Egypt. J. Bot. **Vol. 63,** No. 3, pp. 1127- 1139 (2023)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Convergent Replication and Mobilization Mechanism of *Staphylococcus* Pathogenicity Islands (SaPIbov5) by Interfering with Bacteriophage ϕ12 Production Models

Thamer Bouback [1, 3], Faisal Al-Sarraj[1]#, Ibrahim Alotibi[2], Raed Albiheyri[1, 4], Majid Al-Zahrani [5], Mashail A. Alghamdi[1], Nada M. Nass[1, 6], Bayan H. Sajer[1], Atif A. Bamagoos[1], Sheren Azhari[1], Reem M. Farsi[1]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia; [2]Health Information Technology Department, Applied College, King Abdulaziz University, Jeddah, Saudi Arabia; [3]Princess Dr. Najla Bint Saud Al-Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, Jeddah, 21589, Saudi Arabia; [4]Centre of Excellence in Bio Nanoscience Research, King Abdulaziz University, Jeddah, Saudi Arabia; [5]Biological Science Department, College of Science and Art, King Abdulaziz University, Rabigh, Saudi Arabia; [6]Immunology Unit, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

STAPHYLOCOCCUS aureus can enter the bloodstream, leading to health complications such as sepsis, arthritis, endocarditis, and pneumonia. This study screened for the impact of six replication genes of cos phage 12 (ϕ12) on the evolution and transfer of cos *S. aureus* pathogenicity islands (SaPIbov5). An overnight culture of the *S. aureus* strain RN4220 diluted with fresh TSB was used in the bacteriophage ϕ12 titering assay. Phage-point mutagenesis was achieved using the pMAD vector, which facilitates homologous recombination in a two-step process. Finally, the transduction SaPI titering assays also utilized the *S. aureus* strain RN4220, which was then mixed with $CaCl_2$ and fresh TSB. The study showed that ORF11, ORF12, and VirE mutants did not lyse or produce phage particles after titering into the recipient RN4220 strains. While ORF26, ORF10, and ORF04 mutants generated detectable plaques, the mutations may have an effect on phage replication or packaging after tittering. For instance, complemented ϕ12 ORF26, ORF10, and ORF04 mutant strains had fully restored phage titers. The experiment found that cos phages facilitate inter and intra-generically transfer of cosSaPIs. It was also established that ϕ12 transduces SaPIbov5. However, ϕ12 mutants in VirE, ORF12, and ORF11 did not show SaPI mobilization because of their effects on infective phage particles and lack of replication. The complemented ϕ12 VirE, ORF12, and ORF11 mutants had partly restored phage titers when they were expressed in the recipient and donor strains. Mutations in ORF26, 0RF10, and ORFO4 decrease the ϕ12's ability to transfer SaPIbov5. Generally, this study found a new mechanism that facilitates the transfer of SaPI and cos genes. While the VirE, ORF12, and ORF11 affect packaging and are necessary for replication and phage biology

Keywords: Bacteriophage, Packaging, Pathogenicity islands, Phage ϕ12 replication, *Staphylococcus aureus,* Transduction.

Introduction

Staphylococcus aureus bacterium is spherical-shaped (0.5- 1.4µm) gram-positive cocci that grow in groups of irregular clusters of cells. *S. aureus* can grow either as a commensally bacterium or as an opportunistic pathogen. This bacterium can colonize different tissues, such as the nares, skin,

#Corresponding author email: falsaraj@kau.edu.sa
Received 12/05/2023; Accepted 01/08/2023
DOI: 10.21608/ejbo.2023.210573.2330
Edited by: Dr. Mahmoud S.M. Mohamed, Faculty of Science, Cairo University, Giza 12613, Egypt.
©2023 National Information and Documentation Center (NIDOC)

and mucous membranes of humans (Edwards et al., 2012; Rocha et al., 2013; Lekkerkerk et al., 2015; Chen et al., 2019).

Among the most dangerous community-isolated pathogens, *S. aureus* is found in healthcare settings worldwide. Most of the isolates' rapid emergence of antibiotic resistance, as well as a variety of cell-surface and secreted proteins, are credited with *S. aureus*' relevance (El-baz et al., 2017; Metwally et al., 2020). Genetic traits that mediate virulence, invasiveness, immune evasion, and drug resistance are primarily responsible for the pathogenicity of *S. aureus* (Ahmed et al., 2019; Metwally et al., 2020). *S. aureus* is a commensal bacterium that can colonize the human nose with 20-30% of the population being asymptomatic carriers (Holland et al., 2014; Lowder et al., 2009; Bonneaud et al., 2019). Bacteria can penetrate the bloodstream and cause organ infections, such as pneumonia, endocarditis, arthritis, osteomyelitis, and severe symptoms of sepsis (Gorwitz, et al., 2008; Nienaber et al., 2011; Tenover & Tickler, 2022). Additionally, *S. aureus* is responsible for toxin-mediated diseases such as food poisoning (Baba et al., 2002), toxic shock syndrome (Imanishi et al., 2019; Omar & Mohammed, 2021), and scalded skin syndrome (Kuroda et al., 2001; Yamaguchi et al., 2002).

Bacteriophages are viruses that infect bacterial cells. Phages exist in all environmental conditions in which bacteria can be detected, including soil ecosystems and marine, animal, and human microbiomes (Ashelford et al., 2003; Clokie et al., 2011; Mirzaei & Maurice, 2017). In seawater, the phage population is predicted to be two orders of magnitude higher than the bacterial population by a level of 2 orders of magnitude. Thus, phages are the most abundant biological entity on Earth, with an estimated 1031 phage particles (Perera et al., 2015). Phages encode proteins that shape bacterial host cells for their purpose (Rosenwasser et al., 2016). Phages also mediate the horizontal transfer of genetic elements (HGT) within bacterial populations (Braga et al., 2020).

Phages are key mobile genetic elements that contribute to the emergence of novel pathogenic bacterial strains through the transfer of virulence and fitness factors via HGT. The phenomenon of phage-mediated transfer of bacterial DNA is known as transduction and is crucial for bacterial biology, diversity, and evolution (Saeed et al., 2021).

S. aureus The pathogenicity islands (SaPIs) are distinct phage-inducibe high mobile genomic islands (GIs) of 12 to 18kb with characteristic life cycle. They play significant roles in the pathogenesis, evolution, and biology of organisms (Novick, 2019). SaPIs can interfere with temperate phage (helper phage) reproduction and transfer to a new host for promoting bacterial cell survival, similar to other page defense mechanisms (Hwang & Feiss, 1996; Watson et al., 2018; Rocha & Bikard, 2022; Rouss et et al., 2022). SaPIbov5 subset is a prototypical member of a family of cos-type SaPI prototypes. At least two major families have been identified: SaPI1 and SaPIbov5 (Quiles-Puchalt et al., 2014). They can be packaged by φ12 typical helper.

Tailed phages employ two different packaging systems to cleave and package concatemeric dsDNA (Rao & Feiss, 2008). Both the *pac* and *cos* models are used to describe the cleavage sites of DNA present in each type of phage genome and then continue with the translocation of the phage genome into the procapsid. These two characteristics are sequence-specific cleavage for the first cut and non-specific cleavage for the subsequent ones, and the increased packaging capacity allows *pac* phages to be involved in the transfer of bacterial genes by generalized transduction.

Indeed, bacteriophage ϕ12 is an interesting example to study bacteriophage replication. It encodes six genes with putative roles in replication, a higher number in comparison to other staphylococcal phages. Moreover, the disposition of these genes is not clustered but separated along the phage genome. As the functions of these genes have been attributed bioinformatically, we decided to study their role *in vivo* (Lopes et al., 2010). Thus, the aim of this is to focus on the characterization of the replication module of the *S. aureus* bacteriophage ϕ12. To study the role of these genes, individual mutants of phage ϕ12 on SaPIbov5 transfer.

Materials and Methods

Plasmid and Bacterial strains used in this study
The *S. aureus* strains and plasmids used in this study are presented in (Table 1). *S. aureus* strains were grown in Tryptic soy broth (TSB) and the appropriate antibiotic was used (chloramphenicol 20μg mL^{-1}, erythromycin 10μg mL^{-1}). *S. aureus* strain RN4220 is a nonlysogenic derivative

of a naturally occurring strain of S. aureus (NCTC8325), which expresses SaPIbov5 genomic elements, and is a vital intermediate for laboratory *S. aureus* manipulation, as it can accept plasmid DNA from *E. coli* (Novick, 1967; Kreiswirth et al., 1983). *S. aureus* inocula was grown overnight at 37°C for plasmid replication (Novick, 1991). The growth of *E. coli* strains and transformation of cloned constructs into the *E. coli* DH5α strain were performed using standard procedures. The plasmids obtained were transformed into *S. aureus* strains (Cucarella et al., 2001).

TABLE 1. Plasmid and Bacterial strains used in this study

Strain or plasmid	Relevant characteristic(s)
E. coli	Host for DNA cloning
DH5α	Competent *E. coli* cells, Host for DNA cloning
S. aureus RN4220	Is a mesophilic human pathogen derived from NCTC 8325-4, cloning intermediate, and used in virulence, resistance, and metabolic studies
pCN51	Staphylococcal shuttle cloning vectors, the cloning using pCN51 plasmid achieved under control of the Pcad promoter into *S. aureus* RN4220
pMAD	Vector, Antibiotic Resistance: Ampicillin. Length: 6430 bp, Staphylococcal shuttle vector
S. aureus RN4220	lysogenic for ϕ12
S. aureus RN4220	lysogenic for ϕ12 ΔORF04
S. aureus RN4220	lysogenic for ϕ12 ΔORF10
S. aureus RN4220	lysogenic for ϕ12 ΔORF11
S. aureus RN4220	lysogenic for ϕ12 ΔORF12
S. aureus RN4220	lysogenic for ϕ12 ΔORFVirE
S. aureus RN4220	lysogenic for ϕ12 ΔORF26

Phage propagation and infection

These experiments conducted by induction of *S. aureus* strains containing lysogenic ϕ12 bacteriophages of mitomycin C (MC, Sigma-Aldrich, Germany) to produce a bacteriophage lysate. The lysate generated by DNA damaging by MC that trigger the SOS response. A second technique used for obtaining bacteriophage lysates was ϕ12 bacteriophage infection of susceptible RN4220 *S. aureus* bacteria. Following this, ϕ12 bacteriophages enter the bacteria and progress through the lytic cycle, replicating and increasing the phage population until bacterial cell lysis occurs. The induction of the lysogenic RN4220 *S. aureus* strains was to investigate the effect of these mutations on the biology of the mutant phages. Briefly, bacteriophages infect the bacteria and progress until bacterial cell lysis, and the infection of investigated RN4220 *S. aureus* strains is performed, and analysis of phage lysates according to the (Novick, 1991) method with slight modifications. After lysis, un-lysed bacterial cells were removed by filtration with sterile filters of 0.2µm, and the resulting lysate was stored at 4°C.

Bacteriophage titering assay

The ϕ12 Bacteriophage titering assays were performed in *S. aureus* strain RN4220 as a receptor strain. To determine the phage titers, RN4220 cultures were diluted 1:50 with freshly TSB, and grown until OD540= 0.35. Serial dilutions of the phage lysate using phage buffer, 50µL of this culture were mixed with 100 µl of the serial phage dilutions and incubated at room temperature for 10 min. 3 ml of phage top-agar (PTA; 25g of Nutrient Broth No. 2, Oxoid; 7g Agar) supplemented with 10mM $CaCl_2$ (Kreiswirth et al., 1983). The number of formed plaques was counted and the plaque-forming units (PFU) were calculated as follows:

PFU/mL = Number of plaques /(d x v)

where, d= Dilution of plate counted, v= Volume of lysate dilution plated

To make the phage plaques more visible, 5mL of a 0.1% (w/v) solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) was added to the plates containing plaques and incubated at room temperature for 30min or until stained. The TTC dye is reduced to form red formazan by living bacteria, meaning that plaques remain unstained within a surrounding red background.

SaPI Transduction titering assay

The SaPIs used for this study contained *erm*C antibiotic resistance cassettes and were used as markers to select SaPI integration into the receptor cell on selective TSA plates (10 µg/ml tetracycline, chloramphenicol or erythromycin). Transduction tittering assays were also carried out using the *S. aureus* strain RN4220 as the receptor. The number of transductants in a lysate was quantified by a titering assay. To perform the transduction, assay an overnight culture was diluted 1:50 in fresh TSB and grown to OD_{540} 1.4 according to a previous methods (O'Neill et al., 2007; Penadés & Christie, 2015).

Phage Point mutagenesis using pMAD

The genes of interest were targeted for allelic exchange by homologous recombination using the pMAD vector. The pMAD vector allows homologous recombination in a two-step process; the initial integration of the whole vector, then the deletion of the vector backbone. The plasmid contains a beta-lactamase resistance cassette and two different origins of replication within Gram-negative and Gram-positive bacteria (Clokie et al., 2011). While the origin of replication for *E. coli* is stable at 37°C, pMAD vector contains a pE194 thermo sensitive origin of replication for Gram-positive bacteria.

Data analysis

Raw data was organised using Microsoft® Excel® 2010. SaPI titres were calculated respectively as PFU/mL or CFU/mL. GraphPad Prism® 6 (La Jolla) has been used to analyse the SaPI titres. All SaPI titre assays have been carried out in biological and technical triplicate. Results are shown as the mean.

The NCBI BLAST server program www.ncbi.nlm.nih.gov has been used to compare sequences with the GenBank database for homology (Quiles-Puchalt et al., 2014). Assembling sequences to a reference, multiple nucleotide or protein sequence alignments were performed using CLC Genomics Workbench 7.

Results

Identification of the putative replication proteins encoded in cos phage ϕ12

S. aureus bacteriophages encode the proteins needed to initiate replication of their genome. They also use part of the replication machinery of the host bacteria in the replication process. In the case of *S. aureus* bacteriophage ϕ12, our model to study *cos* phages has an interesting and uncharacterised replicating module. First, we identified *in silico* the six genes by using NCBI BLAST server program with putative domains involved in replication: ORF04, ORF10, ORF11, ORF12, VirE, and ORF26 (Fig. 1 and Table 2). The genes ORF10, ORF11, ORF12, VirE, and ORF26 are encoded in the intermediate transcript of the phage, but contrary to other replication modules, they are not clustered together, suggesting that maybe not all of them are involved in the same process (replication). In the case of the gene ORF04, it is encoded downstream of the repressor cI.

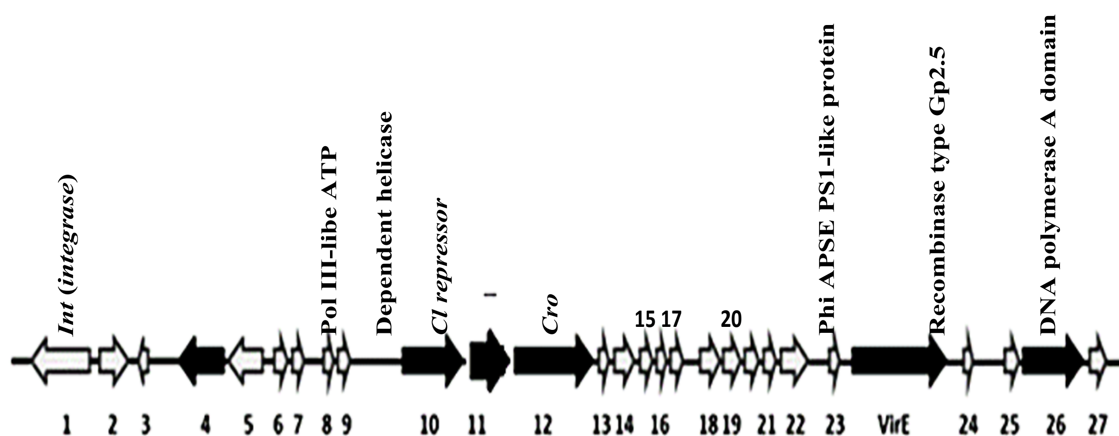

Fig. 1 . Map of bacteriophage ϕ12. Localization of the genes encoded between the integration and replication modules of bacteriophage ϕ12. Arrows indicate the predicted ORFs, and the number for each gene and the putative functions are also indicated, black arrow

TABLE 2. Description of ϕ12 ORF genes analyses in this study

ORFs	Description (including domains identified using BlastP or Pfam)
ORF04	DNA_pol_III_epsilon_like domain. This subfamily is composed of uncharacterised bacterial proteins with similarity to the epsilon subunit of DNA polymerase III (Pol III), a multisubunit polymerase that is the main DNA replicating enzyme in bacteria, functioning as the chromosomal replicase.
ORF10	Pfam10926, protein of unknown function (DUF2800). This is a family of uncharacterised proteins found in bacteria and viruses. Some members of this family are annotated as being Phi APSE P51-like proteins. CRISPR/Cas system-associated protein Cas4; CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and associated Cas proteins comprise a system for heritable host defense by prokaryotic cells against phage and other foreign DNA; Cas4 is RecB-like nuclease with three-cysteine C-terminal cluster.
ORF 11	DUF2815 (pfam10991); Recombinase type Gp2.5. This family of recombinases has single-stranded annealing activity *in vivo*.
ORF12	DNA_pol_A_pol_I_A domain. Polymerase I functions primarily to fill DNA gaps that arise during DNA repair, recombination and replication; Family A polymerase (polymerase I) functions primarily to fill DNA gaps that arise during DNA repair, recombination, and replication.
VirE	VirE, pfam05272; Virulence-associated protein E. This family contains several bacterial virulence-associated protein E-like proteins. These proteins contain a P-loop motif. COG5545; Predicted P-loop ATPase and inactivated derivatives [Mobilome: prophages, transposons]
ORF26	Helicase_C, pfam00271; Helicase conserved C-terminal domain; The Prosite family is restricted to DEAD/H helicases, whereas this domain family is found in a wide variety of helicases and helicase-related proteins. It may be that this is not an autonomously folding unit, but an integral part of the helicase. HepA. Superfamily II DNA or RNA helicase, SNF2 family [Transcription, Replication, recombination, and repair].

These genes were selected according to the putative conserved domains contained in the proteins encoded by these genes (Table 2). Some ORFS (ORF04 and ORF12) encode proteins with putative domains present exclusively in the host bacterial polymerase subunits such as DNA polymerase I (ORF12) and III (ORF04). ORF11 has been identified as a recombinase belonging to the third superfamily of recombinases, the type Gp2.5. This type of recombinase was initially thought to be present only in virulent phages, but recent studies suggest they are present in both virulent and temperate bacteriophages. In the case of ORF26, it encodes a DNA helicase domain, which may have a role during DNA replication, although this has not been tested *in vivo*. The gene VirE was not initially annotated in the genome of ϕ12, and although VirE has a putative conserved domain involved in bacterial virulence, it also carries a P-loop ATPase domain with an unknown role. In the case of ORF10, it has a conserved domain related to the CRISPR/Cas system-associated protein Cas4, which provides acquired resistance to mobile elements and targets invading nucleic acids to the bacterial host. Although this gene may not have a role in the replication process because of its putative conserved domain, it is interesting for this study as it is encoded in the intermediate transcript region of the phage and the same cluster as the replication genes, upstream of the ORFs 11 and 12.

All mutant clones were generated by using *E. coli* DH5α as the intermediate host strain and *S. aureus* RN4220 as the final recipient. All PCR products were cloned into pMAD (gene replacement) and pCN51 plasmid for complementation assay which has a cadmium-inducible promoter that can be induced with different concentrations of cadmium (0.1–2μM) using EcoRI and BamHI restriction sites and generating the resulting plasmids.

Effects of phage ϕ12 mutations on phage biology

By test the biological role of these six genes located in the replication module of phage ϕ12 by introducing a stop codon in the middle of their coding sequence (ochre mutation). Bacteriophage ϕ12 has a *cos*-type packaging system, meaning that the size of the genome of the phage has to be conserved to be packaged correctly. To generate a mutation that also conserves the length of the

phage we decided to introduce stop codons that will disrupt the coding region of the gene but preserve the size of the phage genome. After generating the mutants in a lysogenic strain, the SOS response was induced by the addition of mitomycin C and the lysates generated were tested to investigate the effect of these mutations on the biology of the mutant phages (Table 3).

TABLE 3. Effects of phage mutations on phage titer.

Donor strain	Phage titers
ϕ12	3.6 x 10^9
ϕ12 ΔORF04	6.8 x 10^7
ϕ12 ΔORF10	1.4 x 10^6*
ϕ12 ΔORF11	< 10*
ϕ12 ΔORF12	< 10*
ϕ12 ΔVirE	< 10*
ϕ12 ΔORF26	1.9 x 10^6*

*The means of results from three independent experiments are shown. Variation was within ±5% in all cases. Several phage particles, transductants, or transducing particles per milliliter of induced culture, using RN4220 as the recipient strain.

Different results were obtained for each mutant. The results showed that ϕ12ORF11, ORF12, and VirE mutants did not lyse nor produce phage particles after titering into the recipient *S. aureus* strain RN4220. These results suggested that these three proteins are essential for the phage cycle. Moreover, the ϕ12 ORF10 and ORF26mutants generate detectable plaques but these were smaller compared to the wt ϕ12 (Fig. 2). This indicates that these mutations may affect phage replication or packaging (Table 3).

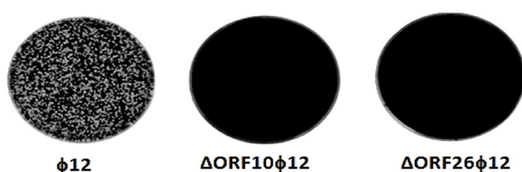

Fig. 2. Effect of ϕ12 mutations on plaque sizes. Approximately 108 CFU of the *S. aureus* RN4220 strain were infected with the indicated phage, plated on phage base agar, and incubated overnight at 37°C. 0.1% TTC was used to stain the plates for photography

Effect of ϕ12 mutations on plaque sizes.

Approximately 10^8 CFU of the *S. aureus* RN4220 strain were infected with the indicated phage, plated on phage base agar, and incubated overnight at 37°C. 0.1% TTC was used to stain the plates for photography. The mutant in ORF04 also has a reduction in the phage titer. Although these mutations are not deleterious for the phage, the fact that the titer is slightly reduced, as is the size of the plaques, indicates that they may have a function for the phage.

The previous results suggested that genes ORF11, ORF12, and VirE were essential for phage biology. To test which genes are involved in the replication process of the phage, we analyzed the replication of the different mutants under study using Southern blot analyses. As shown in (Fig. 3), the three genes that were severely affected in the titering assays, ORF11, ORF12, and VirE, are required for phage replication. The Southern blot analyses also showed that the replication of the mutants ORF10 and ORF26 was slightly reduced compared to the wt ϕ12 (Fig. 3).

Effect of complementation of the ϕ12 mutants

To complete our mutational analyses, we performed complementation experiments to confirm that the observed defects in phage replication were a consequence of the mutated genes. For this, we individually cloned the different genes under study under the control of a cadmium inducible promoter in the expression vector pCN51. These plasmids were introduced into the corresponding mutants, and the mutant phages were induced using MC. Phage titers were then determined using either recipient strain RN4220 carrying an empty pCN51 plasmid or RN4220 carrying the corresponding complementing pCN51 plasmid (Table 4).

The results showed that the complementation of the ORFs 11, 12, and VirE restored the ability of these phages to lyse and produce phage particles in the recipient strain compared with the non-complemented strains, supporting the idea that the ORFs 11, 12, and VirE are essential for phage replication. This complementation did not restore the ability of the phage as the titer of the complemented strains is lower than that observed in the wild type ϕ12. This can be explained by differences in protein expression levels by the plasmid or the fact that these proteins are not expressed in a time-appropriate manner in the phage life cycle. Furthermore, complementation of the ORFs 04, 10, and 26 restored the phage titer.

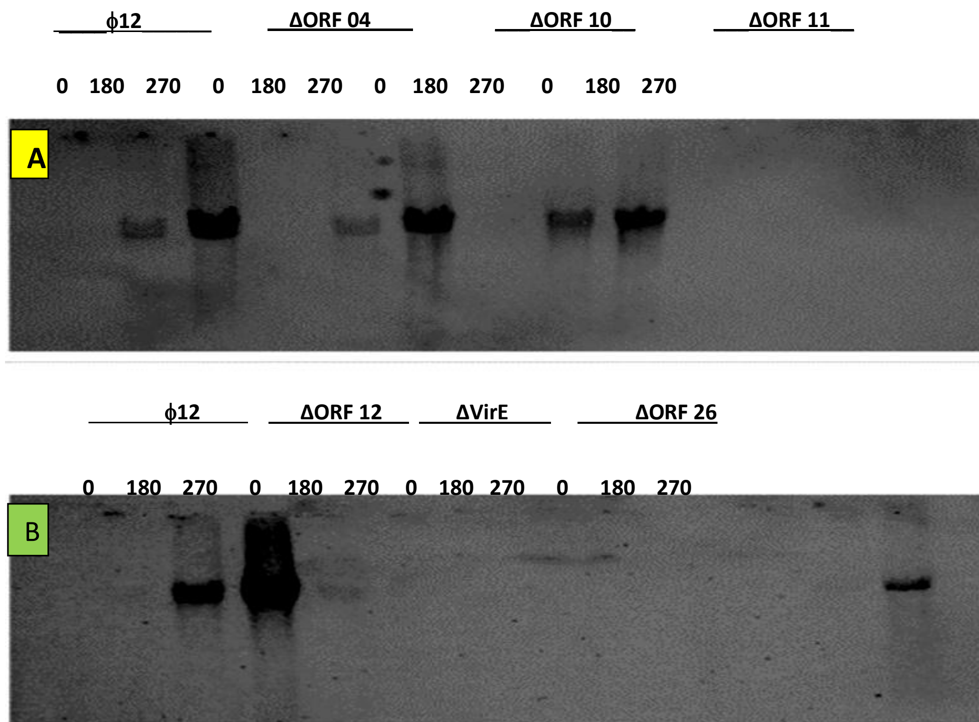

Fig. 3. Replication analyses of ϕ12 mutants. Southern blot from MC induced strains carrying the ϕ12 or the ϕ12 mutant in ORFs 04, 10, 11 (A), 12, VirE, and ORF26 (B). Samples were collected at 0, 180, or 270min after induction with MC, separated on agarose gel

TABLE 4. Effect of complementation on the phage titer for ϕ12 mutants

Donor strain		Receptor strain		
Phage	**Plasmid**	**RN4220**	**RN4220 pCN51 empty**	**RN4220 pCN51 ORF cloned**
ϕ12	pCN51 empty	3.6 x 10^9	3.2 x 10^9	-
ϕ12 ΔORF04	pCN51 empty	6.8 x 10^7	2.7 x 10^7	5.3 x 10^8
	pCN51-ORF04	2.1 x 10^8	4.3 x 10^8	1.5 x 10^9
ϕ12 ΔORF10	pCN51 empty	1.4 x 10^6	2.6 x 10^6	1.8 x 10^7*
	pCN51-ORF10	2.6 x 10^7	3.7 x 10^7	1.6 x 10^9
ϕ12 ΔORF11	pCN51 empty	< 10	< 10	< 10*
	pCN51-ORF11	<10	<10	3.2 x 10^5*
ϕ12 ΔORF12	pCN51 empty	< 10	< 10	< 10*
	pCN51-ORF12	<10	<10	2.3 x 10^5*
ϕ12 ΔVirE	pCN51 empty	< 10	< 10	< 10*
	pCN51-VirE	<10	<10	2.7 x 10^5*
ϕ12 ΔORF26	pCN51 empty	1.9 x 10^6	3.1 x 10^6	1.6 x 10^7*
	pCN51-ORF26	1.5 x 10^7	1.9 x 10^7	2.2 x 10^9

*The means of results from three independent experiments are shown. Variation was within ±5% in all cases. Several phage particles, transductants, or transducing particles per milliliter of induced culture, using RN4220 as the recipient strain.

Effects of phage mutations on SaPIbov5 transfer

The study demonstrated that *cos* phages are able to transfer *cos* SaPIs both intra- and inter-generically and that SaPIbov5 has been shown to be transduced by ϕ12 phages (Costa et al., 2013; Khasheii et al., 2021). To test if the ϕ12 mutants were still able to mobilise this SaPI, different mutant strains carrying SaPIbov5 with the tetracycline marker (*tet*M) were generated and SOS induced with MC. SaPIs and phage particles

are released from the bacteria after induction and potential recipient strains were exposed to the lysate, and then SaPI transduction was detected by tetracycline selection. First, we identified *in silico* six genes with putative domains involved in replication: ORF04, ORF10, ORF11, ORF12, VirE and ORF26 (Fig. 3 and Table 2). The genes ORF10, ORF11, ORF12, VirE and ORF26 are encoded in the intermediate transcript of the phage, but on the contrary to other replication modules, they are not clustered together, suggesting maybe not all of them are involved in the same process (replication). The results showed that the ϕ12 mutants in ORFs 04, 10 and 26 slightly reduced SaPIbov5 transfer, compared with wild type ϕ12. By contrast, mutations of ϕ12 ORFs 11, 12 and VirE completely eliminate SaPIbov5 transfer. Since these mutations completely eliminate phage replication, this explains why SaPIbov5 is not induced and transferred by these mutants (Table 5).

Discussion

Bacteriophages are predicted to contribute to the generation of novel bacterial lineages by spreading MGEs that carrying virulence gene, resistance cassettes, and other important key features. DNA replication at a specific time is a critical step during the phage life cycle. Which are phage-encoded proteins involved in this mechanism, since the replication of the staphylococcal *cos* phages has not been extensively studied, we decided to investigate the role of some genes located in the putative replication module of phage ϕ12. The study was focused on determining the effect of mutations in six putative replication proteins and their role in phage replication. Our mutational analyses showed that phage replication and concatemers DNA formation were affected by the mutations in the ORF11, ORF12, and VirE of ϕ12, which abolished phage replication and the formation of the infective phage particles. However, the mutations of ORF04, ORF10, and ORF26 had no significant effect on the formation of the phage particles, showing a slightly reduced phage titer compared to the phage wild type (Carpena et al., 2016). The gene ORF10 is classified as a CRISPR/Cas system-associated protein Cas4 which is associated with the Cas system of genetic host defence used by prokaryotic cells to limit phage infection and/pr integration of other foreign DNA. The gene ORF11 was previously identified as a recombinase that promotes single-strand annealing *in vivo* and belongs to the Gp2.5 family of recombinases, present in either virulent or temperate bacteriophages (Weigel & Seitz, 2006). Conversely, ORF12 belongs to the Family A polymerases (polymerase I) and can function as recombination, replication, and DNA repair polymerase by filling the DNA gaps that arise during DNA repair. Both mutants are encoded close to each other supporting the idea of a replication core inside the intermediate transcript. The other gene essential for the replication of the phage is *vir*E. This gene is located downstream of ORF12, separated by 4,239 bp and 11 genes. This pattern is very unusual compared to the other staphylococcal phage modules analyzed so far; a possible explanation for this is given below.

TABLE 5. Effects of phage mutations on SaPIbov5 transfer

Donor strain		Phage titer[a]	SaPIbov5 transfer[b]
ϕ12	SaPIbov5	3.6×10^9	2.6×10^5
ϕ12 ΔORF04	SaPIbov5	6.8×10^7	2×10^4
ϕ12 ΔORF10	SaPIbov5	1.4×10^6	2.4×10^4
ϕ12 ΔORF11	SaPIbov5	< 10	< 10*
ϕ12 ΔORF12	SaPIbov5	< 10	< 10*
ϕ12 ΔVirE	SaPIbov5	< 10	< 10*
ϕ12 ΔORF26	SaPIbov5	1.9×10^6	1.7×10^4

*The means of results from three independent experiments are shown. Variation was within ±5% in all cases. Several phage particles, transductants, or transducing particles per milliliter of induced culture, using RN4220 as the recipient strain.

In summary, in this study we have revealed the existence of novel mechanisms involved in the transfer of chromosomal genes: *cos* and SaPI LT. In addition, we also reported the existence of a novel phage-replicating module. Unfortunately, in some cases we could not decipher completely the exact mechanism controlling the process under study. However, we anticipate that the results of this study will be important as they propose the existence of unrecognized mechanisms of gene transfer, which are important for the emergence of novel bacterial virulent clones. Furthermore, it was described in a previous study that the *cos* phage ϕ12 was able to induce and mobilize SaPIbov5. For that reason, in this study we examined the effect of the phage mutations on the transfer of this pathogenicity island. The results showed that mutations in ORF04, ORF10 and ORF26 slightly reduced the ability of ϕ12 to transfer SaPIbov5. However, the ϕ12 mutants in ORF11, ORF12 and VirE showed no transfer of the SaPI due to the impact that these mutations have on the phage replication and on the formation of infective phage particles.

Realized that, by utilizing with high frequency transfer capabilities and excellent packing of antistaphylococcal SaPIs therapeutic islands', we might design a delivery method that would be effective in counter the problem of antibiotic resistance. Antimicrobial resistance genes being transferred by phages is still up for debate (Modi et al., 2013; Colavecchio et al., 2017; Enault et al., 2017; Calero-Ca´ceres et al., 2019), highlighting the need for additional research and improved comprehension of phage-mediated gene transfer, as well as the necessity to create more dependable methods to analyze viral metagenomes.

As a result of the growing issue of *S. aureus* antibiotic resistance around the globe, phage treatment seems to be an effective and safe therapy for fighting bacterial resistance. First and foremost, there is a lack of research that would thoroughly analyze the safety and effectiveness of phage treatment. A further limitation of the current literature is the absence of an established and regulated protocol for phage extraction and purification that leads to differences in the outcomes of the different studies reported. In conclusion, bacteriophages ϕ12 treatment can be an effective and safe alternative therapy against *S. aureus* RN4220 throughout decrease the transferormation of SaPIbov5 p Pathogenicity Island. Further research to characterize the host immune response dynamics upon treatment is required to assure that phage therapy is successful.

Conclusion

In conclusion, bacteriophages ϕ12 treatment can be an effective and safe alternative therapy against *S. aureus* RN4220 throughout decrease the transferormation of SaPIbov5 p Pathogenicity Island. Further research to characterize the host immune response dynamics upon treatment is required to assure that phage therapy is successful.

Funding: The authors received no extramural funding for the study.

Data availability statement: Raw data were generated at Biological Department in King Abdulaziz University. Derived data supporting the findings of this study are available from the corresponding author on request. The data that support the findings of this study are available on request from the corresponding author.

Acknowledgments: I would like to thank the staff of the Microbiology lab in Biology Dep. At King Abdul-Aziz University for your help in collecting data

Conflict of interest: All authors declare no conflict of interest.

Authors' contributions: All the authors contributed to the study's conception and design. Thamer Bouback, Faisal Al-Sarraj, and Ibrahim Alotibi were Identify ultimate objective of study and designed the required analysis and bio-assays. Majed Al-Zahrani, Raed Albiheyri, Mashail A. Alghamdi were involved in funding acquisition, prepare the chemical and performed the practical part of this work. Nada Nass, Raed Albiheyri, and sheren Azhari and Reem M. Farsi were participate in study analysis, obtained raw data collection, supervision, and perform the statistical analysis. Faisal Al-Sarraj, Atif Bamagoos and Bayan H. Sajer were shared in writing the manuscript body, create a strong conclusion and language editing.

Ethics approval: Not applicable.

References

Ahmed, S.H., Tolba, S.T.M., El-Zawahry, Y.A. (2019) Evaluation of the role of *bla* genes in beta lactam and methicillin resistant *Staphylococcus aureus*. *Egyptian Journal of Botany*, **59**(1), 29-38.

Ashelford, K.E., Day, M.J., Fry, J.C. (2003) Elevated abundance of bacteriophage infecting bacteria in soil. *Applied and Environmental Microbiology*, **69**(1), 285-289.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K.-i., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T. (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *The Lancet*, **359**(9320), 1819-1827.

Bonneaud, C., Weinert, L.A., Kuijper, B. (2019) Understanding the emergence of bacterial pathogens in novel hosts. *Philosophical Transactions of the Royal Society* B, **374**(1782), 20180328.

Braga, L.P., Spor, A., Kot, W., Breuil, M.-C., Hansen, L.H., Setubal, J.C., Philippot, L. (2020) Impact of phages on soil bacterial communities and nitrogen availability under different assembly scenarios. *Microbiome*, **8**(1), 1-14.

Calero-Ca´ceres, W., Ye, M., Balca´zar, J.L. (2019) Bacteriophages as environmental reservoirs of antibiotic resistance. *Trends in Microbiology*, **27**, 570–7.

Carpena, N., Manning, K.A., Dokland, T., Marina, A., Penadés, J.R. (2016) Convergent evolution of pathogenicity islands in helper cos phage interference. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **371**(1707), 20150505.

Chen, C.-H., Kuo, K.-C., Hwang, K.-P., Lin, T.-Y., Huang, Y.-C. (2019) Risk factors for and molecular characteristics of methicillin-resistant *Staphylococcus aureus* nasal colonization among healthy children in southern Taiwan, 2005–2010. *Journal of Microbiology, Immunology and Infection*, **52**(6), 929-936.

Colavecchio, A., Cadieux, B., Lo, A. Goodridge, L.D. (2017) Bacteriophages contribute to the spread of antibiotic resistance genes among foodborne pathogens of the Enterobacteriaceae family—a review. *Frontiers in Microbiology*, **8**, 1108.

Costa, S.S., Viveiros, M., Amaral, L., Couto, I. (2013) Multidrug efflux pumps in *Staphylococcus aureus*: An Update. *The Open Microbiology Journal*, **7**, 59–71 0.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I.N.I., Penadés, J.R., Bap, A. (2001) *Staphylococcus aureus* surface protein involved in biofilm formation. *Journal of Bacteriology*, **183**(9), 2888-2896.

Edwards, A.M., Massey, R.C., Clarke, S. (2012) Molecular mechanisms of *Staphylococcus aureus* nasopharyngeal colonization. *Molecular oral Microbiology*, **27**(1), 1-10.

El-baz, R., Rizk, D.E., Barwa, R., Hassan, R. (2017) Virulence characteristics and molecular relatedness of methicillin resistant *Staphylococcus aureus* harboring different staphylococcal cassette chromosome mec. *Microbial Pathogenesis*, **113**, 385-395.

Enault, F., Briet, A., Bouteille, L. Roux, S., Sullivan, M., Petit, M.-A. (2017) Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME Journal*, **11**, 237–47.

Gorwitz, R.J., Kruszon-Moran, D., McAllister, S.K., McQuillan, G., McDougal, L.K., Fosheim, G.E., Jensen, B.J., Killgore, G., Tenover, F.C., Kuehnert, M.J. (2008) Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001–2004. *The Journal of Infectious Diseases*, **197**(9), 1226-1234.

Holland, T.L., Arnold, C., Fowler, V.G. (2014) Clinical management of *Staphylococcus aureus* bacteremia: a review. *JAMA*, **312**(13), 1330-1341.

Hwang, Y., Feiss, M. (1996) Mutations affecting the high affinity ATPase center of gpA, the large subunit of bacteriophage λ terminase, inactivate the endonuclease activity of terminase. *Journal of Molecular Biology*, **261**(4), 524-535.

Imanishi, I., Nicolas, A., Caetano, A.-C. B., Castro, T.L., Tartaglia, N.R., Mariutti, R., Guédon, E., Even, S., Berkova, N., Arni, R.K. (2019) Exfoliative toxin E, a new Staphylococcus aureus virulence factor with host-specific activity. *Scientific Reports*, **9**(1), 1-12.

Behnoush, K., Mahmoodi, P., Mohammad Zadeh, A. (2021) Siderophores: Importance in bacterial pathogenesis and applications in medicine and industry. *Microbiological Research*, **250**, 126790.

Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O'Reilly, M., Schlievert, P.M., Bergdoll, M.S., Novick, R.P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature*, **305,** 709-712.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K.-I., Nagai, Y. (2001) Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *The Lancet*, **357**(9264), 1225-1240.

Lekkerkerk, W., Van Wamel, W., Snijders, S., Willems, R., van Duijkeren, E., Broens, E., Wagenaar, J., Lindsay, J., Vos, M. (2015) What is the origin of livestock-associated methicillin-resistant Staphylococcus aureus clonal complex 398 isolates from humans without livestock contact. An epidemiological and genetic analysis. *Journal of Clinical Microbiology*, **53**(6), 1836-1841.

Lopes, A., Amarir-Bouhram, J., Faure, G., Petit, M.-A., Guerois, R. (2010) Detection of novel recombinases in bacteriophage genomes unveils Rad52, Rad51 and Gp2. 5 remote homologs. *Nucleic Acids Research*, **38**(12), 3952-3962.

Lowder, B.V., Guinane, C.M., Zakour, N.L.B., Weinert, L.A., Conway-Morris, A., Cartwright, R.A., Simpson, A.J., Rambaut, A., Nübel, U., Fitzgerald, J.R. (2009) Recent human-to-poultry host jump, adaptation, and pandemic spread of Staphylococcus aureus. *Proceedings of the National Academy of Sciences*, **106**(46), 19545-19550.

Metwally, M.A., Ali, S.S., Khatab, I.A., El-Sayed, M.K. (2020) Antibacterial potential of some seaweeds species to combat biofilm-producing multi-drug resistant *Staphylococcus aureus* of *Nile tilapia*. *Egyptian Journal of Botany,* **60**(1), 9-24.

Mirzaei, M.K., Maurice, C.F. (2017) Ménage à trois in the human gut: interactions between host, bacteria and phages. *Nature Reviews Microbiology*, **15**(7), 397-408.

Modi, S.R., Lee, H.H., Spina, C.S. Collins, J.J. (2013) Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature*, **499**, 219–22.

Nienaber, J.J., Sharma Kuinkel, B.K., Clarke-Pearson, M., Lamlertthon, S., Park, L., Rude, T.H., Barriere, S., Woods, C.W., Chu, V.H., Marín, M. (2011) Methicillin-susceptible *Staphylococcus aureus* endocarditis isolates are associated with clonal complex 30 genotype and a distinct repertoire of enterotoxins and adhesins. *Journal of Infectious Diseases*, **204**(5), 704-713.

Novick, R. (1967) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology*, **33**, 155166-.

Novick, R.P. (1991) Genetic systems in staphylococci. *Methods in Enzymology*, **204**, 587636-.

Novick, R.P. (2019) Pathogenicity islands and their role in Staphylococcal biology. *Microbiolgy Spectrum*, **7**(3), https://doi.org/10.1128/microbiolspec.gpp3-0062-2019

Omar, N.N. Mohammed, R.K. (2021) A molecular study of toxic shock syndrome toxin gene (tsst-1) in β-lactam resistant Staphylococcus aureus clinical isolates. *Iraqi Journal of Science*, **62**(3), 825-837.

O'Neill, A.J., Larsen, A.R., Skov, R., Henriksen, A.S., Chopra, I. (2007) Characterization of the epidemic European fusidic acid-resistant impetigo clone of *Staphylococcus aureus*. *Journal of Clinical Microbiology,* **45**, 1505–1510.

Penadés, J.R., Christie, G.E. (2015) The phage-inducible chromosomal islands: a family of highly evolved molecular parasites. *Annual Review of Virology,* **2**, 181–201.

Perera, M.N., Abuladze, T., Li, M., Woolston, J., Sulakvelidze, A. (2015) Bacteriophage cocktail significantly reduces or eliminates Listeria monocytogenes contamination on lettuce, apples, cheese, smoked salmon and frozen foods. *Food Microbiology*, **52**, 42-48.

Quiles-Puchalt, N., Carpena, N., Alonso, J.C., Novick, R.P., Marina, A., Penadés, J.R. (2014) Staphylococcal pathogenicity island DNA packaging system involving cos-site packaging and phage-encoded HNH endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 6016–6021.

Rao, V.B., Feiss, M. (2008) The bacteriophage DNA packaging motor. *Annual Review of Genetics*, **42**, 647-681.

Rocha, E.P.C., Bikard, D. (2022) Microbial defenses against mobile genetic elements and viruses: Who defends whom from what. *PLOS Biology*, **20**(1) :e3001514. Epub 20220113. https://doi.org/10.1371/journal.

Rocha, L.A., Ribas, R.M., da Costa Darini, A.L., Gontijo Filho, P.P. (2013) Relationship between nasal colonization and ventilator-associated pneumonia and the role of the environment in transmission of Staphylococcus aureus in intensive care units. *American Journal of Infection Control*, **41**(12), 1236-1240.

Rosenwasser, S., Ziv, C., Van Creveld, S.G., Vardi, A. (2016) Virocell metabolism: metabolic innovations during host–virus interactions in the ocean. *Trends in Microbiology*, **24**(10), 821-832.

Rousset, F., Depardieu, F., Miele, S., Dowding, J., Laval, A-L., Lieberman, E., et al. (2022) Phages and their satellites encode hotspots of antiviral systems. *Cell Host & Microbe*, **30**(5), DOI:10.1016/j.chom.2022.02.018

Saeed, A.F., Chan, C., Guan, H., Gong, B., Guo, P., Cheng, X., Ouyang, S. (2021) Structural insights into gp16 ATPase in the bacteriophage ϕ29 DNA packaging motor. *Biochemistry*, **60**(11), 886-897

Tenover, F.C., Tickler, I.A. (2022) Detection of methicillin-resistant *Staphylococcus aureus* infections using molecular methods. *Antibiotics*, **11**(2), 1-8.

Watson, B.N.J., Staals, R.H.J., Fineran, P.C. (2018) CRISPR-cas-mediated phage resistance enhances horizontal gene transfer by transduction. *mBio*, **9**(1), e02406-17.

Weigel, C., Seitz, H. (2006) Bacteriophage replication modules. *FEMS Microbiology Reviews*, **30**(3), 321-381.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M., Sugai, M. (2002) Identification of the *Staphylococcus aureus* etd pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infection and Immunity*, **70**(10), 5835-5845.

ميكانيكية الاستنتساخ والتعبئة المتقاربة لمسببات المرض من المكورات العنقودية (سابيبوف5) عن طريق التداخل مع البكتيريوفاج نموذج 12

ثامر بوبك[1، 3]، فيصل السراج[1]، إبراهيم العتيبي[2]، رائد البحيري[1، 4]، ماجد الزهراني[5]، مشاعل الغامدي[1]، ندى ناس [1، 6]، بيان ساجر[1]، عاطف بامقوس[1]، شيرين أزهري[1]، ريم فارسي[1]

[1]قسم العلوم البيولوجية- كلية العلوم- جامعة الملك عبد العزيز- جدة- المملكة العربية السعودية، [2]قسم تكنولوجيا المعلومات الصحية- الكلية التطبيقية- جامعة الملك عبد العزيز- جدة- المملكة العربية السعودية، [3]الأميرة الدكتورة نجلاء مركز بنت سعود آل سعود لبحوث التميز في التكنولوجيا الحيوية- جامعة الملك عبدالعزيز- جدة -21589 المملكة العربية السعودية، [4]مركز التميز في علم النانو الحيوي البحوث- جامعة الملك عبد العزيز- جدة- المملكة العربية السعودية، [5] قسم العلوم البيولوجية- كلية العلوم والفنون- جامعة الملك عبد العزيز- رابغ- السعودية، المملكة العربية السعودية ، [6]وحدة المناعة- مركز الملك فهد للبحوث الطبية- جامعة الملك عبد العزيز- جدة- المملكة العربية السعودية.

يمكن أن تدخل المكورات العنقودية الذهبية إلى مجرى الدم ، مما يؤدي إلى مضاعفات صحية مثل الإنتان والتهاب المفاصل والتهاب الشغاف والالتهاب الرئوي. تم فحص هذه الدراسة للتأثير من ستة جينات النسخ المتماثل من كوس فج 12 (1 12) على تطور ونقل كوس س. أوريوس جزر الإمراضية (سابيبوف5). تم زراعة المزرعة البكتيرية طوال الليل لسلالة S. aureus RN4220 مع TSB الجديد تم استخدامه في مقايسة الفاج 12 φ. طفرات الباكتيريوفاج باستخدام ناقل pMAD، مما يسهل إعادة التركيب المتماثل في عملية ذات خطوتين. أخيرًا، استخدمت مقايسات عنوان SaPI التحويل أيضًا المكورات العنقودية الذهبية RN4220 سلالة آر إن والتي تم خلطها بعد ذلك CaCl2, أظهرت الدراسة أن ORF11، ORF12، ومسوخات VirE لم تكذب أو تنتج جزيئات العاثيات بعد العنوان إلى سلالات RN4220 المتلقي. في حين أن ORF26، ORF10، ORF04 المسوخ تولد اللوحات القابلة للكشف، قد يكون للطفرات تأثير على تكرار العاثيات أو التغليف بعد العطاءات. على سبيل المثال، تكمل 12 φ سلالة ORF26 و ORF10 و ORF04 متحولة قد تم ترميمه بالكامل. وجدت التجربة أن الباكتيريوفاج تسهل بين و النقل العام البيني للأرقام القياسية لأسعار الاستهلاك. ثبت أيضًا أن 12 φ تنقل SaPIbov5. ومع ذلك، فإن 12 φ متحولة في VirE و ORF12 و ORF11 لم تظهر تعبئة SaPI لأن لآثارها على جزيئات الباكتيريوفاج المعدية وعدم تكرارها. رابعاً - φ المكملة 12استعاد VirE و ORF12 و ORF11 المتحولين جزئيًا الباكتيريوفاج عندما تم التعبير عنها في سلالات المتلقي والمانح. انخفاض الطفرات في ORFO4 0RF10 ORF26 قدرة 12 φ على نقل SaPIbov5. بشكل عام، وجدت هذه الدراسة آلية جديدة ويسهل نقل SaPI و cos الجينات. بينما يؤثر VirE و ORF12 و ORF11 التغليف وهي ضرورية للتكرار وبيولوجيا الباكتيريوفاج.