Analysis host-recognition mechanism of staphylococcal kayvirus ϕSA039 reveals a novel strategy that protects *Staphylococcus aureus* against infection by *Staphylococcus pseudintermedius Siphoviridae* phages

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Abstract

Following the emergence of antibiotic resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), phage therapy has attracted significant attention as an alternative to antibiotic treatment. Bacteriophages belonging to kayvirus (previously known as Twort-like phages) have broad host range and are strictly lytic in *Staphylococcus* spp. Previous work revealed that kayvirus ϕSA039 has a host-recognition mechanism distinct from those of other known kayviruses: most of kayviruses use the backbone of wall teichoic acid (WTA) as their receptor; by contrast, ϕSA039 uses the β-N-acetylglucosamine (β-GlcNAc) residue in WTA. In this study, we found that ϕSA039 could switch its receptor to be able to infect *S. aureus* lacking the β-GlcNAc residue by acquiring a spontaneous mutation in open reading frame (ORF) 100 and ORF102. Moreover, ϕSA039 could infect *S. pseudintermedius*, which has a different WTA structure than *S. aureus.* By comparison with newly isolated *S. pseudintermedius*–specific phage (SP phages), we determined that glycosylation in WTA of *S. pseudintermedius* is essential for adsorption of SP phages, but not ϕSA039. Finally, we describe a novel strategy of *S. aureus* which protects the bacteria from infection of SP phages. Notably, glycosylation of ribitol phosphate (RboP) WTA by TarM or/and TarS prevents infection of *S. aureus* by SP phages. These findings could help to establish a new strategy for treatment of *S. aureus* and *S. pseudintermedius* infection, as well as provide valuable insights into the biology of phage–host interactions.

Keyword: Kayvirus, phage therapy, host-recognition mechanism, *Staphylococcus pseudintermedius*, *Staphylococcus aureus*

Introduction

Staphylococcus is a Gram-positive bacterium that causes many kinds of infections. Two representative species, *S. aureus* and *S. pseudintermedius*, are coagulase-positive bacteria that are notoriously pathogenic in humans and animals (Kloos and Bannerman 1994; Pompilio et al. 2015). *S. aureus* is a commensal found on the skin and mucosae of humans, whereas *S. pseudintermedius* commonly inhabits dog skin. Both are common bacterial pathogens associated with chronic and recurrent skin infections that require long-term systemic antimicrobial therapy. Infection by *S. aureus* and *S. pseudintermedius* is becoming problematic due to the emergence of antibiotic-resistant strains, including methicillin-resistant (MRSA and MRSP) and vancomycin-resistant strains (VRSA) (Enright et al. 2002; Sakoulas et al. 2005). Virulent bacteriophages that can kill a wide range of *S. aureus* hosts represent promising alternatives to conventional antibiotic treatment (Alves et al. 2014; Iwano et al. 2018; Azam and Tanji 2019a). The success of phage infection depends on its host specificity, which is often determined by the interaction between a phage receptor-binding protein (RBP) and its cognate receptor on the surface of the host cell (Hyman and Abedon 2010).

Staphylococcal kayviruses (previously known as Twort-like phages) have broad host range and high lytic ability, making them suitable candidates for phage therapy (Łobocka et al. 2012). Most phages belonging to this group use the backbone of wall teichoic acid (WTA), the most abundant molecule in the cell wall of *Staphylococcus*, as their receptor (Xia et al. 2011). However, previous work showed that kayvirus ϕSA039 uses the β-GlcNAc moiety in WTA of *S. aureus* SA003, a unique feature within the group (Azam et al. 2018).

The broad host range of kayviruses includes coagulase-negative staphylococci (CoNS) (Cui et al. 2012; Lobocka et al. 2012; Iwano et al. 2018). WTA in *S. aureus* generally consists of

repetitive 1,5-ribitol-phosphate (RboP) modified with a GlcNAc residue and D-alanine. The GlcNAc moieties are transferred onto WTA by the α-GlcNAc transferase TarM and the β-GlcNAc transferase TarS (Xia et al. 2010; Brown et al. 2012). By contrast, WTA of *S. pseudintermedius* and CoNS has glycerol-phosphate (GroP) as the backbone and various glycoepitopes (GlcNAc, GalNAc, or Glc) (Endl et al. 1983; Winstel et al. 2014).

Staphylococcal phages that use glycoepitopes in the WTA as their receptors, e.g., phages from families *Siphoviridae* and *Podoviridae*, target either the RboP or GroP type of WTA. For example, *Siphoviridae* ϕ11 only recognizes GlcNAc of RboP WTA, whereas ϕ187 only recognizes the glycoepitope of GroP WTA (Winstel et al. 2014). Therefore, the unique feature of ϕSA039, a kayvirus that requires the β-GlcNAc residue in the WTA of SA003 (RboP WTA), raises the question of whether this phage can also recognize other types of WTA from different *Staphylococcus* species. In this study, we evaluated the host range of ϕSA039 and its potential for phage therapy toward various strains of *S. pseudintermedius*, including MRSP. In addition, we also screened *S. pseudintermedius*–specific phages, evaluated them as potential antimicrobial agents, and compared them with ϕSA039.

In addition, a previous study showed that kayviruses such as ϕSA012 and ϕK have at least two RBPs (RBP1 and RBP2), which are responsible for these phages' broad host range (O'Flaherty et al. 2004; Takeuchi et al. 2016). Mutant ϕSA012 has a modified RBP2 (ORF103) that enables the phage to infect mutant *S. aureus* SA003R38, which has an altered WTA and overproduces capsular polysaccharide (Takeuchi et al. 2016; Osada et al. 2017; Azam et al. 2018), indicating that mutated RBP2 may allow the phage to counter-adapt to resistant hosts by using an alternative component as a novel receptor (Takeuchi et al. 2016). Like ϕSA012, ϕSA039 also has two RBPs, RBP1 (ORF100) and RBP2 (ORF102) (Azam et al. 2018). Hence,

in light of the unique infection strategy of ϕSA039, we also investigated the ability of the phage to counter-adapt to phage-resistant host and analyzed the underlying mechanism.

Material and Methods

Bacterial strains, bacteriophages, and plasmids

Bacteria, phages, and plasmids used in this study are listed in Table 1. *S. aureus* RN4220 was used with the permission of Professor Richard P. Novick (Skirball Institute of Biomolecular Medicine, New York, NY, USA). *S. aureus* SA003 was isolated from milk of a mastitic cow (Synnott et al. 2009). The *S. aureus* virulent phages ϕSA039 and ϕSA012 were isolated from sewage in Japan (Synnott et al. 2009). All *S. pseudintermedius* isolates were isolated from the skin of dogs with canine pyoderma. Nine coagulase-negative *Staphylococcus* (CoNS) were isolated from a patient at Jichi Medical University hospital, and species identification was performed based on the 16S rRNA gene. *S. aureus* SA003, *S. pseudintermedius* SP015, *S. pseudintermedius* SP070, and *S. pseudintermedius* SP079 were deposited in the culture collection of NITE Biological Research Center, Kisarazu, Japan, under accession numbers NBRC110650, NBRC113855, NBRC113857 and NBRC113858, respectively. All phages used in this study are deposited in the corresponding author's institution and distributed to other researcher by request. All primers used in this study are listed in Supplemental Table S1.

Isolation of S. pseudintermedius

Staphylococcus was isolated based on standard culture and biochemical tests on site. Screening of *S. pseudintermedius* was performed using the API®/ID32 kit (Sysmex-bioMérieux, Tokyo, Japan). To distinguish *S. pseudintermedius* and *S. intermedius*, restriction fragment length polymorphism (RFLP) analysis was performed on the *kat* gene (Blaiotta et al. 2010). *S.*

pseudintermedius strains were distinguished by PCR amplification of the hypervariable X region of the protein A gene (*spa*) (Moodley et al. 2009). Multilocus sequence typing (MLST) was performed following Bannoehr's method, which targets five genes (16SrRNA, *tuf*, *cpn60*, *pta*, and *agrD*) (Bannoehr et al. 2007; Bannoehr et al. 2009).

Bacteriophage isolation and purification

Phage ϕDP001, which is capable of lysing *S. pseudintermedius*, was isolated from dog saliva. Saliva was collected using sterile cotton swabs, which were soaked in 1 ml SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin), and incubated at 4°C for at least 1 h. After centrifugation (9730 *g*, 1 min), 100 µl supernatant was mixed with 100 µl overnight culture of *S. pseudintermedius* SP015 in 3 ml of 0.5% top agar, plated on LB agar, and incubated at 37°C overnight. ϕSP120, ϕSP197, and ϕSP276 were isolated by mitomycin induction; Mitomycin was added into *S. epidermidis* culture (OD = 0.5) at a final concentration of 0.5 µg/ml and incubated for 1 h. Phages were isolated from the supernatant after centrifugation (9730 *g*, 3 minutes). Propagation and purification of the phages was performed as previously described (Synnott et al. 2009).

Plaque assay and efficiency of plating (EOP)

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

Spot test

Two microliters of phage lysate at a titer of 10^8 plaque forming units (PFU) per ml (10^5 PFU) was dropped onto a lawn of bacteria and incubated overnight at 37°C. Experiments were conducted in triplicate.

Adsorption assay

The adsorption efficiency of phages on *S. aureus* strains was measured by titrating the presence of free phages in the supernatant after 20 minutes of cell–phage contact following previous study (Azam et al. 2018). Free phage was collected by centrifugation (9730 × *g*, 1 min) and titrated using SA003 for ϕSA039 and SP070 for SP phages.

Isolation and characterization of spontaneous mutant of ϕSA039

Co-culture experiments were performed with *S. aureus* SA003 and ϕSA039. First, 4.5 ml of LB broth was inoculated with 1% overnight culture of SA003 and cultured until early exponential phase (OD_{660}=0.1) in a TVS062CA BioPhotorecorder (Advantec, Tokyo, Japan). Then, phage ϕSA039 was added at a multiplicity of infection (MOI) = 1. The mixture was cultured at 37°C with shaking at 40 rpm. After 2 days, the culture was transferred to 4.5 ml of fresh LB medium (1% dilution) and cultured under the same condition. Co-cultures were repeated until a spontaneous mutant of ϕSA039 that could infect TarS-null *S. aureus* was isolated. Two spontaneous mutants were isolated from the co-culture and were designated ϕM1 and ϕM2.

Checking for mutations in genes encoding tail and baseplate proteins

Genes encoding tail and baseplate proteins (ORF95–ORF102) in spontaneous mutant phages were amplified by plaque PCR using DirectAmpPCR (TAKARA, Shiga, Japan) and

155 analyzed by Sanger sequencing. Phage plaques were touched with a toothpick and mixed into the
156 PCR mixtures.

157 **Generation and characterization of chimeric phage**

158 Chimeric phage was generated by homologous recombination using plasmid pNL9164
159 (Sigma-Aldrich, MO, USA) in *S. aureus* SA003. Mutated DNA fragments of ORF100 and
160 ORF102 were amplified from spontaneous mutant phages by PCR using KOD-plus Neo enzyme
161 (Toyobo, Shiga, Japan). PCR fragments and the plasmid were digested with appropriate
162 restriction enzymes. The DNA fragment was inserted into plasmid pNL9164 using T4 DNA
163 Ligase (New England BioLabs, Ipswich, MA, USA). The constructed plasmid was cloned into
164 *Escherichia coli* JM109 competent cells (TAKARA, Shiga, Japan) and pre-introduced into
165 restriction-deficient *S. aureus* RN4220 before being transformed into *S. aureus* SA003.

166 To perform homologous recombination, transformant SA003 harboring plasmid with
167 homologous region was infected with phage (MOI = 1). The mixture was incubated at 37°C
168 overnight. Recombinant phages were isolated from the supernatant fraction of the mixture after
169 centrifugation (8000 × g, 3 minutes). SA003ΔTarS was used to screen recombinant phages from
170 homologous recombination of ORF100, and RN4220 was used to screen recombinant phages
171 from homologous recombination of ORF102. Mutated ORF100 from spontaneous mutant phage
172 ϕM1 was introduced into wild-type ϕSA039, yielding chimeric phage ϕrM1/r-100. Mutated
173 ORF102 of ϕM1 was introduced into ϕrM1/r-100, yielding chimeric phage ϕrM1/r-100&102.

174 **Deletion of *oatA* gene in RN4220ΔtarMΔtarS**

175 The gene was deleted by pCasSA plasmid with clustered regularly interspaced short
176 palindromic repeats (CRISPR)-Cas9 system (Chen et al. 2017). Plasmid construction was

177 performed as previously described (Chen et al. 2017; Azam et al. 2018). Spacers were manually
178 selected by searching the protospacer adjacent motif (PAM) region. Two oligos were designed as
179 single-stranded DNA for each spacer, and double-stranded spacer was generated by
180 phosphorylation with T4 PNK (New England BioLabs, Ipswich, MA, USA) and annealed at
181 95°C for three minutes. Plasmid pCasSA was digested with *Bsa*I. The double-stranded spacer
182 and digested pCasSA were ligated with T4 DNA ligase (New England BioLabs, Ipswich, MA,
183 USA). Editing template was amplified by splicing by overlap extension (SOE) PCR using the
184 region flanking the target gene as the DNA template. The resultant plasmid was cloned into
185 *Escherichia coli* JM109 competent cells (TAKARA, Shiga, Japan) and transformed into *S.*
186 *aureus* RN4220ΔtarMΔtarS. Complementation of deletion mutants was performed using plasmid
187 pLI50 (purchased from Addgene [Cambridge, MA]) under the control of the P3 promoter
188 (pLIP3) (Jeong et al. 2011; Takeuchi et al. 2016). The wild-type allele from RN4220 was used as
189 the insert.

190 **Selection of phage-resistant mutants of *S. pseudintermedius* SP015**

191 Plaque assays were performed using SP015 and ϕSA039 at an MOI = 10 and incubated at
192 37°C overnight. Colonies (resistant mutants) were purified, inoculated onto LB plates, and
193 incubated at 37°C overnight. The phage resistance of isolated mutants was determined by spot
194 test and adsorption assay. Phage-resistant SP015-R1 which showed ability to inhibit phage
195 adsorption was further characterized.

196 **DNA extraction, sequencing, and bioinformatics**

197 Phage genome was extracted using phage DNA isolation kit (NORGEN, ON, Canada).
198 Bacterial genome was extracted using DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA).

Whole-genome sequencing was conducted on the Illumina HiSeq platform with genome coverage (sequencing depth) of 100-fold. Genomes were assembled using velvet ver 1.2.10. ORFs were predicted and annotated using the RAST server (http://rast.nmpdr.org/). The presence of toxin or virulence genes in the phage genome was determined using PHASTER (PHAge Search Tool Enhanced Release) server (phaster.ca).

Statistical analysis

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

Accession number (s)

Genome data of three SP phages (ϕSP120, ϕSP197, and ϕSP276) and *S. pseudintermedius* SP079 were submitted to the DNA Data Bank of Japan (DDBJ) database under accession numbers AP019560, AP019561, AP019562, and AP019372, respectively.

Results

ϕSA039 can recognize WTA of *Staphylococcus pseudintermedius* SP015

ϕSA039 exhibited at least a moderate ability to infect various strains of *S. pseudintermedius*, and 12 CoNS (Table 2). A close relative of ϕSA039, ϕSA012, also had a broad host range toward the *Staphylococcus* species we tested. In this study, we also isolated *S. pseudintermedius*–specific phages (SP phages) ϕDP001, ϕSP120, ϕSP197, and ϕSP276, and compared them with ϕSA012 and ϕSA039.

As shown in Table 2, in 20 isolates of *S. pseudintermedius*, ϕSA012 and ϕSA039 exhibited at least a moderate infection (turbid plaque): ϕSA012 infected 95% of isolates (19/20), whereas ϕSA039 infected 90% (18/20). ϕSA012 and ϕSA039 could infect 93% of *S. aureus* isolates (13/14) and the 12 CoNS. Unlike ϕSA012 and ϕSA039, no SP phages could infect the *S. aureus* strains used in this study. Regarding *S. pseudintermedius*, ϕDP001 infected 50% of isolates (10/20), whereas the other three SP phages infected 95% of isolates (19/20). *S. pseudintermedius* SP079 was the only strain that was resistant against all SP phages. Whole-genome analysis revealed that this bacterium encodes CRISPR-Cas9 system in its genome. DNA fragments of ϕSP120 and ϕSP197 were identified in the CRISPR regions of the two SP079 (Supplementary Table 1). Four SP phages formed at least turbid plaques in CoNS, with the exception of *Staphylococcus saprophyticus*.

We speculated that SP phages recognize receptors in *S. pseudintermedius* and CoNS isolates that are absent in *S. aureus*. Because most staphylococcal phages require WTA as their receptor (Azam and Tanji 2019b), we sought to determine whether our SP phages also require WTA. To this end, we generated WTA-free *Staphylococcus* by inhibiting WTA synthesis in the

cell using tunicamycin (Zhu et al. 2018). ϕSA039, ϕSA012, and three SP phages (ϕDP001, ϕSP120, and ϕSP197) had no ability to infect WTA-free *S. pseudintermedius* and *S. epidermidis* (data not shown). The phages could not form a plaque and failed to adsorb onto WTA-free isolates. By contrast, ϕSP276 did not completely lose its infectivity toward WTA-free isolates, indicating that phage can use another component as a receptor. These findings indicated that all phages in this study utilize WTA in *S. pseudintermedius* and *S. epidermidis* as their receptor, but that the WTA of these bacteria is likely distinct from that of *S. aureus.*

Accumulation of point mutations in ORF100 enables ϕSA039 to infect TarS-null *S. aureus*

ϕSA039 requires β-GlcNAc glycosylation of WTA by TarS (Azam et al. 2018). In this study, we found that ϕSA039 could generate spontaneous mutants capable of infecting TarS-null *S. aureus.* Mutants of ϕSA039 that could infect TarS-null *S. aureus* were obtained from the fifth batch of two co-cultures. One spontaneous mutant phage (ϕM1and ϕM2) was purified from each of co-culture and further characterized. In SA003ΔtarS, adsorption of ϕM1and ϕM2 was around 8-fold than that of wild-type ϕSA039 (Fig 1a). ϕM1and ϕM2 exhibited similar adsorption toward SA003ΔtarS. Because the phage tail fiber and baseplate region are thought to be involved in phages' adsorption specificity, we amplified the genomic region encoding the tail and baseplate proteins (ORF94 until ORF102) using primers described in Supplemental Table S1. Spontaneous mutations were detected in ORF100 and ORF102. ϕM1 harbors three point mutations in ORF100 and one point mutation in ORF102, whereas ϕM2 only has three point mutations in ORF100. All mutations changed the amino acid sequence of the protein. The mutations in ORF100 were distributed among five locations (one near the N-terminus, two in the middle, and two near the C-terminus). A point mutation located at base 623 (TCT, S → TAT, Y) was detected in both mutant phages. Point mutations at bases 907 (GAT, D → TAT, N) and 1850 (ACG, T → AGG,

R) were detected in ϕM1, whereas mutations at bases 1012 (GAA, E → AAA, K) and 1844 (ACA, T → AAA, K) were detected in ϕM2. The mutation in ORF102 was located in the C-terminus at base 1116 (AAA, K → ACA, T).

Because all ϕSA039 mutants had spontaneous base changes in ORF100, we speculated that these mutations enabled the phages to infect SA003ΔtarS. We hypothesized that by introducing the point mutation in ORF100 into wild type ϕSA039, we should be able to construct chimeric ϕSA039 capable of infecting SA003ΔtarS. Hence, we introduced ORF100 of ϕM1 into wild-type ϕSA039 by homologous recombination, yielding the chimeric phage ϕrM1/r-100.

Spot tests revealed that ϕrM1/r-100 could infect SA003ΔtarS (data not shown). We then performed an adsorption assay to determine whether the ability of the recombinant phages to infect SA003ΔtarS was due to an effect of adsorption as a result of replacement of ORF100. Indeed, adsorption of ϕrM1/r-100 on SA003ΔtarS was significantly elevated relative to wild-type ϕSA039 (Fig. 1a).

The α-GlcNAc residue in WTA blocks the infection of mutant ϕSA039 lacking a mutation in ORF102

S. aureus SA003 naturally lacks the gene encoding glycosyltransferase TarM (genome accession number: AP018376). Because certain *S. aureus* strains have *tarM* and *tarS* in their genome (Brown et al. 2012), in this study, we also used *S. aureus* RN4220, which has a complete set of WTAs (genome accession number: GCA_000212435.2), to characterize the mutant phage. As shown in Fig. 1b, wild-type ϕSA039 could not infect RN4220ΔtarS or RN4220ΔtarMΔtarS. However, mutant phages ϕM1 and ϕM2 exhibited completely different patterns of infectivity in the presence of *tarM*. Infection of ϕM2 was severely impaired toward RN4220, which has *tarM*.

Only 3.86% of ϕM2 could adsorb onto RN4220, whereas 38.93% of ϕM1 could do so. Deletion of the *tarM* gene in RN4220 (RN4220ΔTarM) improved adsorption of ϕM1 and ϕM2 to 63.97% and 28.04%, respectively. A similar pattern was observed in RN4220ΔTarS. The presence of *tarM* in RN4220ΔTarS decreased the adsorption efficiency of both ϕM1 and ϕM2, and conversely, deletion of *tarM* in RN4220ΔTarS (RN4220ΔTarMΔTarS) improved the adsorption of both mutant phages. Overall, the mutations in ϕM1 and ϕM2 decreased the ability of the phages to adsorb onto WTA glycosylated by TarM; in particular, adsorption of ϕM2 was almost completely abolished.

As with ϕM2, the chimeric phage ϕrM1/r-100 was unable to adsorb onto RN4220 (6.06%), but was infectious toward RN4220ΔTarM (14.60% adsorbed phage). Based on this observation, we hypothesized that the absence of a point mutation in ORF102 in ϕM2 and ϕrM1/r-100 may make these phages unable to infect RN4220. To test this idea, we introduced a point mutation in ORF102 into ϕrM1/r-100 by homologous recombination, yielding the chimeric phage ϕrM1/r-100&102. Indeed, adsorption of the chimeric phage ϕrM1/r-100&102 improved three times higher than that of ϕrM1/r-100 (fig 1b).

Whole-genome sequencing of SP phages

Because ϕSP120, ϕSP197, and ϕSP276 had a broad host range in *S. pseudintermedius*, we further investigated their potential as antimicrobial agents. To this end, we first sequenced the entire genomes of all three phages. In doing so, our primary goal was to investigate the presence of toxin, virulence, and antibiotic-resistance genes that could make them inappropriate for phage therapy.

The whole-genome sequencing analysis revealed that ϕSP120, ϕSP197, and ϕSP276 belong to *Siphoviridae*, with genome sizes of 40530, 41149, and 40711 bp, respectively. The GC content of ϕSP120, ϕSP197, and ϕSP276 was similar to that of *S. pseudintermedius*, but somewhat higher that of *S. aureus* (36%, 35%, and 36%, respectively). The genomes of the phages had a low degree of identity relative to one another. Their genomes were organized into six functional modules; lysogeny, DNA replication, packaging, head, tail, and lysis. The presence of integrase (CI) and repressor (Cro) genes in the genome indicate that these phages can undergo a lysogenic cycle (Xia and Wolz 2014). The integrases of the three phages were identical. Toxin, virulence, and antibiotic-resistance genes were absent from all three genomes, according to the PHASTER server.

Using the ViPTree online tool (https://www.genome.jp/viptree), we found that these phages are phylogenetically close to ϕ187 (Genome accession number: NC_007047). ϕ187 is a *Siphoviridae* phage isolated from the *S. aureus* ST395 lineage by mitomycin C induction (Asheshov and Jevons 1963). Other phages evolutionarily close to our SP phages were Stb27 (Genome accession number: NC_019914) and StB12 (Genome accession number: NC_020490), both of which were isolated from CoNS *Staphylococcus hominis* trough mitomycin C induction (Deghorain et al. 2012). The WTA structure of the *S. aureus* ST395 lineage and CoNS are similar to that of *S. pseudintermedius* (Winstel et al. 2014).

WTA of *S. pseudintermedius* is different to that of *S. aureus*

Because SP phages cannot infect *S. aureus*, we speculated that the WTA of *S. pseudintermedius* is distinct from that of *S. aureus*. To date, the known WTAs of *Staphylococcus* bacteria are of at least two types, GroP and RboP (Brown et al. 2013; Winstel et al. 2014). Using

primers targeting the *tagF* gene, which encodes the GroP polymerase of *S. pseudintermedius* ED99, we determined that all *S. pseudintermedius* in our study likely possess GroP type WTA. Most of the isolates had WTA clusters similar to that of ED99 (genome accession number: NC_017568). Genes encoding glycosyl transferases TarM and TarS were absent from *S. pseudintermedius* isolates. As with *S. pseudintermedius*, CoNS also have GroP WTA (Endl et al. 1983; Winstel et al. 2014). Therefore, our SP phages may specifically recognize GroP WTA, whereas ϕSA012 and ϕSA039 recognize both GroP and RboP WTA.

ϕ187 can utilize WTA of *S. aureus* PS187, which belongs to the ST395 lineage, but not that of the common *S. aureus* lineage (Winstel et al. 2014). Unlike other *S. aureus*–type strains, *S. aureus* ST395 has GroP WTA with α-GalNAc glycosylation mediated by TagN (Winstel et al. 2013, Winstel et al. 2014). In this study, we detected the presence of *tagN* in most *S. pseudintermedius* isolates. However, *S. pseudintermedius* SP070 and SP190 lacked *tagN* but had a gene encodes glycosyltransferase that absent from other isolates (unpublished data).

SP phages can recognize non-glycosylated RboP WTA in *S. aureus*

WTA sugar modifications are highly variable in *Staphylococcus* species, and have been implicated in bacteriophage susceptibility and immunogenicity (Brown et al. 2013). The WTA of *S. aureus* contains α-GlcNAc and/or β-GlcNAc. In this study, we found that although no SP phages could infect *S. aureus* (Table 2), the absence of α-GlcNAc and β-GlcNAc in the WTA enabled the SP phages to infect this bacterium. SP phages could infect *S. aureus* RN4220ΔTarMΔTarS and SA003ΔTarS, both of which lack the GlcNAc modification in their WTA (Fig 2). Because SA003 naturally lacks TarM, SA003ΔTarS has a WTA similar to that of RN4220ΔTarMΔTarS. Although the observed plaques were slightly turbid, SP phages could

adsorb onto RN4220ΔTarMΔTarS (Fig 2). Adsorption efficiencies of ϕSP120, ϕSP197, and ϕSP276 onto RN4220ΔTarMΔTarS were 40.34%, 32.69%, and 43.75%, respectively. Complementation of either TarM (α-GlcNAc) or TarS (β-GlcNAc) blocked infection by SP phages; however, the complete absence of WTA in RN4220dtagO also prevented infection. Thus, SP phages can utilize non-glycosylated WTA as their recognition site on *S. aureus* cells.

6-O acetylation of muramic acid residues in peptidoglycan of *S. aureus* decreases the adsorption ability of staphylococcal *Siphoviridae* phages ϕ11 and ϕ52A (Li et al. 2016). To determine whether peptidoglycan acetylation is involved in SP phage adsorption onto RN4220 ΔTarMΔTarS, we generated a deletion mutant of *oatA* and used this strain as a host for the adsorption assay. We did not observe a change in the adsorption of SP phages relative to RN4220ΔTarMΔTarS (data not shown), suggesting that peptidoglycan acetylation is not essential for SP phage adsorption onto *S. aureus*.

Glycoepitope of WTA in SP015 is essential for SP phages

Staphylococcal *Siphoviridae* phages require glycosylated WTA as their receptor (Xia et al. 2011). In RboP WTA, *Siphoviridae* phages recognize the GlcNAc residue, regardless of its stereochemistry. However, the host recognition mechanism of *Siphoviridae,* which recognizes GroP WTA, remains poorly understood. To analyze the host recognition mechanism of our SP phages, we generated phage-resistant *S. pseudintermedius* SP015.

Reduced adsorption of three SP phages was observed in phage-resistant SP015 R1 (Fig 3). Using primers targeting the WTA gene cluster in SP015, we identified a point mutation in *tagN* of R1 that caused a premature stop codon at amino acid 629 (Fig 3a). Complementation of *tagN* using a wild-type allele restored adsorption of SP phages around 50%. Notable, mutation in

R1 improved adsorption of ϕSA039 (56.20%), whereas complementation of *tagN* in R1 decreased adsorption of ϕSA039 (31.70%). In SP015 and its mutant derivatives, adsorption did not differ significantly between mutant ϕSA039 (ϕM1, ϕM2, ϕrM1/r-100, and ϕrM1/r-100&102) and wild-type ϕSA039 (Supplemental Figure S2).

Discussion

Alteration of RBP enables ϕSA039 to switch its receptor

Although ϕSA039 requires the β-GlcNAc moiety in WTA of *S. aureus*, it can switch its receptor by acquiring spontaneous mutation in its RBP. Alteration of phage receptors is a bacterial strategy used to prevent the initial step of phage infection (Capparelli et al. 2010; Hyman and Abedon 2010; Azam and Tanji 2019a). A previous study showed that spontaneous mutant *S. aureus* lacking the β-GlcNAc moiety in WTA can be easily obtained by co-culturing the bacteria with phage (Azam et al. 2018), suggesting that the emergence of phage-resistant *S. aureus* lacking β-GlcNAc moiety in WTA is possible in a real-world setting. However, in contrast to the situation with antibiotic resistance, ϕSA039 can counter-adapt phage-resistant host by acquiring mutations in ORF100. We confirmed that mutation in ORF100 is key to ϕSA039's ability to infect TarS-null *S. aureus*. Spontaneous mutants with three point mutations in ORF100 could infect a TarS-null host. Chimeric phages harboring mutant ORF100, produced by homologous recombination, also exhibited similar infectivity. However, a phage with mutated ORF100 could not infect *S. aureus* RN4220, which has TarM (an enzyme responsible for glycosylation of α-GlcNAc of WTA). This residue is likely important for the infection of RN4220 by ϕSA039. Mutation in ORF100 caused the loss of binding ability of ϕSA039 onto

RN4220. Our hypothesis was consistent with these observations: ϕM2 and ϕrM1/r-100 could infect TarM-null RN4220 (RN4220ΔTarM), and complementation of TarM in RN4220ΔTarM blocked the infection of the phages.

Subsequent analysis revealed that a point mutation in ORF102 enabled the mutant phage to adsorb onto RN4220. Phage ϕM1, which has mutations in ORF100 and ORF102, was able to bind WTA of RN4220. Our hypothesis is consistent with the ability of a chimeric phage harboring point mutations in ORF100 and ORF102 (ϕrM1/r-100&102) to infect RN4220.

A similar phenomenon has been documented in phages infecting *Escherichia coli*: mutation in *gp38*, which encodes the tail protein of coliphage PP01, enables PP01 to infect *E. coli* O157:H7 lacking the receptor OmpC (Morita et al. 2002). Via such mutations, ϕSA039 and PP01 may acquire the ability to target new receptors other than their original cognate receptors. These indicate that even if the phage-resistant bacteria may appear, new infectious phage will nevertheless be available.

Staphylococcal kayvirus from family *Myoviridae* is a suitable candidate for phage therapy

Staphylococcal *Myoviridae* phages have a broad host range (Lobocka et al. 2012; Cui et al. 2017). In particular, kayvirus can infect multiple species of *Staphylococcus* (Lobocka et al. 2012). *Staphylococcus* species harbor various glycoepitopes (GlcNAc, GalNAc, or Glc) and two types of WTA backbone (RboP and GroP) (Endl et al. 1983; Brown et al. 2013). Commonly, the infectivity of staphylococcal phages that use glycoepitopes in the WTA as their receptors, e.g. phages from families *Siphoviridae* and *Podoviridae*, depend on the WTA type of the host. For example, *Siphoviridae* phage ϕ187 (a *S. aureus* ST395 specific phage) strictly requires the GalNAc residue in GroP WTA. The phage is not infective toward *S. aureus*, which has RboP

409 WTA (Winstel et al. 2014; Li et al. 2015). The phage can infect coagulase-negative
410 staphylococci (CoNS) including animal-related CoPS, *S. pseudintermedius* (Winstel et al. 2014).
411 The same study also showed that *Siphoviridae* phage ϕ11 and ϕ80α (*S. aureus*–specific phages)
412 can infect *S. aureus* but not *Staphylococcus* species with GroP WTA. Therefore, the host range
413 of phages belong to this group likely depends on the type of WTA.

414 In this study, we found that ϕSA039 could infect different *Staphylococcus* species with
415 GroP or RboP WTA. The phage exhibited at least moderate infectivity toward *S.*
416 *pseudintermedius* isolates and strong infection toward CoNS, all of which lack of the β-GlcNAc
417 residue in their WTA and have a GroP backbone. We confirmed that ϕSA039 can utilize GroP-
418 or RboP WTA in *Staphylococcus* bacteria as its receptor. TagN-mediated glycosylation of WTA
419 in *S. pseudintermedius* SP015 likely inhibits the infection of ϕSA039.

420 Staphylococcal *Siphoviridae* phage often exhibit broad host range, but are specific for
421 bacteria within the same species (Xia et al. 2011; Li et al. 2015). In this study, we demonstrated
422 that SP phages can engage in inter-species infectivity in *Staphylococcus* that possess GroP WTA.
423 Three SP phages (ϕSP120, ϕSP197, and ϕSP276) exhibited strong infectivity toward 19 isolates
424 of *S. pseudintermedius* and 11 CoNS isolates. Unlike other CoNS, *S. saprophyticus* i.e. CCM883
425 strain has RboP-type WTA (Endl et al. 1983); hence, SP phages cannot infect this species. The
426 absence of non-beneficial genes (toxin, virulence, and antibiotic resistance) in the genomes of
427 ϕSP120, ϕSP197, and ϕSP276 lead us to consider them as potential antimicrobial agents for
428 control of *S. pseudintermedius* infection.

429 Phage host range is an important criterion when considering a candidate phage for
430 therapeutic application. According to our results, staphylococcal *Myoviridae*, especially those of

kayvirus group, are the best candidates in terms of host range; however, to establish efficient treatment, it will be necessary to precisely determine the bacteria responsible for each infection. For example, if one desires to treat *S. pseudintermedius* infection without affecting *S. aureus*, the use of a SP *Siphoviridae* phage represents an alternative. For practical phage therapy, the use of strictly lytic phages that lack non-desirable genes would preferable. Although our newly isolated SP phages are lysogenic, non-desirable genes are absent from their genome; thus, selection of spontaneous mutant phages that are exclusively lytic would be beneficial for practical applications. A recent report described a practical method for screening for spontaneous mutant lytic phages from a lysogenic strain (Gutiérrez et al. 2018), suggesting promising future applications of such phages in therapy.

Glycosylation of RboP WTA protects *S. aureus* cell from infection by SP phages

Surface carbohydrate moieties are essential for bacterial communication and phage–bacteria and host–pathogen interactions (Weidenmaier et al. 2005; Brown et al. 2013). Most *S. aureus* strains produce RboP WTA substituted with α- and/or β-GlcNAc residues. Many *S. aureus* strains have lost a major genetic barrier against phage infection, the CRISPR-Cas system (Brussow et al. 2004). Consequently, *S. aureus* frequently exchanges genetic material via phage-mediated horizontal gene transfer (HGT) events (Xia and Wolz 2014; Li et al. 2015). The difference in WTA structure determines efficient HGT among *Staphylococcus* bacteria. Staphylococcal *Siphoviridae* mediates HGT among *Staphylococcus* (Xia and Wolz 2014). Host recognition of phages from this family depends on WTA structure (Azam and Tanji 2019b). For example, *Siphoviridae* phages capable of infecting *S. aureus* (SA *Siphoviridae*), such as ϕ11 and ϕ80A, can recognize GlcNAc of RboP WTA, but cannot recognize GroP WTA of CoNS (see

453 illustration in Fig 4). On the other hand, *Siphoviridae* that infect other *Staphylococcus* species
454 have rarely been reported.

455 Within *S. aureus* species, the ST395 lineage harbors a unique WTA containing 1,3-
456 glycerol-phosphate (GroP) modified with α-GalNAc and D-alanine (Winstel et al. 2013; Winstel
457 et al. 2014). The WTA structure of the ST395 lineage resembles that of CoNS and *S.*
458 *pseudintermedius*. *Siphoviridae* phages capable of infecting PS187 (a representative strain of the
459 ST395 lineage) can also infect CoNS and other species with similar WTA structure (Winstel et
460 al. 2014). In this study, we characterized *Siphoviridae* phages capable of infecting *S.*
461 *pseudintermedius* (termed SP *Siphoviridae*). We demonstrated that the *S. pseudintermedius*
462 strains used in our study have GroP WTA. In a representative strain (SP015), we observed that
463 our SP *Siphoviridae* phages required glycosylated GroP WTA. A nonsense mutation resulting in
464 deletion of the C-terminus of *tagN*, encoding glycosyl transferase for GroP WTA, caused a
465 significant decrease in adsorption of SP phages onto SP015 but improved adsorption of ϕSA039.
466 Adsorption of phages was not observed in the present of tunicamycin (an antibiotic which
467 inhibits WTA synthesis), suggesting that SP phages and ϕSA039 required WTA for infection but
468 glycosylation by TagN was dispensable for ϕSA039.

469 Interestingly, we showed that SP phages could recognize *S. aureus* harboring non-
470 glycosylated WTA (due to lack of TarM and TarS). Although *S. aureus* lacking both TarM and
471 TarS is not likely to be present in nature, our finding indicates that glycosylation of RboP WTA
472 of *S. aureus* is a strategy used by the bacteria to protect themselves against infection by SP
473 *Siphoviridae*, thereby limiting the HGT across different species with different WTA backbones.
474 Similar strategies have been documented in other reports (Li et al. 2015) showing that TarM
475 protects *S. aureus* against the lytic activity of *Podoviridae*. Staphylococcal *Podoviridae* require

476 precise WTA glycosylation pattern for infection. TarM-mediated α-GlcNAc glycosylation in
477 RboP WTA prevents infection of *Podoviridae* while TarS-mediated β-GlcNAc glycosylation is
478 important for *S. aureus* susceptibility to *Podoviridae*. Our findings reveal a novel strategy by
479 which *S. aureus* protects itself against infection by phages capable of specifically infecting
480 phylogenetically distant species. These finding provide novel insight into biology of
481 staphylococcal phage. We suggested that host recognition mechanism of staphylococcal phage is
482 likely more complex than our current understanding.

483

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487

488 **Compliance with ethical standards**

489 Conflict of interest: The authors declare that they have no competing interests.

490 Ethical approval: This article does not contain any studies with human participants or animal
491 performed by any of the authors.

492

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631

632

633 **Figure legends**

634 Fig 1. Adsorption of ϕSA039 and its derivative mutants (spontaneous mutant ϕM1 and ϕM2,
635 recombinant phage harboring point mutations in ORF100 [ϕrM1/r-100], and recombinant
636 phage harboring point mutations in ORF100 and ORF102 [ϕrM1/r-100&102]) onto (a)
637 SA003 and *tarS* deletion mutant (SA003ΔTarS), (b) wild-type RN4220, *tarM* deletion
638 mutant (RNΔTarM), *tarS* deletion mutant (RNΔTarS), and *tarM*/*tarS* double deletion
639 mutant (RNΔTarMΔTarS). Statistical significance ($P<0.05$) is indicated with *.

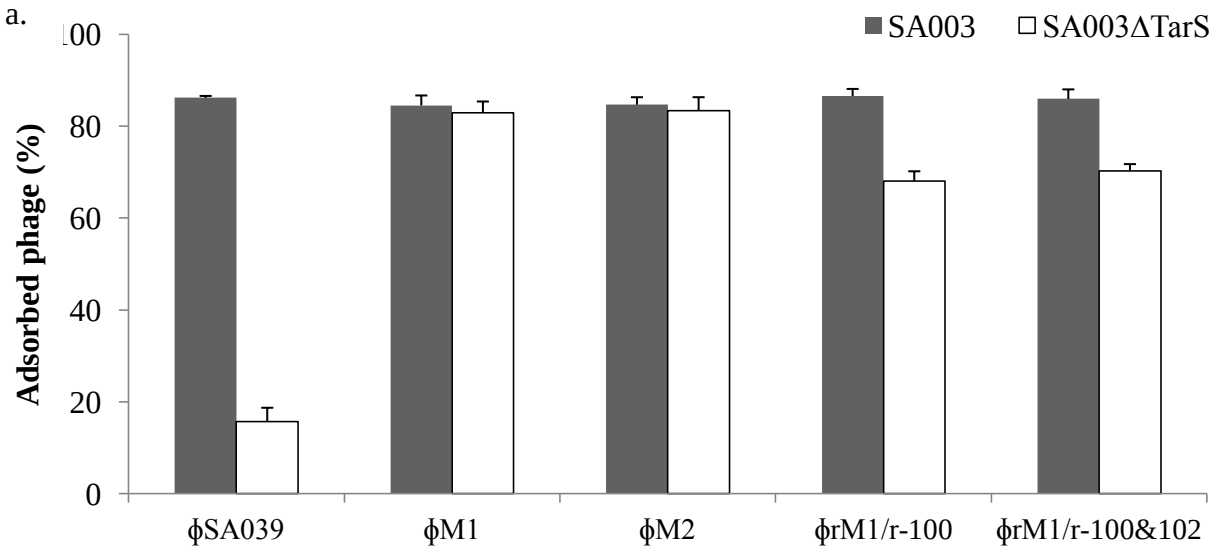
640 Fig 2. Infectivity of SP phages (ϕSP120, ϕSP197, and ϕSP276) toward RN4220 and its deletion
641 mutants; *tarM* deletion mutant (RNΔTarM), *tarS* deletion mutant (RNΔTarS), and
642 *tarM*/*tarS* double deletion mutant (RNΔTarMΔTarS), *tagO* deletion mutant (RNΔTagO),
643 *tarM* complemented mutant RNΔTarMΔTarS::pLIP3.TarM, and *tarS* complemented mutant
644 RNΔTarMΔTarS::pLIP3.TarS, as determined by adsorption assay.

645 Fig 3. (a) Mutation that causes an early stop codon in *tagN* of SP015-R1. Susceptibility of SP015
646 wild-type, tunicamycin-induced Sp015 (final concentration 5μg/ml), *tagN* spontaneous
647 mutant (SP015-R1), and TagN-complemented SP015-R1 to all phages, as determined by
648 spot test (b); to phage ϕSP120, as determined by adsorption assay (c); and against phage
649 ϕSA039, as determined by adsorption assay.

650 Fig 4. Mechanism of recognition of WTA by staphylococcal phages: (a) SA *Siphoviridae* and
651 *Myoviridae* recognize glycosylated RboP WTA; (b) SP *Siphoviridae* and *Myoviridae*
652 recognize glycosylated GroP WTA; and (c) SP *Siphoviridae* and *Myoviridae* recognize non-
653 glycosylated RboP WTA. *Glycosylation by TagN is based on a report in the *S. aureus*
654 ST395 lineage (Winstel et al. 2014).

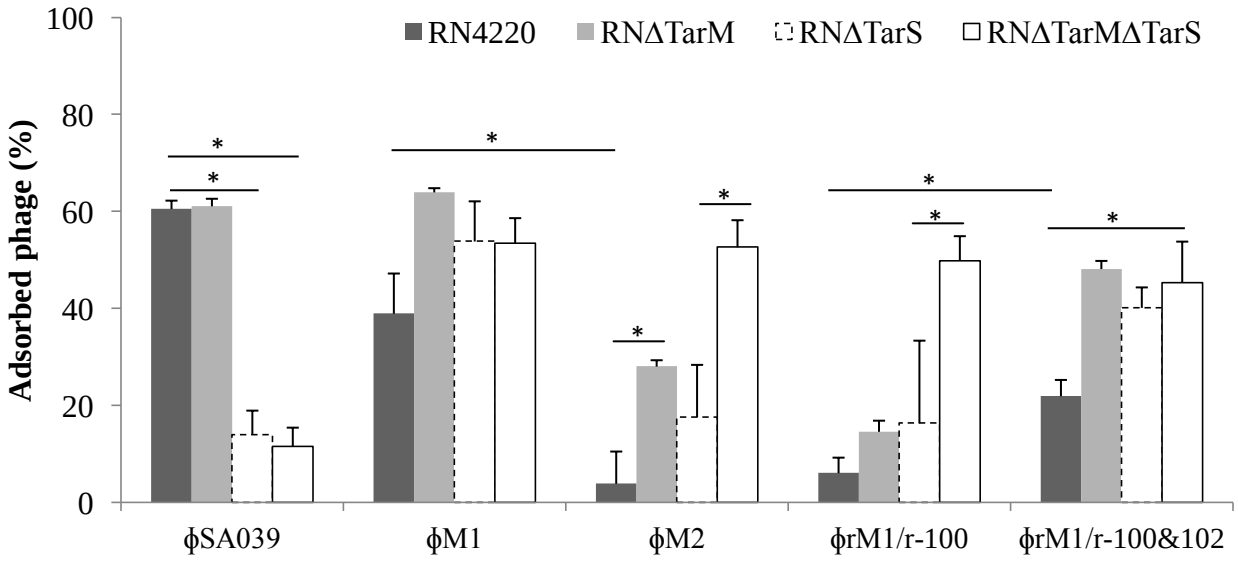
655

a.



656

657 b.



658

659 Fig 1.

660

661

662

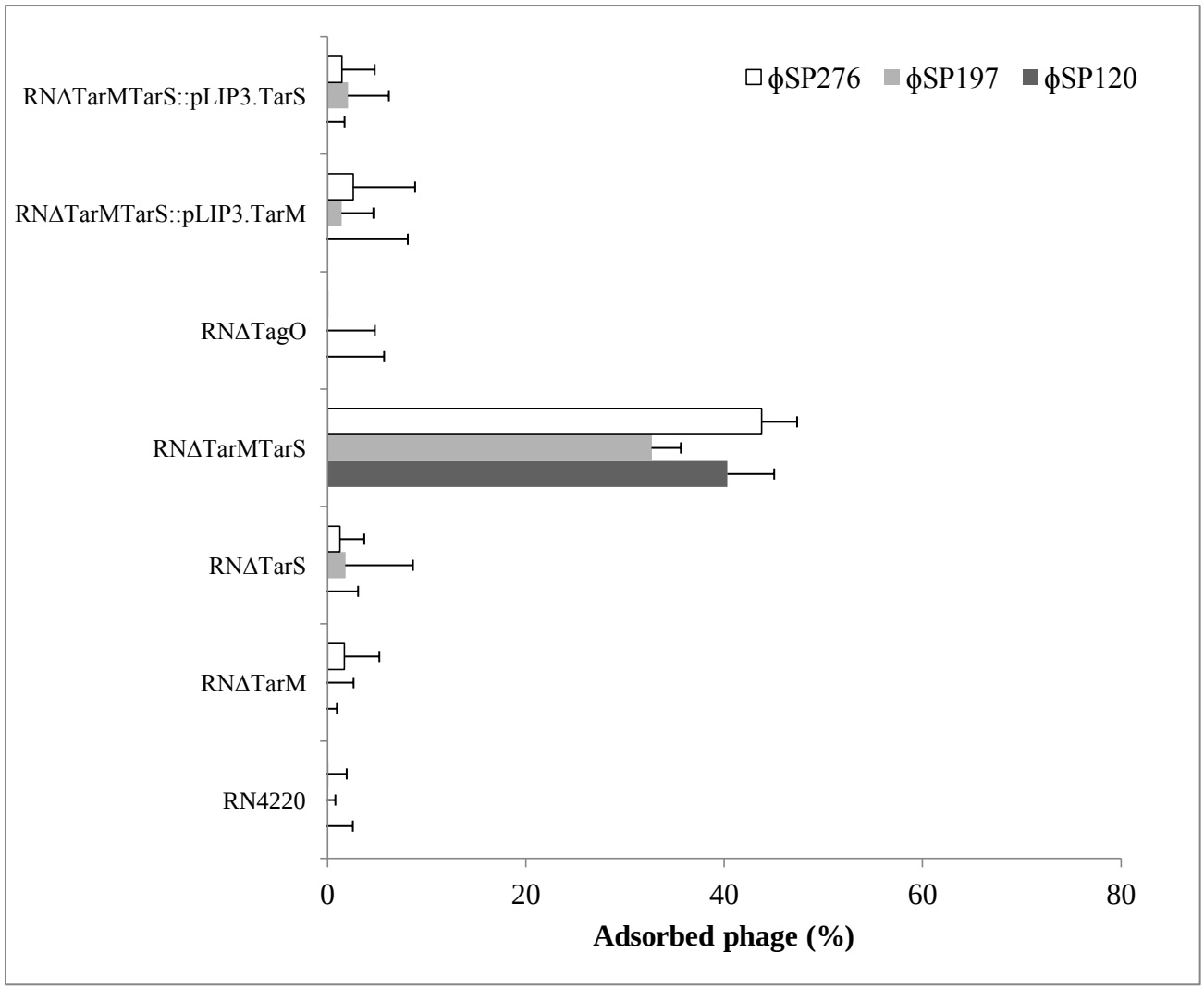


Fig. 2

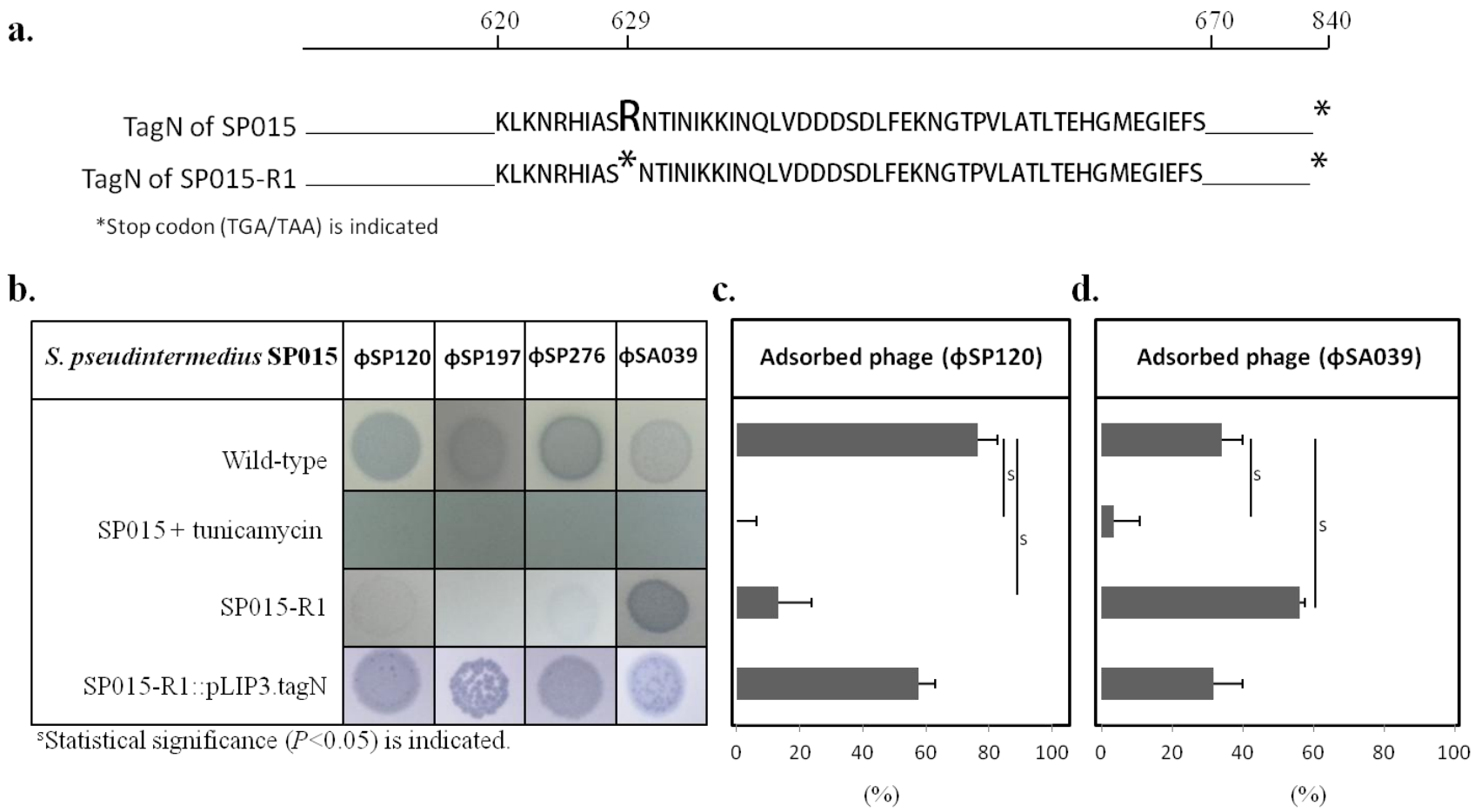
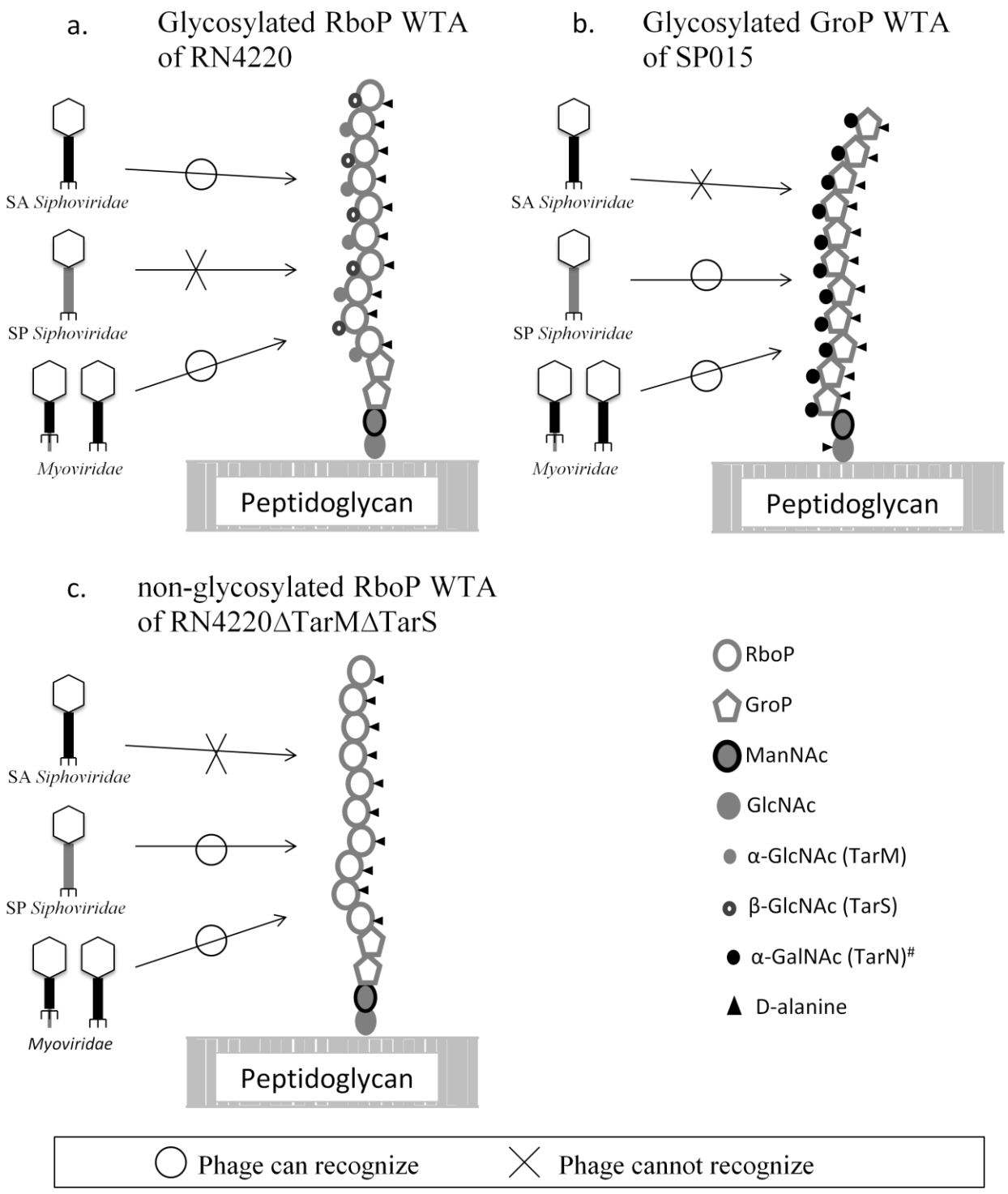

a.
620
629
670
840
TagN of SP015 KLKNRHIASRNTINIKKINQLVDDDSDLFEKNGTPVLATLTEHGMEGIEFS*
TagN of SP015-R1 KLKNRHIAS*NTINIKKINQLVDDDSDLFEKNGTPVLATLTEHGMEGIEFS*
*Stop codon (TGA/TAA) is indicated
b.
S. pseudintermedius SP015
ϕSP120
ϕSP197
ϕSP276
ϕSA039
Wild-type
SP015 + tunicamycin
SP015-R1
SP015-R1::pLIP3.tagN
sStatistical significance (P<0.05) is indicated.
c.
Adsorbed phage (ϕSP120)
0
20
40
60
80
100
(%)
d.
Adsorbed phage (ϕSA039)
0
20
40
60
80
100
(%)

Fig. 3

675



676

677 Fig. 4

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	(Azam et al. 2018)
S. aureus RNΔOatA	*S. aureus* RN*4220* with deleted *oatA* gene	(Azam et al. 2018)
S.aureus RNΔTarS	*S. aureus* RN4220 lacking *tarS* gene	(Kurokawa et al. 2013)
S.aureus RNΔTarMΔTarS	*S. aureus* RN4220 lacking *tarM* and *tarS* genes	(Kurokawa et al. 2013)
RNΔTarMΔTarS.pLIP3.TarM	*S. aureus* RNΔTarMΔTarS harboring pLIP3.TarM	This study
RNΔTarMΔTarS.pLIP3.TarS	*S. aureus* RNΔTarMΔTarS harboring pLIP3.TarS	This study
S.aureus RNΔTarMΔTarSΔOatA	*S. aureus* RN4220 lacking *tarM, tarS* and *oatA* genes	This study
S.aureus SA003	*S. aureus* isolated from milk of mastitic cow	(Synnott et al. 2009)
SA003ΔtarS	*S. aureus* SA003 deficient in *tarS*	(Azam et al. 2018)
SA003ΔtarS::pLIP3.TarS	Complemented SA003ΔtarS with pLIP3.TarS	(Azam et al. 2018)
S. aureus SA001, 002, 003, 009, 019,020, 021,026, 028,029,031,033	*S. aureus* isolated from milk of mastitic cow	(Synnott et al. 2009)
S. pseudintermedius SP015, 017, 023, 040, 055, 042, 070, 079, 092, 120, 145, 188, 190, 195, 197, 251, 253, 276, 294, 296	*S. pseudintermedius* isolated from skin of Canine pyoderma	This study
SP015.R1	*S. pseudintermedius* SP015 with premature stop codon in *tagN*	This study
SP015.R1.pLIP3.TagN	Complemented SP015.R1 with pLIP3.TagN	This study
S. epidermidis NBRC 100911	*S. epidermidis* isolated from nose	(NBRC culture collection, Shizuoka, Japan)
S. warneri IAM-1296	*S. warneri* isolated from skin lesion	(JCM culture collection, Tsukuba, Japan)
S. epidermidis NBRC12993	Standard strain for Japanese pharmacopeia (microbial assay for antibiotics)	(NBRC culture collection, Shizuoka, Japan)
S. caprae JMUB-50	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. epidermidis JMUB-51	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. haemolyticus JMUB-57	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection

Table 2. Spot test of phages toward *Staphylococcus aureus*, *S. pseudintermedius* and 12 CoNS.

S. caprae JMUB-145	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. hominis JMUB-323	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. warneri JMUB-339	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. saprophyticus JMUB-344	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. lugdunensis JMUB-1729	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
S. capitis JMUB-1992	Strain isolated from patient at Jichi Medical University hospital	Jichi Medical University culture collection
Phages		
ϕSA012, ϕSA039	*S. aureus* phage isolated from sewage influent in Japan.	(Synnott et al. 2009)
ϕM1	Spontaneous mutant of ϕSA039	This study
ϕM2	Spontaneous mutant of ϕSA039	This study
ϕrM1/r-100	Chimeric phage of ϕSA039 harboring mutated ORF100 of ϕM1	This study
ϕM1/r-100&102	Chimeric phage of ϕSA039 harboring mutated ORF100 and ORF102 of ϕM1	This study
ϕSP120	*S. pseudintermedius* specific phage isolated by Mitomycin induction	This study
ϕSP197	*S. pseudintermedius* specific phage isolated by Mitomycin induction	This study
ϕSP276	*S. pseudintermedius* specific phage isolated by Mitomycin induction	This study
Plasmid		
pNL9164	*E. coli/S. aureus* shuttle vector, AmpR (*E. coli*), ErmR (*S. aureus*).	(Sigma-Aldrich, St. Louis, MO, USA)
pNL.m1.100	Plasmid pNL9164 with inserted mutated-ORF100 from ϕM1	This study
pNL.m1.102	Plasmid pNL9164 with inserted mutated-ORF102 from ϕM1	This study
pLI50	*E. coli/S. aureus* shuttle vector, AmpR (*E. coli*),CmR (*S. aureus*).	(Addgene, MA, 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.	(Azam et al. 2018)
pLIP3.TagN	pLIP3 with inserted *tagN* gene from SP015.	This study
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.	(Azam et al. 2018)
pLIP3.oatA	pLIP3 with inserted *oatA* gene from RN4220	(Azam et al. 2018)

678

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		S. pseudintermedius																		
		MSSP							MRSP											
		SP017	SP023	SP042	SP070	SP079	SP092	SP120	SP015	SP040	SP055	SP145	SP188	SP190	SP195	SP197	SP251	SP253	SP276	SP294	SP296
MLST		NT	NT	NT	NT	NT	NT	NT	71	110	194	71	36	NT	NT	NT	NT	NT	5	NT	NT
WTA type		GroP																			
Phage	ϕSA039	C	T	T	C	N	T	T	C	T	T	T	T	N	T	C	C	C	C	T	T
	ϕSA012	C	T	T	C	N	T	T	C	T	T	C	C	T	C	C	C	C	C	T	T
	ϕSP0120	C	C	C	C	N	C	T	C	C	C	C	T	C	T	C	T	T	C	C	C
	ϕSP0197	C	C	C	C	N	C	C	C	C	C	C	C	C	C	T	C	C	C	C	C
	ϕSP276	C	C	C	C	N	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C
	ϕDP001	C	T	T	T	N	N	N	C	T	T	N	N	N	N	C	C	C	N	N	N

		S. aureus													*S. epidermidis*			*S. warneri*		*S. capitis*
		SA003	RN420	ATCC6538	*S. warneri*	SA002	SA009	SA019	SA020	SA021	SA026	SA028	SA029	SA031	SA033	NBRC100911	NBRC12993	JMUB-51	IAM-1296	JMUB-339	JMUB-1992
WTA type		RboP													GroP			GroP		GroP
Phage	ϕSA039	C	C	N	T	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
	ϕSA012	C	C	T	C	N	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C
	ϕSP120	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C
	ϕSP197	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C
	ϕSP276	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C
	ϕDP001	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	T	T	C	C	C

		S. caprae		*S. hominis*	*S. haemolyticus*	*S. lugdunensis*	*S. saprophyticus*
		JMUB 50	JMUB-145	JMUB-323	JMUB-57	JMUB-1729	JMUB-344

		GroP					RboP[#]
Phage	ϕSA039	C	C	C	C	C	C
	ϕSA012	C	C	C	C	C	C
	ϕSP120	C	C	C	C	C	N
	ϕSP197	C	C	C	C	C	N
	ϕSP276	C	C	C	C	C	N

MRSA= Methicillin-resistant *S. pseudintermedius*
MSSP= Methicillin-susceptible *S. pseudintermedius*
NT means Non type
*C = Clear, T = Turbid, and N = No plaque
#WTA was likely similar to the strain reported by Endl et al (1983)

681

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684

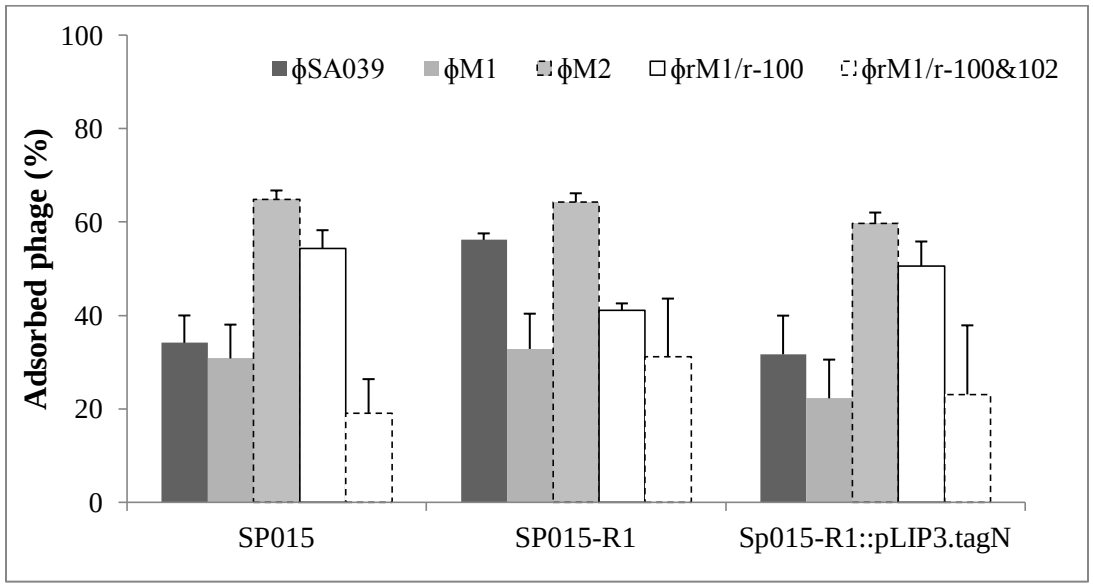
Supplemental Table S1. Primers used in this study.

Primer name	Sequence	Description	Reference
F_orf94	ACAGTCTATTGTACTTGAAGC	Primer to amplified *orf94* of ϕSA039	This study
R_orf94	CTAATGTTTCAAATTGCTGAGG	Primer to amplified *orf94* of ϕSA039	This study
F_orf95	CCTCAGCAATTTGAAACATTAG	Primer to amplified *orf54* of ϕSA039	This study
R_orf95	GTGCATCTATGAGAGCAAAG	Primer to amplified *orf95* of ϕSA039	This study
F_orf96	CAGACCTTATTCAAGCTATAATG	Primer to amplified *orf96* of ϕSA039	This study
R_orf96	GGTGGGTTTGTTTCATTAGAC	Primer to amplified *orf96* of ϕSA039	This study
F_orf97-98	CCATACGATAGTGAAGTAGTAAC	Primer to amplified *orf97* of ϕSA039	This study
R_orf98-97	CTTGTTGATACGGGTTTATCT	Primer to amplified *orf98* of ϕSA039	This study
R1_orf98	CTGTTGTATCTCCTGGAATAATC	Primer to amplified *orf98* of ϕSA039	This study
R2_orf98	GTTTCAGCACTAGCATTATTAG	Primer to amplified *orf98* of ϕSA039	This study
R3_orf98	CATAAGTCTACGAACACGAG	Primer to amplified *orf98* of ϕSA039	This study
F_orf99	CTCGTGTTCGTAGACTTATG	Primer to amplified *orf99* of ϕSA039	This study
R_orf99	CTTGTACTGGTAACTTTCTTATC	Primer to amplified *orf99* of ϕSA039	This study
R_orf101	GAGCTGTTATGTTTCCATACG	Primer to amplified *orf101* of ϕSA039	This study
F_orf100	GCAAAGCTTGGGTTGATTGACCCCTCTTT	Primer to amplified *orf100* of ϕSA039 with *Hind*III site for insertion into pNL9164 and used for homologous recombination	This study
R_orf100	ATATGTACACTCCTAAAGTGTATTAATTCCTGC	Primer to amplified *orf100* of ϕSA039 with *BsrG*I site for insertion into pNL9164 and used for homologous recombination	This study
R_orf100	CCATCGTCTCTGATACTACTTAG	Primer to amplified *orf100* of ϕSA039	This study
M_orf100	CAGCAAGTGAGATAACAACTG	Primer to amplified *orf100* of ϕSA039	This study

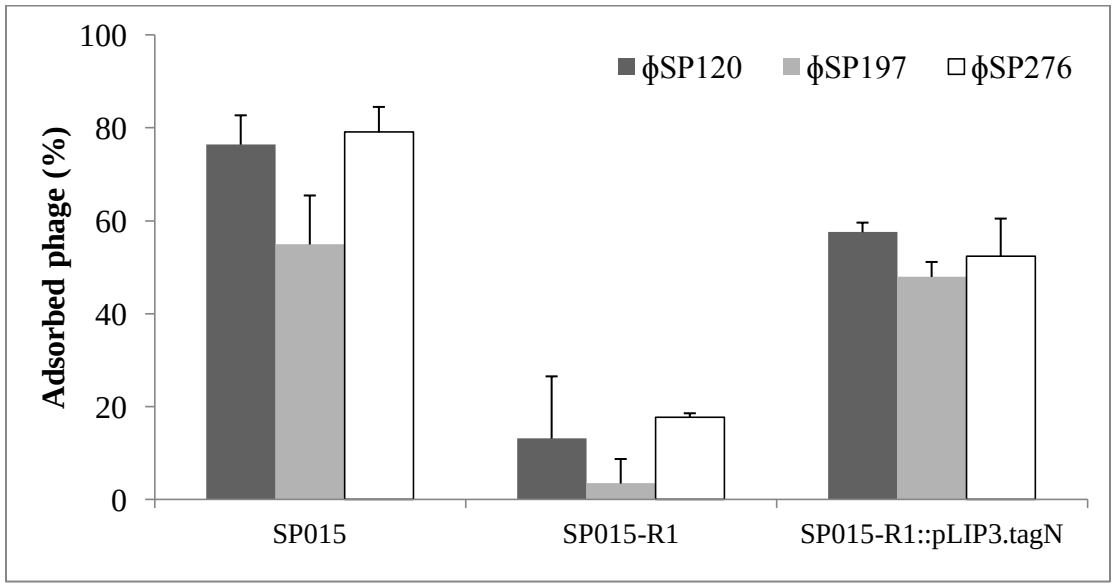
F_orf102	GCAAAGCTTCAAGAAGCTATGATGGAAA TGC	Primer to amplified *orf102* of ϕSA039 with *Hind*III site for insertion into pNL9164 and used for homologous recombination	This study
R_orf102	ATATGTACAGTCTACATACCCATCCCAAC	Primer to amplified *orf102* of ϕSA039 with *BsrG*I site for insertion into pNL9164 and used for homologous recombination	This study
F_tagN	GCAGGATCCGGGATGTACTAAGAAAGAG TC	Primer to amplified *tagN* of *S. pseudintermedius* with *BamH*I site for insertion into pLIP3	This study
R_tagN	ATAAAGCTTGTTCACCCTCTTTTCCATACA T	Primer to amplified *tagN* of *S. pseudintermedius* with *Hind*III site for insertion into pLIP3	This study
F_tagF1	CAGGGTTTGGAGGGATGATTG	Primer to amplified *tagF1* of *S. pseudintermedius*	This study
R_tagF1	AGTTAGTTGAAGGGCAGCCC	Primer to amplified *tagF1* of *S. pseudintermedius*	This study
F_tagF2	GATTTCAAACACGAAACATATAGC	Primer to amplified *tagF2* of *S. pseudintermedius*	This study
R_tagF2	CAATCATCCCTCCAAACCCTG	Primer to amplified *tagF2* of *S. pseudintermedius*	This study
M_tagF2	CTGTAGAATAACGGTTCGGTG	Primer to amplified *tagF2* of *S. pseudintermedius*	This study
F_tagVN	GATATGATTAAGGAAGTGGTTAC	Primer to amplified *tagVN* of *S. pseudintermedius*	This study
R_tagN	CGATGATTGGTCTGTTGTTG	Primer to amplified *tagN* of *S. pseudintermedius*	This study
F_tagO	GATTGCGAATGAATGAGACAG	Primer to amplified *tagO* of *S. pseudintermedius*	This study
R_tagO	GTACTTTCCATGTGGCATTC	Primer to amplified *tagO* of *S. pseudintermedius*	This study
SIspa-F	AACCTGCGCCAAGTTTCGATGAAG	Primer for spa typing of *S. pseudintermedius*	Moodley et al. 2009
SIspa-R	CGTGGTTTGCTTTAGCTTCTGGC	Primer for spa typing of *S. pseudintermedius*	Moodley et al. 2009
spaA-F	AAGTAGTGATATTCTTGCT	Primer for spa typing of *S. pseudintermedius*	Ruscher et al. 2010
spA-R	CCAGGTTGAACGACATGCAT	Primer for spa typing of *S. pseudintermedius*	Ruscher et al. 2010
SPspa1F	CCGCTCTATTTTTAGGTTAATC	Primer for spa typing of *S. pseudintermedius*	Perreten et al. 2010

SIspaFlkR 1	CGTAACAACTCAATGCTACATA	Primer for spa typing of *S. pseudintermedius*	Perreten et al. 2010
tuf-F	CAATGCCACAAACTCG	Primer for typing of *S. pseudintermedius*	Bannoehr et al. 2007
tuf-R	GCGACTGTACTTGCACAAGCA	Primer for typing of *S. pseudintermedius*	
cpn60-F	GCGACTGTACTTGCACAAGCA	Primer for typing of *S. pseudintermedius*	
cpn60-R	AACTGCAACCGCTGTAAATG	Primer for typing of *S. pseudintermedius*	
pta-F	GTGGGTATCGTATTACCAGAAGG	Primer for typing of *S. pseudintermedius*	
pta-R	GCAGAACCTTTTGTTGAGAAGC	Primer for typing of *S. pseudintermedius*	
agrD-F	GGGGTATTATTACAATCATTC	Primer for typing of *S. pseudintermedius*	
agrD-R	GTGATGGGAAAATAAAGGATTG	Primer for typing of *S. pseudintermedius*	
F_seqA.oatA	GCATCTAGAGAAATTATATGAACCTGCTTGG	Primer to amplify A region of *oatA* gene with *Xba*I site for pCasSA	Azam et al. 2018
R_seqA.oatA	CAAATCCCATAGTAACGGTG	Primer to amplified A region of *oatA* gene	Azam et al. 2018
F_seqB.oatA	CACCGTTACTATGGGATTTGGTTGATTTCATTACTGCAAAC	Primer to amplified B region of *oatA* gene	Azam et al. 2018
R_seqB.oatA	ATACTCGAGCTCTTCCCATAGAAATTGGG	Primer to amplify B region of *oatA* gene with *Xho*I site for pCasSA	Azam et al. 2018

a.



b.



Supplemental Figure S2

716

Supplemental Table S3. Spacers present in CRISPR of *S. pseudintermedius* SP079

Spacer	Sequence	Closest phage sequence in database*	Phage genome accession number
1	TTTTAATCACTATACGATTATGAAAGCCT	NA	
2	GAACAGTTTGATTTACCTTATCGCTATATT	NA	
3	TGTTTCTGTTTGTTACTTTTATTCTGTAC	NA	
4	TGAACATTACCGAACCCTCTTGTGATTAA	NA	
5	GCGAACAACGGTATGCTTACGATGTTAGGT	ϕSP120	AP019560
6	TCGCATTAATCAAGAATCAAAAGGGTTGC	NA	
7	AAAAAGCCAGTACAAGGTTCTATGGTTTCA	NA	
8	ATTAAGAACAATATAAATGTATCGATTTCA	Phage SN8	MF428481
9	GCAATGGATCAATTGAAGGCTCGTCAACAA	NA	
10	GTGTGTCATCAGACTTTACACCTTCAAATAC	NA	
11	GAACTCTTAAAGTATCTTTTAGGACACTT	NA	
12	AAAGATTATGCCATCTTCAGTTACTTTAAC	NA	
13	GATGAGAGGTGTAAAAGCACAATTGCATGA	NA	
14	TGTACGTTCAAACGGTTCTCTTTTAATTT	NA	
15	CGGCACAGATTGTGTGATTTGTTGACCTGC	NA	
16	TCAAAAGTATTTCGTCGAGATGGAGCGACT	ϕSP197	AP019561
17	TGAAGATTTTCCACGCCAATGTATTTTTAT	Phage 2638A	AY954954
18	TAAAATCAGAAACAAAAGGGCAAGCGGTT	NA	
19	CATTACCTTTGTTTGCGTTGTTTGATTCT	NA	
20	GTTAACTGAACTCGAGTGTTGGCGCTTGA	NA	
21	ACAGGCTTAAAGAATCATTACAGTTTGCTA	NA	
22	TTGACCGTGCAAAAATTAATCACAAGGCGC	NA	
23	GTTATGATGTTTGGCGATATGGGTCGTCGT	NA	

*National Center for Biotechnology Information (NCBI) database was used in this study.
NA means the data was not available in the database.

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