

The Presence of Two Receptor-Binding Proteins Contributes to the Wide Host Range of Staphylococcal Twort-Like Phages

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ABSTRACT

Thanks to their wide host range and virulence, staphylococcal bacteriophages (phages) belonging to the genus *Twortlikevirus* (staphylococcal Twort-like phages) are regarded as ideal candidates for clinical application for *Staphylococcus aureus* infections due to the emergence of antibiotic-resistant bacteria of this species. To increase the usability of these phages, it is necessary to understand the mechanism underlying host recognition, especially the receptor-binding proteins (RBPs) that determine host range. In this study, we found that the staphylococcal Twort-like phage Φ SA012 possesses at least two RBPs. Genomic analysis of five mutant phages of Φ SA012 revealed point mutations in *orf103*, in a region unique to staphylococcal Twort-like phages. Phages harboring mutated ORF103 could not infect *S. aureus* strains in which wall teichoic acids (WTAs) are glycosylated with α -N-acetylglucosamine (α -GlcNAc). A polyclonal antibody against ORF103 also inhibited infection by Φ SA012 in the presence of α -GlcNAc, suggesting that ORF103 binds to α -GlcNAc. In contrast, a polyclonal antibody against ORF105, a short tail fiber component previously shown to be an RBP, inhibited phage infection irrespective of the presence of α -GlcNAc. Immunoelectron microscopy indicated that ORF103 is a tail fiber component localized at the bottom of the baseplate. From these results, we conclude that ORF103 binds α -GlcNAc in WTAs, whereas ORF105, the primary RBP, is likely to bind the WTA backbone. These findings provide insight into the infection mechanism of staphylococcal Twort-like phages.

IMPORTANCE

Staphylococcus phages belonging to the genus *Twortlikevirus* (called staphylococcal Twort-like phages) are considered promising agents for control of *Staphylococcus aureus* due to their wide host range and highly lytic capabilities. Although staphylococcal Twort-like phages have been studied widely for therapeutic purposes, the host recognition process of staphylococcal Twort-like phages remains unclear. This work provides new findings about the mechanisms of host recognition of the staphylococcal Twort-like phage Φ SA012. The details of the host recognition mechanism of Φ SA012 will allow us to analyze the mechanisms of infection and expand the utility of staphylococcal Twort-like phages for the control of *S. aureus*.

Staphylococcus aureus, a Gram-positive coccus, is a commensal and pathogenic bacterium that causes opportunistic infections in humans and animals. Currently, antibiotic resistance in this species poses a threat to public health. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infections around the world (1). The emergence of such antibiotic-resistant bacteria requires development of alternatives to antibiotic-based therapies. One promising alternative is phage therapy, in which bacteriophages (phages) are used to treat bacterial infections (2, 3). In preclinical trials performed in mice, *S. aureus* infections (including MRSA infections) were successfully treated by use of phages (4). Therefore, phage therapy has attracted great interest as an alternative to antibiotics.

Previously, we isolated the virulent staphylococcal phage Φ SA012, which exerts lytic effects on a wide range of *S. aureus* isolates from bovine mastitis cases (5). Genomic analysis of Φ SA012 revealed that it belongs to the genus *Twortlikevirus*, which contains the *Staphylococcus* phages Twort, K, and G, whereas the genus *SPO1likevirus* contains the *Bacillus* phage SPO1 as well as *Listeria* phages P100 and A511. *Lactobacillus* phage LP65 and *Enterococcus* phage Φ EF24C were classified as orphans within the subfamily (6). Representatives of the two genera share the following features: they (i) have large genomes (127 to 140 kb), (ii) are strictly virulent, (iii) infect Gram-positive, low-GC-content bacteria, (iv) have a wide host range among strains of the susceptible bacterial genus, and (v) share considerable similarities at the

protein sequence level (7). Thanks to their wide host range and highly lytic capabilities, phages in these genera, including Φ SA012, are considered to be promising candidates for therapeutic use and as detection agents (8, 9).

The infection process of phages can be divided into the following steps: phage adsorption to the host, DNA injection into the host cell, DNA replication, assembly of phage particles, and lysis of the host cell. Because adsorption of a phage to the host, caused by the interaction between a phage receptor on the bacterial surface and receptor-binding proteins (RBPs) in the tips of the tail fibers or tail spikes, is extremely specific, RBPs determine the target bacteria for phage infection (10). Therefore, application of phages as

Received 30 May 2016 Accepted 13 July 2016

Accepted manuscript posted online 15 July 2016

Citation Takeuchi I, Osada K, Azam AH, Asakawa H, Miyanaga K, Tanji Y. 2016. The presence of two receptor-binding proteins contributes to the wide host range of staphylococcal Twort-like phages. *Appl Environ Microbiol* 82:5763–5774. doi:10.1128/AEM.01385-16.

Editor: M. J. Pettinari, University of Buenos Aires

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.01385-16>.

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TABLE 1 Bacterial strains, phages, and plasmids

Bacterial strain, phage, or plasmid	Relevant feature(s)	Reference or source
Bacterial strains		
<i>S. aureus</i> strains		
SA003	Host strain, isolated from bovine mastitis, lacks <i>tarM</i>	5
SA003R11	ΦSA012-resistant derivative	60
SA003R20	ΦSA012-resistant derivative	60
RN4220	Restriction-deficient, transformable strain	61
RN4220 Δ <i>tarM</i>	In-frame deletion mutant of <i>tarM</i> in RN4220	This study
RN4220 Δ <i>tarM</i> ::pLIP3_Δ <i>tarM</i>	RN4220 Δ <i>tarM</i> complemented with <i>tarM</i> via the plasmid pLIP3_Δ <i>tarM</i>	This study
<i>E. coli</i> strains		
JM109	Used for plasmid construction	TaKaRa Bio
Rosetta-gami 2(DE3)	Used for expression of recombinant proteins	Novagen
Phages		
ΦSA012	<i>S. aureus</i> lytic phage	5
ΦSA012M1	Spontaneous mutant phage	This study
ΦSA012M2	Spontaneous mutant phage	This study
ΦSA012M11	Spontaneous mutant phage	This study
ΦSA012M20	Spontaneous mutant phage	This study
ΦSA012M38	Spontaneous mutant phage	This study
ΦSA012TM103	Phage harboring three mutations in <i>orf103</i>	This study
Plasmids		
pKOR1	<i>E. coli</i> - <i>S. aureus</i> shuttle vector, temperature sensitive	33
pLI50	<i>E. coli</i> - <i>S. aureus</i> shuttle vector	Addgene
pLIP3_Δ <i>tarM</i>	<i>tarM</i> expression plasmid driven by pLI50	This study
pNL9164	<i>E. coli</i> - <i>S. aureus</i> shuttle vector	Sigma
pET-29a	Expression vector for production of recombinant proteins	Novagen

therapy necessitates an understanding of the interaction between RBPs and phage receptors on the bacterial surface.

The interaction of RBPs with phage receptors on the bacterial surface has been studied widely with *Escherichia coli* phages T4 and lambda (λ). In T4, gp37, located in the tips of long tail fibers, reversibly binds to lipopolysaccharides (LPS) or OmpC in *E. coli* K-12 (11). This first adsorption activates a second adsorption, in which gp12 (the short tail fiber) irreversibly binds to LPS on the cell surface (12). Although many studies of RBPs in phages of Gram-negative bacteria have been carried out, knowledge of RBPs in the phages of Gram-positive bacteria remains limited. To date, RBPs have been identified for *Bacillus subtilis* phages SPP1 and φ29, *Streptococcus thermophilus* phages DT1 and MD4, *Lactococcus lactis* phages bIL67 and CHL92 of the c2 species, sk1, bIL170, and p2 of the 936 species, and TP901-1 and Tuc2009 of the P335 species, and *Listeria* phage A118 (13–22). Among *S. aureus* phages, RBPs have also been identified in *Siphoviridae* phage φSLT and *Podoviridae* AHJD-like phages S24-1 and S13' (23, 24). Remarkably, Habann et al. identified that the RBPs of *Listeria* phage A511 and staphylococcal Twort-like phages ISP and Twort (Gp108, gp40, and gp17, respectively) are located in short tail fibers (25). Among Gram-positive bacteria, cell wall-associated carbohydrates, such as teichoic acids, are regarded as phage receptors of *B. subtilis*, *S. aureus*, and *Listeria monocytogenes* (15, 25–29). In *S. aureus*, siphoviruses need *N*-acetylglucosamine (GlcNAc) in wall teichoic acids (WTAs) for adsorption, whereas myoviruses, such as staphylococcal Twort-like phages, adsorb to the WTA backbone (27). A recent study suggested that staphylococcal podoviruses require α-GlcNAc, but not β-GlcNAc, for adsorption (30). Nevertheless, the mechanism of host recognition by staphylococ-

cal Twort-like phages remains unclear. In this study, we focused on the mechanism of host recognition by staphylococcal Twort-like phage ΦSA012 at the molecular level.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and culture conditions. Bacterial strains, phages, and plasmids are listed in Table 1. *S. aureus* strain RN4220 was kindly supplied by Motoyuki Sugai (Hiroshima University Graduate School of Biomedical & Health Sciences, Hiroshima, Japan), with the permission of Richard P. Novick (Skirball Institute of Biomolecular Medicine, New York, NY), and was used for gene manipulations (31). *S. aureus* strain SA003, which was isolated from raw milk samples from a mastitic cow, was used for propagation and enumeration of staphylococcal phages. The *S. aureus* virulent phage ΦSA012 was isolated from sewage in a previous study (5). ΦSA012 and SA003 were deposited in the culture collection of the NITE Biological Resource Center, Kisarazu, Japan (accession no. NBRC110649 and NBRC110650, respectively). Shuttle vectors pNL9164 and pLI50 were purchased from Sigma-Aldrich (St. Louis, MO) and Addgene (Cambridge, MA), respectively. The shuttle vector pKOR1 was kindly supplied by Taeok Bae (Indiana University School of Medicine-Northwest, Indianapolis, IN). All *S. aureus* and *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium overnight, unless otherwise stated.

Phage preparation. All phages were propagated by the plate lysate method (32). Briefly, 100 μl of phage lysate (>10⁵ PFU/ml) was mixed with 100 μl of overnight bacterial culture in 3 ml of 0.5% top agar, plated on LB agar, and incubated at 37°C overnight. After 5 ml of salt magnesium (SM) buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin) was added to the plate and the overlayer was scraped off to extract phage, the supernatant was collected by centrifugation (5,000 × *g*, 15 min, 4°C). The obtained phage lysate was purified by polyethylene

TABLE 2 Primers used in this study

Primer	Direction	Sequence (5' → 3') ^a	Purpose
PKOR1 insert_c_F	Forward	CACAGGAAACAGCTATGACATAG	Insert check in pKOR1
PKOR1 insert_c_R	Reverse	CAGGTACATCATTCTGTTTGTG	Insert check in pKOR1
tarM_A_EagI	Forward	AAACGGCCGTGAAATTGAAGAGAGTAAAGGTATTTTC	SOE PCR for deletion of <i>tarM</i>
tarM_B	Reverse	TTTTGGAAAACCTCCCTGGTCC	SOE PCR for deletion of <i>tarM</i>
tarM_C	Forward	GGACCAGGGAGTTTTCCAAAAGGTCAAGGGTTAAGTATGATAGAAG	SOE PCR for deletion of <i>tarM</i>
tarM_D_EcoRV	Reverse	AAAGATATCAGTAGTTACAGCTGGAAGAAA	SOE PCR for deletion of <i>tarM</i>
tarM_Fw	Forward	AATGGATCGAAGAACGAAAATGT	Check for deletion of <i>tarM</i>
tarM_Rv	Reverse	ACGCCTTATGTTAATGTTTTTATATTTG	Check for deletion of <i>tarM</i>
pLI50-insertcheck-fw	Forward	TAATACCGCGCCACATAGCAGAAC	Insert check in pLI50 or pLIP3
pLI50-insertcheck-rv	Reverse	TAGCTCACGCTATGCCGACATTC	Insert check in pLI50 or pLIP3
P1075_EcoRI	Forward	AAAGAATTCGAGTGGTATAAGTGGTTTTTTCG	Amplify DNA fragment of P3 promoter
P697_KpnI	Reverse	AAAGGTACCTTCACCTCTGTTCTTACGACCTC	Amplify DNA fragment of P3 promoter
TarM_e_p3_Fw	Forward	AAACCCGGGGAGGTAAAGGAATAATTATAATGAAAAAAA	Complementation of <i>tarM</i>
TarM_e_Rv	Reverse	AAAGTCGACTTAGCTATTGAAAAGATTTAACCATTTTTC	Complementation of <i>tarM</i>
ORF103M-F	Forward	CCC <u>AAGCTT</u> GGGGGGTTGATTGACCCCTCTTT	Introduction of mutations into a recombinant phage
ORF103M-R	Reverse	CCC <u>AAGCTT</u> GGGCCCTAGCTCCTTGTCATACCC	Introduction of mutations into a recombinant phage
ORF103b-F	Forward	TGAATCCACAACCTCAATATGCAAC	Check of mutations in a recombinant phage
ORF103c-F	Forward	TCATCTAGTAAAGGTAATGGTGC	Check of mutations in a recombinant phage
ORF103r-F	Forward	GGGAATTC <u>CATATG</u> GCATTTAACTACACGCCTC	Expression of ORF103
ORF103r-R	Reverse	CCG <u>CTCGAGT</u> CCTCTATTAATCCCATAATATTGTATACC	Expression of ORF103
ORF105r-F	Forward	GGGAATTC <u>CATATG</u> GCATTTAACTACACGCCTC	Expression of ORF103
ORF105r-R	Reverse	CCG <u>CTCGAGT</u> CCTCTATTAATCCCATAATATTGTATACC	Expression of ORF103
pET29a_Insert-c_F	Forward	CATGAGCCCGAAGTGGCGAGCCCGATCTTC	Insert check in pET29a
pET29a_Insert-c_R	Reverse	CGTGCGCGTAACCACCACACCCGCGCGCGC	Insert check in pET29a

^a Bold underlined residues indicate restriction sites.

glycol (PEG) sedimentation and CsCl density gradient centrifugation (32). Each phage culture was titrated and stored at 4°C until use.

Molecular cloning in *S. aureus*. The primers used in this study are shown in Table 2. Deletion of *tarM* in *S. aureus* RN4220 was performed using the shuttle vector pKOR1 as described before (33). A DNA fragment for allelic exchange was prepared by splicing by overlap extension (SOE) PCR, digested with EagI and EcoRV, and inserted into pKOR1 (New England BioLabs, Ipswich, MA). The resultant plasmid was constructed in *E. coli* JM109 and electroporated into *S. aureus* RN4220 (34). An *S. aureus* strain in which the plasmid had integrated into the genome was selected at 43°C in the presence of chloramphenicol (10 µg/ml). Subsequently, the plasmid was excised and cured by culturing the strain in brain heart infusion (BHI) medium at 30°C or 43°C. To screen for plasmid-free strains, colonies were replica plated onto tryptic soy broth (TSB) agar, with or without chloramphenicol (10 µg/ml). Anhydrotetracycline (1 µg/ml) was used for counterselection. Gene deletion was confirmed by PCR with the appropriate primer set. For complementation of *tarM*, the plasmid pLIP3_tarM for ectopic expression of *tarM* was constructed by integrating the P3 promoter, which is constitutive in *S. aureus*, and the *tarM* gene into the shuttle vector pLI50 (35, 36). DNA fragments corresponding to the P3 promoter and *tarM* were amplified from genomic DNA of RN4220 and inserted into pLI50. The plasmid pLIP3_tarM was constructed in *E. coli* JM109 and then electroporated into *S. aureus* RN4220 Δ tarM (34).

Isolation of mutant phages from coculture experiments. In order to isolate Φ SA012-resistant derivatives and mutant phages, SA003 and Φ SA012 were cocultured. SA003 was inoculated into 4.5 ml of LB medium and cultured until early exponential phase (optical density at 660 nm [OD₆₆₀] = 0.1) in a TVS062CA compact rocking incubator (Advantec, Tokyo, Japan). Approximately 4.5×10^8 PFU of Φ SA012 was added (multiplicity of infection [MOI] of 1) and cultured at 37°C with shaking at 40 rpm. After 2 to 10 days, bacterium-phage mixed cultures were collected. Forty-five microliters of bacterium-phage mixed culture was trans-

ferred to 4.5 ml of fresh LB medium (1:100 dilution) and cultured under the same conditions. After 2 to 10 days, the bacterium-phage mixed culture was collected, and a 1:100 dilution was performed again.

For isolation of Φ SA012-resistant derivatives and mutant phages, 1.5 ml of mixed culture was separated by centrifugation ($9,730 \times g$, 5 min, 4°C) at each passage step. After washing four times with phosphate-buffered saline (PBS) to remove free phage, the pellet was resuspended in PBS and spread onto LB plates; one colony was picked as a Φ SA012-resistant derivative after overnight incubation. Supernatant from the coculture was used for a plaque assay with SA003. After overnight incubation, one plaque was also picked as a mutant phage.

This procedure was repeated continuously until the 38th passage. Φ SA012-resistant derivatives and mutant phages were defined as SA003R1 to SA003R38 and Φ SA012M1 to Φ SA012M38, respectively. Each number in the names of the Φ SA012-resistant derivatives and mutant phages represents the number of passages in coculture (e.g., SA003R11 refers to the phage-resistant derivative isolated from the coculture at the 11th passage, and Φ SA012M20 refers to the mutant phage isolated from the coculture at the 20th passage).

Extraction and analysis of phage genomic DNA. Genomic DNAs of Φ SA012 and five mutant phages (Φ SA012M1, Φ SA012M2, Φ SA012M11, Φ SA012M20, and Φ SA012M38) were extracted from purified phages by use of a phage DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada). Whole-genome sequencing was performed on a Genome Sequencer FLX+ system (Roche, Basel, Switzerland). Sequencing results were assembled and aligned using GS De Novo Assembler v2.8 (Roche, Basel, Switzerland) and Tablet (The James Hutton Plant Bioinformatics Group, Invergowrie, Scotland), respectively. Open reading frames (ORFs) were predicted with myRAST (<http://blog.theseed.org/servers/presentations/t1/running-a-job-with-the-desktop-rast.html>). Nucleotide and amino acid sequences were scanned for homologs by BLAST

searches (37). Phage-carried tRNA genes were identified using Aragorn and tRNA Scan SE ver. 1.21 software (38, 39).

Generation and isolation of a recombinant phage harboring three mutations in *orf103*. In order to construct a plasmid harboring three mutations in *orf103*, a DNA fragment was amplified from the genomic DNA of Φ SA012M20 by a PCR using primers ORF103M-F and ORF103M-R. This PCR fragment contained the nucleotide sequence between 200 bp upstream and 110 bp downstream of *orf103*. The recombinant fragment was digested with HindIII (TaKaRa Bio) and inserted into the shuttle vector pNL9164 (40). The plasmid was constructed and cloned in *E. coli* JM109. The constructed plasmid (named pNL9164::TMorf103) was then electroporated into *S. aureus* strain RN4220 (34).

A recombinant Φ SA012 phage harboring three mutations in *orf103* (Φ SA012TM103) was generated by the following procedure. Transformed RN4220 harboring pNL9164::TMorf103 was grown to early exponential phase ($OD_{660} = 0.1$; 10^8 CFU/ml) at 32°C with shaking at 120 rpm in 10 ml of LB medium. Φ SA012 (10^6 PFU/ml) was then added at an MOI of 0.01. During phage infection, homologous recombination between phage DNA and the plasmid in transformed RN4220 harboring ORF103 of Φ SA012M20 might happen at a low frequency. After overnight incubation at 32°C with shaking, the supernatant was collected by centrifugation ($6,230 \times g$, 10 min, 4°C) and plated for a plaque assay for isolation of Φ SA012TM103. Briefly, 500 μ l of phage lysate and 250 μ l of overnight culture of phage-resistant derivative SA003R11 (susceptible to Φ SA012M20 but not to Φ SA012) were added to 6 ml of 0.5% top agar and poured onto LB agar plates. After incubation overnight at 37°C, single plaques were picked and resuspended in 100 μ l of SM buffer. These suspensions were purified by two rounds of plaque assay with SA003R11. Mutations in *orf103* of Φ SA012TM103 were identified by Sanger sequencing using primers ORF103b-F and ORF103c-F.

Protein expression and purification. C-terminally His-tagged recombinant ORF103 and ORF105 were expressed in *E. coli* Rosetta-gami 2(DE) from vector pET-29a and purified by immobilized-metal affinity chromatography (IMAC). To construct plasmids for expression of ORF103 and ORF105, DNA fragments were amplified by PCR with the appropriate primer sets (Table 2). Recombinant fragments for protein expression were digested with NdeI and XhoI (New England BioLabs) and inserted into pET-29a. The plasmids were constructed and cloned into *E. coli* JM109, and the clones were then electroporated into *E. coli* Rosetta-gami 2(DE).

To produce the ORF103 and ORF105 proteins, expression was induced by addition of 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) when the culture reached an OD_{600} of 0.5. After incubation at 28°C with shaking at 160 rpm overnight, the cells were collected by centrifugation ($6,230 \times g$, 10 min, 4°C), resuspended in phosphate buffer (20 mM phosphate [pH 7.4], 0.5 M NaCl), and disrupted by sonication for 40 min in a VP60-S sonicator (Taitec, Koshigaya, Japan). Target proteins were purified using a HisTrap HP column (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom). After His-tagged proteins were eluted from the Ni column with elution buffer (40 to 500 mM imidazole in phosphate buffer), the imidazole was removed by dialysis against phosphate buffer.

Preparation of antibodies. Polyclonal rabbit antibodies against the recombinant ORF103 and ORF105 proteins were generated by Japan Bio Serum (Hiroshima, Japan). Briefly, rabbits were immunized with 0.3 mg of protein once every 2 weeks for 8 weeks; in total, 1.5 mg of protein was used. After blood was collected in week 10, anti-ORF103 serum was purified on a protein A column and stored at -20°C until use. The binding ability of the anti-ORF103 and anti-ORF105 polyclonal antibodies was confirmed by Western blotting.

Immunoelectron microscopy. Immunoelectron microscopy was conducted as described previously (23). A freshly purified phage sample (10^{10} PFU/ml) was mixed with purified anti-ORF103 antibody diluted in SM buffer (1:100) and incubated at room temperature for 30 min. The samples were loaded onto ester-carbon-coated copper grids (EMJapan,

Tokyo, Japan). The copper grids were washed twice with SM buffer and incubated with 12-nm colloidal gold-labeled AffiniPure goat anti-rabbit IgG(H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS (1:50) at 37°C for 30 min. After washing with SM buffer and Milli-Q water twice each, the grids were stained with 2% uranyl acetate and observed on a JEM-1400 Plus microscope (JEOL, Akishima, Japan).

Spot test and assay of the efficiency of plating (EOP) with antibodies. The infectivity of phages was evaluated as previously described (5). Briefly, 2- μ l aliquots of serially diluted phage lysate (10^7 to 10^{10} PFU/ml) were dropped onto LB plates overlaid with *S. aureus* strains mixed with 0.5% top agar and then incubated overnight to assess plaque formation.

The EOP of phages on *S. aureus* strains with antibodies was measured by plaque assay, with the phage lysate adjusted with SA003. For assay with an antibody to evaluate the role of ORF103 or ORF105 in infection, 10 μ l of anti-ORF103 or anti-ORF105 serum was added to 100 μ l of phage lysate. After incubation for 1 h at room temperature, the number of infectious phage was enumerated by a plaque assay with SA003. Serum collected from a rabbit before immunization (preimmune serum) was used as a control.

Adsorption assay. The adsorption efficiency of phages on *S. aureus* strains was measured by titrating free phage present in the supernatant after defined periods of cell-phage contact. *S. aureus* cells were prepared by 10% inoculation of overnight culture into 4.5 ml of LB medium; the culture was then incubated at 37°C with shaking at 120 rpm to an OD_{660} of 1.0 ($\sim 10^9$ CFU/ml). Phage lysate (10^7 PFU/ml) was then added to the bacterial culture. After infection at 37°C with shaking at 120 rpm, free phage was collected by centrifugation ($9,730 \times g$, 1 min) at defined times and titrated using SA003. For longer incubation, 50 μ g/ml of chloramphenicol or erythromycin was added, and cells were equilibrated for 10 min at 37°C before infection to inhibit cell growth and phage development during incubation with phages (41). Adsorption efficiency was calculated by dividing the number of adsorbed phage by the initial number of phage.

Statistical analysis. Two-tailed Student's *t* test was used to determine statistical significance.

Accession number(s). The complete genome of Φ SA012 has been deposited in the GenBank database under accession number [AB903967](https://www.ncbi.nlm.nih.gov/nuccore/AB903967).

RESULTS

Genomic analysis of Φ SA012 and its mutant phages. Whole-genome sequencing of Φ SA012 revealed that its genome is 142,094 bp long and contains 207 ORFs (see Table S1 in the supplemental material). Terminally redundant regions, a common feature of *Twortlikevirus* and *Spounalikevirus*, were found at both ends of the genome (7, 62–65). Three tRNA genes (Met-tRNA gene, bp 8,180 to 8,109; Asp-tRNA gene, bp 30,496 to 30,423; and Phe-tRNA gene, bp 30,416 to 30,344) are carried in the Φ SA012 genome.

Mutant phages isolated from cocultures with SA003 showed infectivities different from that of wild-type Φ SA012 by the spot test with Φ SA012-resistant derivatives (data not shown). In order to find the candidate genes responsible for host recognition, all mutations in five mutant phages isolated from cocultures were identified by whole-genome sequencing (Table 3). Mutations were found in six ORFs (*orf33*, *orf58*, *orf79*, *orf99*, *orf103*, and *orf133*) and the terminally redundant regions located at both ends of the genome.

Comparisons of structural proteins among Twort-like viruses and relatives. Comparisons of structural proteins (from capsid to helicase) among Φ SA012, K, and ISP (*Twortlikevirus*), SPO1 (*Spounalikevirus*), and Φ EF24C revealed that the region from ORF103 to ORF105 is unique to staphylococcal Twort-like

TABLE 3 Distribution of mutation sites in mutant phages

Phage	Presence of terminally redundant region (bp)	Sequence at indicated position(s) (bp) in indicated ORF (predicted function) ^a										Presence of terminally redundant region (bp 141673–142094) (<i>orf207</i>)
		<i>orf33</i> (unknown)	<i>orf58</i> (hypothetical membrane protein)	<i>orf79</i> (tail sheath protein)	<i>orf99</i> (tail morphogenic protein)	<i>orf103</i> (virion component [part of tail fiber])	79071	79167	<i>orf133</i> (tail morphogenic protein [lg-like])			
ΦSA012 wild type or derivative	1–1045) (<i>orf1</i> to <i>orf3</i>) ^b	16908	33020–(A) ^c 33021	50380	71775	71909	78605	79071	79167	107746		
ΦSA012 (wild type)	+	GAA	ACA-TCT-TAC	TTC	AAT	TCT	AAT	GGT	ACA	GTA	+	
	+	E	T-S-Y	F	N	S	N	G	T	V		
ΦSA012M1	+	GAA	ACA-TCT-TAC	TTC	AAT	TCT	AAT	GGT	AGA	GTA	+	
	+	E	T-S-Y	F	N	S	N	G	R	V		
ΦSA012M2	+	AAA	ACA-TCT-TAC	TTC	AAT	TCT	GAT	GGT	AGA	GTA	+	
	+	K	T-S-Y	F	N	S	D	G	R	V		
ΦSA012M11	Deleted	GAA	ACA-TCT-TAC	TTA	AAT	TCT	GAT	GGT	AGA	GTA	Deleted	
	Deleted	E	T-S-Y	L	N	S	D	G	R	V	Deleted	
ΦSA012M20	Deleted	GAA	AA-C-ATC-TTA	TTA	AA-G	TCT	GAT	GAT	AGA	GTA	Deleted	
	Deleted	E	N-I-L	L	K	S	D	D	R	V	Deleted	
ΦSA012M38	Deleted	GAA	AA-C-ATC-TTA	TTA	AA-G	TAT	GAT	GAT	AGA	TTA	Deleted	
	Deleted	E	N-I-L	L	K	Y	D	D	R	L	Deleted	

^a All positions are relative to the genome of ΦSA012. For each phage variant, upper sequences are nucleotide sequences, and lower sequences are amino acid sequences. Bold underlined residues indicate nucleotide mutations, and gray boxes represent mutations in amino acids.

^b +, the region is present.

^c An insertion of adenine (A) was discovered between bp 33020 (adenine) and bp 33021 (tyrosine), resulting in a premature stop codon and partial deletion of the product (aa 85 to 108).

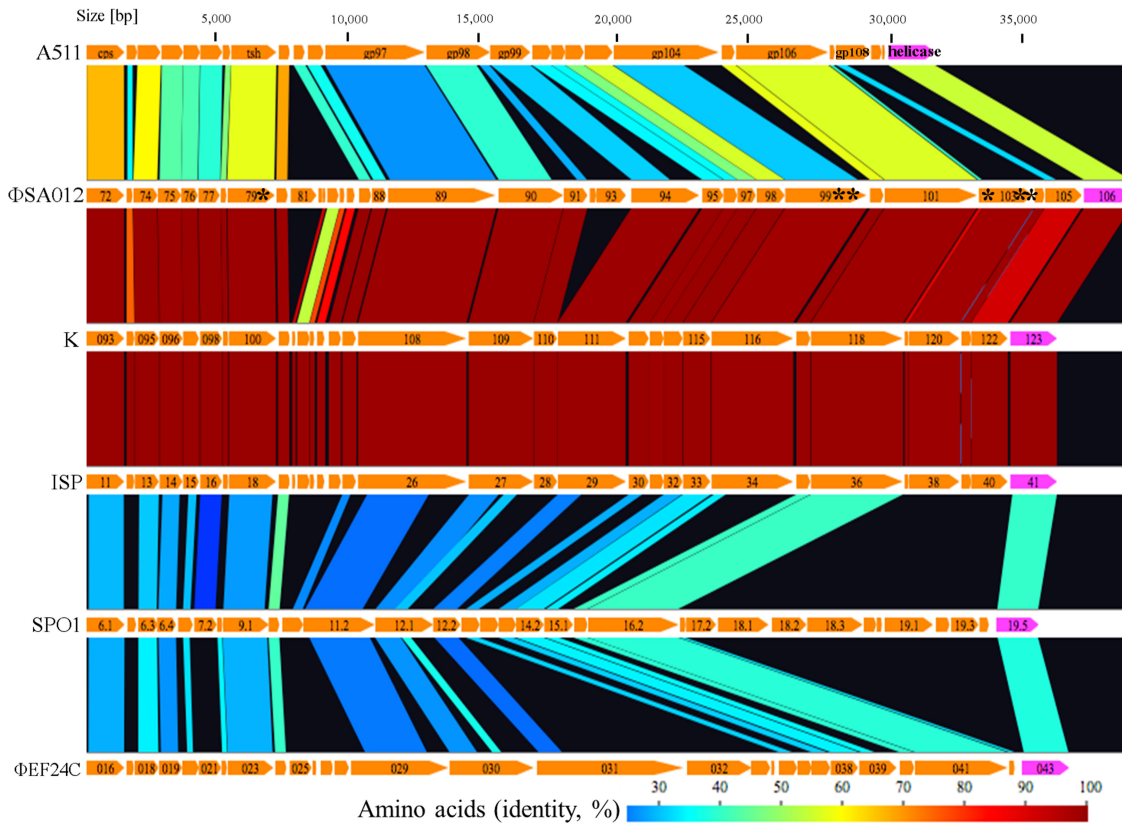


FIG 1 Comparisons of main structural proteins among *Twortlikevirus* and related phages. Host bacteria for each phage are as follows: A511, *Listeria monocytogenes*; ΦSA012, K, and ISP, *Staphylococcus aureus*; SPO1, *Bacillus subtilis*; and ΦEF24C, *Enterococcus faecalis*. Asterisks (*) represent the locations of mutations. Gp108 in A511 is an RBP (25). The figure was generated using GenomeMatcher (59).

phages (ΦSA012, K, and ISP) at the protein level (Fig. 1). The basic structural components, such as the capsid (ORF72 in ΦSA012), tail sheath (ORF79 in ΦSA012), and tape major protein (ORF89 in ΦSA012), as well as a helicase involved in DNA

replication (ORF106 in ΦSA012), share higher degrees of similarity, probably due to functional similarities between the respective proteins. ORF101 in ΦSA012, which was predicted to be an adsorption-associated tail protein facilitating infection of Gram-positive bacteria by digesting sialic acid residues in their

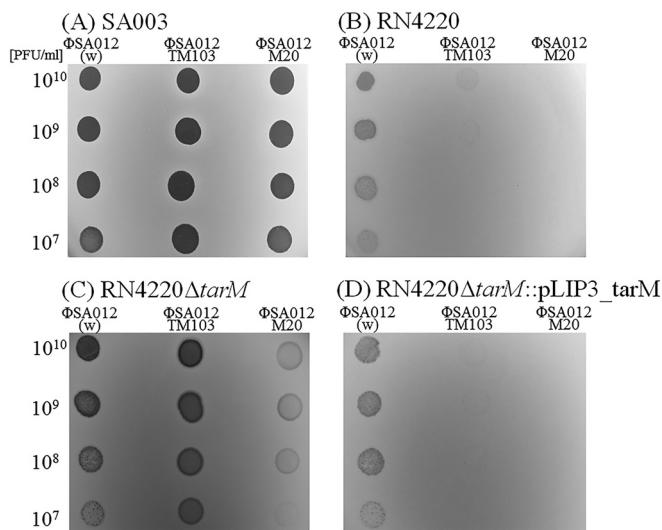


FIG 2 Spot tests of phages on *S. aureus* strains. Two microliters of concentrated phage lysate (10^7 to 10^{10} PFU/ml) was dropped onto an LB plate overlaid with *S. aureus* cells. (A) SA003 ($\Delta tarM$); (B) RN4220; (C) RN4220 $\Delta tarM$; (D) RN4220 $\Delta tarM::pLIP3_tarM$. w, wild type.

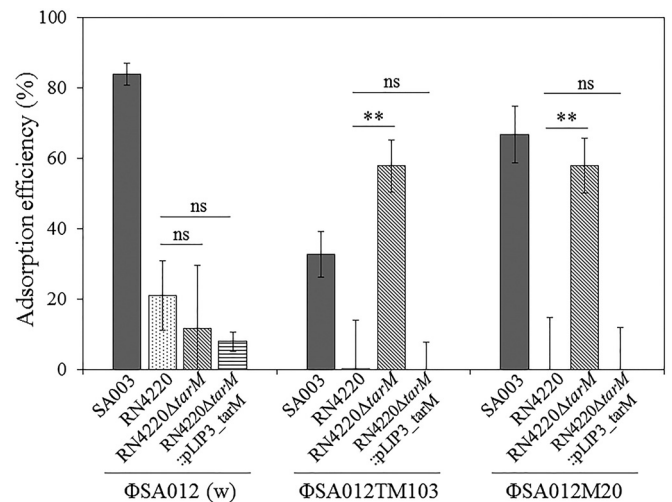


FIG 3 Adsorption efficiencies with 10^8 CFU/ml of *S. aureus* strains in 60 min. Error bars indicate standard deviations (SD). Three biological replicates were conducted. **, $P < 0.01$; ns, not significant.

slime or capsules due to the presence of a putative conserved neuraminidase/sialidase domain, was also conserved among related phages (7, 45).

Infectivity of a recombinant phage harboring three mutations in *orf103*. The results of the spot test revealed that the infectivities of the recombinant phage Φ SA012TM103 and the mutant phage Φ SA012M20 differed from that of the wild-type phage Φ SA012 (Fig. 2). All three phages (Φ SA012, Φ SA012TM103, and Φ SA012M20) could produce plaques in *S. aureus* SA003. However, Φ SA012TM103 produced very turbid plaques, whereas Φ SA012M20 could not produce plaques in RN4220, indicating that the three mutations in *orf103* inhibited infection of RN4220. Because ORF103 is likely to bind the WTA polymer due to the existence of a carbohydrate-binding domain in ORF103, we compared the genes related to WTA synthesis between SA003 and RN4220. We found that SA003 lacks the *tarM* gene, whose product is responsible for glycosylation of α -GlcNAc of WTAs, whereas RN4220 has this gene (46). To investigate the importance of the encoded protein, we knocked out *tarM* in RN4220. Deletion of *tarM* made RN4220 susceptible to Φ SA012TM103 and Φ SA012M20, harboring mutated ORF103. Thus, TarM-mediated α -GlcNAc modification of WTAs inhibited infection by Φ SA012TM103 and Φ SA012M20 but not that by Φ SA012. Complementation of *tarM* in the deletion mutant restored the infectivity of phages, indicating that the role of ORF103 is related to α -GlcNAc on WTAs.

The behaviors of *S. aureus* strains in adsorption assays corresponded with the results of spot tests (Fig. 2 and 3). All phages (Φ SA012, Φ SA012TM103, and Φ SA012M20) had the ability to adsorb to SA003, although the adsorption efficiency of Φ SA012TM103 was lower than those of Φ SA012 and Φ SA012M20. For RN4220, on the other hand, Φ SA012TM103 and Φ SA012M20 had lower adsorption efficiencies than that of Φ SA012. Thus, mutations in *orf103* changed the adsorption efficiency, indicating that ORF103 is involved in adsorption. Deletion of *tarM* in RN4220 enhanced the adsorption efficiencies of the ORF103 mutant phages Φ SA012TM103 and Φ SA012M20 but did not affect adsorption of Φ SA012. Complementation of *tarM* confirmed that α -GlcNAc of WTAs inhibited adsorption of Φ SA012TM103 and Φ SA012M20 but not that of Φ SA012.

From these results, we concluded that ORF103 is the RBP that binds to α -GlcNAc on WTAs and that mutations in ORF103 altered the protein's function, resulting in deficient adsorption of ORF103 mutant phages to *S. aureus* strains whose WTAs contain α -GlcNAc due to the activity of TarM. It is plausible that the absence of α -GlcNAc in WTAs did not contribute to phage resistance due to the presence of ORF105, previously shown to be an RBP.

Effects of anti-ORF103 and anti-ORF105 antibodies on phage infection. To test our hypothesis, we evaluated the effects of polyclonal antibodies raised against ORF103 and ORF105 on phage infection (Fig. 4). Infection of RN4220 by Φ SA012 was drastically inhibited by anti-ORF103 serum, but infection of SA003 was not. Inhibition by anti-ORF103 serum was not observed for infection of RN4220 Δ *tarM* by Φ SA012, whereas anti-ORF103 serum inhibited infection of a *tarM*-complemented strain. This indicated that the anti-ORF103 serum inhibited the interaction between ORF103 and α -GlcNAc, supporting the idea that ORF103 binds to α -GlcNAc on WTAs and plays an important

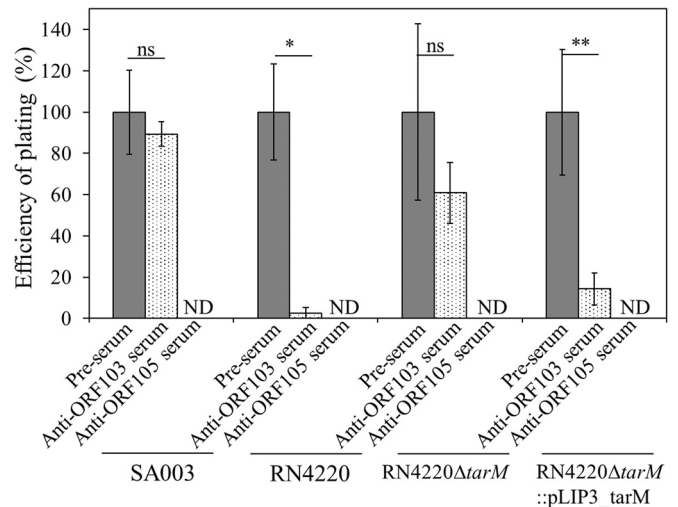


FIG 4 EOP of Φ SA012 with anti-ORF103 and anti-ORF105 antibodies. Pre-immune serum (pre-serum) was used in controls. The number of plaques observed in the presence of preimmune serum was set as 100%. Plaques were not detected (ND) in the presence of anti-ORF105 serum. Three biological replicates were conducted. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

role in infection only in *S. aureus* strains whose WTAs contain α -GlcNAc.

In contrast, inhibition by anti-ORF105 serum was observed for all *S. aureus* strains (SA003, RN4220, RN4220 Δ *tarM*, and RN4220 Δ *tarM*::pLIP3_*tarM*) irrespective of the presence of α -GlcNAc, indicating that ORF105 is the primary RBP for infection by Φ SA012.

Location of ORF103 in Φ SA012. Immunoelectron microscopy using anti-ORF103 antibody and a gold-conjugated anti-rabbit antibody revealed that ORF103 in Φ SA012 is localized on the tail fiber at the bottom of the baseplate (Fig. 5). This observation supported the idea that ORF103 interacts with components on the cell surface during infection.

Role of the three mutations in *orf103* in mutant phages during coevolution. Apart from the function of ORF103, i.e., binding to α -GlcNAc, mutations in ORF103 seemed to be important during coevolution between Φ SA012 and SA003. The results of spot tests and adsorption assays confirmed that Φ SA012TM103 and Φ SA012M20 could infect the Φ SA012-resistant derivatives SA003R11 and SA003R20 due to the increase in adsorption efficiency caused by the three mutations in *orf103* (Fig. 6A). The inconsistency in adsorption efficiency between Φ SA012TM103 and Φ SA012M20 suggested that mutations in other genes besides *orf103* also affected adsorption.

The inhibitory effect of anti-ORF103 serum on infection by Φ SA012M20 also suggested that mutated ORF103 is essential for infection of SA003R11 and SA003R20. In contrast, anti-ORF103 serum did not inhibit infection of SA003 (Fig. 6B). Because WTAs in SA003R11 and SA003R20 do not contain α -GlcNAc due to the absence of *tarM* in these strains, this finding implied that mutations in ORF103 changed the original function of ORF103 to enhance adsorption on Φ SA012-resistant derivatives. Thus, mutations in ORF103 were necessary to adapt to phage resistance arising during coevolution.

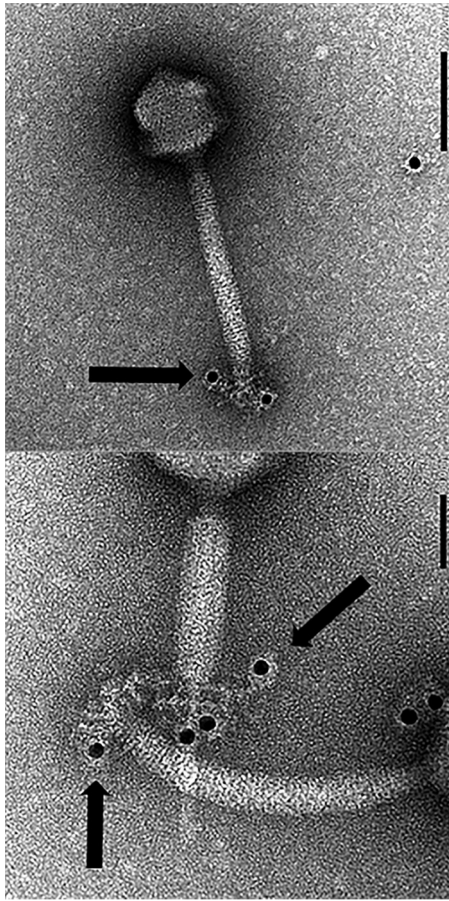


FIG 5 Localization of ORF103 in Φ SA012 stained with a gold-conjugated secondary antibody. Bars, 100 nm (top) and 50 nm (bottom). The size of the gold particles is 12 nm. Black spots in the images represent gold particles conjugated to the secondary antibody.

DISCUSSION

In silico analysis of Φ SA012 and its mutant phages suggests that ORF103 is responsible for host recognition. Deletions of bp 1 to 1,045 (containing *orf1* to *orf3*) and bp 141,673 to 142,094 (containing *orf207*) were detected in Φ SA012M11 to Φ SA012M38 (Table 3). In SPO1, these terminally redundant regions contain the host takeover module (47). For staphylococcal Twort-like phages, it has also been suggested that some or all genes located in these regions play a role in host takeover, by analogy to the corresponding regions of SPO1 (7). Insertion of one nucleotide in *orf58* caused a frameshift and generated a premature stop codon, leading to partial deletion of the gene product (amino acids [aa] 85 to 108). *orf58* encodes a protein homologous to the membrane protein MbpC of staphylococcal Twort-like phage A5W; it is predicted to play a role in attachment of the complex of replicating phage DNA to a cell membrane, analogously to membrane protein p16.7 of *Bacillus* phage ϕ 29 (7, 48). Although substitutions of amino acids in ORF33 were observed only in Φ SA012M2, they did not accumulate among later mutant phages. Other ORFs in which mutations were observed (*orf79*, *orf99*, *orf103*, and *orf133*) encode putative tail proteins. *orf79* is predicted to encode the tail sheath protein and has homologs in a number of phages. The functions of *orf99* and *orf103*, predicted to encode tail morphogenetic proteins, remain unknown. Due to the presence of an immunoglobulin (Ig)-like domain conserved among hundreds of phages, *orf133* is predicted to encode a tail protein that plays an accessory role by interacting weakly with carbohydrates on the bacterial surface (7, 49, 50).

Given that the largest number of mutations accumulated in *orf103*, we hypothesized that *orf103* plays a crucial role in determining infectivity. Due to the selection pressures imposed by phage-bacterium coevolution, phage RBP genes are among the most diverse genes (51). It is worth mentioning that a carbohydrate-binding domain (1,4- β -glucanase CenC from *Cel-lulomonas fimi*) was identified in the central position of ORF103 (aa 221 to 337) by InterPro 56.0, and one of the three mutations was located within this domain (52, 53). This observation im-

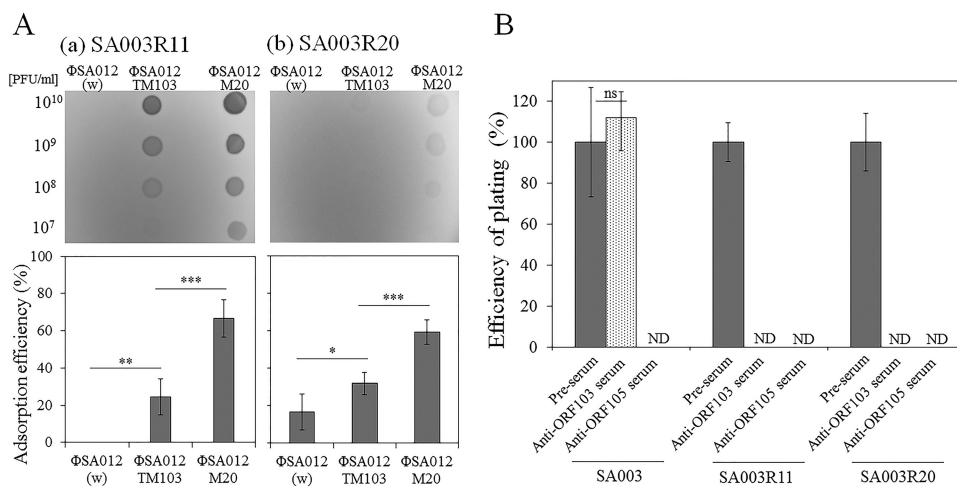


FIG 6 Effects of mutations in ORF103. (A) Spot tests and adsorption assays with Φ SA012-resistant strains. Upper and lower panels show the results of adsorption assays (10^9 CFU/ml of cells in 270 min) and spot tests, respectively, with SA003R11 (a) and SA003R20 (b). Three or five biological replicates were conducted. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) EOP of Φ SA012M20 with anti-ORF103 and anti-ORF105. Preimmune serum was used in controls. The number of plaques formed in the presence of preimmune serum was defined as 100%. ND, not detected. Three biological replicates were conducted. Error bars indicate SD. ns, not significant.

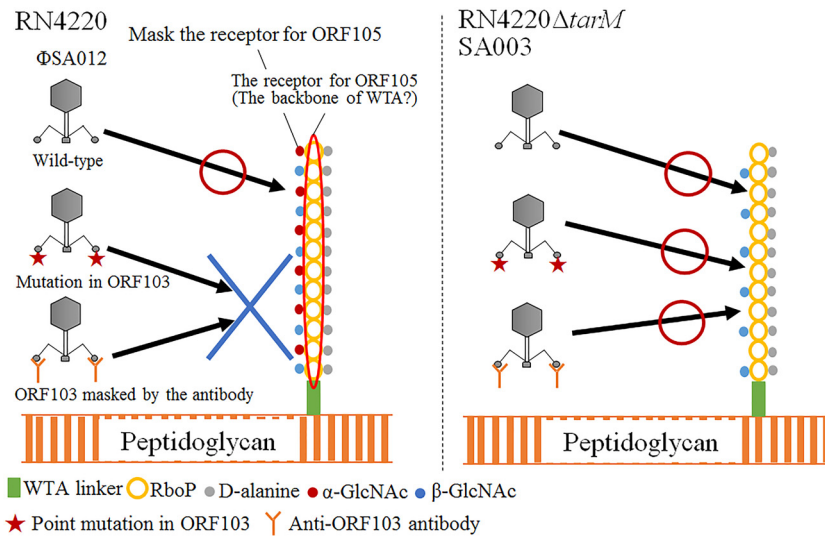


FIG 7 Scheme of putative adsorption mechanism of Φ SA012. In most *S. aureus* strains, the WTA polymer consists of 11 to 40 ribitol phosphate (RboP) repeats substituted with D-alanine and α - and β -GlcNAc, covalently attached to a peptidoglycan with a WTA linkage unit. Φ SA012 recognizes the WTA backbone. α -GlcNAc masks the WTA backbone, which is the binding site of the primary RBP ORF105, and ORF103 helps the phage to bind the WTA backbone by binding α -GlcNAc. Hence, a phage in which ORF103 is disabled by point mutations or antibodies cannot infect *S. aureus* strains in the presence of α -GlcNAc, whereas the loss of ORF103 function does not affect phage infection in the absence of α -GlcNAc.

plied that ORF103 encodes a possible RBP that binds to sugar components in WTAs on the cell surface. Since ORF105, a homolog of gp40 in ISP that acts as an RBP (25), is located in the unique region from ORF103 to ORF105 revealed by comparisons of structural proteins among Φ SA012 and its related phages, this unique region from ORF103 to ORF105 determines the host range for staphylococcal Twort-like phages (Fig. 1). Thus, we focused mainly on *orf103* as a host determinant gene in this study.

Presence of two RBPs in staphylococcal Twort-like phages.

Our findings showed that at least two adsorption apparatuses (ORF103 and ORF105) are present in Φ SA012. Due to the carbohydrate-binding domain in ORF103, ORF103 recognizes sites in WTAs different from those recognized by ORF105. The N-terminal 37 residues of ORF103 and ORF105 are 41% similar. In most cases, the N termini of RBPs are conserved due to the connection to the virus particle, whereas the C termini, responsible for recognition of the host receptor, are diverse. The conserved N termini of ORF103 and ORF105 may suggest that ORF103 and ORF105 are conjugated to the same virion component.

Several phages are known to possess multiple adsorption apparatuses (51). The *E. coli* lytic myovirus phi92 has at least five different tail spikes and tail fiber proteins identified by cryoelectron microscopy, allowing it to infect a wide range of *E. coli* and *Salmonella* strains (54). Two different RBPs, LtfA and LtfB of the T5-like siphoviruses DT57C and DT571/2, recognize different O antigens, i.e., the O22 or O87 type and the O81 type, respectively (55). Two different carbohydrate-binding modules have been identified in *Lactococcus lactis* phage Tuc2009; the first is in a classical *bona fide* RBP (BppL), and the other is in an accessory protein, BppA (56). BppA enhances adsorption to cells, although its true contribution is not fully understood (57).

The distribution of *tarM* in *S. aureus* strains has been addressed in previous studies. Many human-associated *S. aureus* lineages lost *tarM* over the course of evolution (30). However, the presence

of multiple RBPs may explain the wide host range of staphylococcal Twort-like phages.

Role of ORF103 in infection. From the results of spot tests, EOP tests with antibodies, and adsorption assays, it was clear that ORF103 is involved in adsorption and binds to α -GlcNAc in WTAs (Fig. 2 to 4). SA003 and RN4220 $\Delta tarM$ were still susceptible to Φ SA012 upon inhibition of ORF103 by anti-ORF103 antibody (Fig. 4); thus, in the absence of α -GlcNAc, the function of ORF103 is not essential for infection of host cells by Φ SA012. Deletion of *tarM* or *tarS*, encoding an enzyme that glycosylates α -GlcNAc or β -GlcNAc on WTAs, respectively, did not affect infection of *S. aureus* strains by phage K, whereas the lack of WTAs by deletion of *tagO*, a gene related to the initial step of WTA synthesis, rendered *S. aureus* strains resistant to phages (30). Therefore, staphylococcal myovirus is thought to recognize the backbone of WTAs (27). In an infection by *S. aureus* podovirus, the presence of α -GlcNAc inhibited the adsorption of phages to β -GlcNAc, the podovirus phage receptor, and deletion of *TarM* enhanced phage adsorption (30). Likewise, it is possible that α -GlcNAc masks the backbone of WTAs, which is the binding site for the primary RBP ORF105, and that ORF103 helps the phage to bind the WTA backbone by binding α -GlcNAc (Fig. 7). Hence, phages that lacked wild-type ORF103 function due to point mutations or antibodies were not able to infect *S. aureus* strains in the presence of α -GlcNAc, whereas a loss of ORF103 function did not affect phage infection in the absence of α -GlcNAc. The influence of β -GlcNAc on adsorption may be minor, as no inhibition of infection by the anti-ORF103 antibody was observed in SA003 irrespective of the presence of *tarS*, whose product is responsible for glycosylation of β -GlcNAc.

Changes in ORF103 function by point mutations arising during coevolution. Three mutations in ORF103 contributed to adsorption to the Φ SA012-resistant derivatives SA003R11 and SA003R20, whereas adsorption to RN4220 was inhibited by these

mutations. The effect of these three mutations on adsorption enhanced the infectivity of Φ SA012TM103, as shown in the spot test and the adsorption assay. It is intriguing that mutant phages derived from Φ SA012 acquired mutations in *orf103* to adapt to Φ SA012-resistant derivatives of SA003, despite the absence of α -GlcNAc. Mutations in RBPs enable phages to recognize a new phage receptor and to circumvent inhibition of adsorption during coevolution (51). A recent study on phage λ and its host, *E. coli* B, demonstrated that this phage can evolve to target a new receptor, OmpF, when expression of the cognate receptor, LamB, is reduced by mutations (58). In order to survive during coevolution, it is reasonable for the phage to expand its host range by acquiring point mutations in ORF103 without altering ORF105, allowing mutant phages to infect both wild-type and resistant derivatives. The results of adsorption assays confirmed the strong inhibition of adsorption of Φ SA012 to SA003R11 and SA003R20, suggesting that the structure of the cell surface was altered during coevolution (Fig. 6). Thus, it is plausible that the three mutations altered the function of ORF103 to allow the phage to survive during coevolution. From the complete genomes of SA003R11 and SA003R20, we also identified point mutations in genes responsible for WTA and peptidoglycan synthesis (A. H. Azam, I. Takeuchi, K. Miyayama, and Y. Tanji, unpublished data). This implies that alterations in the structure of the cell surface induced mutations in *orf103*, resulting in acquisition of a new function by ORF103: specifically, in SA003R11 and SA003R20, the protein acquired the ability to bind mutated WTA structures at the expense of the ability to bind α -GlcNAc. However, further study of the effects of point mutations in ORF103 is still necessary.

ACKNOWLEDGMENT

This research was financially supported by grant 24246133 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

FUNDING INFORMATION

This work, including the efforts of Yasunori Tanji, was funded by Ministry of Education, Culture, Sports, Science, and Technology (MEXT) (24246133).

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