of phage or capsid-based vaccines for antigen delivery, use in the establishment of the capsid library for the phage therapy cocktail, applicable for targeting toxin genes and for development of simple and cost-effective gene detection or SCCmec typing kit for bacterial diagnosis.

## **5. ACKNOWLEDGMENTS**

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