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Authors	Aa Haeruman Azam, Fumiya Hoshiga, Ippei Takeuchi, Kazuhiko Miyanaga, Yasunori Tanji
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Analysis of phage resistance in *Staphylococcus aureus* SA003 reveals different binding mechanisms for the closely related Twort-like phages ϕSA012 and ϕSA039

Aa Haeruman Azam[1] · Fumiya Hoshiga[1] · Ippei Takeuchi[1] · Kazuhiko Miyanaga[1] · Yasunori Tanji[1]

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Abstract
We have previously generated strains of *Staphylococcus aureus* SA003 resistant to its specific phage ϕSA012 through a long-term coevolution experiment. However, the DNA mutations responsible for the phenotypic change of phage resistance are unknown. Whole-genome analysis revealed eight genes that acquired mutations: six point mutations (five missense mutations and one nonsense mutation) and two deletions. Complementation of the phage-resistant strains by the wild-type alleles showed that five genes were linked to phage adsorption of ϕSA012, and two mutated host genes were linked to the inhibition of post-adsorption. Unlike ϕSA012, infection by ϕSA039, a close relative of ϕSA012, onto early coevolved phage-resistant SA003 (SA003R2) was impaired drastically. Here, we identified that ϕSA012 and ϕSA039 adsorb to the cell surface *S. aureus* SA003 through a different mechanism. ϕSA012 requires the backbone of wall teichoic acids (WTA), while ϕSA039 requires both backbone and the β-GlcNAc residue. In silico analysis of the ϕSA039 genome revealed that several proteins in the tail and baseplate region were different from ϕSA012. The difference in tail and baseplate proteins might be the factor for specificity difference between ϕSA012 and ϕSA039.

Keywords *Staphylococcus aureus* · Twort-like phage · Phage-resistance mechanism · Bacteriophage receptor · Bacteriophage therapy

Introduction

Staphylococcus aureus is a Gram-positive bacterium that causes a wide variety of clinical manifestations. In the dairy industry, for instance, *S. aureus* is one of the most frequent causative agents of bovine mastitis, with prevalence rates as high as 50% in some countries (Leitner et al. 2003). The infection of this bacterium in humans and animals is of worldwide concern due to the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Enright et al. 2002; Sakoulas et al. 2005). One alternative strategy to control *S. aureus* is to exploit lytic phages as an agent to kill the bacteria. Various efforts are currently made to evaluate the potential of phage therapy (Matsuzaki et al. 2005; Maciejewska et al. 2018). However, the use of phages often creates pressure for selection of phage-resistant bacteria. The emergence of phage-resistant bacteria is a significant obstacle to realizing phage therapy (Denes et al. 2015; Osada et al. 2017). A deeper understanding of phage resistance mechanisms is critical before we apply phage therapy in the real world.

In response to phage-resistant bacteria, phages have an ability to counter-adapt through a process called the coevolution of phage and bacteria (Golais et al. 2013). Coevolution is a process of reciprocal adaptation and counter-adaptation between ecologically interacting species. Each party in a coevolutionary relationship exerts selective pressure on the other, thereby they influence each other's evolution (Hall et al. 2011; Golais et al. 2013). Phage-bacteria coevolution studies can contribute to our understanding of the phage-bacteria dynamic. For example, the coevolution study of *Escherichia coli* O157:H7 with its specific phage PP01 led us to identify the outer membrane protein (Omp) C as the receptor of this phage, while alteration of long tail fiber (Gp38) recovered the

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✉ Yasunori Tanji
ytanji@bio.titech.ac.jp

[1] School of Life Science and Technology, Tokyo Institute of Technology, 4259 J2-15 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

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adsorption ability of PP01 against OmpC-null strain (Mizoguchi et al. 2003; Fischer et al. 2004). Likewise, the coevolution study on *S. aureus* and its phage might give us a deep understanding of their interaction.

Previously, two virulent staphylococcal phages (ϕSA012 and ϕSA039), which display broad, yet distinct, host ranges against *S. aureus* isolates from bovine mastitis, were isolated from sewage influent (Synnott et al. 2009). Genomic analysis of ϕSA012 and ϕSA039 revealed that these phages belong to the genus Twort-like phage which includes *Staphylococcus* phage Twort, K, G, and JD007 (Cui et al. 2012; Łobocka et al. 2012). The phages from this group are considered to be promising candidates for phage therapy in *S. aureus* and other *Staphylococcus* species (Loessner et al. 1996; Alves et al. 2014). Phages belonging to this genera have been reported to be strictly virulent and infect a broad host range (Łobocka et al. 2012). In preclinical experiment using mice, ϕSA012 has shown effective and promising results to treat *S. aureus* infection (Iwano et al. 2018).

We previously performed a batch co-culture experiment between *S. aureus* SA003 (an isolate from milk of mastitic cow) and ϕSA012 (Osada et al. 2017). The coevolution of *S. aureus* SA003 and ϕSA012 was observed, and analysis of coevolved mutant phage has provided an understanding about the presence of two receptor-binding proteins (RBP) that contribute to the wide host range of Twort-like phage (Takeuchi et al. 2016; Osada et al. 2017). In the current study, we focused on genetic analysis of the coevolved resistant bacteria by whole genome sequencing (WGS).

The current study enables us to identify the host genes that contribute to phage resistance during coevolution. Understanding host genes that endow phage resistance may help us design a better strategy for applying phage therapy under real-world conditions. By utilizing the genotypic change of phage-resistant SA003, we also identified the different host-recognition mechanisms of ϕSA012 and ϕSA039 and analyzed the WGS of the phages to determine the genes that contribute to the host-specificity differences. Elucidating the details of the host recognition mechanism of these two different Twort-like phage species will expand our understanding of how closely related phages exhibit different host preferences. Our finding provides substantial information for expanding the utility of staphylococcal Twort-like phages in the future for practical use.

Materials and methods

Bacteria, phages, and plasmids

Bacteria, phages, and plasmids used in this study are listed in Table 1. *S. aureus* SA003 was previously isolated from milk of a mastitic cow and used for propagation of phages. *S. aureus* strain RN4220 was kindly provided by Prof. Motoyuki Sugai (Hiroshima University Graduate School of Biomedical & Health Science, Hiroshima, Japan) with the permission of Prof. Richard P. Novick (Skirball Institute of Biomolecular Medicine, New York, NY) and used for genetic manipulation. The *S. aureus* virulent phages, ϕSA012 and ϕSA039, were isolated from sewage in Japan (Synnott et al. 2009). Plasmid pLI50 was purchased from Addgene (Cambridge, MA, USA). Plasmid pLIP3 was previously constructed using pLI50 and the P3 promoter, which is constitutive in *S. aureus* (Lee et al. 1991; Jeong et al. 2011; Takeuchi et al. 2016). Plasmid pKOR1-mcs was kindly provided by Dr. Taeok Bae (Indiana University School of Medicine—Northwest, Indianapolis, IN). Plasmid pCasSA was a gift from Dr. Quanjiang Ji (ShanghaiTech University, School of Physical Science and Technology, Shanghai, China). All bacteria and phages were stored in 15% glycerol at − 80 °C. Luria Bertani (LB), brain heart infusion broth, and trypticase soy broth are used as a liquid media. For growth on agar, the medium was solidified by adding 1.5% (*w*/*v*) agar. ϕSA012 and *S. aureus* SA003 were deposited in the culture collection of the NITE Biological Research Center, Kisarazu, Japan under accession numbers NBRC110649 and NBRC110650, respectively (Takeuchi et al. 2016). The phage ϕSA039 is only preserved in our laboratory. For contact details and to acquire channel, refer to the address of the corresponding author.

Passage co-culture experiment of *S. aureus* SA003 and ϕSA012

Serial passaging of ϕSA012 co-cultured with SA003 was carried out in the previous study (Takeuchi et al. 2016; Osada et al. 2017). Briefly, SA003 was inoculated into LB medium and cultured until early exponential phase. Phage ϕSA012 was added with multiplicity of infection (MOI) = 0.1. After 2 to 10 days, bacterium-phage mixed culture was transferred into new LB medium (1:100 dilution) and cultured under the same conditions. The co-culture was repeated until the 38th passage. A phage-resistant strain of SA003 and mutant ϕSA012 phage were collected at the end of each cycle. The resistant strain was named SA003R**n**, whereas the mutant phage was named ϕSA012M**n**, where **n** represents the passage number (e.g., SA003R11 refers to the phage-resistant SA003 derivative isolated from co-culture at the 11th passage, while ϕSA012M11 refers to the mutant phage ϕSA012 isolated from the co-culture at the 11th passage).

Molecular cloning in *S. aureus*

The primers used in this study are listed in Supplemental Table S1. T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Plasmid and PCR product were purified using the Nucleospin® kit

Table 1 Bacterial strains, phages, and plasmids used in this study

Bacterial strain, phage or plasmid	Description	Reference
Bacteria		
E. coli JM109	Competent cells. Genotype *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_K^- m_K^+), *e14*$^-$ (*mcrA*$^-$), *supE44*, *relA1*, Δ (*lac-proAB*)/F′[*traD36*, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15]	TAKARA, Shiga, Japan
S. aureus RN4220	Transformable strain: restriction-deficient (*hsdR*$^-$), *rsbU*$^-$, *agr*$^-$.	DSMZ culture collection, Braunschweig, Germany
S. aureus RNΔTarM	*S. aureus* RN4220 lacking *tarM* gene	Takeuchi et al. (2016)
S. aureus RNdTO	*S. aureus* RN4220 disruptant of *tagO* gene	This study
S. aureus RNΔOatA	*S. aureus* RN*4220* with deleted *oatA* gene	This study
S. aureus SA003	*S. aureus* isolated from milk of mastitic cow	Synnott et al. (2009)
S. aureus SA003R2	Phage-resistant mutant of *S. aureus* SA003 from 2nd round co-culture	Osada et al. (2017)
S. aureus SA003R11	Phage-resistant mutant of *S. aureus* SA003 from 11th round co-culture	Osada et al. (2017)
R2pLIP3.TO	*S. aureus* SA003R2 harboring plasmid pLIP3.TO	This study
R2pLIP3.TarS	*S. aureus* SA003R2 harboring plasmid pLIP3.TarS	This study
R2pLIP3.ScdA	*S. aureus* SA003R2 harboring plasmid pLIP3.ScdA	This study
R11pLIP3.TO	*S. aureus* SA003R11 harboring plasmid pLIP3.TO	This study
R11pLIP3.TarS	*S. aureus* SA003R11 harboring plasmid pLIP3.TarS	This study
R11pLIP3.ScdA	*S. aureus* SA003R11 harboring plasmid pLIP3.ScdA	This study
R11pLIP3.GK	*S. aureus* SA003R11 harboring plasmid pLIP3.GK	This study
R11pLIP3.MurA2	*S. aureus* SA003R11 harboring plasmid pLIP3.MurA2	This study
R11pLIP3.RapZ	*S. aureus* SA003R11 harboring plasmid pLIP3.RapZ	This study
R11pLIP3.Pol	*S. aureus* SA003R11 harboring plasmid pLIP3.Pol	This study
SA003ΔtarS	*S. aureus* SA003 deficient in *tarS*	This study
SA003ΔtarS::pLIP3.TarS	Complemented SA003ΔtarS with pLIP3.TarS	This study
Phages		
ϕSA012, ϕSA039	*S. aureus* phage isolated from sewage influent in Japan	Synnott et al. (2009)
ϕSA012M2	Spontaneous mutant ϕSA012 from 2nd round of co-culture	Takeuchi et al. (2016)
ϕSA012M11	Spontaneous mutant ϕSA012 from 11th round of co-culture	Takeuchi et al. (2016)
Plasmid		
pKOR1-mcs	Plasmid pKOR1 with inserted multiple cloning site, harboring the temperature sensitive origin *repF*, and a selection marker anti-*secY* gene	Bae and Schneewind (2006)
pNL9164	Plasmid for Targetron® system	Sigma-Aldrich, St. Louis, MO, USA
pLIP3	Plasmid pLI50 with promoter P3 from *S. aureus*, CmR (*S. aureus*), AmpR (*E. coli*)	Takeuchi et al. (2016)
pLIP3.TO	pLIP3 with inserted *tagO* gene from SA003	This study
pLIP3.GK	pLIP3 with inserted guanylate kinase-encoding gene of SA003	This study
pLIP3.RapZ	pLIP3 with inserted *rapZ* gene from SA003	This study
pLIP3.MurA2	pLIP3 with inserted *murA2* gene from SA003	This study
pLIP3.TarS	pLIP3 with inserted *tarS* gene from SA003	This study
pLIP3.ScdA	pLIP3 with inserted *scdA* gene from SA003	This study
pLIP3.Pol	pLIP3 with inserted with DNA-directed RNA polymerase alpha subunit-encoding gene of SA003	
pCasSA	Plasmid for genome editing in *S. aureus* by CRISPR/Cas9 system	Chen et al. (2017)
pCasSA-oatA	pCasSA plasmid with spacer and editing template of the *oatA* gene from RN4220	This study
pCasSA-tarS	pCasSA plasmid with spacer and editing template of the *tarS* gene from SA003	This study

(Macherey-Nagel, Düren, Germany). Colony or plaque PCR was performed using DirectAmpPCR® (TAKARA, Shiga, Japan) and processed as recommended by the manufacturer. For PCR using pure DNA, *Taq* DNA polymerase (TAKARA, Shiga, Japan) was used. Deletions of the genes *S2356* and *S2121* were generated by allelic exchange using plasmid pKOR1-mcs (Bae and Schneewind 2006). Gene disruption in *S497* was performed using the Targetron® gene knockout

system (Sigma-Aldrich, St. Louis, MO, USA) (Yao et al. 2006). Deletions of the gene encoding *O*-acetyltransferase (OatA), *oatA*, and of gene *S156* were performed by CRISPR/Cas9 system using plasmid pCasSA (Chen et al. 2017). The complementation experiment was carried out using *E. coli*/*S. aureus* shuttle vector pLIP3 (Takeuchi et al. 2016). Wild-type alleles of SA003 were used as the insert. Electrocompetent *S. aureus* cells were generated and transformed as previously reported (Monk et al. 2012). To transform SA003R11, a large mass of plasmid (< 20 μg) was first concentrated by Pellet Paint® (Novagen®, Billerica, MA, USA) before being introduced into electrocompetent cells. The constructed plasmid was cloned in *E. coli* JM109 and pre-introduced into the *S. aureus* restriction-deficient strain, RN4220, before transforming it into SA003.

Plasmid construction

Complementation plasmids: Target genes were amplified using appropriate primers with restriction site. Plasmid pLIP3 and an amplified PCR product were digested using the same restriction enzyme and ligated by T4 DNA ligase.

Reconstruct plasmid pNL9164 for gene disruption by the Targetron® gene knockout system (Sigma-Aldrich, St. Louis, MO, USA): The plasmid which targets the *S497* (*tagO*) gene was designed following the company's recommendation. Briefly, three primers (IBS, EBS2, and EBS1d) were algorithm-designed to mutate the intron by using the target gene as template sequence (conducted through the company's website). The re-targeted intron was generated through PCR using three algorithm-designed primers and one universal primer which was provided by the company. The re-targeted intron was first digested by *Dpn*I enzyme then further digested by *Hin*dIII and *Bsr*GI, while pNL9164 was digested by *Hin*dIII and *Bsr*GI. Following digestion, the intron was ligated into pNL9164 by T4 DNA ligase.

Reconstruct plasmid pCasSA for gene deletion using CRISPR/Cas9 system: Plasmid construction was performed following a previous report (Chen et al. 2017). Briefly, candidate spacers were searched from the target genes (*oatA* and *S156*) by using online software (https://chopchop.rc.fas.harvard.edu/dev/) or manually selected by searching the protospacer adjacent motif (PAM) region. Two oligos were designed as single strand DNA for each spacer. The double-stranded spacer was generated by phosphorylation using T4 PNK enzyme (New England BioLabs, Ipswich, MA, USA) and annealed at 95 °C for 3 min. Plasmid pCasSA was digested by *Bsa*I enzyme. The double stranded spacer and digested pCasSA were ligated by T4 DNA ligase. Editing template was amplified from the region flanking the target gene. The editing template and the plasmid pCasSA carrying the spacer were digested by *Xho*I and *Xba*I enzymes and subsequently ligated by T4 DNA ligase.

Reconstruct plasmid pKOR1-mcs for gene deletion using allelic exchange: A DNA fragment was prepared by splicing by overlap extension (SOE) PCR using regions flanking the target genes (*S2356* and *S2121*) as the template DNA, digested with appropriate enzymes, and inserted into pKOR1-mcs.

Phage preparation

All phages were propagated by the plate lysate method (Stephenson 2011). Briefly, 100 μl of phage lysate (10^5 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 $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin) was added to the plate and the over layer was scraped off to extract phage, the supernatant was collected by centrifugation (5000*g*, 15 min, 4 °C). The obtained phage lysate was purified by polyethylene glycol (PEG) precipitation and CsCl density gradient centrifugation (Stephenson 2011). Each phage culture was titrated and stored at 4 °C until used.

Spot testing

Two microliters of phage lysate at a titer 10^5 plaque forming units (PFU) was dropped on a lawn of bacteria and incubated overnight at 37 °C. We did not use a high titer of phage lysate to avoid lysis from without (Abedon 2011). The experiment was conducted in triplicate. Efficiency of plating (EOP) test was performed for further confirmation of each tested bacteria. As the EOP of each tested strains (relative to wild-type) was calculated, the number of plaque in the spot test of this study could be validated according to the EOP value.

Efficiency of plating

One-hundred microliters of phage (10^4 PFU/ml) was mixed with 100 μl overnight culture of tested bacteria in 3 ml of 0.5% top agar, plated on LB agar, and incubated at 37 °C overnight. The experiment was conducted in triplicate. The EOP value was measured as a percentage of the number of observed plaque in the tested bacteria divided by the number of observed in the wild-type strain.

Adsorption assay

The adsorption efficiency of phages on *S. aureus* strains was measured by titrating free phages present in the supernatant after 25 min 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 subsequently incubated at 37 °C with shaking at 120 rpm to an OD_{660} of 1.0 (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 for 25 min, free phages were collected by centrifugation (9730*g*, 1 min) and titrated using SA003. Fifty micrograms per milliliter of either 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 (Baptista et al. 2008). Adsorption efficiency was calculated by dividing the number of adsorbed phages by the initial number of phages. We selected 25 min for the cell-phage contact because the adsorption of phage onto phage-resistant bacteria tended to be delayed as compared to the adsorption onto wild-type bacteria.

DNA extraction, sequencing, and bioinformatic

DNA was extracted from bacteria using a DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA). Whole-genome sequencing of bacteria was conducted with the Illumina Miseq platform with genome coverage (sequencing depth) = 300-fold. Genomes were assembled by Platanus de novo assembly ver1.2.1 (Kajitani et al. 2014). Open reading frames (ORFs) were predicted and annotated by using the RAST server (http://rast.nmpdr.org/). Genome was mapped by Bowtie2 (Langmead and Salzberg 2012), and SNPs were detected by SAMtools/BCFtools (Li et al. 2009). Mutated regions were confirmed by PCR and Sanger-sequencing. DNA extraction and genome analysis of phage was performed as previously reported (Takeuchi et al. 2016).

Measuring phage inhibition by peptidoglycan

Pure peptidoglycan from *S. aureus* (PGN-SA, InvivoGen, Shatin, Hong Kong) was used for our phage inhibition assay. The teichoic acid was removed from peptidoglycan by treatment of 48% hydrofluoric acid at 4 °C for 48 h (De Jonge et al. 1992). Efficiency of plating with the addition of peptidoglycan was performed as follows: 10 μl of peptidoglycan solution (concentration [ng/μl]; 250, 100, 25, and 0) was added into 100 μl (10^4 PFU/ml) phage and incubated at room for 1 h. Next, 100 μl of the mixture was mixed with 100 μl *S. aureus* overnight culture in 3 ml of 0.5% top agar, plated on LB agar, and incubated at 37 °C overnight.

Phosphate quantification of wall teichoic acids (WTA) and total sugar quantification of whole cell

WTA was extracted from bacteria culture that had been adjusted to same colony-forming unit (CFU). WTA extraction was conducted following previous method (Meredith et al. 2008). The extraction of WTA was performed using high alkalinity (0.1 M NaOH) which strongly removed the WTA from peptidoglycan. The consistency of WTA isolation was measured by continuously checking the phosphate content during extraction until the phosphate content was constant. The strains from the early round of co-culture did not show to have a slimy texture (capsule); therefore the extraction was consistent. The strain which has sliminess suggestive of capsular polysaccharide (such as SA003R38) was not further documented for the WTA quantification as the sliminess structure was likely inhibiting the WTA extraction.

Phosphate content of WTA was determined following Chen et al. (1956). Same volume of extracted WTA sample and freshly prepared reagent C (mixture of one volume of 6 N sulfuric acid with 2 volumes of distilled water, one volume of 2.5% ammonium molybdate, and one volume of 10% ascorbic acid) were mixed, covered with parafilm, and incubated at 37 °C for 1.5–2 h; KH_2PO_4 was used as phosphate standard. The mixture was cooled down at room temperature for a few minutes and read at the absorbance of 820 nm. Total carbohydrate of bacterial cells was measured by anthrone reagent (Viles and Silverman 1949); 2 ml bacteria culture (2×10^9 CFU) was washed by phosphate buffer saline (PBS) twice and mixed with anthrone solution (1 mg/ml in concentrated H_2SO_4 [95–98% *w*/*v*]), the mixture was incubated at 90 °C for 10 min and the OD_{630} of sample was measured. D(+)-Glucose was used as standard.

Phage inhibition by antibodies

EOP with antibodies specific for ϕSA012 ORF103 (anti-ORF103) and ORF105 (anti-ORF105) was conducted according to a previous study (Takeuchi et al. 2016).

Statistical analysis

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

Accession number(s)

The genome data of *S. aureus* SA003 and ϕSA039 were submitted to the DDBJ database under the accession number AP018376 and AP018375, respectively. Genome of ϕSA012 was available in the GenBank database under accession number AB903967.

Results

Whole genome analysis of phage-resistant SA003

The phenotypic change in the phage-resistant strains such as the change of growth rate and colony size was observed and

well explained in our previous study (Osada et al. 2017). We also observed a turbid or completely no plaque by spot testing and a significant reduction of phage adsorption by adsorption assay (Osada et al. 2017; Takeuchi et al. 2016). We believe that these observations were enough to justify the claim of phage resistance.

The whole genomes of five phage-resistant mutant strains, as well as parent strain SA003, were sequenced on an Illumina Miseq platform. Mutations detected in the sequenced strains were mapped against the wild-type SA003 reference genome (Table 2). A clustered regularly interspaced polindromic repeats (CRISPR) system, which is well-known as a major genetic barrier against phage infection (Hyman and Abedon 2010), is absent in the genome of SA003.

A total of eight mutations were identified. One-point mutation (AAA → ATA) in *S2356*, which encodes ferredoxin glutamate synthase, was found in the phage-resistant isolate from the first round, SA003R1. The mutation was preserved until the last round of co-culture. However, compared to wild-type SA003, the sensitivity of SA003R1 toward ϕSA012 phage did not differ significantly according to plating efficiency and adsorption assays (data not shown). Deleting *S2356* also did not alter the sensitivity of *S. aureus* (data not shown). We assumed that the mutation in *S2356* did not contribute to phage resistance.

Table 2 Mutations of SA003 resulting in phage resistance

Gene	*MasSA_00497* (+)	*MasSA_00156* (+)	*MasSA_00157* (+)	*MasSA_00515* (+)	*MasSA_00692* (+)	*MasSA_00768* (+)	*MasSA_02121* (−)	*MasSA_02190* (−)	*MasSA_02356* (−)
Arbitrary name	*S497*	*S156*	*S157*	*S515*	*S692*	*S768*	*S2121*	*S2190*	*S2356*
Function	Undecaprenyl-phosphate *N*-acetylglucosaminyl 1-phosphate transferase (TagO)	Glycosyltransferase (TarS)	Iron-sulfur repair protein ScdA	RNase adapter protein Z (RapZ)	Hypothetical membrane protein YozB	Guanylate kinase	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase 2 (MurA2)	DNA-directed RNA polymerase alpha subunit	Ferredoxin-dependent glutamate synthase
Position in the gene [a]	118	523…1722 [b]	1…455	142 799	196	122	346	806	821
SA003	**CAC** **H**	+ +	+ +	**CCT CAT** **P H**	**AAT** **N**	**ACA** **T**	**GGA** **G**	**GCA** **A**	**AAA** **K**
SA003 R1	**CAC** **H**	+ +	+ +	**CCT CAT** **P H**	**AAT** **N**	**ACA** **T**	**GGA** **G**	**GCA** **A**	**ATA** **I**
SA003 R2	**TAC** **Y**	+ **Deleted**	+ **Deleted**	**CCT CAT** **P H**	**AAT** **N**	**ACA** **T**	**GGA** **G**	**GCA** **A**	**ATA** **I**
SA003 R11	**TAC** **Y**	+ **Deleted**	+ **Deleted**	**GCT CAT** **A H**	**AAT** **N**	**ATA** **I**	**TGA** **Stop**	**GUA** **V**	**ATA** **I**
SA003 R20	**TAC** **Y**	+ **Deleted**	+ **Deleted**	**GCT GAT** **A D**	**GAT** **D**	**ATA** **I**	**TGA** **Stop**	**GUA** **V**	**ATA** **I**
SA003 R38	**TAC** **Y**	+ **Deleted**	+ **Deleted**	**GCT GAT** **A D**	**GAT** **D**	**ATA** **I**	**TGA** **Stop**	**GUA** **V**	**ATA** **I**

[a] Position of the nucleotide from start codon. Upper sequences are nucleotide sequence, and lower sequences are amino acid sequences. Bold underlined residues indicate nucleotide mutations, and gray boxes represent mutations in amino acid

[b] "+" indicates the region is present

The phage-resistant strain in the second round (SA003R2) acquired a point mutation (CAC → TAC) in *S497* encoding undecaprenyl-phosphate *N*-acetylglucosaminyl 1-phosphate transferase, which is well-known as teichoic acid ribitol (*tagO*) gene. This strain also acquired a 1779-bp deletion in the WTA *tar* gene cluster (Brown et al. 2013) consisting of: a 1200-bp C-terminal region of *S156* (which encodes glycosyltranferase, TarS); a 125-bp non-coding region; and a 454-bp N-terminal region of *S157* (which encodes iron-sulfur repair protein, ScdA) (Fig. 1A).

Four more genes, *S2121*, *S768*, *S515*, and *S2190*, were mutated in the resistant strain, SA003R11, isolated from the 11th round. *S2121*, which encodes UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase-2 MurA2 (an enzyme which catalyzes the first step of peptidoglycan synthesis together with MurA) (Blake et al. 2009) showed a nonsense mutation in a glycine-encoding codon (GGA → TGA). *S768*, which encodes a guanylate kinase (Oeschger 1978), and *S2190*, which encodes the alpha subunit of DNA-directed RNA polymerase (Ishihama 1992), showed point mutations that changed a threonine into isoleucine (ACA → ATA) and an alanine into valine (GCA → GUA), respectively. Mutations in *S156*, *S157*, *S497*, *S2121*, *S768*, and *S2190* were preserved until later generations, including the 20th and 38th rounds, from which we isolated SA003R20 and SA003R38, respectively. In SA003R11, we also identified one point mutation in *S515*, which encodes for the RNAse adapter protein RapZ (Komatsuzawa et al. 2004). The mutation in this gene developed into two point mutations in SA003R20 and SA003R38. In SA003R20 and SA003R38, we also identified a point mutation (AAT → GAT) *S692*, which encodes the putative membrane protein YozB.

To evaluate the role of mutated host genes in phage resistance, we performed complementation in SA003R2 and SA003R11 by using the wild-type alleles in trans with each mutated gene. As we had identified a nonsense mutation in *S2121*, we also constructed a deletion mutant of this gene. The *S2121* deletion mutant was unchanged in its sensitivity toward ϕSA012 (data not shown).

In the resistant strain SA003R2, among three mutated genes (*S156, S157,* and *S497*), only complementation of *S157* and *S497* can restore phage susceptibility of SA003R2 to ϕSA012M2 (Fig. 1B). However, another *Myoviridae* Twort-like phage, ϕSA039, showed completely different infectivity toward SA003R2 and complemented mutant. ϕSA039 formed faint plaques on SA003R2 plates (Fig. 1B) with the EOP value = 0.01 ± 0.00%, and surprisingly, complementation of *S156* allele in SA003R2 was able to restore the susceptibility to ϕSA039 (Fig. 1B, D) with an EOP value = 86.02 ± 1.25%. We suggested that ϕSA039 and ϕSA012 recognize *S. aureus* cell wall by different mechanisms. The mutation of SA003R2 was associated with the reduction in WTA production as total phosphate in extracted WTA from SA003R2 decreased significantly compared to wild-type SA003 (Fig. 2). However, we could not determine the reduction of WTA in SA003R38 because of the production of sliminess suggestive of capsular polysaccharide (not further documented).

In the resistant strain SA003R11, complementation of each mutated gene, except *S156*, can significantly restore phage susceptibility toward ϕSA012M11 (Fig. 1E). The complemented mutant of *S156* has an EOP value (%) 5.53 ± 2.52, while complementation of other genes: *S157*, *S497, S515, S768*, *S2121*, and *S2190*, shows EOP values 63.70 ± 1.15, 77.64 ± 0.58, 65.38 ± 0.58, 78.85 ± 0.58, 63.70 ± 1.15, and 43.12 ± 0.58, respectively. Complementation of *S768* and *S497* shows the highest EOP value among other genes. Excluding the *S156* complemented mutant, ϕSA039 failed to form plaques on plates of SA003R11 or its complemented mutants. This observation provides stronger evidence on the importance of *S156* gene for the infection of ϕSA039 as the complementation of other mutated genes did not significantly restore host susceptibility toward ϕSA039.

Adsorption of ϕSA012 and ϕSA039 to complemented mutants

To determine the adsorption of phages to the complemented mutants, we conducted an adsorption assay for phages ϕSA012M2 and ϕSA012M11 toward wild-type SA003, their phage-resistant counterpart, as well as the complemented mutants. For the complementation in SA003R2 (Fig. 1C), adsorption of ϕSA012M2 onto wild-type SA003 and SA003R2 was 93.64 ± 0.44% and 26.83 ± 2.29%, respectively. Complementation of *S156* (R2pLIP3.TarS) did not significantly restore the phage adsorption (29.35 ± 1.15%), while *S497* (R2pLIP3.TO) and *S157* (R2pLIP3.ScdA) restored the phage adsorption to 47.86 ± 1.97% and 42.00 ± 2.30%, respectively. Interestingly, adsorption of ϕSA039 was significantly restored to 76.20 ± 1.49% in R2pLI.TarS indicating the importance of the *S156* gene for the adsorption of ϕSA039 onto SA003.

Next, we analyzed the adsorption of ϕSA012M11 toward the complemented mutants of SA003R11. The complemented mutants of SA003R11 harbor *S156* (R11pLIP3.TarS), *S157* (R11pLIP3.ScdA), *S497* (R11pLIP3.TO), *S515* (R11pLIP3.RapZ), *S768* (R11pLIP3.GK), *S2121* (R11pLIP3.MurA2), and *S2190* (R11pLIP3.Pol). As shown in Fig. 1F, after 25 min of co-incubation, 93.23 ± 8.79% of ϕSA012M11 was adsorbed onto wild-type SA003 while only 1.92 ± 3.59% was adsorbed onto SA003R11. After complementation of the mutated genes by their respective wild-type alleles, the adsorption of ϕSA012M11 to the complemented mutants of *S157*, *S497, S515, S768,* and *S2121* was restored. However, even though we observed plaques, the adsorption

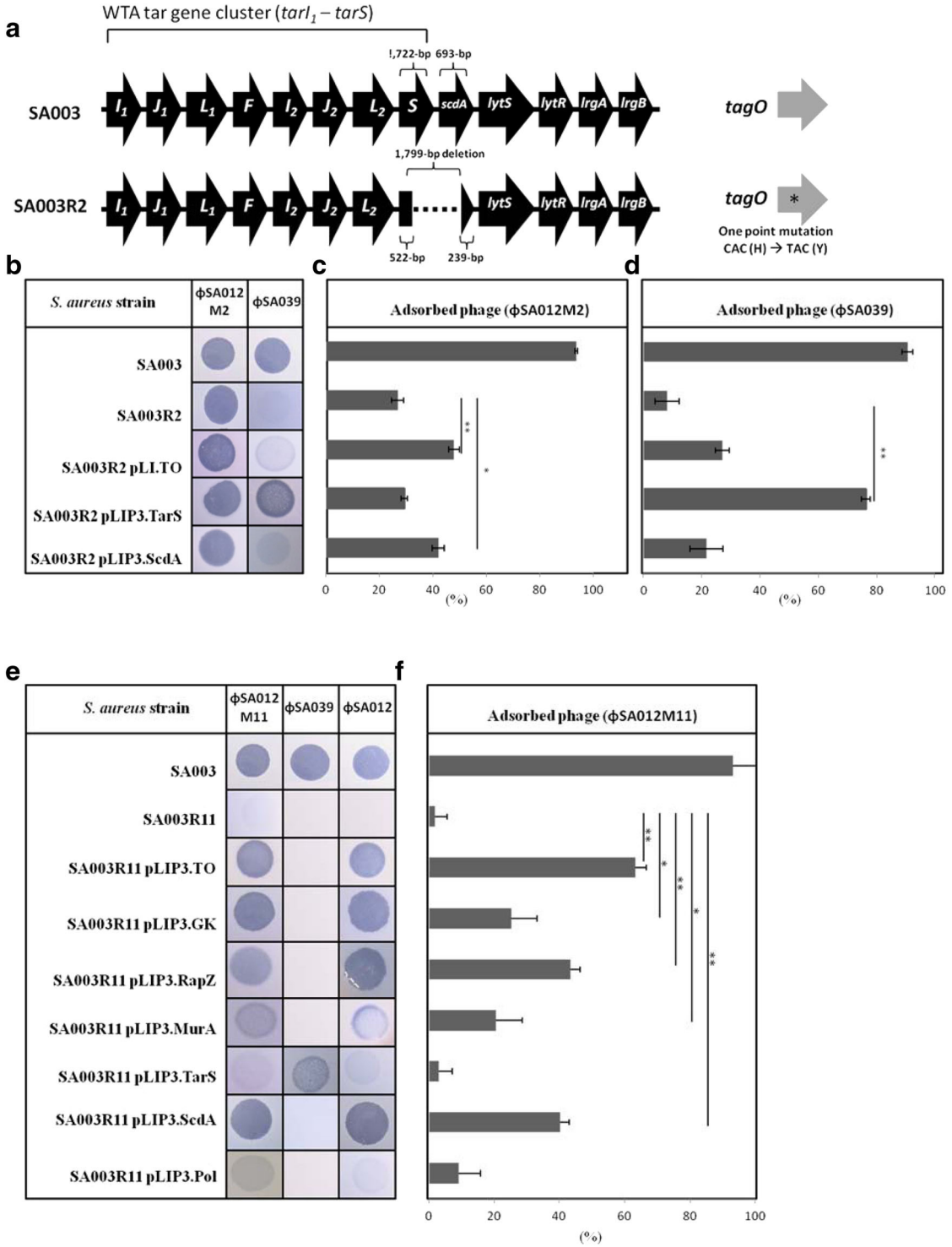

Fig. 1 (**A**) Spontaneous mutations in SA003R2; deletion of the *tarS* and *scdA* genes, one point mutation in the *tagO*. (**B**) Spot test of ϕSA012M2 and ϕSA039 toward SA003R2 and its phage resistance derivative. Adsorption assay of ϕSA012M2 (**C**) and ϕSA039 (**D**) toward SA003R2 and its phage resistance derivative. Spot test (**E**) and adsorption assay (**F**) of ϕSA012M11 toward SA003R11 and its phage resistance derivative. Statistical significance is indicated by *($P < 0.05$) or **($P < 0.01$)

onto *S2190*-complemented mutant did not change significantly. Interestingly, on the case of *S768* gene, although the EOP value of *S768*-complemented mutant (R11pLIP3.GK) was the highest among other genes, the adsorption of phage onto this complemented mutant was relatively low compared to the complemented mutant of *S497, S515,* and *S2121*. We speculated that the mutation in *S2190* (which encodes DNA-directed RNA polymerase alpha subunit), and *S768* (which

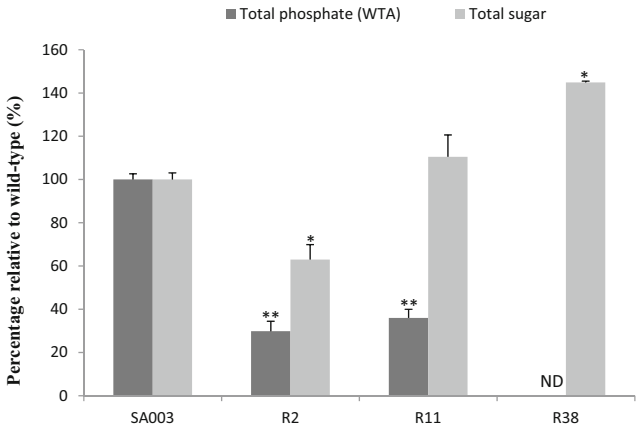

Fig. 2 Percentage of total phosphate from extracted wall teichoic acid (WTA) (dark) and whole-cell sugar (gray) relative to the wild-type strain SA003. Values are given as means ± standard deviations (SD, $n = 3$). Statistical significance is indicated by *($P < 0.05$) or **($P < 0.01$). ND = not determined

encodes guanylate kinase) contributes to the inhibition of post-adsorption.

Adsorption of ϕSA012M11 to R11pLIP3.TO, R11pLIP3.ScdA, R11pLIP3.GK, R11pLIP3.RapZ, and R11pLIP3.MurA was restored to 63.20 ± 3.30, 40.25 ± 2.77%, 25.17 ± 7.88%, 43.44 ± 2.95%, and 20.55 ± 7.96%, respectively. The mutation in SA003R11 was associated with an increase in whole-cell sugar amount according to total carbohydrate quantification using anthrone reagent (Fig. 2). Relative to SA003, the whole-cell sugar amount of SA003R2 decreased to 62.98 ± 6.92%. Meanwhile, the total sugar amounts of SA003R11 and SA003R38 increased to 110.49 ± 10.10% and 144.89 ± 0.63%, respectively. A slimy texture was visible on the overnight liquid culture of SA003R38 (data not shown).

The infection of wild-type ϕSA012 to the complemented mutant of R11 is similar to ϕSA012M11 according to the EOP test (data not shown) and a spot test (Fig. 1E). The adsorption of ϕSA012 toward the SA003R11-complemented mutant was not significantly different compared to ϕSA012M11 (Supplemental Table S2). However, for most of the genes (except R11pLIP3.ScdA and R11pLIP3.Pol), the wild-type phage's adsorption showed a tendency to be stronger than ϕSA012M11.

Peptidoglycan from *S. aureus* inhibits infection of ϕSA012 but not ϕSA039

As some mutated genes (e.g., *S2121* and *S515*) are related to peptidoglycan synthesis, we assume that peptidoglycan may play an important role in inhibiting phage adsorption. For this reason, we performed a plating efficiency test with the addition of peptidoglycan. Different concentrations of peptidoglycan from *S. aureus* (InvivoGen) were added to 100 μl of 10^3 PFU/ml phages prior to the plaque assay against SA003. ϕSA012 and ϕSA039 were used. We used the *E. coli* phage SP-21 (Tanji et al. 2005) as a negative control. As shown in Fig. 3, peptidoglycan inhibited the infection of ϕSA012 but not ϕSA039. To confirm if *O*-acetylation in C6 of the muramic acid residue is important for binding of ϕSA012, we deleted the *oatA* gene by a CRISPR/Cas9 system using the pCasSA plasmid. However, the adsorption of ϕSA012 onto the *oatA* deletion mutant did not change (Fig. 5).

Genome comparison of ϕSA012 and ϕSA039

According to morphological observation under TEM, ϕSA039 and ϕSA012 belong to *Myoviridae* phages. Both of them show broad, yet different, host ranges against *S. aureus* isolated from the milk of mastitic cows (Synnott et al. 2009). As these two phages show different infectivity, we analyzed the whole genome of ϕSA039 and mapped it to the genome of ϕSA012 as a reference. The whole genome of ϕSA012 has been explained in a previous report (Takeuchi et al. 2016). Whole genome sequencing of ϕSA039 revealed that its genome size is 141,038 bp long and contains 217 ORFs. The ϕSA039 genome encodes three tRNAs (pseudo Trp-tRNA gene, bp 27,174 to 27,103; Phe-tRNA gene, bp 27,253 to 27,181; and Asp-tRNA gene, bp 27,333 to 27,260). At the nucleotide level, ϕSA039 and ϕSA012 share 96% identity where several genes are missing or partially deleted in either ϕSA012 or ϕSA039 (listed in Supplemental Table S3). The genome of ϕSA039 shares 99% identity with staphylococcal phage JD007 (Cui et al. 2012).

As ϕSA039 and ϕSA012 differ in their host preference, we selected the region of the genome encoding the putative tail and baseplate proteins and analyzed ORFs in that region by amino acid alignment of the encoded products by basic local alignment tool (blastp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Most of the ORFs in the selected region share a high degree of similarity (Fig. 4); most of the ORFs have more than 99% identity and three

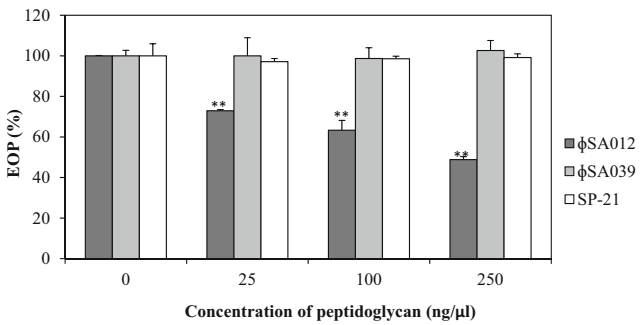

Fig. 3 Dose-dependent inhibition by peptidoglycan of phage ϕSA012 (black bar), phage ϕSA039 (gray bar) and *E. coli* phage SP-21 (white bar). Data represent means ± standard deviations (SD, n = 3). Statistical significance was indicated by *($P < 0.05$) or **($P < 0.01$)

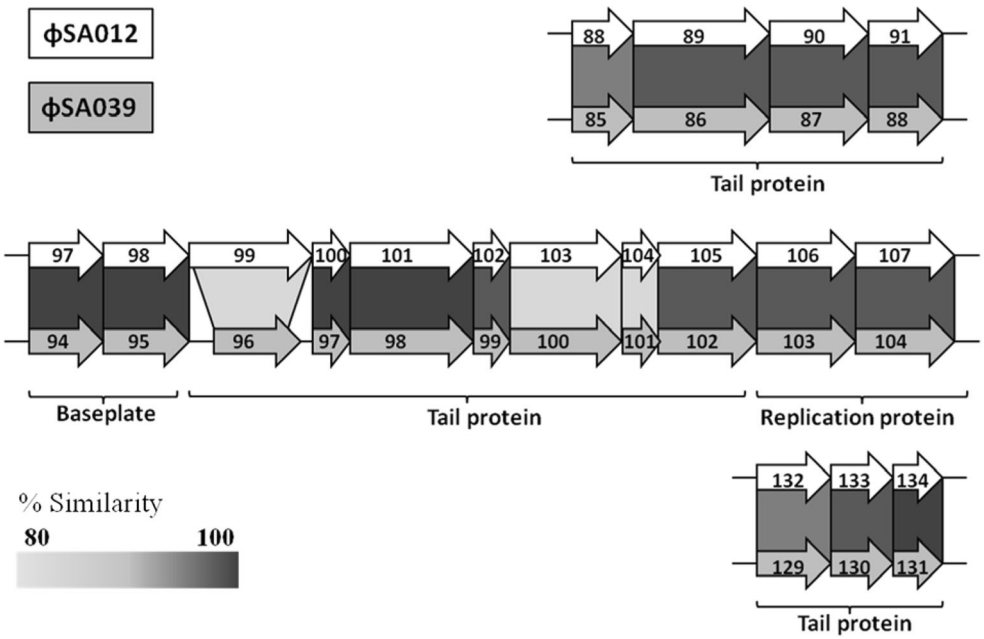

Fig. 4 Alignment of putative tail and baseplate region between ϕSA012 and ϕSA039

ORFs share 91% identity. Three ORFs share relatively limited level of identity with all the others: *orf100* (86%), *orf101* (88%), and *orf96* (83%). Moreover, *orf96* of ϕSA039 lacks codons for 195 amino acid residues compared to ϕSA012. *Orf100* and *orf102* of ϕSA039 are homologs of *orf103* and *orf105* of ϕSA012 which were previously reported as genes for receptor-binding protein (RBP) (Takeuchi et al. 2016).

To determine whether proteins of *orf100* and *orf 102* of ϕSA039 have the same activity as those of homolog genes, we performed a neutralization test in ϕSA039 by EOP with antibodies against the *orf103* protein of ϕSA012 (anti-ORF103 serum) and the *orf105* protein of ϕSA012 (anti-ORF105) by using *S. aureus* RN4220. Infection by ϕSA039 was completely inhibited by anti-ORF105 serum but not by anti-ORF103 serum (Supplemental Fig. S1).

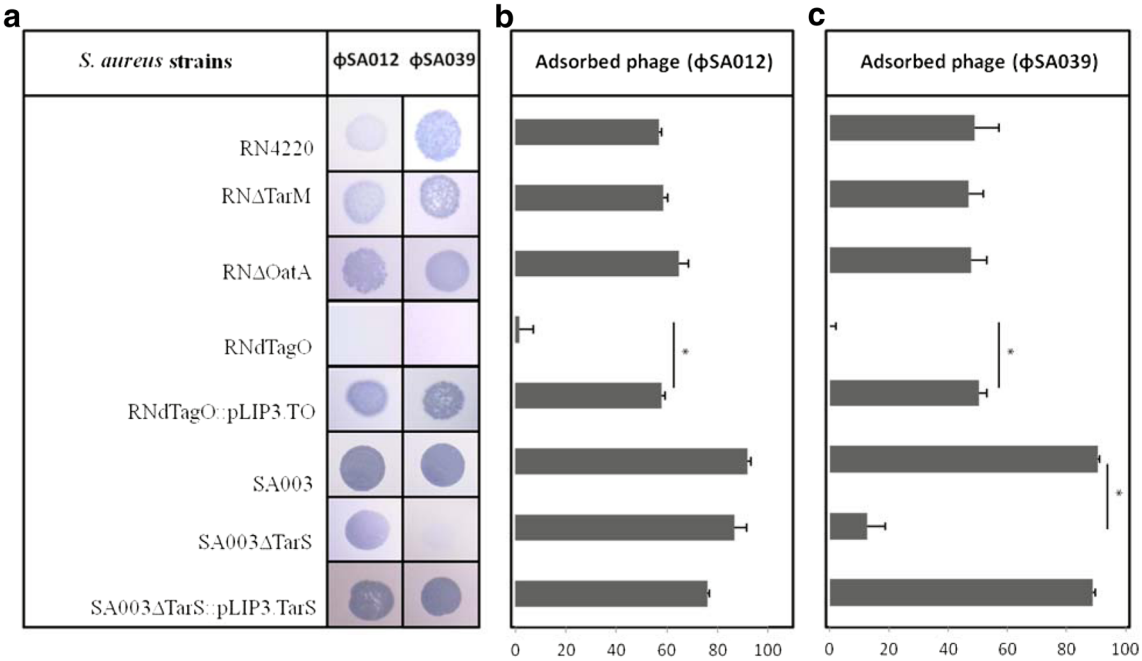

Fig. 5 Infectivity of ϕSA012 and ϕSA039 toward RN4220, *tarM* deletion mutant (RNΔTarM), *oatA*-deletion mutant (RN ΔOatA), *tagO*-deletion mutant (RNdTagO), and complemented *tagO* (RNdTagO::pLIP3.TO). *tarS*-deletion mutant (SA003ΔTarS) and complemented *tarS* (SA003ΔTarS::pLIP3.TarS). The infectivity assay is based on spot test (**A**) and adsorption assay for ϕSA012 (**B**) and ϕSA039 (**C**). Data represent means ± standard deviations (SD, n = 3). Statistical significance is indicated by *($P < 0.05$) or **($P < 0.01$)

Artificial deletion of the *tarS* gene in SA003 significantly reduces the adsorption of ϕSA039 but not ϕSA012

As described in "Adsorption of ϕSA012 and ϕSA039 to complemented mutants", phage susceptibility of SA003R2 toward ϕSA039 was decreased drastically, and complementation of the *S156* gene for glycosyltransferase TarS, the enzyme that glycosylates β-GlcNAc on WTA (Brown et al. 2012), restores the adsorption of ϕSA039. We hypothesized that β-GlcNAc on WTA is crucial for binding of ϕSA039. To test our hypothesis, we first generated a deletion and complementation of *tagO* in *S. aureus*. As shown in Fig. 5, our spot test and adsorption assay failed to detect infection of the *tagO* deletion mutant (RNΔTagO) either by ϕSA039 or ϕSA012. Complementation of the *tagO* deletion (RNΔTagO::plIP3.TO) restored the adsorption of ϕSA039 and ϕSA012 to 50.55 ± 2.50% and 57.75 ± 1.57%, respectively. On the other hand, deleting *tarS* caused a significant reduction only on the adsorption of ϕSA039. Complementation of the *tarS* deletion mutant (SA003ΔTarS::plIP3.TarS) restored the adsorption of ϕSA039 to 88.69 ± 0.98%. This result indicated that ϕSA039 requires β-GlcNAc on WTA. Since the adsorption of ϕSA039 was not completely inhibited in TarS-null SA003, this phage may also use the backbone of WTA for its binding.

Discussion

Most mutated host genes are linked to phage adsorption

The infection pathway of phages can be divided into several steps: phage adsorption onto the cell surface of the host, DNA injection into the host cell, DNA replication, assembly of phage particles, and lysis of the host cell. To prevent the first infection process, phage-resistant bacteria typically evolve phage adsorption inhibition by altering their phage receptor (Bohannan and Lenski 2000; Denes et al. 2015) or blocking the receptor by, for example, production of capsular polysaccharide (Bernheimer and Tiraby 1976; Scholl et al. 2005).

Our finding showed that SA003 also developed phage resistance through inhibition of phage adsorption as the first defense system. In the early round of co-culture (SA003R2), the bacteria acquired spontaneous mutation in TagO (*S497*) and ScdA (*S157*) which are involved in the initial synthesis of WTA polymer (Soldo et al. 2002) and in cell morphogenesis (Brunskil et al. 1997), respectively. These spontaneous mutations were manifested by the reduction of WTA production in SA003R2. We proved that the mutation of these two genes correspond to the inhibition of ϕSA012 adsorption. In response to the mutation in SA003R2, two point mutations were identified in *orf103* of the mutant phage counterpart, ϕSA012M2. The mutation in *orf103* was consistent with the increase in the infectivity toward SA003R2. Therefore, mutant *orf103* likely fastens the phage binding onto SA003R2 (Takeuchi et al. 2016). Interestingly, in SA003R2, we also found a deletion of *S156* (TarS), which glycosylates β-GlcNAc on WTA (Brown et al. 2012). This deletion was shown not to affect ϕSA012 adsorption but significantly impaired ϕSA039 adsorption. Further development of mutations was observed in SA003R11. The bacteria overproduced their capsular polysaccharide or altered their peptidoglycan by spontaneous mutation in MurA2 (*S2121*), which has been reported to catalyze the first step of peptidoglycan synthesis together with MurA (Blake et al. 2009), and RapZ (*S515*), which modulates the expression of GlmS (the key enzyme that feeds glucose into cell wall synthesis in *S. aureus*) (Komatsuzawa et al. 2004). The spontaneous mutations in RapZ accumulated in later mutant bacteria (SA003R20 and SA003R38) which was consistent with the increasing of capsular production. This observation indicated in this study that the *rapZ* gene was involved in capsular polysaccharide production.

When the phage adsorption and DNA injection are inevitable, major defenses against invading DNA are the restriction-modification (R-M) system and the clustered regularly interspaced short polindromic repeats (CRISPR) system (Murray 2000; Levin et al. 2013). However, phages could potentially escape from the R-M system by the absence of endonuclease recognition sites in their genome. For example, *Staphylococcus* phage K (a well-known polyvalent Twort-like phage) has no *Sau*3A (which recognizes the '5-GATC-3' sequence) site in its genome. Like phage K, phages in our study (ϕSA012 and ϕSA039) have no *Sau*3A site in their genome, and none of the mutations identified in phage-resistant SA003 mapped to a R-M system present in these bacteria. Therefore, we concluded that during coevolution, SA003 did not use the R-M system to combat ϕSA012. Furthermore, the CRISPR/Cas9 system is absent in SA003, leading us to conclude that mutant SA003 did not develop CRISPR-mediated phage resistance. It is well-known that in many pathogenic bacteria, including *S. aureus*, many of the clones have lost CRISPR/Cas9 during evolution. As a result, *S. aureus* frequently exchange their genetic material via phage-mediated horizontal gene transfer (Brussow et al. 2004; Lindsay 2010).

Two mutated genes in phage-resistant SA003 involved in inhibition of post-adsorption

We defined the "inhibition of post-adsorption" as the defense system of the host after the phage is able to absorb. Because the phage resistance mechanism consists of several systems (such as blocking the phage DNA entry, cutting the phage DNA), we simplified them by using the term "inhibition of post-adsorption" in this study.

To defend against mutant ϕSA012 that can escape the adsorption inhibition system, the resistant bacteria (SA003R11) evolved spontaneous mutations in the genes *S768* (guanylate kinase) and *S2190* (DNA-directed RNA polymerase). Our study shows that the complemented mutant of guanylate kinase shows a high EOP value compared to other complemented mutants, but a relatively low adsorption rate. We interpreted that the mutation of this gene is related to both inhibition of phage adsorption and post-adsorption. This gene is involved in a crucial intermediate step in RNA/DNA synthesis and is essential for cellular GMP recycling and nucleotide equilibrium (Oeschger 1978; Konrad 1992). The product of this gene participates in purine metabolism (Weber et al. 1992). Purine biosynthesis has been shown to be associated with the survival of *S. aureus* under conditions of stress, such as in the presence of vancomycin and daptomycin (Keer et al. 2001; Mongodin et al. 2003). Yee et al. (2015) reported that a defect in the purine biosynthesis pathway may be related to downstream energy production (i.e., increased purine biosynthesis fuels the generation of polymers, the most energy-demanding metabolic process in bacteria). Two of the most abundant polymers in *S. aureus* and *Bacillus subtilis* are peptidoglycan (Mongodin et al. 2003) and WTA (Ellwood 1970). By affecting purine metabolism, the mutation in this gene is likely involved in the recycling of injected phage-DNA, and subsequently fueling the generation of polymers in the cell wall.

Unlike guanylate kinase, the complementation in *S2190* (DNA-directed RNA polymerase) did not restore phage adsorption but did increase the EOP value. RNA polymerase alpha subunit is well-known to be involved in transcription activation (Ishihama 1992). Most phages with a genome size less than 200 kbp commonly did not harbor a complete set of genes responsible for genome replication and nucleotide metabolism. Only a large-genome size phage (termed Jumbo phage), with a genome larger than 200 kbp, has been reported to have more than one paralogous gene for DNA polymerase and RNA polymerase (RNAP) (Yuan and Gao 2017). Our phage in this study has a genome size around 142 kbp and the complete set of paralogous genes of the RNA polymerase subunits was not found (Takeuchi et al. 2016). Therefore this phage may use host RNA polymerase as machinery for its DNA transcription. Early studies using phages has broadened our understanding on how phages modulate the transcription process to favor viral production over host cell function. For example, gp67 protein of *Staphylococcus* phage G1, one of the Twort-like phages, has been identified to block the transcription of *S. aureus* by its binding to the host RNA polymerase (Osmundson et al. 2012; Osmundson and Darst 2013). We speculated that in order to prevent phage acquisition, SA003 acquired a spontaneous mutation in *S2190*, thereby, inhibiting the transcription of phage DNA. This finding is supported by a previous observation (Osada et al. 2017) that SA003R11 resists phage infection not only by inhibiting phage adsorption but also by suppressing phage genome replication.

In this study, we also observed that the infection of wild-type ϕSA012 onto the complemented mutant of SA003R11 was not significantly different compared to ϕSA012M11. However, excluding R11pLIP3.ScdA and R11pLIP3.Pol, the adsorption of wild-type ϕSA012 showed a tendency to be stronger than ϕSA012M11. Fitness cost might be detected in ϕSA012M11 as it experienced long-term coexistence with SA003. Therefore, the infection of ϕSA012M11 toward the SA003R11-complemented mutants is weaker than that of wild-type ϕSA012. Such phenomena have also been observed in other studies that demonstrated that coevolved phages reduced their infectivity toward the wild-type host and limited the host range expansion (Hall et al. 2011).

Two *Myoviridae* Twort-like phages (ϕSA012 and ϕSA039) use different receptors to infect *S. aureus*

Our data showed that although the ϕSA012 and ϕSA039 genomes share high similarity, these two phages infect *S. aureus* by different mechanisms. ϕSA039 infectivity toward SA003R11 and SA003R2 and its complemented mutant is lost or significantly lessened, except in the instance of *S156* (TarS) complementation. This suggests that TarS is important for ϕSA039. Most *S. aureus* strains synthesize repeating units of ribitol-phosphate (RboP) WTA with the three tailoring modifications D-alanine, α-GlcNAc, and β-GlcNAc (Brown et al. 2013). The GlcNAc moieties are attached to RboP by two independent enzymes namely α-GlcNAc WTA glycosyltransferase TarM (Xia et al. 2010) and β-GlcNAc WTA glycosyltransferase TarS (Brown et al. 2012). Most *S. aureus* phages target WTA and its GlcNAc moieties for adsorption. Notably, it has been reported that *Siphoviridae* phages use α-GlcNAc moieties as receptors (Xia et al. 2011; Li et al. 2016), while *Podoviridae* phages, such as ϕ44AHJD, ϕ66, and ϕP68, use β-GlcNAc moieties (Li et al. 2015). In contrast, *Myoviridae* phages have been reported to simply require WTA polymer, regardless of GlcNAc acetylation (Xia et al. 2011). According to our observation, the deletion mutant of *tagO* is resistant to both phages. Deletion of TarM has no affect to both phages, while deletion of TarS significantly reduces the adsorption of ϕSA039. As a result, we conclude that both phages (ϕSA012 and ϕSA039) utilize WTA as the receptor. ϕSA012 uses the backbone of WTA while ϕSA039 uses the backbone and the β-*N*-acetyl glucosamine residue of WTA. As β-*N*-acetyl glucosamine has been reported to serve as a binding site of PBP2a, the enzyme responsible for β-lactam antibiotic resistance in *S. aureus* (Brown et al. 2012), the application of ϕSA039 and β-lactam antibiotic may give a synergetic effect for the treatment of *S. aureus* infection. In addition, we found that ϕSA012 is inhibited by peptidoglycan. However, both

phages did not use *O*-acetylated peptidoglycan for adsorption. ϕSA012 may utilize another component in the peptidoglycan polymer.

In silico analysis of ϕSA039 and ϕSA012 genomes reveal potential viral proteins that contribute to different adsorption mechanisms

Two terminal repeat regions (*orf195* and *orf200*) that exist in ϕSA012 genome are missing in ϕSA039. Łobocka et al. (2012) reported that all genes located in the terminal redundant region play a role in a host takeover that is analogous to the *B. subtilis* phage, SPO1, which also possesses a terminal redundant region (Stewart et al. 1998). O*rf81* of ϕSA012, which contains a sequence for a putative intron-encoded nuclease, and the gene of *orf39* of ϕSA012, which encodes a DNA ligase are also missing in ϕSA039. Interestingly, a partial deletion is found in *orf96* of ϕSA039, a homolog of *orf99* of ϕSA012, which encodes a tail morphogenetic protein. By using the HHpred search tool (https://toolkit.tuebingen.mpg.de/#/tools/hhpred), this ORF is predicted to have a similar function as the wedge protein gp7 (in the baseplate) of the T4 phage, which involves in sheath contraction (Yap et al. 2016). In our previous study, the mutant phage ϕSA012M20 carried a point mutation in *orf99*, and subsequently accumulated two more point mutations to become the mutant phage ϕSA012M38 (Takeuchi et al. 2016), which may indicate the importance of *orf99* during phage-host coevolution.

In the tail or baseplate region of ϕSA039, compared to ϕSA012, several ORFs have been shown to have limited level of identity (83–88%): i.e., *orf99*, *orf103*, and *orf104,* whereas *orf103* and *orf104* are located in the unique region of the Twort-like tail/baseplate module (Fig. 4) (Takeuchi et al. 2016). During phage-host interaction, the product of *orf103* of ϕSA012 had been reported to bind onto α-GlcNAc of WTA, while the product of *orf105* is likely to bind onto the backbone of WTA (Takeuchi et al. 2016). Since anti-ORF105 of ϕSA012 can neutralize the ϕSA039 infection, the binding activity of the product of *orf102* of ϕSA039, a homolog of *orf105* of ϕSA012, must be similar. Thus, in ϕSA039, the product of *orf102* may bind onto the backbone of WTA. However, the binding of the product of *orf100*, a homolog of *orf103* of ϕSA012, onto bacteria cell remains unknown. Taken together, the difference in tail and baseplate proteins are likely to be factors responsible for specificity difference between ϕSA012 and ϕSA039. However, further analysis of those potential genes might be necessary.

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Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest in this article.

Ethical approval This article does not contain any studies with human participants and animals performed by any of the authors.

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