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Isolation and Genome Characterization of the Virulent Staphylococcus aureus Bacteriophage SA97

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Abstract: A novel bacteriophage that infects S. aureus, SA97, was isolated and characterized. The phage SA97 belongs to the Siphoviridae family, and the cell wall teichoic acid (WTA) was found to be a host receptor of the phage SA97. Genome analysis revealed that SA97 contains 40,592 bp of DNA encoding 54 predicted open reading frames (ORFs), and none of these genes were related to virulence or drug resistance. Although a few genes associated with lysogen formation were detected in the phage SA97 genome, the phage SA97 produced neither lysogen nor transductant in S. aureus. These results suggest that the phage SA97 may be a promising candidate for controlling S. aureus.

Keywords: bacteriophage; genome analysis; Staphylococcus aureus

1. Introduction

Bacteriophages are natural antibacterial agents that lyse a specific bacterial host strain [1]. Based on their bacterial host specificity and bacteriolytic activity, the use of bacteriophages has been suggested as a potential biocontrol strategy for various pathogenic bacteria. Staphylococcus aureus is a major foodborne and clinical pathogen that causes food poisoning, endocarditis, staphylococcal scalded skin syndrome and toxic shock syndrome [2]. Furthermore, the emergence of antibiotic-resistant S. aureus, including methicillin-resistant S. aureus (MRSA) [3], has complicated the treatment of S. aureus infections [4]. Bacteriophage treatment could be an alternative method for controlling S. aureus. Various bacteriophages have been used as alternative antibacterial therapies for human patients in eastern Europe for several decades [5]. In addition, the US Food and Drug Association (FDA) has approved the application of bacteriophages as a safe food preservative [6].

Virulent phages are generally preferred as biocontrol agents as they cannot integrate their genome into the bacterial chromosome to form lysogens and will always lyse and kill infected target bacterial cells [7]. Staphylococcal lytic phages have demonstrated their potential as biocontrol agents of food [1] and phage therapy of human or animal infections [8–10]. For example, Myovirus phages S25-3 [11], K [9] and the Podoviruses S13’ [11], S24-1 [11] are virulent phages that have been applied to food samples or mice. However, most reported S. aureus phages are siphoviruses and known as temperate phages that contain lysogeny modules in their genomes [12,13]. Enterobacteria
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phage lambda [14–16] and S. aureus phage phi11 [14] are well-known temperate phages that form lysogens. These phages all contain typical lysogen modules including cro-like repressor, antirepressor, integrase, excisionase, cl-like repressor, and lambda contains additional cII and cIII regulatory proteins. Integrase mediates site-specific recombination between two DNA recognition sequences in phage and host bacteria, while excisionase is involved in excisional recombination by excising the phage genome from the bacterial chromosome. CI-like repressor represses lytic functions; Cro-like repressor, which is a repressor of cl, leads the phage to the lytic infection cycle, while the antirepressor blocks phage repressors. Among the lysogen formation-related genes, cl-like repressor gene is the key regulator that maintains the phage in the lysogenic life cycle [14–16].

Here, the phage SA97, which specifically infects S. aureus without lysogen formation, was isolated and characterized. An analysis of the whole genome of the phage SA97 revealed part of the genes encoding a lysogeny module but no genes related to virulence or drug resistance. An analysis of lysogen formation or transduction by SA97 showed that SA97 produced neither lysogen nor transductant, suggesting that the phage SA97 may be applicable as a biocontrol agent against S. aureus.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Growth Conditions

The bacterial strains used in this study are summarized in Table 1. Food isolates 1 to 4 were from vegetable, pork, beef and Korean meal kimbab (rice with laver, ham, egg, cucumber, pickled radish and crab meat), respectively. They were isolated and purified by Baird-Parker agar medium (Difco, Detroit, MI, USA) and identified by VITEK® 2 Compact (bioMerieux, Inc., Hazelwood, MO, USA). These strains were all grown in tryptic soy broth (TSB) medium (Difco) at 37 °C with agitation. Tryptic soy agar (Difco) containing 1.5% (w/v) agar was used to count the bacteria.

2.2. Bacteriophage Isolation and Propagation

Bacteriophage SA97 was isolated from the skin using the strain S. aureus RN4220 as a bacterial host strain. To isolate the phage, 0.5 cm² of skin was rubbed with a cotton swab that was subsequently homogenized with 1 mL of sodium chloride and magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM MgSO₄, and 50 mM Tris-HCl, pH 7.5). The sample was then mixed with TSB broth supplemented with 10 mM CaCl₂, sub-cultured with S. aureus RN4220 and incubated at 37 °C for 12 h with shaking. After incubation, the sample was centrifuged at 8000× g for 10 min and filtered to remove bacterial cells and obtain the supernatant that contained the bacteriophage. For phage propagation, TSB broth containing 10 mM CaCl₂ was first sub-inoculated with S. aureus RN4220, and the culture was incubated at 37 °C for 1.5 h. Subsequently, the phage was added at a multiplicity of infection (MOI) of 1, and the culture was incubated for 3 h at the same temperature with shaking. To prepare a high-titer phage, the phages were precipitated with polyethylene glycol (PEG) 6000 and concentrated using CsCl density gradient ultracentrifugation [17]. Finally, to confirm the phage plaque formation, the supernatant was overlaid on 0.4% TSA soft agar containing 10 mM CaCl₂ and S. aureus RN4220. Plaques were evident after incubation in at 37 °C for 12 h.

2.3. Transmission Electron Microscopy (TEM)

The phage SA97 was analyzed using transmission electron microscopy (TEM). The phage suspension was placed on a carbon-coated copper grid and negatively stained with 2% uranyl-acetate (pH 4.0). The sample was examined under an energy-filtering transmission electron microscope at an operating voltage of 120 kV [18]. Phage SA97 was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses [19].
2.4. Bacteriophage Host Range

The bacterial strains listed in Table 1 were incubated overnight at 37 °C. Each bacterial culture was added to 5 mL of the 0.4% TSA soft agar and overlaid on TSA agar plates. Subsequently, the phage SA97 containing diluted lysates was spotted onto the prepared plate and incubated at 37 °C for at least 6 h to obtain single plaques. After incubation, we could determine the infectivity based on the clarity of the spots, and efficiency of plating (EOP) was calculated by dividing the phage titer on the test strain by the phage titer on the reference strain. All the experiments were performed in triplicate.

2.5. Bacterial Challenge Assay

Fifty milliliters of TSB broth containing 10 mM CaCl$_2$ was sub-inoculated with S. aureus RN4220, and the culture was incubated at 37 °C until it reached the early exponential growth phase ($3.5 \times 10^8 \pm 0.2 \times 10^8$). The culture was then infected with the phage at a MOI of 1 or 10. The OD$_{600}$ was measured each hour after phage infection for 15 h [20]. An un-infected culture was used as a control. All the experiments were performed in triplicate.

2.6. Lysogen Formation

To evaluate the ability of the phage SA97 to form lysogens, the phage-resistant bacteria were isolated 10 h after phage infection, and the presence of SA97 gene fragments in the fifty of SA97-resistant colonies was analyzed by PCR using primers specific to the transcriptional regulator gene (SA97_044) or antirepressor gene (SA97_045). In addition, the phage-resistant strains grown to exponential phase were induced with mitomycin C at a final concentration of 0.5 µg/mL and centrifuged for 5 min at 10,000 $\times$ g after incubation at 37 °C. The supernatants were filtered using 0.22-µm filter and spotted onto S. aureus overlay.

2.7. One-Step Growth Curve Assay

One-step growth curve analysis was performed as previously described [21]. Briefly, phage was mixed with S. aureus RN4220 in its early exponential growth phase at MOI of 0.001 in the presence of 10 mM CaCl$_2$ and allowed to adsorb for 10 min at room temperature, then it was centrifuged at 6000 $\times$ g for 10 min. The pellet containing infected cells was suspended in 50 mL of fresh TSB liquid medium and incubated at 37 °C with shaking. Two sets of samples were taken every 5 min up to 1 h. To release the intracellular phages, CHCl$_3$ was added to one of them, and the eclipse period was determined. Subsequently, each sample was immediately titered by the double-layer agar plate method and latent period, eclipse period and burst size were calculated. All the experiments were performed in triplicate.

2.8. Receptor Analysis

S. aureus RN4220, a strain free of prophages, restriction mechanisms and capsules [22,23], was used in this study (Table 1). To identify the phage receptor, we obtained the ΔtagO/RN4220 mutant, which is deficient in the peptidoglycan-anchored wall teichoic acid (WTA) [24], and its complemented strain using plasmid pRB474-tagO, which was constructed by sub-cloning the tagO gene into an Escherichia coli-S. aureus shuttle expression vector [25]. We carried out a spotting assay with the wild type RN4220, ΔtagO/RN4220 mutant and the tagO complemented strains.

2.9. Adsorption Assay

The phage adsorption ability was assayed as previously described [26]. Overnight cultures of the bacterial strains were diluted 1:100 in TSB and incubated until the OD$_{600}$ reached 2.0. One milliliter of the strain cultures was diluted 10-fold in fresh TSB, and the phage was added to the each diluted culture at an MOI of 0.01. The cultures were incubated at 37 °C for 10 min. A sample was
collected immediately after reaction and 10 min after incubation for each culture and immediately centrifuged at 14,000 \( \times g \) and 4 °C for 1 min. The samples were then filtered using 0.22-µm filters. Subsequently, the supernatants were serially diluted and overlaid on TSA plates to determine the titers of unabsorbed phages. All the experiments were performed in triplicate.

2.10. Determination of the Frequency of the Bacteriophage-Insensitive Mutants (BIMs)

The frequency of the BIMs was determined as previously described [27]. Phage was mixed with \( S. \text{aureus} \) \((10^9 \text{ CFU/mL})\) cell suspension containing 10 mM of CaCl\(_2\) with a MOI of 100. After incubation at 37 °C for 10 min, serial dilutions of the mixtures were plated. The BIMs frequency was determined by dividing remaining viable counts by the initial viable counts. All the experiments were performed in triplicate.

2.11. Transduction Assay

A transduction assay was performed to determine the ability of phage SA97 to induce horizontal gene transfer between the host bacteria cells and bacteriophages. Donor phage lysate was prepared by infecting SA97 phage to the host bacteria containing the erythromycin antibiotic-resistant cassette (\(\Delta\text{ypfP::erm}, \) the glycolipid-deficient mutant) [28]. After infection, the cultures were incubated in TSB for 8 h at 37 °C. After incubation, the cultures were treated with chloroform, and the supernatant lysate was harvested. To determine the ability of the phage to transduce host bacteria, supernatant phage lysate was added to the recipient host bacteria culture (\( S. \text{aureus RN4220}, \) \(10^{10} \text{ CFU/mL}\)) at an MOI of 10 and the culture was incubated for 15 min at 37 °C. TSB containing ethylene glycol tetraacetic acid (EGTA) was added to the culture (final concentration of 20 mM) to stop the reaction. After incubation for 20 min under the same conditions, the phage-infected bacterial cells were harvested, spread on TSA agar media containing erythromycin and incubated at 37 °C. All the experiments were performed in triplicate, and the transduction frequencies were calculated as the quotient of the number of transductants divided by the number of input PFU per plate [29].

2.12. Bacteriophage Genomic DNA Purification

Bacteriophage genomic DNA was purified as previously described [30]. Prior to purification, the phage lysates were treated with DNase and RNaseA at 37 °C for 1 h to remove bacterial DNA and RNA contamination, respectively. The phage lysate was then treated with lysis buffer containing 0.5 M of EDTA, 10 mg/mL of proteinase K and 1% of sodium dodecyl sulfate (SDS) for 15 min at 65 °C. Finally, ethanol precipitation was performed, followed by phenol-chloroform DNA purification.

2.13. Full-Genome Sequencing of Bacteriophage SA97 and Bioinformatics Analysis

The extracted SA97 phage genomic DNA was sequenced with a Genome Sequencer FLX titanium sequencer (Roche, Mannheim, Germany) and assembled with the GS de novo assembler software (Roche) at Macrogen Inc., South Korea. Open reading frames (ORFs) were predicted using the FGENESB (http://www.softberry.com), Glimmer v3.02 [31] and GeneMark.hmm [32] software packages. The ORFs were annotated using the InterProScan [33] and BLASTP [34] programs. Comparative genomic analysis of SA97 with other phietalikevirus Subgroup2 phages and visualization were conducted with BLASTN [35] and ACT12 [36]. The complete genome sequence of \( S. \text{aureus} \) phage SA97 was deposited in GenBank under accession number KJ716334.
3. Results and Discussion

3.1. Phage SA97 Isolation and Its Morphological Analysis

The *S. aureus* infecting bacteriophage SA97 was newly isolated. This phage formed clear plaques against the *S. aureus* RN4220 bacterial host strain. Transmission electron microscopy (TEM) revealed an icosahedral capsid (63 ± 2 nm, n = 5) with a long, flexible and non-contractile tail (171 ± 6 nm, n = 5) for phage SA97, indicating that this phage belongs to the *Siphoviridae* family (Figure 1A). The baseplate structure of the phage SA97 consists of multiple disc baseplates and is similar to those of phages StB12, StB27 and StB20 [37]. However, the tail of phage SA97 is shorter than those of the above-mentioned phages (206 nm for StB12, 213 nm for StB27, and 250 nm for StB20) [37]. According to Xia and Wolz [38], phage SA97 belongs to serogroup B, which contains phages with a tail length of less than 200 nm.

![Figure 1](image)

**Figure 1.** General characterizations of bacteriophage SA97. (A) Electron micrograph of phage SA97. The phage belongs to the *Siphoviridae* family. The scale bar represents 100 nm; (B) One-step growth curve analysis of phage SA97 on exponential culture of *S. aureus* RN4220 incubated in tryptic soy broth (TSB) medium at 37 °C under agitation. Cells were chloroform treated (○) or untreated (■). E, eclipse period; L, latent period; B, burst size; (C) Inhibition assays of *S. aureus* RN4220 with phage SA97 at 37 °C. ○, control group without phage; ■, ▲, experimental groups with phage SA97 (MOI of 1 and 10, respectively). The data shown are the mean values from three independent measurements and the error bars represent the standard deviations.

3.2. One-Step Growth Curve of SA97 and Bacterial Challenge Assay

The one-step growth kinetics of SA97 showed a latent period time of 20 min, an eclipse period time of 15 min and a burst size of 32 PFU/infected cell when infecting *S. aureus* RN4220 (Figure 1B).

To determine the bacteriolytic activity of the phage SA97, a bacterial growth inhibition assay was performed in the presence of SA97 (Figure 1C). The growth inhibition of *S. aureus* by SA97 persisted for up to 10 or 11 h after infection with MOI of 1 or 10, respectively. SA97 showed a longer inhibitory
effect than most other *S. aureus* phages such as CS1, DW2 [10] and SAH-1 [39], all of which showed only 3 h of growth inhibition. These results demonstrate that SA97 possesses strong bacteriolytic activity, which is essential in phage therapy. Re-growth of the culture 10 h after infection with SA97 suggested the possibility of development of lysogen or resistant mutant. However, PCR analysis of the 50 *S. aureus* colonies resistant to SA97 obtained 10 h after phage infection using primers specific to the transcriptional regulator gene (SA97_044) or antirepressor gene (SA97_045) detected neither gene. In addition, no plaques were observed after induction of the resistant strains with mitomycin C, indicating that the resistant strains were not SA97 lysogens but resistant mutants.

### 3.3. Receptor Analysis

The only known host receptor of *S. aureus* phages is peptidoglycan-anchored wall teichoic acid (WTA). Xia *et al.* [40] demonstrated that WTA is required for siphovirus and myovirus infection of *S. aureus*; thus, a ∆tagO mutant of *S. aureus* (RN4220∆tagO) [41] was tested (Figure 2A). The RN4220∆tagO strain was resistant to SA97, and the phage sensitivity was recovered by complementing the strain with tagO [42]. The phage adsorption assay also showed that phage adsorption was severely reduced in the RN4220∆tagO strain (Figure 2B), indicating that the host receptor for phage SA97 is peptidoglycan-anchored WTA.

![Figure 2](image_url)

**Figure 2.** (A) Wall teichoic acid (WTA)-dependent infection of *S. aureus* phage SA97. The phage SA97 lysate was spotted onto lawns of wild-type RN4220 (WT), ∆tagO mutant (∆tagO), and tagO-complemented (∆tagO pBR474::tagO) strains. Plaque formation indicates successful adsorption and infection by the phage SA97. EOP relative to WT strain was 7.5 × 10^{-2} ± 0.2 × 10^{-2} on ∆tagO pBR474::tagO strain; (B) WTA-dependent adsorption ability of *S. aureus* phage SA97. The adsorption efficiency relative to the adsorption to wild-type strain RN4220, which was set to 100%, is indicated. The data shown are the mean values from three independent measurements and the error bars represent the standard deviations.

### 3.4. Host Range of Bacteriophage SA97

Plaque formation efficiency of the phage SA97 was determined for 19 *S. aureus* strains and several other strains of both Gram-positive and Gram-negative bacteria as shown on Table 1. SA97 phage could infect only *S. aureus* strains. Six out of 13 *S. aureus* type strains and all six *S. aureus* local isolates tested were sensitive to SA97. It is interesting to note that nine of these 12 sensitive strains were isolated in Korea, indicating that SA97 phage may be more effective for controlling *S. aureus* strains isolated in Korea.
3.5. Bacteriophage SA97 Genomic Analysis

The phage SA97 genome was sequenced and analyzed. The complete genome sequence of the phage SA97 revealed that the genome consists of 40,592 bp with a GC content of 34.25% encoding 54 predicted open reading frames (ORFs) and no tRNA genes (Figure 3).

The functional analysis of the predicted ORFs revealed that they can be classified into five functional groups: phage structure (major capsid protein, head morphogenesis protein, head-tail connector protein, major and minor tail proteins, tape measure protein, minor structure proteins, tail fiber protein and endo-β-N-acetylglucosaminidase), packaging (portal protein and terminase small/large subunits), host lysis (holin and endolysin), DNA regulation (single-stranded DNA-binding protein, dUTP diphosphatase, transcriptional activators RinAB and transcriptional regulators) and lysis/lysogeny module (integrate and antirepressor) (Table 2).

Table 1. The antimicrobial spectrum of bacteriophage SA97

| Bacteria                                      | Plaque Formation of SA97 | E.O.P b | Reference or Source c |
|-----------------------------------------------|--------------------------|---------|-----------------------|
| **Staphylococcus strains**                    |                          |         |                       |
| Staphylococcus aureus RN4220                   | C 1.0 × 10^9 ± 0.0 × 10^8 | [42]    |                       |
| Staphylococcus aureus KCTC 1916                | C 1.1 × 10^9 ± 0.0 × 10^4 | KCTC    |                       |
| Staphylococcus aureus ATCC 29213               | C 1.7 × 10^4 ± 2.1 × 10^−5 | ATCC    |                       |
| Staphylococcus aureus ATCC 33593               | C 1.2 × 10^4 ± 2.1 × 10^−2 | ATCC    |                       |
| Methicillin resistant Staphylococcus aureus CCARM 3089 | C 1.0 × 10^9 ± 8.1 × 10^−2 | CCARM   |                       |
| Methicillin resistant Staphylococcus aureus CCARM 3090 | C 7.4 × 10^−2 ± 3.1 × 10^−2 | CCARM   |                       |
| Staphylococcus aureus food isolate 1 (from vegetable) | C 4.9 × 10^−2 ± 1.6 × 10^−2 | This study |                       |
| Staphylococcus aureus food isolate 2 (from pork) | C 1.2 × 10^9 ± 1.0 × 10^−1 | This study |                       |
| Staphylococcus aureus food isolate 3 (from beef) | C 1.2 × 10^9 ± 2.8 × 10^−1 | This study |                       |
| Staphylococcus aureus food isolate 4 (from Kimbab) | C 1.0 × 10^9 ± 4.0 × 10^−1 | This study |                       |
| Staphylococcus aureus clinical isolate 1       | C 2.0 × 10^−4 ± 5.0 × 10^−5 | This study |                       |
| Staphylococcus aureus clinical isolate 0055    | C 1.1 × 10^−3 ± 5.5 × 10^−5 | GNUH-NCCP |                       |
| Staphylococcus aureus Newman                   | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 6538                | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 23235               | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 25923               | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 12600               | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 13301               | -                        | ATCC    |                       |
| Staphylococcus aureus ATCC 27664               | -                        | ATCC    |                       |
| Staphylococcus xylosis ATCC 29971              | -                        | ATCC    |                       |
| Staphylococcus cohnii ATCC 29974               | -                        | ATCC    |                       |
| Staphylococcus warneri ATCC 10209              | -                        | ATCC    |                       |
| Staphylococcus intermedius ATCC 29663          | -                        | ATCC    |                       |
| **Other Gram positive bacteria**               |                          |         |                       |
| Enterococcus faecalis ATCC 29212               | -                        | ATCC    |                       |
| Bacillus cereus ATCC 14579                     | -                        | ATCC    |                       |
| Bacillus subtilis ATCC 23837                   | -                        | ATCC    |                       |
| Listeria monocytogenes ATCC 19114              | -                        | ATCC    |                       |
| **Gram negative bacteria**                     |                          |         |                       |
| Salmonella enterica serovar Typhimurium SL1344 | -                        | ATCC    |                       |
| Escherichia coli MG1655 ATCC 47076             | -                        | ATCC    |                       |
| Escherichia coli O157:H7 ATCC 35150            | -                        | ATCC    |                       |
| Cronobacter sakazakii ATCC 29544               | -                        | ATCC    |                       |
| Pseudomonas aeruginosa ATCC 27385              | -                        | ATCC    |                       |

a C, clear plaques; -, no plaque. b The EOP is relative to an average result obtained for RN4220. c ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; GNUH-NCCP, Gyeongsang National University Hospital Branch of National Culture Collection for Pathogens; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

Interestingly, three genes in the category of host lysis may be responsible for the host lysis activity of phage SA97. Holin (SA97_035) is generally known to create a hole in the host inner membrane to expose the host peptidoglycan layer to endolysin. Endolysin (SA97_036) may then
lyse the host peptidoglycan to allow phage burst-out. In general, Gram positive endolysins form in a modular structure, consisting of enzymatically active domains (EADs) and a cell wall binding domain (CBD) [44] which leads the enzyme specifically to the substrate bacterial peptidoglycan. The BLASTP analysis of endolysin revealed two major protein domains containing CHAP (PF05257) and amidase_3 (PF01520), which may be involved in host lysis via the activities of endopeptidase and amidase. However, a host cell wall-binding domain (e.g., SH3 domain) was not detected, suggesting that this phage endolysin may contain a new type of CBD. Endo-\(\beta\)-N-acetylglucosaminidase (SA97_032), which is known as a tail-associated cell wall hydrolase, may degrade the peptidoglycan of the bacterial cell wall during infection [45].

A few putative genes associated with lysogen formation [antirepressor (SA97_045) and integrase (SA97_040)] were detected in the category of DNA manipulation and regulation; however, the major components of the lytic/lysogenic decision gene cluster, \(\lambda\)- or \(\lambda\)-like repressors, were missing.

A phylogenetic analysis of the terminase large subunits in the staphylococcal bacteriophages, including phage SA97, was performed (Figure 4). According to a previous study [46], Siphovirus staphylococcal phages cluster into three groups: “3alikevirus”, “77likevirus” and “phietalikevirus”. “phietalikevirus” includes 31 staphylococcal phages, which are theoretically divided again into three subgroups based on DNA similarity. The terminase large subunit of phage SA97 is similar to those of phage 85 [47] and phiMR25 [48], so that SA97 could be classified into the phietalikevirus subgroup2 as shown in Figure 4. Terminase large subunit of SA97 (SA97_011) has a terminase_3 domain like \(B.\ subtilis\) phage SPP1, which is known to use the \(pac\) site for DNA packaging [49], suggesting the possibility that SA97 would also use a \(pac\) mechanism for packaging DNA. Moreover, the tail morphogenesis module of SA97 consists of the SGNH hydrolase superfamily (SA97_026), tail protein (SA97_027), virion-associated peptidoglycan hydrolase (SA97_032) and tail fiber protein (SA97_033). The composition is the same as that of phages involved in “phietalikevirus subgroup2” as analyzed by Gutierrez et al [46]. Additional comparative genomic analysis of phage SA97 with phietalikevirus subgroup2 phages such as phage 85 and phage phiMR25 (Figure 5) revealed that DNA packaging and structure (heads and tails) gene clusters are highly conserved but the lysis/lysogeny control regions (Integrase, Repressor, Antirepressor and Cro-like protein) are not, suggesting that phage SA97 may have distinctive lytic/lysogenic decision mechanism even though phage SA97 is grouped in the phietalikevirus subgroup2 with phage 85 and phage phiMR25. The host cell lysis mechanism of SA97 is also expected to be different from those of phage 85 and phiMR25 because the endolysin and holin genes of SA97 differ from those of phage 85 and phiMR25.
Table 2. Functional categories of predicted open reading frames (ORFs) in bacteriophage SA97.

| Group          | Subgroup | Locus_tag  | Function                  | BLASTP Best Match                                      | Identity (%) | Accession no. |
|----------------|----------|------------|---------------------------|--------------------------------------------------------|--------------|--------------|
| packaging      |          | SA97_010   | terminase small subunit   | phage terminase small subunit [Staphylococcus aureus subsp. aureus [H1] | 164/164 (100) | YP_001316047.1 |
| packaging      |          | SA97_011   | terminase large subunit   | PBSX family phage terminase large subunit [Staphylococcus aureus subsp. aureus [H9] | 431/431 (100) | YP_001246264.1 |
| packaging      |          | SA97_012   | portal protein            | SPP1 family phage portal protein [Staphylococcus aureus subsp. aureus [H9] | 512/512 (100) | YP_001246265.1 |
| structure      | head     | SA97_013   | head morphogenesis protein| phage head morphogenesis protein [Staphylococcus aureus subsp. aureus [H9] | 331/331 (100) | YP_001246266.1 |
| hypothetical   |          | SA97_014   | hypothetical protein      | hypothetical protein phiETA2 gp45 [Staphylococcus phage phiETA2] | 56/56 (100)  | YP_001004305.1 |
| structure      | head     | SA97_015   | capsid protein            | phage capsid protein [Staphylococcus aureus] | 206/206 (100) | WP_000392146.1 |
| structure      | head     | SA97_016   | major capsid protein      | Phage major capsid protein [Staphylococcus aureus subsp. aureus DSM 20231] | 324/324 (100) | WP_001836233.1 |
| hypothetical   |          | SA97_017   | hypothetical protein      | phage protein [Staphylococcus aureus subsp. aureus DSM 20231] | 95/95 (100)  | ELP28728.1    |
| structure      | head-tail| SA97_018   | head-tail adapter protein | head-tail connector protein [Staphylococcus phage SA12] | 110/110 (100) | YP_008241892.1 |
| hypothetical   |          | SA97_019   | hypothetical protein      | hypothetical protein NWMN_1023 [Staphylococcus aureus subsp. aureus str. Newman] | 115/115 (100) | YP_001332057.1 |
| structure      | head     | SA97_020   | head protein              | hypothetical protein SAV0897 [Staphylococcus aureus subsp. aureus Mu50] | 127/127 (100) | NP_571421.1   |
| structure      | tail     | SA97_021   | major tail protein        | phage major tail protein [Staphylococcus phage phiNM] | 193/193 (100) | YP_873998.1   |
| hypothetical   |          | SA97_022   | hypothetical protein      | hypothetical protein SaurJH9_0899 [Staphylococcus aureus subsp. aureus [H9] | 121/121 (100) | YP_001246276.1 |
| hypothetical   |          | SA97_023   | hypothetical protein      | hypothetical protein T874_02808, partial [Staphylococcus aureus SJO6126] | 114/114 (100) | EVG26589.1    |
| structure      | tail     | SA97_024   | tape measure protein      | putative tape measure protein [Staphylococcus phage phiSauS-PLA88] | 1130/1154 (98) | YP_002332525.1 |
| structure      | tail     | SA97_025   | putative minor tail protein | putative minor tail protein [Staphylococcus phage TEM2123] | 314/315 (99)  | YP_006382275.1 |
| structure      | tail     | SA97_026   | endopeptidase tail protein | SGNH hydrolase superfamily [Staphylococcus aureus subsp. aureus 21283] | 632/632 (100) | EHO98287.1    |
| Group   | Subgroup | Locus_tag  | Function                                      | BLASTP Best Match                              | Identity (%) | Accession no.   |
|---------|----------|------------|-----------------------------------------------|-----------------------------------------------|--------------|----------------|
| structure | tail     | SA97_027   | putative minor tail protein                   | minor structural protein [Staphylococcus aureus] | 635/636 (99) | WP_000369023.1 |
|         |          |           |                                               | hypothetical protein NWMN_0306                 | 607/607 (100) | YP_001331340.1 |
|         |          | SA97_028   | hypothetical protein                          | hypothetical protein [Staphylococcus aureus subsp. aureus str. Newman] | 125/125 (100) | YP_001316067.1 |
|         |          | SA97_029   | hypothetical protein                          | SLT orf 129-like protein [Staphylococcus aureus JH1] | 57/57 (100)  | YP_003857088.1 |
|         |          | SA97_030   | hypothetical protein                          | hypothetical protein SAP26_gp20              | 99/99 (100)  | YP_001004323.1 |
|         |          | SA97_032   | mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase | mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase [Staphylococcus aureus DSM 20231] | 632/632 (100) | WP_001836236.1 |
|         |          | SA97_033   | putative tail fiber protein                   | tail fiber [Staphylococcus aureus subsp. aureus JH1] | 412/412 (100) | YP_001316071.1 |
|         |          | SA97_034   | hypothetical protein                          | hypothetical protein SASA_03740              | 131/131 (100) | ELP28745.1     |
| host    | lysis    | SA97_035   | holin                                         | holin, SPP1 family [Staphylococcus aureus subsp. aureus 21267] | 91/91 (100)  | EZH90125.1     |
| host    | lysis    | SA97_036   | amidase                                       | CHAP domain-containing protein               | 470/470 (100) | YP_001246290.1 |
|         |          | SA97_037   | hypothetical protein                          | hypothetical protein SaurJH1_0932           | 185/185 (100) | YP_001316075.1 |
|         |          | SA97_038   | hypothetical protein                          | hypothetical protein SAOUHSC_02018          | 50/50 (100)  | YP_500515.1    |
| regulation |          | SA97_039   | putative transcriptional regulatory protein   | XRE family transcriptional regulator [Staphylococcus aureus] | 61/61 (100)  | WP_000382163.1 |
| Group         | Subgroup | Locus_tag | Function                          | BLASTP Best Match                      | Identity (%) | Accession no. |
|--------------|----------|-----------|-----------------------------------|----------------------------------------|--------------|--------------|
| lysis/lysogeny |          | SA97_040  | putative integrase                 | integrase [Staphylococcus aureus subsp. aureus DSM 20231] | 349/349 (100) | WP_001145725.1 |
| hypothetical |          | SA97_041  | hypothetical protein               | hypothetical protein SAOUHSC_02087 [Staphylococcus aureus subsp. aureus NCTC 8325] | 301/301 (100) | YP_500579.1 |
| hypothetical |          | SA97_042  | hypothetical protein               | hypothetical protein phiETA2_gp04 [Staphylococcus phage phiETA2] | 241/241 (100) | YP_001004264.1 |
| hypothetical |          | SA97_043  | hypothetical protein               | hypothetical protein SaurJH1_0868 [Staphylococcus aureus subsp. aureus JH1] | 224/224 (100) | YP_001316011.1 |
| regulation   |          | SA97_044  | transcriptional regulator           | XRE family transcriptional regulator [Staphylococcus aureus] | 98/110 (89)  | WP_001055143.1 |
| lysis/lysogeny |          | SA97_045  | anti-repressor                     | anti-repressor protein [Staphylococcus phage phiETA2] | 255/255 (100) | YP_001004268.1 |
| hypothetical |          | SA97_046  | hypothetical protein               | hypothetical protein WWS_01005 [Staphylococcus aureus M1510] | 154/155 (99)  | ENN21654.1 |
| hypothetical |          | SA97_047  | hypothetical protein               | hypothetical protein SAV0856 [Staphylococcus aureus subsp. aureus Mu50] | 74/74 (100)  | NP_371380.1 |
| hypothetical |          | SA97_048  | hypothetical protein               | hypothetical protein phiETA2_gp10 [Staphylococcus phage phiETA2] | 148/149 (99)  | YP_001004270.1 |
| hypothetical |          | SA97_049  | hypothetical protein               | hypothetical protein phiETA3_gp11 [Staphylococcus phage phiETA3] | 73/73 (100)  | YP_001004340.1 |
| hypothetical |          | SA97_050  | hypothetical protein               | hypothetical protein SaurJH1_0876 [Staphylococcus aureus subsp. aureus JH1] | 100/100 (100) | YP_001316019.1 |
| hypothetical |          | SA97_051  | hypothetical protein               | hypothetical protein SASA_03250 [Staphylococcus aureus subsp. aureus DSM 20231] | 86/86 (100)  | ELP28696.1 |
| hypothetical |          | SA97_052  | hypothetical protein               | hypothetical protein phiSLTp16 [Staphylococcus phage phiSLT] | 177/178 (99)  | NP_075479.1 |
| regulation   |          | SA97_053  | DNA-binding protein 1             | ssDNA-binding protein Staphylococcus aureus SA_ST125_MupR | 216/216 (100) | EPR23947.1 |
Table 2. Cont.

| Group       | Subgroup | Locus_tag  | Function                               | BLASTP Best Match                      | Identity (%) | Accession no. |
|-------------|----------|------------|----------------------------------------|----------------------------------------|--------------|---------------|
| regulation  |          | SA97_054   | single-stranded DNA-binding protein 2  | ssDNA-binding protein [*Staphylococcus* phage phiETA3] | 104/105 (99) | YP_001004346.1 |
| hypothetical|          | SA97_001   | hypothetical protein                   | hypothetical protein U622_02779, partial [*Staphylococcus aureus* F53399] | 61/61 (100)  | EWV07862.1    |
| hypothetical|          | SA97_002   | hypothetical protein                   | hypothetical protein SaurJH9_0873 [*Staphylococcus aureus* subsp. aureus JH9] | 101/101 (100)| YP_001246250.1 |
| hypothetical|          | SA97_003   | hypothetical protein                   | hypothetical protein phiETA3_gp28 [*Staphylococcus phage phiETA3*] | 118/118 (100)| YP_001004357.1 |
| hypothetical|          | SA97_004   | hypothetical protein                   | hypothetical protein [*Staphylococcus aureus* subsp. aureus DSM 20231] | 80/80 (100)  | ELP28710.1    |
| hypothetical|          | SA97_005   | hypothetical protein                   | hypothetical protein phiETA3_gp28 [*Staphylococcus aureus* M0157] | 110/110 (100)| EUU37240.1    |
| regulation  |          | SA97_006   | dUTP diphosphatase                     | dUTP diphosphatase [*Staphylococcus aureus* A9765] | 175/176 (99) | WP_001797468.1 |
| hypothetical|          | SA97_007   | hypothetical protein                   | hypothetical protein [*Staphylococcus phage phiMR25*] | 67/68 (99)   | YP_001949833.1 |
| regulation  |          | SA97_008   | transcriptional activator RinB         | transcriptional activator RinB [*Staphylococcus aureus* subsp. aureus CM05] | 57/57 (100)  | WP_001657250.1 |
| regulation  |          | SA97_009   | transcriptional activator RinA         | transcriptional activator RinA [*Staphylococcus phage phiETA3*] | 140/140 (100)| YP_001004368.1 |

*a* Each predicted ORFs of SA97 phage was labeled with locus_tag. *b* Identities were calculated between amino acid sequences. *c* Genbank accession numbers.
**Figure 3.** Genome map of phage SA97. Representation of the ORF (ORF 1 to 54) organization of phage SA97. The predicted genes are indicated as arrows. Blue arrows, DNA regulation module; purple arrows, packaging module; yellow arrows, phage structural proteins; red arrows, host lysis proteins; green arrows, lysis/lysogeny module; black arrows, hypothetical proteins.
Figure 4. Phylogenetic analysis of terminase large subunits in the staphylococcal bacteriophages. The terminase large subunits were compared using the ClustalW program, and the phylogenetic tree was generated with the neighbor-joining method with $P$ distance values using the MEGA5 program.

3.6. Determination of the Frequency of BIMs and Horizontal Gene Transfer by SA97

The frequencies of BIMs were calculated with SA97 and SA12 phages. *S. aureus* temperate phage SA12 [17] was compared because the temperate phage is generally known to show a higher frequency of BIMs compared to the lytic phages [27]. The frequency of BIMs of *S. aureus* RN4220 by SA97 was $4.4 \times 10^{-6} \pm 2.9 \times 10^{-6}$, which was much lower than $2.8 \times 10^{-3} \pm 2.79 \times 10^{-3}$ observed by SA12 phage. These results indicate that the possibility of lysogen formation by SA97 is very low compared with SA12.
To evaluate the ability of the SA97 to transduce, frequencies of erythromycin-resistant gene transduction by SA97 were calculated with *S. aureus* RN4220 and four isolates (Table 3). SA12 phage was used as a positive control. No erythromycin-resistant colonies were found from any strain tested after transduction with SA97, suggesting that the horizontal gene transfer by SA97 may have hardly occurred. However, the transduction was possible with SA12 with transduction frequencies ranging from $1.7 \times 10^{-6} \pm 1.2 \times 10^{-8}$ to $6.0 \times 10^{-7} \pm 1.6 \times 10^{-7}$. We do not understand the cause of this difference because most *pac*-type phages such as SA12 and SA97 are known to be able to transduce bacterial DNA, but it is speculated that SA12 may transduce better than SA97 because SA12 can produce lysogen while SA97 cannot. Genome analysis revealed that SA12 has intact lysogeny-related genes [recombinase (SA12\_ORF07), *cI*-like repressor (SA12\_ORF54), *cro*-like repressor (SA12\_ORF55) and antirepressor (SA12\_ORF57)] but that *cI*-like repressor, a key gene of the lysogeny module, is missing in the phage SA97 genome.

**Table 3.** Transduction frequencies with phages SA97 and SA12.

| Recipient Strain              | Transduction Frequency |
|------------------------------|------------------------|
|                               | SA97                   | SA12                   |
| *S. aureus* RN4220            | 0.0 ± 0.0              | $1.7 \times 10^{-6} \pm 1.2 \times 10^{-8}$ |
| *S. aureus* food isolate 1    | 0.0 ± 0.0              | $2.6 \times 10^{-7} \pm 1.5 \times 10^{-7}$ |
| *S. aureus* food isolate 2    | 0.0 ± 0.0              | $6.0 \times 10^{-7} \pm 1.6 \times 10^{-7}$ |
| *S. aureus* food isolate 3    | 0.0 ± 0.0              | $5.1 \times 10^{-6} \pm 1.2 \times 10^{-8}$ |
| *S. aureus* clinical isolate 1| 0.0 ± 0.0              | $1.3 \times 10^{-7} \pm 0.5 \times 10^{-7}$ |

In conclusion, SA97 could not cause horizontal gene transfer despite containing genes encoding a lysogeny module, likely because the phage lacks a key gene (**cI**) important for lysogen formation. Our results suggest that even phages containing a lysogen module should be examined for their ability...
to transduce or lysogenize because they can be used as biocontrol agents if they can form neither lysogen nor transductant.

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