Food Microbiology 82 (2019) 523–532

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Mutation of a *Staphylococcus aureus* temperate bacteriophage to a virulent one and evaluation of its application

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ARTICLE INFO

Keywords:
Staphylococcus aureus
Bacteriophage
Phage mutation
Virulent phage

ABSTRACT

Bacteriophages have been suggested as alternative antimicrobial agents based on their host specificity and lytic activity. Therefore, it is necessary to obtain a virulent phage from a temperate one using molecular techniques to control *Staphylococcus aureus* efficiently. SA13, a novel temperate phage infecting *S. aureus,* was isolated and characterized. From this phage, mutant phages were generated by random deletion mutations, and a virulent mutant phage SA13m was selected. Comparative genome analysis revealed that the SA13m genome contains various nucleotide deletions in six genes encoding three hypothetical proteins and three lysogeny-associated proteins, including putative integrase, putative CI, and putative anti-repressor proteins. Mitomycin C induction of SA13m-resistant strains revealed that this mutant phage does not form lysogen, suggesting that SA13m is a virulent phage. In addition, SA13m showed rapid and long-lasting host cell growth inhibition activity. Furthermore, application of SA13m in sterilized milk showed that *S. aureus* was reduced to non-detectable levels both at refrigerator temperature (4 °C) and room temperature (25 °C), suggesting that SA13m can efficiently control the growth of *S. aureus* in foods. The virulent mutant phage SA13m could be used as a promising biocontrol agent against *S. aureus* without lysogen formation.

1. Introduction

Staphylococcus aureus is widespread in various environmental habitats (Oller et al., 2010). *S. aureus* infection causes various clinical symptoms (purulent inflammation, septicemia, endocarditis, and toxic shock syndrome, etc.) and food-borne poisoning (abdominal cramps, diarrhea, and vomiting, etc.) (Chang et al., 2013; Le Loir et al., 2003). From 1998 to 2008, *S. aureus* caused 167 confirmed food-borne outbreaks with 296 hospitalizations in the United States (Bennett et al., 2013). It has been reported that methicillin-resistant *S. aureus* is frequently detected in contaminated dairy and meat products all over the world (Aung et al., 2017; Chang et al., 2017; Chon et al., 2017; Garcia et al., 2009; Hennekinne et al., 2012; Kümmel et al., 2016). Therefore, control of *S. aureus* in foods is important to reduce *S. aureus* outbreaks. However, the use of antibiotics and artificial preservatives are not preferred in foods and related products, meaning that alternative and safe approaches for the biocontrol of *S. aureus* in foods are required.

Bacteriophages (phages) are viruses that infect only prokaryotes (bacteria and archaea) with host specificity and growth inhibition activity for its replication and propagation. In general, the life cycle of the phage consists of two alternatives: following phage infection of the host strain, the phage chooses to enter either a lytic or lysogenic cycle (Skurnik and Strauch, 2006; Young, 1992). A virulent phage only undergoes the lytic cycle for host lysis and thus has the potential to be developed as a novel biocontrol agent against a specific host pathogen (Bai et al., 2016). In addition, the phage takes advantage of the infection and lysis of antibiotic-resistant strains without affecting other bacteria or human cells.

As of October 2018, 200 complete genome sequences of *S. aureus*-infecting phages have been reported (*Myoviridae* 54 phages; *Siphoviridae* 127 phages; *Podoviridae* 19 phages) and are available in the National Center for Biotechnology Information (NCBI) database. Bioinformatic analysis of *Siphoviridae* phages revealed that majority of them (125 phages, 98.4%) had lysogen decision gene clusters in their genomes, implying that these phages are temperate. The remaining two phages, SA97 and YMC/09/04/R1988 MRSA BP, do not have CI− or Cro-like

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https://doi.org/10.1016/j.fm.2019.03.025
Received 12 December 2018; Received in revised form 27 March 2019; Accepted 28 March 2019

repressors in the lysogen decision gene clusters (Chang et al., 2015; Jeon et al., 2014), suggesting that these two phages may be virulent. However, only phage SA97 was experimentally characterized and proven to be a virulent phage (Chang et al., 2015). Virulent phages are generally preferred as biocontrol agents due to low frequency of gene transfer compared to the temperate phages (Chang et al., 2015; Guenther et al., 2009). Therefore, genetic engineering of the lysogen decision gene cluster in *S. aureus*-infecting *Siphoviridae* temperate phages to obtain virulent ones is a useful strategy for further various applications to control *S. aureus*. A previous study reported that two temperate phages in the *Siphoviridae* family were converted to virulent ones by random gene mutations using sodium pyrophosphate and that the virulent mutant phages lysed the host strain efficiently, suggesting they may have potential as novel biocontrol agents against *S. aureus* (Garcia et al., 2007).

In this study, a novel *Siphoviridae* phage, SA13, was isolated and characterized to control *S. aureus*. SA13 is a temperate phage which forms lysogen but has strong host growth inhibition activity. To develop SA13 as an efficient biocontrol agent, phage genes were randomly deleted by incubation with sodium pyrophosphate and mutant phages forming clear plaques were isolated. Among them, a mutant phage SA13m was selected and its whole genome was sequenced and analyzed to figure out mutated genes. Characterization of SA13m showed that it is a virulent phage showing rapid and long-lasting host cell growth inhibition activity. Subsequent food application tests in *S. aureus*-contaminated milk were conducted to evaluate the potential of SA13m as a novel biocontrol strategy against *S. aureus* in foods.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 2. All of these strains were grown with shaking at 220 rpm in tryptic soy broth (TSB) medium (Difco, Detroit, MI) at 37 °C for 12 h. The tryptic soy agar (TSA) plate was prepared with TSB supplemented with 1.5% Bacto agar (Difco) and soft top agar containing TSB was prepared with 0.4% agar for SA13 and SA13m phages plaque confirmation. The Baird-Parker medium (Difco, Detroit, MI) containing 1.5% (w/v) agar was used for selective enumeration of *S. aureus* in food application test.

2.2. Bacteriophage isolation and propagation

Bacteriophage SA13 was isolated from a goat fecal sample using *S. aureus* ATCC 29213 as a host strain. To isolate the phage, twenty-five grams of the goat fecal sample was mixed with 225 ml of sodium chloride and magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM $MgSO_4$, and 50 mM Tris-HCl, pH 7.5) and homogenized (Park et al., 2012). Twenty-five milliliters of the mixture were diluted with the same volume of 2X fresh TSB supplemented with 10 mM $CaCl_2$ (final concentration in total 50 ml). After 1% subinoculation of the host strain *S. aureus* ATCC 29213, the culture was incubated at 37 °C for 12 h. The sample was centrifuged at 8000 x g for 10 min, and the supernatant was filtered using a 0.22-μm filter (Millipore, MA) to remove bacterial cells. A hundred microliters of the supernatant were mixed with 5 ml of 0.4% molten soft agar containing 1% *S. aureus* ATCC 29213 and 10 mM $CaCl_2$ (final concentration) and the mixture was overlaid on a TSA plate. To isolate the pure individual phage, a single phage plaque was selected and resuspended in 200 μl of fresh SM buffer. These steps for isolation and purification of a single phage were performed five times. For phage propagation, the host strain *S. aureus* ATCC 29213 was inoculated (1%) and incubated with fresh TSB supplemented with 10 mM $CaCl_2$ (final concentration) at 37 °C for 1.5 h. After incubation of the culture, the phage was added to the culture at a multiplicity of infection (MOI) of 1 and incubated at 37 °C for 3 h. This phage propagation method was serially performed with three different culture volumes (3, 30, and 300 ml of the culture) to obtain more phages, followed by centrifugation at 8000 × g for 10 min and subsequent filtration with 0.22-μm filters for removal of host cells. To obtain a high-titer phage, the isolated phage particles were precipitated for overnight at 4 °C with polyethylene glycol (PEG) 6000 (10%, final concentration) (Junsei, Japan), and concentrated using CsCl gradient density ultracentrifugation (Himac CP 100β; Hitachi, Japan) with a concentration gradient (densities = 1.3, 1.45, 1.5 and 1.7 g/ml) at 78,500 × g for 2 h at 4 °C (Park et al., 2012). The obtained phage particles were purified with dialysis for 1 h. To confirm the phage titer, 10 μl of each serially diluted phage was spotted onto TSA plates covered with 0.4% soft agar containing 1% (final concentration) *S. aureus* ATCC 29213 and 10 mM (final concentration) $CaCl_2$ and the number of plaques was counted. Purified phage was stored at 4 °C.

2.3. Transmission electron microscopy (TEM)

Morphological analysis of the SA13 phage was performed with TEM (JEM-2100, JEOL, Tokyo, Japan). Staining of the phage and morphological observation were performed as previously described by Shin et al. (2014). Based on the observed morphological characteristics of the SA13 phage, it was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (Rodhain, 1995).

2.4. Lysogen confirmation test

A prophage-free *S. aureus* RN4220 was used for lysogen test (Bae et al., 2006). To evaluate the ability of the phage to form lysogen, isolation of resistant colonies and induction of prophages was performed after SA13 infection as described previously (Goffart-Roskam, 1965) with some modifications. After phage infection (MOI of 1, 10^8 PFU/ml) of *S. aureus* RN4220, the surviving colonies were isolated after 32 h incubation. Among the resistant bacteria, 50 colonies were randomly selected and induced with mitomycin C (0.5 μg/ml, final concentration) and incubated with shaking at 220 rpm at 37 °C for 2 h

Table 1
Gene annotation of the lysogen decision gene cluster in the SA13 genome.

ORF[a]	Predicted function	Length[b]	BLASTP best matches	% identity[c]	Accession no.
SA13_01	integrase	461	Putative phage recombinase/integrase [*Staphylococcus* phage P954]	100 over 461 aa	ACV04942.1
SA13_02	hypothetical protein	226	Hypothetical protein [*S. aureus*]	100 over 226 aa	WP_000392109.1
SA13_03	hypothetical protein	241	Hypothetical protein phiETA2_gp04 [*Staphylococcus* phage phiETA2]	100 over 241 aa	YP_001004264.1
SA13_04	hypothetical protein	224	Hypothetical protein [*S. aureus*]	100 over 224 aa	WP_000775187.1
SA13_05	CI repressor	110	Cro/CI family helix-turn-helix domain protein [*S. aureus* CBD-635]	100 over 110 aa	EOR90015.1
SA13_06	Cro repressor	64	Cro protein [*Staphylococcus* phage phiETA2]	100 over 64 aa	YP_001004267.1
SA13_07	antirepressor	254	Phage antirepressor protein KilAC domain protein [*S. aureus* 21272]	99 over 254 aa	EHO97181.1

[a] Each predicted ORF of the SA13 phage was labeled with locus_tag.
[b] Number of amino acids.
[c] Amino acid (aa) sequence identity.

Table 2
Host range of bacteriophages SA13 and SA13m.

Bacterial strain	Plaque formation of SA13[a]	Plaque formation of SA13m[a]	Reference or Source[b]
Staphylococcus aureus strains			
ATCC 6538	+ + +	+ + +	ATCC
ATCC 23235	+	+	ATCC
ATCC 29213	+ +	+ +	ATCC
Newman	+ +	+ +	Oku et al. (2009)
RN4220	+	+	Park et al. (2010)
ATCC12600	+	+	ATCC
MRSA CCARM 3089	+ +	+ +	CCARM
MRSA CCARM 3090	+ +	+ +	CCARM
Other Gram-positive bacteria			
Enterococcus faecalis ATCC 29212	–	–	ATCC
Bacillus cereus ATCC 14579	–	–	ATCC
Bacillus subtilis ATCC 23857	–	–	ATCC
Listeria monocytogenes ATCC 19114	–	–	ATCC
Gram-negative bacteria			
Salmonella enterica serovar Typhimurium SL1344	–	–	ATCC
Escherichia coli O157:H7 ATCC 35150	–	–	ATCC
Cronobacter sakazakii ATCC 29544	–	–	ATCC
Pseudomonas aeruginosa ATCC 27853	–	–	ATCC

[a] , + + +, efficiency of plating (EOP) of 2 to 1; + +, EOP of 1 to 0.2; +, EOP $<$ 0.2; -, not susceptible to phage.
[b] , ATCC, American Type Culture Collection; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

(Chang et al., 2015). After incubation, the culture was centrifuged at 10,000 × g for 5 min, and the supernatant was transferred to a new tube. Residual bacterial cells were removed by filtration of the supernatant with a 0.22 μm syringe filter and the filtered supernatant was spotted onto TSA plates covered with 0.4% molten soft agar containing *S. aureus* RN4220 (1% inoculum). The number of resistant colonies that were able to reproduce plaques by mitomycin C induction were regarded as SA13 lysogenized colonies and counted (Goffart-Roskam, 1965). The rate of lysogen formation was calculated by dividing the number of lysogenized colonies with the initial number of randomly selected colonies (fifty colonies). These experiments were performed in triplicate, and the rate of lysogen formation is indicated as a % value with standard deviations.

2.5. Bacteriophage genome sequencing and bioinformatics analysis

Prior to isolation and purification of the phage genomic DNA, phage particles were treated with DNase I and RNase A at 37 °C for 1 h to remove contamination of bacterial DNA and RNA. Phage genomic DNA was isolated and purified as previously described by Wilcox et al. (1996). The genomic DNA was sequenced using a Genome Sequencer FLX Titanium Sequencer (Roche, Mannheim, Germany) and assembled with GS de novo assembler software (Roche) at Macrogen Inc. (Seoul, Korea). The ORFs were predicted and compared with FgenesB software (http://www.softberry.com), Glimmer v3.02 (Delcher et al., 2007), and GeneMarkS (Lukashin and Borodovsky, 1998). Predicted ribosome binding sites (RBSs) by RBSfinder (J. Craig Venter Institute, Rockville, MD, USA) were used to confirm the predicted ORFs. Conserved protein domain analysis using InterProScan5 (Jones et al., 2014) and BLASTP (Mount, 2007) were performed for functional analysis of predicted ORFs. Putative tRNAs were predicted using tRNAscan-SE 1.21.

2.6. Preparation of a virulent mutant phage SA13m

The virulent mutant of the temperate SA13 phage was obtained by the DNA random deletion method using pyrophosphate as previously described (Ladero et al., 1998) with some modifications. Briefly, the wild-type phage SA13 (10^6 PFU) was mixed with 100 mM sodium pyrophosphate (pH 7.4) and incubated for 60 min at 37 °C. After incubation, phages forming clear plaques were selected, picked, and suspended in fresh SM buffer. The phage in SM buffer was treated again with the same concentration of sodium pyrophosphate and incubated under the same conditions. The overall mutation process was repeated 25 times. After repetitive mutations, a virulent mutant phage forming clear plaques, SA13m, was then selected.

2.7. Bacterial challenge assay

The host strain *S. aureus* ATCC 29213 (1%) was subinoculated into 50 ml of fresh TSB and the culture was aerobically incubated with shaking at 220 rpm at 37 °C until it reached the early exponential growth phase (OD = 0.35 at 600 nm wavelength, 10^8 CFU/ml). The phage at an MOI of 1 (10^8 PFU/ml) was added to the culture and incubated at 37 °C for up to 32 h. Aliquots were collected after every 0.5–4 h. Growth of the host strain was monitored by its optical density with a spectrophotometer (Amersham Biosciences Ultrospec 3100 Pro UV/Visible spectrophotometer, GE Healthcare, Chicago, IL, USA) at a wavelength of 600 nm. All experiments were performed in triplicate.

2.8. Determination of the frequency of bacteriophage-insensitive mutants (BIMs)

Frequency of bacteriophage-insensitive mutants (BIMs) was determined as previously described (O'Flynn et al., 2004). Briefly, either SA13 or SA13m phage was mixed with *S. aureus* (10^9 CFU/mL) wild-type cell suspension containing 10 mM of $CaCl_2$ at an MOI of 100 (10^{11} PFU/ml). The initial viable cells were counted before mixing of *S. aureus* cell suspension and phage. After incubation at 37 °C for 10 min, mixtures were serially diluted and plated on a TSA plate. After overnight incubation at 37 °C, the number of remaining cells were counted, and the BIMs frequency was determined by dividing the number of remaining cells by the initial viable cell number. All experiments for formation of BIMs were conducted in triplicate.

2.9. One-step growth curve assay

One-step growth curve analysis was performed as previously described (Chang et al., 2015). Briefly, phage was mixed with *S. aureus* ATCC 29213 (10^7 CFU/ml) in its early exponential growth phase at an MOI of 0.001 (10^4 PFU/ml) in the presence of 10 mM $CaCl_2$ and allowed to adsorb for 10 min at room temperature, followed by centrifugation at 6000 x g for 10 min. The pellet containing infected cells was suspended in 50 mM of fresh TSB liquid medium and incubated at 37 °C with aeration. Two sets of samples were taken every 5 min up to 1 h and $CHCl_3$ was added to one duplicate to release the intracellular phages. Subsequently, each sample was immediately titered by the double-layer agar plate method. Briefly, *S. aureus* ATCC 29213 grown overnight (50 μL) and the obtained phage in each time period (100 μL) were mixed with 5 mL of 0.4% TSA molten soft agar and overlaid on TSA agar plates. After incubation at 37 °C for 12 h, the phage plaques were counted and the eclipse period, latent period, and burst size were determined. All experiments were performed in triplicate.

2.10. Host range analysis

The bacterial indicator strains were incubated at 37 °C for 12 h. Each bacterial culture (1%) was added to 5 ml of 0.4% molten soft agar, and the mixture was overlaid on TSA plates and dried for 20 min. Then serially diluted phage solutions (10^1 to 10^5 PFU/ml) were dotted on the plate. After incubation, the clarity of the plaques was determined, and efficiency of plating (EOP) was calculated by comparing the phage titer on the test strain and the phage titer on *S. aureus* ATCC 29213.

2.11. Determination of host receptor

The host strain *S. aureus* RN4220 was used for determination of the host receptor of the SA13 and SA13m phages (Wann et al., 1999). The *ΔtagO*/RN4220 knockout mutant strain and *ΔtagO*/RN4220::pBR474-*tagO* complementation strain were obtained from Oku et al. (2009). To identify the phage receptor, spotting assays with the wild type RN4220 strain, *ΔtagO*/RN4220 mutant, and *ΔtagO*/RN4220::pBR474-*tagO* complementation strain were conducted and compared.

2.12. Food application

Pasteurized whole milk was purchased at a local market (Seoul, South Korea). The milk sample was inoculated with *S. aureus* ATCC 29213 (10^5 CFU/ml) and pre-incubated at either 4 °C or 25 °C for 1 h. Subsequently, the phage was added to *S. aureus*-contaminated milk (5 mL) at a MOI of 10^2 or 10^3 (10^7 or 10^8 PFU/ml). The milk samples were incubated in a static state at 4 °C and 25 °C for up to 48 h, respectively. The number of viable cells were enumerated on Baird-Parker agar (Difco) at 3, 6, 12, 24 and 48 h time points. All tests were conducted in triplicate. One-way analysis of variance (ANOVA) with Duncan's multiple range tests was used for the comparison in each condition.

2.13. Nucleotide sequence accession number

The complete genome sequence and annotation results of *S. aureus* phage SA13 are available in the GenBank database under accession number NC_021863.1.

2.14. Statistical analysis

Statistical analysis was conducted using SAS software (version 9.4, SAS Inst., Cary, NC., USA). The one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test (95% confidence interval) were done. The data are presented as means with standard deviations. A *P*-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Isolation and morphological characteristics of bacteriophage SA13

S. aureus-infecting phage SA13 was isolated from a goat fecal sample (Seoul National University Farm, Suwon, Korea) using *S. aureus* host strain ATCC 29213. Transmission electron microscopy (TEM) analysis revealed that the SA13 phage has an icosahedral capsid (60 ± 2 nm) with a long, flexible and non-contractile tail (150 ± 3 nm), indicating that it belongs to the family *Siphoviridae* (Fig. 1). As SA13 forms turbid plaques on the host strain and most *Siphoviridae* family phages are known to have a lysogenic cycle (Hsieh et al., 2011), SA13 was expected to be a temperate phage. To elucidate if SA13 forms lysogen, the SA13-resistant strains were isolated after SA13 infection to *S. aureus* RN4220 strain and were induced with mitomycin C. The SA13 phage was regenerated from the SA13-resistant strain (data not shown), substantiating that it is a lysogen and SA13 is a temperate phage. Despite being a temperate phage, SA13 showed relatively strong growth inhibition activity against *S. aureus*. Therefore, SA13 was subjected to further experiments to obtain a virulent mutant phage.

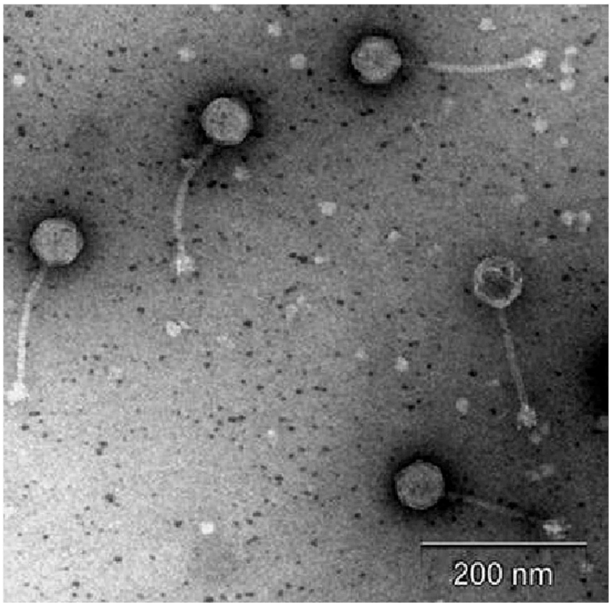


Fig. 1. TEM image of the SA13 phage from the *Siphoviridae* family. The phage was negatively stained with 2% (w/v) uranyl acetate and observed using TEM JEM-2100 (JEOL, Tokyo, Japan) at 200 kV.

3.2. Genome sequence analysis of SA13

The complete genome sequence of the SA13 phage was determined. The genome of SA13 is comprised of double-stranded chromosomal DNA consisting of 42,652 bp with 62 predicted open reading frames (ORFs) and no tRNA gene with a GC content of 35.4%, which is slightly higher than that of *S. aureus* host strains (33.0% average) (Muto and Osawa, 1987). In addition, no known toxin production- and bacterial virulence-associated genes were detected in the genome, suggesting that this phage may be safe to be used as a biocontrol agent. All annotated ORFs can be categorized into five functional groups: (i) lysogen decision gene cluster for lysogen formation (integrase, repressor, CI repressor, Cro protein, and antirepressor), (ii) DNA synthesis and replication (single-stranded DNA-binding (SSB) protein, phage replication protein, dUTP diphosphatase, rho-termination factor domain-containing protein, and transcriptional regulator RinAB), (iii) DNA packaging (phage terminase small/large subunits and phage portal protein), (iv) phage structure/morphogenesis (head morphogenesis protein, major capsid protein, head-tail connector/adaptor protein, tape measure protein, major tail protein, putative tail fiber proteins, putative tail tip protein, tail lysin, and tail assembly protein), and (v) host lysis proteins (putative holin and endolysin) (Fig. 2). Presence of lysogen decision gene cluster in SA13 genome indicated that SA13 phage is a temperate phage.

3.3. Construction and selection of a virulent mutant from phage SA13

It is widely known that virulent phages are suitable to be developed as biocontrol agents. Although the SA13 phage showed very efficient *S.*

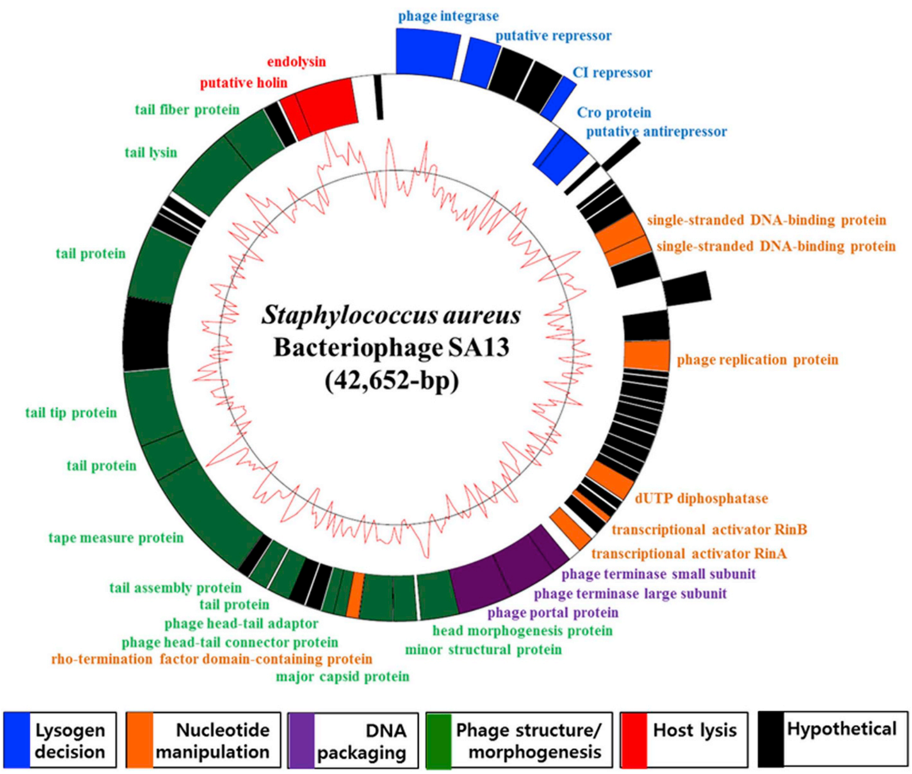


Fig. 2. Genome map of the SA13 phage. The inner circle with the red line indicates the GC content. The outer circle indicates the locations of predicted ORFs with different colors: Blue, lysogen decision; orange, replication and recombination; purple, DNA packaging; green, phage structure/morphogenesis; red, host lysis; black, hypothetical proteins. Annotated proteins are also indicated with different colors in the same way. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

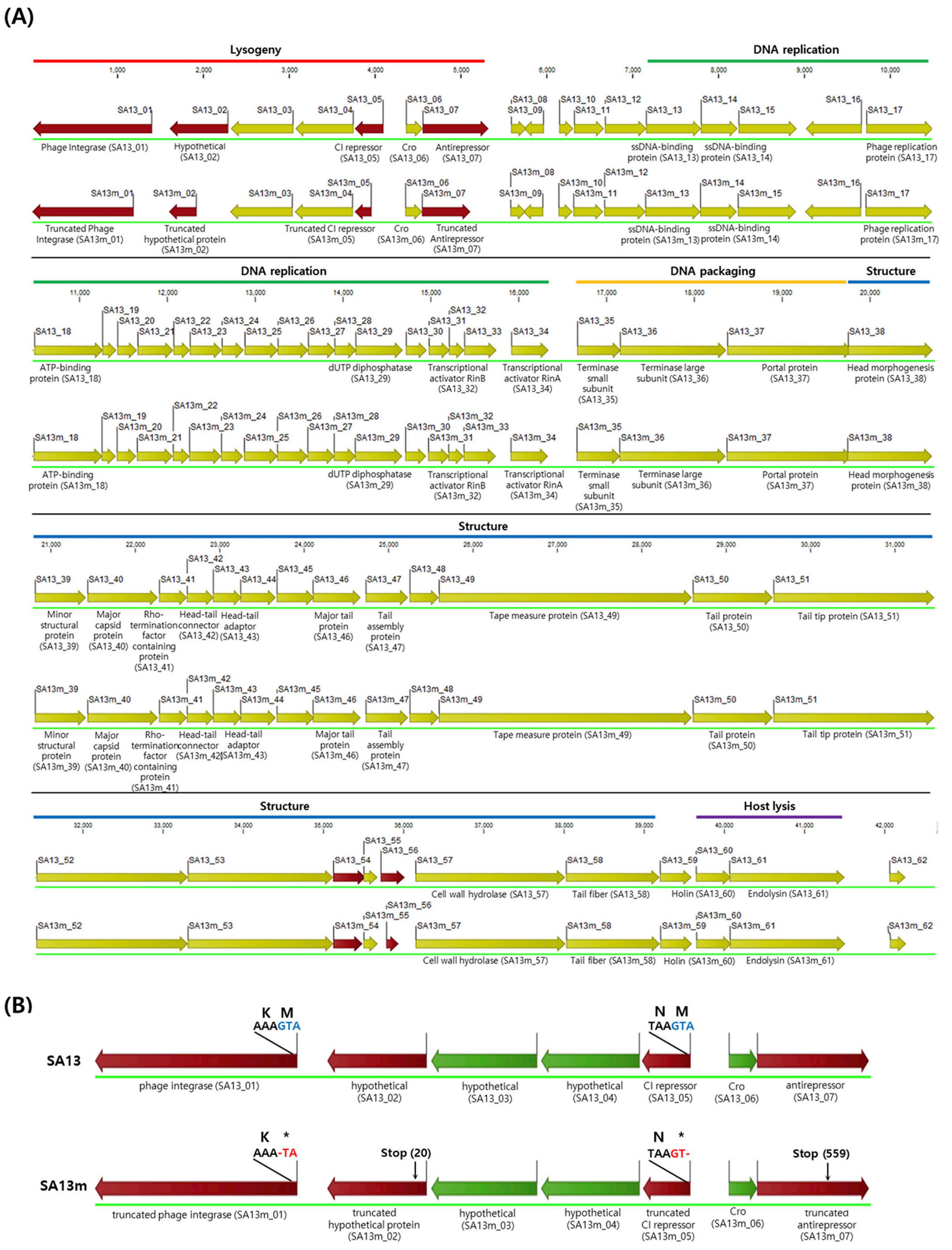
aureus growth inhibition activity, its lysogenic life cycle makes it unsuitable to be used as a biocontrol agent. It has been shown that pyrophosphate can be used for DNA mutation of extrachromosomal elements including plasmids and viruses (Quinn et al., 2011). Previous studies have reported that sodium pyrophosphate destabilizes the phage capsid by chelating the cations in the head structure and resulting in the generation of mutant phages, which contain spontaneously deleted DNA fragments. As the deleted DNA genomes are shorter than the intact ones, packaging of the deleted DNA fragments would lessen the pressure inside the phage head structure, and consequently, only mutant phage particles containing the deleted DNA fragments would remain stable in the presence of sodium pyrophosphate (Garcia et al., 2007; Gutiérrez et al., 2018). Based on this, random deletion of SA13 DNA using sodium pyrophosphate and subsequent mutant phage selections was performed in order to obtain a mutant phage SA13m that generates clear plaques. As mentioned above, the combinational mitomycin C induction experiment with PCR analysis and genome sequence analysis confirmed that SA13 is a temperate phage. However, subsequent phage induction of a SA13m-resistant strain using mitomycin C did not reproduce phage SA13m (data not shown), substantiating that the host strain infected by SA13m does not form lysogens.

3.4. Comparative genome analysis of SA13 and the virulent mutant phage SA13m

To verify the mutation site in the genome of the selected virulent mutant phage, whole genome sequence analysis of SA13m was performed and compared with that of SA13. Four genes (SA13m_01, SA13m_02, SA13m_05, and SA13m_07) in the lysogen decision gene cluster of SA13m phage were truncated. (Table 1 and Fig. 3A). These truncated genes encode putative integrase, hypothetical protein, putative CI repressor, and putative antirepressor proteins, respectively, which are important for lysogen formation (Table 1). Genome sequence analysis revealed that deletion of nucleotides (nt) occurred in all four genes (putative integrase, 4 nt deletions at positions 3, 41, 190, and 207; hypothetical protein, 13 nt deletions at positions 18, 23, 24, 43, 44, 45, 46, 53, 54, 166, 231, 286, and 356; putative CI repressor, 4 nt deletions at positions 1, 34, 129, and 319; and 5 nt deletions at positions 508, 533, 592, 610, and 662 for the putative antirepressor, respectively; all numberings are based on the ORF start site of each gene as 1). Nucleotide deletions resulted in the loss of start codons of two genes (SA13m_01 and SA13m_05), and the truncated ORFs were predicted by using alternative start codons (TTG for SA13m_01 and GTG for SA13m_05) (Fig. 3B). Deletion of nucleotides in the other two genes (SA13m_02 and SA13m_07) caused frameshift mutations to generate internal stop codons (Fig. 3B). Therefore, overall deletion mutations in those four genes may produce nonfunctional proteins. In particular, mutations in the putative integrase (SA13m_01) would result in no insertion of the phage genome into the host chromosome (Susskind and Botstein, 1978; Vander Byl and Kropinski, 2000). Even if the phage genome is inserted, the presence of the truncated putative CI repressor (SA13m_05) would prevent the lysogenic cycle (Garcia et al., 2007). In addition, nucleotide deletions were observed in two other hypothetical genes (SA13m_54 and SA13m_56) (Fig. 3A). However, SA13m has intact tail structure genes and a lysis gene cluster including putative endolysin and putative holin (Fig. 3A), which are known to be important for host recognition and host lysis, respectively. Therefore, it is speculated that truncation of two hypothetical genes (SA13m_54 and SA13m_56) would have little effect on the phage host range and growth inhibition nature.

3.5. Comparative host growth inhibition activity by SA13 and SA13m

As some genes associated with the lysogen decision gene cluster were truncated, it was expected that SA13 and SA13m would show different growth inhibition patterns against the host strain *S. aureus* ATCC 29213. To investigate this, a challenge assay of SA13 and SA13m against *S.*



(caption on next page)

Fig. 3. Whole genome comparison between the two phages, SA13 and SA13m. (A) Red and yellow arrows represent truncated and non-truncated genes, respectively. (B) Comparative sequence analysis of genes in lysogen decision gene clusters in SA13 and SA13m phages. Red and green arrows represent truncated and non-truncated genes, respectively. Intact start codon sequences in the SA13 genome and nucleotide deletions (indicated as '-') in start codons of the SA13m genome are colored blue and red, respectively. Translated amino acid sequences are shown above the nucleotide sequences and asterisks indicate non-translated start codons. Generation of internal stop codons are indicated as 'stop'. Nucleotide positions where the internal stop codons started are shown with parentheses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

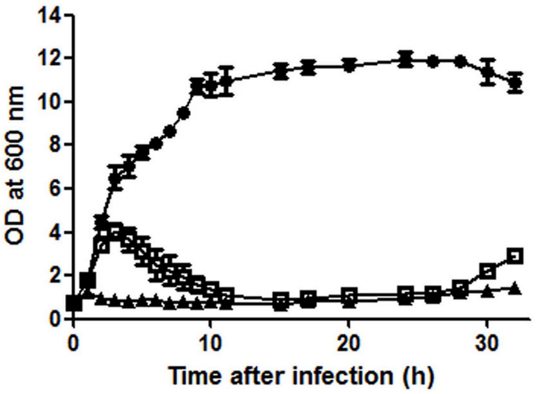


Fig. 4. Bacterial challenge test of SA13 and SA13m phages (10^8 PFU/ml each) with *S. aureus* ATCC 29213 (MOI of 1, 10^8 CFU/ml). The closed circles (●) indicate non-phage-treated *S. aureus* ATCC 29213 as a control; the open squares (□) indicate SA13-treated *S. aureus* ATCC 29213; the closed triangles (▲) indicate SA13m-treated *S. aureus* ATCC 29213. The data shown are the mean values from three independent measurements and the error bars represent the standard deviation.

aureus ATCC 29213 was conducted and results were compared. After SA13 infection, the bacterial host strain grew like the non-phage treated host strain for 3 h. After that, host cell lysis was initiated, and the host growth inhibition persisted for 28 h; however, development of phage-insensitive strains was observed shortly thereafter (Fig. 4). Addition of SA13m to the host cell showed rapid cell lysis within 1 h, while host cell lysis started 3 h after treatment with SA13. Moreover, in contrast with the SA13 phage, inhibition of the host cell growth was sustained up to 32 h by treatment with SA13m (Fig. 4). The experiments were repeated three times. The host growth inhibition activity of SA13m may be more persistent than that of the virulent phage SA97, which inhibits the growth of the host strain up to 7 h (Chang et al., 2015). Among the many resistance mechanisms, bacteria can develop resistance (i.e. bacteriophage-insensitive mutants; BIM) against phage infection by modifying or blocking host receptors or lysogen formation in the host cells (Hancock and Reeves, 1976; Labrie et al., 2010). Given that the rate of lysogen formation by SA13 is relatively high (78.02 ± 8.36%), development of BIMs by SA13 treatment is most likely due to lysogen formation. It has been previously reported that the frequency of BIMs of virulent phages is much lower than that of temperate ones because of the lysogenic state of the host cells by the temperate phages (Garcia et al., 2007). Further bacteriophage-insensitive mutants (BIMs) frequency tests of SA13 and SA13m, which showed that the BIMs frequency of SA13 ($2.4 \times 10^{-2} \pm 7.88 \times 10^{-3}$) was approximately 3.6×10^4-fold higher than that of SA13m ($6.67 \times 10^{-7} \pm 5.77 \times 10^{-7}$), support this conclusion. The one-step growth analysis results showed that the eclipse periods of both phages lasts 15 min, but the SA13m phage has shorter latent periods and a larger burst size than SA13. (Fig. 5). The latent periods of SA13m and SA13 were 20 and 25 min and the average burst sizes were 95 and 85 PFU per cell, respectively (Fig. 5). The results of the one-step growth analysis suggest that SA13m has better multiplication efficiency than SA13, which is most likely due to the inability of SA13m to form lysogens (Shin et al., 2014). Consequently, these results showed the potential of SA13m as an antimicrobial agent against *S. aureus*.

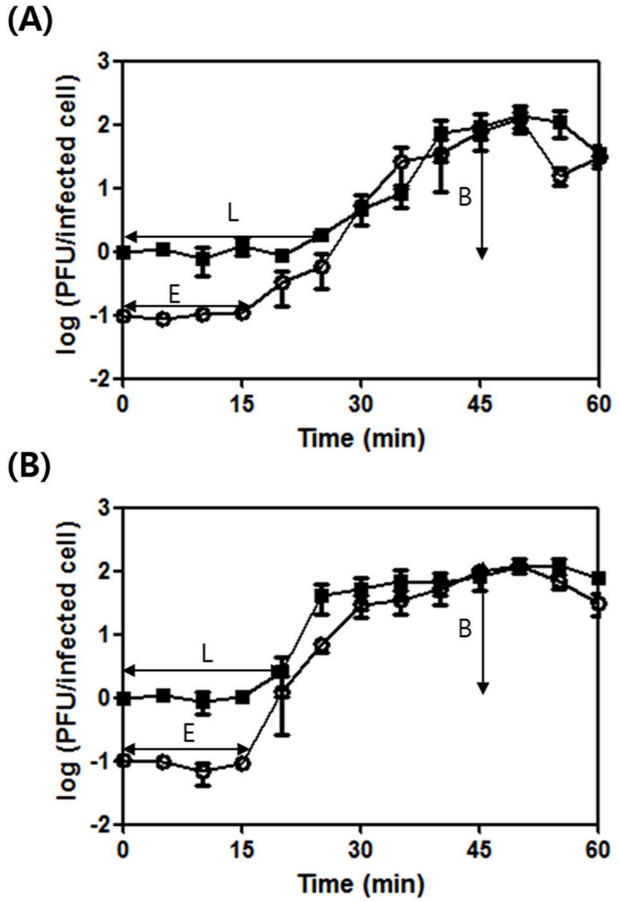


Fig. 5. One-step growth curve analysis of the SA13 and SA13m phages on an exponential culture of *S. aureus* ATCC 29213. E, eclipse period; L, latent period; B, burst size. Closed squares indicated non-chloroform-treated samples, and opened circles indicated chloroform-treated samples. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation.

3.6. Identification of the phage receptor on the host strain

To date, only one host receptor of *S. aureus* has been reported: peptidoglycan-anchored wall teichoic acid (WTA) (Xia et al., 2011). Among the gene products associated with the WTA biosynthesis pathway, TagO is an enzyme that transfers *N*-acetylglucosamine-1-phosphate to undecaprenyl phosphate, producing a base compound for elongation of glycerol phosphates as an initial step of WTA biosynthesis (Atilano et al., 2010). Therefore, a *ΔtagO* mutant of *S. aureus* (RN4220*ΔtagO*) and its complementation strain (*ΔtagO*/RN4220::pBR474-*tagO*) (Park et al., 2010) were subjected to phage SA13 infection to determine the host receptor of this phage. Infection of the *ΔtagO* mutant strain with the SA13 phage showed insensitivity to phage infections. Subsequent phage infections of the *tagO*-complementation strain showed a recovery of sensitivity, indicating that the host receptor of this phage is WTA (Fig. 6A). Subsequent receptor analysis of the virulent mutant phage SA13m revealed that SA13m also utilized WTA for infection similar to the SA13 phage (Fig. 6B).

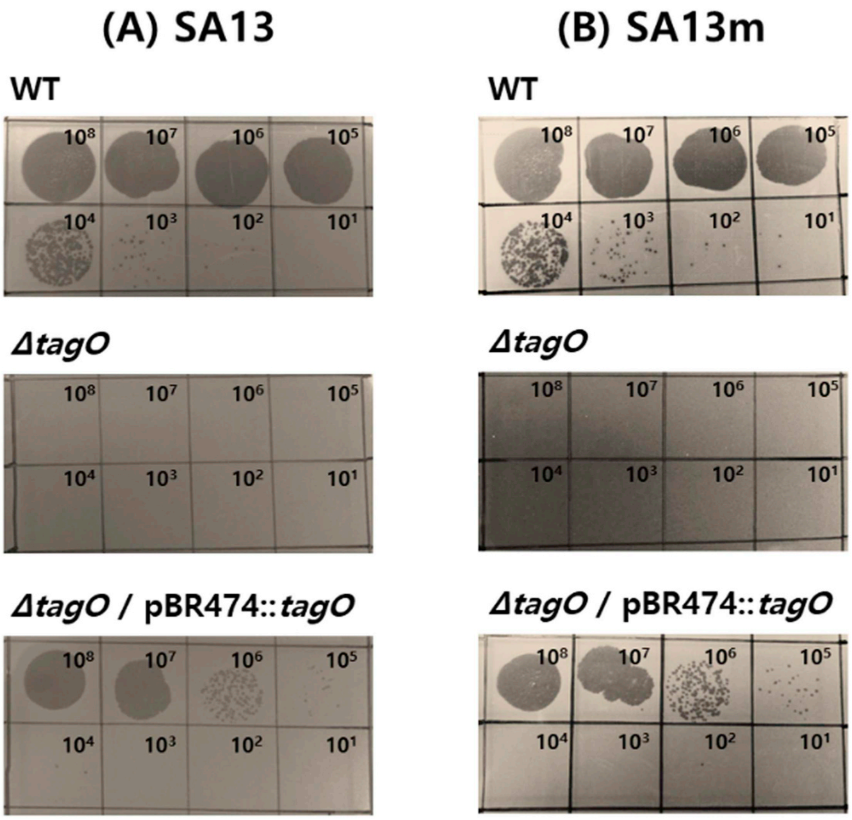


Fig. 6. Determination and confirmation of *S. aureus* RN4220 host receptor of (A) SA13 and (B) SA13m using the wild-type RN4220 strain, the mutant strain (*ΔtagO*/RN4220), and its complementation strain (*ΔtagO*/RN4220::pBR474-*tagO*). Ten-fold serially diluted phage samples (from 10^1 to 10^8 PFU/ml) were dotted.

3.7. Host range analysis

The host ranges of the SA13 and SA13m phages were determined using *S. aureus* strains as well as other Gram-positive and Gram-negative bacteria (Table 2). The results showed that both phages have the same host range, substantiating that the mutations found in 6 genes of SA13m do not affect its host recognition and infection ability. In addition, both phages can infect MRSA strains, suggesting the virulent mutant phage SA13m may also be an efficient biocontrol agent to control MRSA (Table 2). Furthermore, they cannot infect other Gram-positive and Gram-negative bacteria, indicating these phages have high host specificity to *S. aureus*.

3.8. Phage application in milk

There are few studies about phage applications to reduce *S. aureus* in milk (Garcia et al., 2009; Tabla et al., 2012). However, these studies were based on temperate phages (Garcia et al., 2009) or a virulent phage cocktail treatment (Tabla et al., 2012). To evaluate the potential of the virulent mutant phage SA13m as a novel biocontrol agent against *S. aureus,* a food application experiment with milk was conducted. When the SA13m phage (10^7 PFU/ml) at an MOI of 10^2 was added to *S. aureus* (10^5 CFU/ml)-contaminated milk samples at refrigerator temperature (4 °C), the bacterial strain was totally lysed to non-detectable levels, achieving a 4.1-log CFU/ml reduction in 24 h with no recovery at all up to 48 h (Fig. 7A). In a previous study, approximately 3.0-log reduction of *Salmonella* Typhimurium cells was shown in 1 day after the FO1-E2 phage (MOI of 3×10^5) treatment in chocolate milk at 8 °C (Guenther et al., 2012). SA13m showed strong bactericidal effect even in low MOI treated condition, suggesting that it is a good candidate for *S. aureus* biocontrol agent at refrigerator temperature. Meanwhile, addition of the same amount of SA13m (MOI of 10^2, 10^7 PFU/ml) at room temperature (25 °C) achieved more than 4.0-log CFU/ml reduction in viable cell number after 48 h (Fig. 7B). When the SA13m phage at a MOI of 10^3 (10^8 PFU/ml) was added to the sample and incubated at 4 °C, the bacterial strain was lysed to non-detectable levels in 12 h and achieved a 4.2-log CFU/ml reduction in viable cell number, which did not recover up to 48 h (Fig. 7A). Viable cell counts in the SA13m-treated milk samples stored at 25 °C were reduced to non-detectable levels (4.2-log reduction in CFU/ml) in 3 h and did not recover until 48 h, achieving an 8.7-log CFU/ml reduction compared to the non-treated sample (Fig. 7B). Previously, it was reported that treatment of *S. aureus*-contaminated milk with a phage cocktail comprised of two phages at a MOI of 10^2 resulted in an approximate 2.0-log reduction in CFU/ml of bacteria cell number at room temperature (Tabla et al., 2012). Therefore, high host lysis activity of the SA13m phage in milk samples implies that it is a good candidate for a novel biocontrol agent to control *S. aureus* in milk.

4. Conclusions

In this study, we obtained and characterized a virulent mutant (SA13m) by random deletion mutation of temperate phage SA13. Comparative genomic analysis confirmed the deletion of several nucleotides in six genes including the genes in the lysogeny-decision gene cluster and two hypothetical genes. In addition, SA13m phage did not to form lysogens and showed rapid host cell lysis activity compared with SA13 supporting that the mutant SA13m phage is a virulent phage. The virulent mutant phage SA13m could be used as a promising biocontrol agent as it efficiently controlled the growth of *S. aureus* in milk. Our results confirm that the introduction of random mutations into temperate phage genomes to obtain a virulent phage would be a useful strategy to develop an efficient biocontrol agent against *S. aureus*.

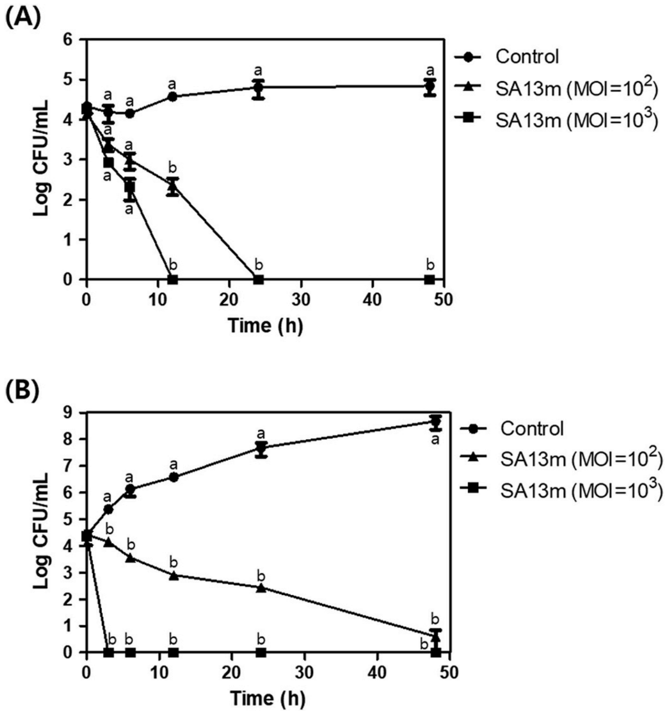


Fig. 7. Application of the virulent mutant phage SA13m in milk. Bactericidal ability of the SA13m phage against *S. aureus* ATCC 29213. The assay was performed in pasteurized whole milk at (A) 4 °C and (B) 25 °C for 48 h. Phages were added at a MOI of 10^2 or 10^3. ●, *S. aureus* ATCC 29213; ▲, MOI of 10^2 SA13m phage (10^7 PFU/ml) treated sample; ■, MOI of 10^3 SA13m phage (10^8 PFU/ml) treated sample. Each point represents the mean of triplicate experiments and error bars indicate the standard deviation. The lowercase letters indicate significant differences ($P < 0.05$) between the test groups.

Acknowledgements

This work was support by the National Research Foundation of Korea (NRF) grant funded by the MSIP (NRF-2017R1A2A1A17069378), Republic of Korea, and Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry (IPET) through the Agriculture, Food and Rural Affairs Research Center Support Program, funded by the Ministry of Agriculture, Food and Rural Affairs (710012-03-1-SB110).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.025.

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