1 **Title:**

2 Analysis of phage-resistant mechanisms in *Staphylococcus aureus* SA003 reveals a

3 different binding mechanism for the closely related Twort-like phages ϕSA012 and

4 ϕSA039

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ABSTRACT

We have previously generated strains of *Staphylococcus aureus* SA003 resistant to its specific phage ϕSA012 through long-term coevolution experiment. However, the DNA mutations responsible for the phenotypic change of phage resistance are unknown. Whole-genome analysis revealed six genes that acquired unique point mutations: five missense mutations and one nonsense mutation. Moreover, one deletion, 1.779-bp, resulted in the deletion of the genes encoding glycosyltransferase, TarS, and iron-sulfure repair protein, ScdA. The deletion occurred from the second round of coculture (SA003R2) and remained through the last round. The ϕSA012 infection toward SA003R2 had decreased to 79.77±7.50% according to plating efficiency. Complementation of the phage-resistant strain by the wild-type allele showed two mutated host genes were linked to the inhibition of post-adsorption, and five genes were linked to phage adsorption of ϕSA012. Unlike ϕSA012, infection by ϕSA039, a close relative of ϕSA012, onto SA003R2 was impaired drastically. Complementation of SA003R2 by wild-type *tarS* restores the infectivity of ϕSA039. Thus, we concluded that ϕSA039 requires β-GlcNAc in Wall Teichoic Acid (WTA) for its binding. In silico analysis of the ϕSA039 genome revealed that several proteins in the tail and baseplate region were different from ϕSA012; notably the partial deletion of *orf96* of ϕSA039, a homolog of *orf99* of ϕSA012. *Orf100* of ϕSA039, a homolog of *orf103* of ϕSA012, a previously reported receptor binding protein (RBP), had low similarity (86%) to that of ϕSA012. The difference in tail and baseplate proteins might be the factor for specificity difference between ϕSA012 and ϕSA039.

KEYWORDS

43   **Introduction**

44   *Staphylococcus aureus* infection in humans and animals is of worldwide concern due to

45   the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and

46   vancomycin-resistant *S. aureus* (VRSA) (Enright et al. 2002; Sakoulas et al. 2005). In dairy

47   industry, for instance, *S. aureus* is one of the most frequent causative agent of bovine mastitis,

48   with prevalence rates as high as 50% in some countries (Leitner et al. 2003). Due to its negative

49   effect on milk quality, high quantities of expensive antibiotic are frequently used. Alternatively,

50   using bacteriophage as a means of controlling *S. aureus* is a promising treatment because of the

51   high specificity and lytic activity of the phage. Various efforts are currently made to evaluate the

52   potential of phage therapy (Matsuzaki et al. 2005; Maciejewska et al. 2018). In order to develop

53   phage therapy for mastitis, we isolated several *S. aureus* stains, including *S. aureus* SA003, from

54   the milk of mastitic cows in our previous study (Synnott et al. 2009). We also isolated two lytic

55   phages with wide host ranges, ϕSA012 and ϕSA039, from sewage influent (Synnott et al. 2009).

56   However, in batch co-culture experiment, *S. aureus* developed resistance to ϕSA012 (Synnott et

57   al. 2009). The emergence of phage-resistant bacteria is a big obstacle to realizing phage therapy.

58   A deeper understanding of phage resistance mechanisms is critical before we apply phage

59   therapy in real world.

60   Phage-resistant bacteria typically evolve phage adsorption inhibition by, for example,

61   altering their phage receptor (Bohannan and Lenski 2000; Denes et al. 2015). Though, there is a

62   report that showed most phage-resistant strains of *Streptococcus thermophilus* used the Clustered

63   Regularly Interspaced Short Polindrom Repeat (CRISPR) system to defend against phage

64   infection (Levin et al. 2013). At the genomic level, mechanisms of phage adsorption inhibition

65   have been well-studied in several Gram-negative bacteria (Morona et al. 1985; Marston et al.

3

66   2012; Meyer et al. 2012; Seed et al. 2012; Castillo et al. 2015) and a few Gram-positive bacteria,

67   such as *Listeria monocytogenes* (Denes et al. 2015) and *Streptococcus pneumoniae* (Leprohon et

68   al. 2015). However, phage resistance mechanism of *S. aureus* and the specific genes that

69   contribute to the phage resistance are not well studied.

70   In response to phage resistance bacteria, phage has ability to counteradapt which is called

71   as coevolution of phage and bacteria (Golais et al. 2013). This phenomenon was also observed in

72   *E. coli* O157:H7 co-cultured with phage PP01 (Mizoguchi et al. 2003; Fischer et al. 2004) and

73   O157:H7 co-cultured with coliphages SP-21, SP-22, and SP-15 (Tanji et al. 2005). Previously,

74   we demonstrated the coevolution of *S. aureus* SA003 and phage ɸSA012 during their long-term

75   co-culture (Osada et al. 2017). We observed cumulative development of phage resistance of the

76   host and corresponding infectivity of the phages during this coevolution (Osada et al. 2017). The

77   coevolution of *S. aureus* and phage is unique and should be taken into account when considering

78   the result of clinical trials. In this paper, we presented the DNA mutation responsible for

79   phenotypic change during coevolution experiment of *S. aureus* SA003 and phage ɸSA012.

80   The phenotype as a result of coevolutionary adaptation of host and phage has previously

81   been observed and explained in our previous report (Takeuchi et al. 2016; Osada et al. 2017). In

82   this study, we focused on genetic analysis of the host cell by whole genome sequencing (WGS).

83   WGS of wild-type SA003 and its phage resistance derivatives enables us to identify the host

84   genes that contribute to the phage resistance during coevolution. Understanding host genes that

85   endow phage resistance may help us design a better strategy for applying phage therapy under

86   real-world conditions.

87   For the treatment of *S. aureus* and other *Staphylococcus* species, *Myoviridae* phages are

88   considered to be promising candidates for phage therapy (Loessner et al. 1996; Alves et al.

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89   2014). Phages belonging to this genera have been reported to have the following features: being

90   strictly virulent, genome size of 127 to 140 kbp, share considerable similarity at the protein

91   sequence level, and infecting Gram positive bacteria with low GC content (Łobocka et al. 2012).

92   Our phages ɸSA012 and ɸSA039 (from our collection), which belongs to *Myoviridae* genus

93   Twort-like phage, displayed to have different host preferences and morphology (Synnott et al.

94   2009). In mice, the application of ɸSA012 has shown an effective and promising outcome

95   (Iwano et al. 2018). However, little is known about why these two closely related phages

96   (ɸSA012 and ɸSA039) have difference host preferences. In this study, we identify the different

97   binding mechanism of these two phages and analyze WGS of the phages to find out the gene that

98   contribute to the host-specificity different. Elucidating the details of the host recognition

99   mechanism of these two different Twort-like phage species will expand our understanding of

100   how closely related phages exhibit different host preferences. Our finding provides substantial

101   information for expanding the utility of staphylococcal Twort-like phages in the future for

102   practical use.

103

104   **Materials and Methods**.

105   **Bacteria, phages, and plasmids**

106   Bacteria, phages, and plasmids used in this study are listed in **Table 1**. *S. aureus* SA003

107   was previously isolated from milk of mastitic cow and used for propagation of phages. *S. aureus*

108   strain RN4220 was kindly provided by Prof. Motoyuki Sugai (Hiroshima University Graduate

109   School of Biomedical & Health Science, Hiroshima, Japan) with the permission of Richard P,

110   Novick (Skirball Institute of Biomolecular Medicine, New York, NY) and used for genetic

111   manipulation. The *S. aureus* virulent phage ɸSA012 and ɸSA039 were isolated from sewage in

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112   Japan (Synnott et al. 2009). Plasmid pLI50 was purchased from Addgene (Cambridge, MA,

113   USA). Plasmid pLIP3 was constructed using pLI50 and P3 promoter, which is constitutive in *S.*

114   *aureus* (Lee et al. 1991; Jeong et al. 2011; Takeuchi et al. 2016). Plasmid pKOR1-mcs was

115   kindly provided by Dr. Taeok Bae (Indiana University School of Medicine—Northwest,

116   Indianapolis, IN). Plasmid pCasSA was a gift from Dr. Quanjiang Ji (ShanghaiTech University,

117   School of Physical Science and Technology, Shanghai, China). All bacteria and phages were

118   stored in 15% glycerol at −80°C. Luria Bertani (LB), brain heart infusion broth, and Trypticase

119   soy broth are used as a liquid media. For growth on agar, the medium was solidified by adding

120   1.5% (w/v) agar. ϕSA012 and *S. aureus* SA003 were deposited in the culture collection of the

121   NITE Biological Research Center, Kisarazu, Japan under accession numbers NBRC110649 and

122   NBRC110650, respectively (Takeuchi et al. 2016). The phage ϕSA039 is only preserved in our

123   laboratory. For contact details and acquire channel, refer to the address of corresponding author.

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

125   Serial passage of ϕSA012 co-cultured with SA003 was carried out in previous study

126   (Takeuchi et al. 2016; Osada et al. 2017). Briefly, SA003 was inoculated into LB medium and

127   cultured until early exponential phage. Phage ϕSA012 was added with multiplicity of infection

128   (MOI) =0.1. After 2 to 10 days, bacterium-phage mixed cultures were transferred into new LB

129   medium (1:100 dilution) and cultured under the same conditions. The co-culture was repeated

130   until 38th passage. A phage-resistant strain of SA003 and mutant ϕSA012 phage were collected

131   at the end of each cycle. The resistant strain was named SA003R**n**, whereas the mutant phage

132   was named ϕSA012M**n**, where **n** represents the passage number (e.g., SA003R11 refers to the

133   phage-resistant SA003 derivative isolated from co-culture at the 11th passage, while ϕSA012M11

134   refers to the mutant phage ϕSA012 isolated from the co-culture at the 11th passage).

Molecular cloning in *S. aureus*

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136   The primers used in this study are listed in **Table 2**. The primers were purchased from

137   Hokkaido System Science (Japan) and Integrated DNA Technologies (Singapore). T4 DNA

138   ligase and restriction enzymes were purchased from New England Biolabs (New England

139   BioLabs, Ipswich, MA, USA). Plasmid and PCR product were purified using the Nucleospin® kit

140   (Macherey-Nagel, Duren, Germany). Colony or plaque PCR was performed using

141   DirectAmpPCR® (TAKARA, Shiga, Japan) and processed as recommended by the manufacturer.

142   For PCR using pure DNA, Taq DNA polymerase (TAKARA, Shiga, Japan) was used. Deletions

143   of the genes *S2356* and *S2121*were generated by allelic exchange using plasmid pKOR1-mcs

144   (Bae and Schneewind 2006). Gene disruption in *S497* was performed using the Targetron® gene

145   knockout system (Sigma-Aldrich, St. Louis, MO, USA) (Yao et al. 2006). Deletion of the gene

146   encoding O-acetyltransferase (OatA), *oatA*, and *S156* were performed by CRISPR/Cas9 system

147   using plasmid pCasSA (Chen et al. 2017). The complementation experiment was carried out

148   using *E. coli* / *S. aureus* shuttle vector, pLI50, under P3 promoter (Lee et al. 1991; Jeong et al.

149   2011). Wild-type alleles of SA003 were used as the insert. Electrocompetent *S. aureus* cells were

150   generated and transformed as previously reported (Monk et al. 2012). To transform SA003R11, a

151   large mass of plasmid (< 20 μg) was first concentrated by Pellet Paint® (Novagen®, Billerica,

152   MA, USA) before being introduced into electrocompetent cells. Constructed plasmid was cloned

153   in *E. coli* JM109 and pre-introduced into the *S. aureus* restriction-deficient strain, RN4220,

154   before transforming it into SA003.

Phage preparation

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156   All phages were propagated by the plate lysate method (Stephenson 2010). Briefly, 100

157   μl of phage lysate (10^5 PFU/ml) was mixed with 100 μl of overnight bacterial culture in 3 ml of

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158   0.5% top agar, plated on LB agar, and incubated at 37°C overnight. After 5 ml of salt magnesium

159   (SM) buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin) was

160   added to the plate and the over layer was scraped off to extract phage, the supernatant was

161   collected by centrifugation (5,000 g, 15 min, 4°C). The obtained phage lysate was purified by

162   polyethylene glycol (PEG) precipitation and CsCl density gradient centrifugation (Stephenson

163   2010). Each phage culture was titrated and stored at 4°C until used.

164

165   **Spot testing**

166   Two microliters of phage lysate at a titer 10^5 plaque forming units (PFU) was dropped on

167   a lawn of bacteria and incubated overnight at 37°C. Infectivity was determined by assessing the

168   turbidity of plaques in the spots where the phage was dropped.

169   **Adsorption assay**

170   The adsorption efficiency of phages on *S. aureus* strains was measured by titrating free

171   phage present in the supernatant after defined periods of cell-phage contact. *S. aureus* cells were

172   prepared by 10% inoculation of overnight culture into 4.5 ml of LB medium. The culture was

173   subsequently incubated at 37°C with shaking at 120 rpm to an OD_{660} of 1.0 (10^9 CFU/ml). Phage

174   lysate (10^7 PFU/ml) was then added to the bacterial culture. After infection at 37°C with shaking

175   at 120 rpm, free phage was collected by centrifugation (9730 g, 1 min) at defined times and

176   titrated using SA003. For longer incubation, 50 μg/ml of either chloramphenicol or erythromycin

177   was added, and cells were equilibrated for 10 min at 37°C before infection to inhibit cell growth

178   and phage development during incubation with phages (Baptista et al. 2008). Adsorption

179   efficiency was calculated by dividing the number of adsorbed phages by the initial number of

180   phages.

181 **DNA extraction, sequencing, and bioinformatic**

182   DNA was extracted from bacteria using a DNA extraction kit (Sigma-Aldrich, St. Louis,

183 MO, USA). Whole-genome sequencing of bacteria was conducted with the Illumina Miseq

184 platform. Genomes were assembled by Platanus *de novo* assembly ver1.2.1 (Kajitani et al. 2014).

185 ORFs were predicted and annotated by using the RAST server (http://rast.nmpdr.org/). Genome

186 was mapped by Bowtie2 (Langmead and Salzberg. 2012), and SNPs were detected by

187 SAMtools/BCFtools (Li et al. 2009). Mutated regions were confirmed by PCR and Sanger-

188 sequencing. DNA extraction and genome analysis of phage was performed as previously

189 reported (Takeuchi et al. 2016).

190 **Measuring phage inhibition by peptidoglycan**

191   Pure peptidoglycan from *S. aureus* (PGN-SA, InvivoGen, Shatin, Hong Kong) was used

192 for our phage inhibition assay. The teichoic acid was removed from peptidoglycan by treatment

193 of 48% hydrofluoric acid at 4°C for 48 hours (De Jonge et al. 1992). Efficiency of plating (EOP)

194 with the addition of peptidoglycan was performed as follows: 10 μl of peptidoglycan solution

195 was added into 100 μl (10^4 PFU/ml) phage and incubated at room for 1 h. Next, 100 μl of the

196 mixture was mixed with 100 μl *S. aureus* overnight culture in 3 ml of 0.5% top agar, plated on

197 LB agar, and incubated at 37°C overnight.

198 **Phosphate quantification of WTA and total sugar quantification of whole cell**

199   Wall Teichoic Acid (WTA) was extracted from bacteria culture that has been adjusted to

200 same Colony Forming Unit (CFU). WTA extraction was conducted following previous method

201 (Meredith et al. 2008). Phosphate content of WTA was determined following Chen et al (1956).

202 Same volume of extracted WTA sample and freshly prepared reagent C (mixture of one volume

203 of 6N sulfuric acid with 2 volumes of distilled water, one volume of 2.5% ammonium moybdate,

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204   and one volume of 10% ascorbic acid) was mixed, cap with parafilm, and incubated at 37°C for

205   1.5 – 2 hours, KH_2PO_4 was used as phosphate standard. The mixture was cooled down at room

206   temperature for few minutes and read at the absorbance of 820 nm. Total carbohydrate of

207   bacterial cell was measured by Anthrone reagent (Viles and Silverman 1949); 2 ml bacteria

208   culture ($2x10^9$CFU) was washed by Phosphate Buffer Saline (PBS) twice and mixed with

209   Anthrone solution (1 mg/ml in concentrated H_2SO_4 [95-98% w/v]), the mixture was incubated at

210   90°C for 10 minutes and the OD_{630} of sample was measured. D(+)-Glucose was used a standard.

211   **Phage inhibition by antibodies**

212   EOP with antibodies specific for ϕSA012 ORF103 (anti-ORF103) and ORF105 (anti-

213   ORF105) was conducted according to a previous study (Takeuchi et al. 2016).

214   **Statistical analysis**

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

216   **Accession number(s)**

217   *S. aureus* SA003 and ϕSA039 were submitted to the DDBJ database under the

218   accession number **AP018376** and **AP018375**, respectively. While genome of ϕSA012 was

219   available in the GenBank database under accession number: **AB903967**.

220

221   **Results**

222   **Whole genome analysis of phage-resistant SA003**

223   The whole genomes of five phage-resistant mutant strains, as well as parent strain

224   SA003, were sequenced on an Illumina Miseq platform. Mutations detected in the sequenced

225   strains were mapped against the wild-type SA003 reference genome (**Table. 3**). Clustered

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226 regularly interspaced polindromic repeat (CRISPR/cas), which is well known as a major genetic

227 barrier against phage infection, is likely absent in the genome of SA003.

228   A total of eight mutations were identified. One point mutation (AAA→ATA) in *S2356*,

229 which encodes Ferredoxyn glutamate synthase, was found in the phage resistant isolate from the

230 first round, SA003R1. The mutation is preserved until the last round. However, compared to

231 wild-type SA003, the sensitivity of SA003R1 towards ɸSA012 phage did not differ significantly

232 according to plating efficiency and adsorption assays (data not shown). Deleting *S2356* also did

233 not alter the sensitivity of *S. aureus* (data not shown). We assumed that the mutation in *S2356*

234 did not contribute to phage resistance.

235   The phage-resistant strain in the second round (SA003R2) acquired a point mutation in

236 (CAC→TAC) in *S497* encoding Undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate

237 transferase, which is well known as teichoic acid ribitol (*tagO*) gene. This strain also acquired an

238 1779-bp deletion in WTA tar gene cluster gene consisting of: a 1200-bp C-terminal region of

239 *S156* (which encodes glycosyltranferase, TarS); a 125-bp noncoding region; and a 454-bp N-

240 terminal region of *S157* (which encodes iron-sulfur repair protein, Scd) (**Fig. 1A**).

241   Four more genes, *S2121*, *S768*, *S515*, and *S2190*, were mutated in the resistant strain,

242 SA003R11, isolated from the 11th round. *S2121*, which encodes UDP-N-acetylglucosamine 1-

243 carboxyvinyltransferase-2 MurA2 (an enzyme which catalyzes the first step of peptidoglycan

244 synthesis together with MurA) showed a nonsense mutation in a glycine-encoding codon

245 (GGA→TGA). *S768*, which encodes a guanylate kinase, and *S2190*, which encodes the alpha

246 subunit of DNA-RNA polymerase, showed point mutations that changed a threonine into

247 isoleucine (ACA→ATA) and an alanine into valine (GCA → GUA), respectively. Mutations in

248 *S156*, *S157*, *S497*, *S2121*, *S768*, and *S2190* were preserved until later generations, including the

11

249   20th and 38th rounds, from which we isolated SA003R20 and SA003R38, respectively. In

250   SA003R11 we also identified one point mutation in *S515*, which encodes for the RNAse adaptor

251   protein RapZ. The mutation in this gene developed into two point mutations in SA003R20 and

252   SA003R38. In addition, a point mutation (AAT → GAT) was identified in *S692*, which encodes

253   the putative membrane protein YozB, in SA003R20 and SA003R38.

254   To evaluate the role of mutated host genes in phage resistance, we performed

255   complementation in SA003R2 and SA003R11 by using wild-type allele in *trans* with each

256   mutated gene. To do so, we employed the *E. coli*/SA shuttle vector pLI50 (Addgene) under

257   promoter P3(Lee et al. 1991; Jeong et al. 2011). As we had identified a nonsense mutation in

258   *S2121*, we also constructed a deletion mutant of this gene. The *S2121* deletion mutant was

259   unchanged in its sensitivity towards ɸSA012 (data not shown).

260   In resistant strain SA003R2, among three mutated genes (*S156, S157,* and *S497*), only

261   complementation of *S157* and *S497* can restore phage susceptibility of SA003R2 to ɸSA012M2

262   (**Fig. 1B**). However, another *Myoviridae* Twort-like phage, ɸSA039, showed completely

263   different infectivity towards SA003R2 and its complemented mutant. ɸSA039 formed faint

264   plaques on SA003R2 plates (**Fig. 1B**), and surprisingly, complementation of *S156* allele in

265   SA003R2 was able to restore the susceptibility to ɸSA039 (**Fig. 1B and 1D**). We suggested that

266   ɸSA039 and ɸSA012 recognize *S. aureus* cell wall by different mechanism. The mutation of

267   SA003R2 is associated with the reduction in WTA production as total phosphate in extracted

268   WTA from SA003R2 decreased significantly compared to wild-type SA003 (**Fig. 2**). WTA-

269   PAGE also showed an invisible ladder of WTA from SA003R2 and all subsequent resistant

270   strains (data not shown).

12

271   In resistant strain SA003R11, complementation of each mutated genes, except *S156*, can

272   significantly restore phage susceptibility toward φSA012M11 (**Fig. 1E**). The complemented

273   mutant of *S156* has EOP value 5.53±2.52, while complementation of other gene: *S157*, *S497,*

274   *S515, S768*, *S2121*, and *S2190*, show EOP value 63.70±1.15, 77.64±0.58, 65.38±0.58,

275   78.85±0.58, 63.70±1.15, and 43.12±0.58 respectively. Complementation of *S768* and *S497* shows

276   the highest EOP value among other genes. Excluding the *S156* complemented mutant, φSA039

277   failed to form plaques on plates of SA003R11 or its complemented mutants. This observation

278   provides stronger evidence on the importance of *S156* gene for the infection of φSA039 as the

279   complementation of other mutated genes did not significantly restore host susceptibility toward

280   φSA039.

281

282   **Adsorption of φSA012 and φSA039 to complemented mutant**

283   To determine the adsorption of phages to the complemented mutants, we conducted an

284   adsorption assay for phage φSA012M2 and φSA012M11 towards wild-type SA003, their phage-

285   resistant counterpart, as well as the complemented mutant. For the complementation in SA003R2

286   (**Fig. 1C**), adsorption of φSA012M2 onto wild-type SA003 and SA003R2 was 93.64±0.44% and

287   26.83±2.29%, respectively. Complementation of *S156* (R2pLIP3.TarS) did not significantly

288   restore the phage adsorption (29.35±1.15%), while *S497* (R2pLIP3.TO) and *S157*

289   (R2pLIP3.ScdA) restored the phage adsorption to 47.86±1.97% and 42.00±2.30%, respectively.

290   Interestingly, adsorption of φSA039 was significantly restored to 76.20±1.49% in R2pLI.TarS

291   indicating the importance of the *S156* gene for the adsorption of φSA039 onto SA003.

292   Next we analyzed the adsorption of φSA012M11 toward the complemented mutants of

293   SA003R11. The complemented mutants of SA003R11 harbor *S156* (R11pLIP3.TarS), *S157*

294 (R11pLIP3.ScdA), *S497* (R11pLIP3.TO), *S515* (R11pLIP3.RapZ), *S768* (R11pLIP3.GK), *S2121*

295 (R11pLIP3.MurA2), and *S2190* (R11pLIP3.Pol). As shown in **Fig. 1F**, after 25 min of co-

296 incubation, 93.23±8.79% of φSA012M11 was adsorbed onto wild-type SA003 while only

297 1.92±3.59% was adsorbed onto SA003R11. After complementation of the mutated genes by their

298 respective wild-type alleles, the adsorption of φSA012M11 to the complemented mutants of

299 *S157, S497, S515, S768,* and *S2121* was restored. However, even though we observed plaque, the

300 adsorption onto *S2190* complemented mutant did not change significantly. Interestingly, In the

301 case of *S768* gene, even though the EOP value of *S768* complemented mutant (R11pLIP3.GK)

302 was the highest among other genes, the adsorption of phage onto this complemented mutant was

303 relatively low compare to complemented mutant of *S497, S515,* and *S2121.*We speculated that

304 the mutation in *S2190* (which encodes DNA-RNA polymeras alpha subunit), and *S768* (which

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

306   Adsorption of φSA012M11 to R11pLIP3.TO, R11pLIP3.ScdA, R11pLIP3.GK,

307 R11pLIP3.RapZ, and R11pLIP3.MurA was restored to 63.20±3.30, 40.25±2.77%, 25.17±7.88%,

308 43.44±2.95%, and 20.55±7.96%, respectively. Mutation in SA003R11 was associated with an

309 increase in whole-cell sugar according to total carbohydrate quantification using Anthrone

310 reagent (**Fig. 2**). Relative to SA003, the whole-cell sugar of SA003R2 decreased to

311 62.98±6.92%. Meanwhile, the total sugar of SA003R11 and SA003R38 increased to

312 110.49±10.10% and 144.89±0.63% relative to SA003, respectively. A slimy texture was visible

313 on the overnight liquid culture of SA003R38 (data not shown).

314   The infection of wild-type φSA012 to the complemented mutant of R11 is similar to

315 φSA012M11 according to spot test (**Fig. 1E**). The adsorption of φSA012 towards the SA003R11

316 complemented mutant was not significantly different compared to φSA012M11 (**Supplemental**

14

317   **Table S1**). However, in most of the genes (except R11pLIP3.ScdA and R11pLIP3.Pol), the wild-

318   type phage's adsorption showed a tendency to be stronger than ϕSA012M11.

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

320   As some mutated genes (e.g., *S2121* and *S515*) are related to peptidoglycan synthesis, we

321   assume that peptidoglycan may play an important role in inhibiting phage adsorption. For this

322   reason, we performed a plating efficiency test with the addition of peptidoglycan. Different

323   concentrations of peptidoglycan from *S. aureus* (Invivogen) were added to 100 μl of 10^3 PFU/ml

324   phage prior to the plaque assay against SA003. Two different phages, ϕSA012 and ϕSA039,

325   belonging to the same group of *Myoviridae* Twort-like phages, were used. We used the *E. coli*

326   phage SP-21(Tanji et al. 2005) as a negative control. As shown in **Fig. 3**, peptidoglycan inhibited

327   the infection of ϕSA012 but not ϕSA039. To confirm if O-acetylation in C6 of the muramic acid

328   residue is important for binding of ϕSA012, we deleted *oatA* gene by CRISPR/Cas9 system

329   using pCasSA plasmid. However, the adsorption of ϕSA012 onto *oatA* deletion mutant did not

330   change (**Fig. 5**).

Genome comparison of ϕSA012 and ϕSA039

332   According to morphological observation under TEM, ϕSA039 and ϕSA012 belong to

333   *Myoviridae* phages. Both of them show broad host ranges against *S. aureus* isolated from the

334   milk of mastitic cows (Synnott et al. 2009). As these two phages show different infectivities, we

335   analyzed the whole genome of ϕSA039 and mapped it to the genome of ϕSA012 as a reference.

336   Whole genome of ϕSA012 has been explained in previous report (Takeuchi et al. 2016). Whole

337   genome sequencing of ϕSA039 revealed that its genome size is 141,038 bp long and contains

338   217 ORFs. The ϕSA039 genome encodes three tRNAs (pseudo Trp-tRNA gene, bp 27,174 to

339   27,103; Phe-tRNA gene, bp 27,253 to 27,181; and Asp-tRNA gene, bp 27,333 to 27,260). At the

15

340   nucleotide level, φSA039 and φSA012 share 96% similarity where several genes are missing or

341   partially deleted in either φSA012 or φSA039 (listed in **Supplemental Table S2**). The genome

342   of φSA039 shares 99% similarity with staphylococcal phage JD007 (Cui et al. 2012), which is

343   classified as a member of the *Myoviridae* phages that are serotype D *S. aureus*–specific lytic

344   phages that include the well-known phage K (O'Flaherty et al. 2004).

345   As φSA039 and φSA012 differ in their host preference, we select the region of genome

346   encoding the putative tail and baseplate protein and aligned the open reading frames (ORFs) in

347   that region for amino acid alignment by basic local alignment tool (blastp). Most of the ORFs in

348   the selected region share a high degree of similarity (**Fig. 4**); most of the ORFs have more than

349   99% similarity and three ORFs share 91% similarity. Three ORFs share relatively low similarity

350   with all the others: *orf100* (86%), *orf101* (88%), and *orf96* (83%). Moreover, *orf96* of φSA039

351   lacks 195 amino acid residues compared to φSA012. *Orf100* and *orf102* of φSA039 are homolog

352   of *orf103* and *orf105 of* φSA012 which were previously reported as receptor-binding protein

353   (RBP).

354   To determine whether protein of *orf100* and *orf 102* of φSA039 has same activity with

355   their homolog genes, we performed neutralization test in φSA039 by EOP with antibodies

356   against *orf103* protein of φSA012 (anti-ORF103 serum) and *orf105* protein of φSA012 (anti-

357   ORF105). Infection in SA003 by φSA039 was completely inhibited by anti-ORF105 serum but

358   not by anti-ORF103 serum (data not shown).

359   **Artificial deletion of *tarS* gene in SA003 significantly reduce the adsorption of φSA039 but**

360   **not φSA012**

361   WTA has been well reported as a phage receptor in *S. aureus* (Xia et al. 2011). Phage K

362   belongs to the same group as φSA012 and φSA039 is able to bind to the backbone of WTA.

16

363 Phage K and the other *S. aureus* phages lost their infectivity towards the *tagO* deletion mutant.

364 As described earlier, phage susceptibility of SA003R2 toward ɸSA039 was decreased

365 drastically, and complementation of *S156* gene for glycosyl transferase TarS, enzyme that

366 glycosylate β-GlcNAc on WTA, restore the adsorption of ɸSA039. We hypothesized that β-

367 GlcNAc on WTA is crucial for binding of ɸSA039. To test our hypothesis, we first generated a

368 deletion and complementation of *tagO* in *S. aureus*. As shown in **Fig. 5**, our spot test and

369 adsorption assay failed to detect infection of the *tagO* deletion mutant (RNΔTagO) either by

370 ɸSA039 or ɸSA012. Complementation of the *tagO* deletion (RNΔTagO::plIP3.TO) restored the

371 adsorption of ɸSA039 and ɸSA012 to 50.55±2.50% and 57.75±1.57%, respectively. On the other

372 hand, deleting *tarS* caused the significant reduction only on the adsorption of ɸSA039.

373 Complementation of *tarS* deletion mutant (SA003ΔTarS::plIP3.TarS) restored the adsorption of

374 ɸSA039 to 88.69±0.98%. This result indicated that ɸSA039 requires β-GlcNAc on WTA. Since

375 the adsorption of ɸSA039 was not completely loss in TarS null SA003, this phage may also use

376 backbone of WTA for its binding.

377

378 **Discussion**

379 Our results show that genotypic changes occurred during coevolution of bacteria and

380 phage. Most of the mutated genes in phage resistant SA003 were linked to phage adsorption

381 inhibition. In the early round of co-culture, SA003R2 altered its WTA which is well known as a

382 phage receptor in *S. aureus*. To counter the mutant host, ɸSA012 phage altered its Receptor-

383 binding protein (RBP) (Takeuchi et al. 2016). However, in later round, in response to the

384 counteradaption of phage, coevolved SA003 from 11th round (SA003R11) changed its cell wall

385 structure, overproduced the capsular polysaccharides, and inhibited the post-adsorption step by

17

386   acquiring a point mutation in the DNA/RNA polymerase alpha subunit and guanylate kinase.

387   Finally, the later coevolved SA003R38 acquired a point mutation in another gene encoding

388   putative membrane protein which seems to be involved in the excessive production of

389   polysaccharide.

390

391   **Most mutated host genes are linked to phage adsorption**

392   In the early round of coevolution, phage resistant strain SA003R2 significantly reduces

393   the adsorption of ɸSA012. Wall Teichoic Acid (WTA) has been previously characterized as a

394   phage receptor for *S. aureus*. Consistent with these observations, three mutated genes (*S497*,

395   *S156*, and *S157*) are associated with WTA. *S497* (TagO) is involved in the initial synthesis of

396   WTA particularly in the attachment of WTA to peptidoglycan (Soldo et al. 2002), *S156* (TarS)

397   glycosilates β-GlcNAc on WTA (Brown et al. 2012), and *S157* (ScdA) affect cell wall

398   morphology. Deletion in *scdA* has been reported to increase the peptidoglycan cross-linking

399   (Brunskil et al. 1997). However, as the complementation of *S156* did not significantly restore

400   susceptibility of SA003R2 to ɸSA012, we assumed that ɸSA012 does not require β-GlcNAc

401   moieties in WTA. The mutation in SA003R2 was followed by a decrease in total phosphate from

402   extracted WTA, which may indicate the alteration of WTA production. However, ɸSA012 has

403   been shown to have at least two RBPs (ORF103 and ORF105) (Takeuchi et al. 2016). Therefore,

404   the phage may alter one of them to enhance the binding ability to its host. The mutant phage

405   counterpart of SA003R2, ɸSA012M2, harbors two point mutations in *orf103* consistent with the

406   increase in infectivity towards SA003R2. We concluded that ɸSA012M2 developed mutation in

407   *orf103* to fasten the binding onto SA003R2.

18

408   In response to the evolution of φSA012M2, SA003R11 acquired point mutations in four

409   other genes: *S515* (*rapZ*)*, S768* (Guanylate kinase)*, S2121* (*murA*)*, and *S2190* (DNA-RNA

410   polymerase alpha subunit). Three of them (*S2121*, *S515*, and *S768*) are associated with phage

411   adsorption. MurA2 (*S2121*) has been reported to catalyze the first step of peptidoglycan

412   synthesis together with *murA* (Blake et al. 2009). A nonsense mutant of this gene may alter

413   peptidoglycan synthesis and inhibit phage adsorption to *S. aureus* either directly or indirectly.

414   However, our study shows that deletion mutant of *murA2* did not change their susceptibility

415   toward our tested phage. It is likely that the effect of mutation in *MurA2* gene is related to

416   another mutated gene.

417   RapZ (*S515*) modulates the expression of GlmS which was reported to be the key enzyme

418   that feeds glucose into cell wall synthesis in *S. aureus* (Komatsuzawa et al. 2004). It is indirectly

419   involved in the assembly of cell wall components such as peptidoglycan, and either

420   lipopolysaccharide in Gram-negative bacteria or lipoteichoic acid in Gram-positive bacteria. It is

421   also involved in capsule or exopolysaccharide production (Coley and Baddiley 1972; Plumbridge

422   et al. 1993). As whole-cell sugar was higher in SA003R11 (which harbors a point mutation in

423   *S515*) than in SA003R2, the host's cell wall may change due to either overproduction of capsular

424   polysaccharide or alteration of peptidoglycan. Moreover, SA003R38, which harbors two point

425   mutations in RapZ (*S515*), had much higher whole-cell sugar than SA003R11. Mutations in *S515*

426   are therefore likely to be related to the overproduction of capsular polysaccharide. Our result is

427   consistent with previous observations that a capsule inhibits bacteriophage infection (Bernheimer

428   and Tiraby 1976; Scholl et al. 2005). Our finding provides deeper insight on the analysis of the

429   *rapZ* gene involved in the capsular polysaccharide production of coevolved *S. aureus* in their

430   defense against phage infection. In addition, in the later round (SA003R38), a point mutation

19

431   was found in *S692* encoding putative membrane protein YozB. As slimy texture and significant

432   increase of whole-sugar were observed in SA003R38, gene *S692* may also be involved in

433   capsule production.

434   Guanylate kinase (*S768*) is involved in a crucial intermediate step in RNA/DNA

435   synthesis by catalyzing guanosinemonophosphate (GMP) phosphorylation to form guanosine

436   diphosphate (GDP) (Konrad 1992). This enzyme is broadly distributed nucleotide kinase and

437   essential for cellular GMP recycling and nucleotide equilibrium (Oeschger 1978). This enzyme

438   participates in purine metabolism (Weber et al. 1992). In other studies, purine biosynthesis has

439   been shown to be associated with the survival of *S. aureus* under conditions of stress, such as the

440   presence of vancomycin and daptomycin (Keer et al. 2001; Mongodin et al. 2003). Yee et al.

441   (2015) reported that a defect in purine biosynthesis pathway may be related to downstream

442   energy production (i.e., increased purine biosynthesis fuels the generation of polymers, the most

443   energy-demanding metabolic process in bacteria). Two of the most abundant polymers in *S.*

444   *aureus* and *B. subtilis* are peptidoglycan (Mongodin et al. 2003) and WTA (Ellwood 1970). Our

445   study shows that the complement mutant of this gene show high EOP value compared to other

446   complement mutant but relatively low adsorption rate. We interpreted that the mutation of this

447   gene is related to both inhibition of phage adsorption and post-adsorption. By affecting purin

448   metabolism, the mutation is likely involved in the recycling of injected phage-DNA, and

449   subsequently fueling the generation of polymers in the cell wall such as teichoic acid and

450   capsular polysaccharide.

451   Complementation in *S2190* (DNA–RNA polymerase) did not restore phage adsorption

452   but did increase the EOP value. Mutations in *S2190* may not be associated with phage

453   adsorption, but with post-adsorption. RNA polymerase alpha subunit has been reported to be

454   involved in transcription activation (Ishihama 1992). Most phages with a genome size less than

455   200 kbp commonly did not harbor a complete set of genes responsible for genome replication

456   and nucleotide metabolism. Only large-genome size phage (termed Jumbo phage), with genome

457   larger than 200 kbp, have been reported to have more than one paralogous gene for DNA

458   polymerase and RNA polymerase (RNAP) (Yuan and Gao 2017). Our phage in this study has a

459   genome size around 142 kbp and the complete set paralogous of RNA polymerase subunit was

460   not found (Takeuchi et al. 2016). Therefore this phage may use host RNA polymerase as

461   machinery for their DNA transcription. Hence, the mutation that we observed in this gene is

462   likely to be involved in the inhibition of phage DNA transcription. This finding is supported by a

463   previous observation (Osada et al. 2017) that SA003R11 resists phage infection not only by

464   inhibiting phage adsorption but also by suppressing phage genome replication.

465   In this study, we also observed that the infection of wild-type ɸSA012 onto the

466   complemented mutant of R11 was not significantly different compared to ɸSA012M11.

467   However, excluding R11pLIP3.ScdA and R11pLIP3.Pol, the adsorption of wild-type ɸSA012

468   showed a tendency to be stronger than ɸSA012M11. Fitness cost might be detected in

469   ɸSA012M11 as it experienced long term coexistence with SA003. Therefore, the infection of

470   ɸSA012M11 toward the R11 complemented mutants is weaker than that of wild-type ɸSA012.

471   Another report has also demonstrated a fitness cost for phage (which experienced coevolution)

472   by the reduction of its infectivity toward wild-type host, or by the limitation of host range

473   expansion (Hall et al. 2011).

474   CRISPR/cas motif was not found in the genome of wild-type SA003 and short fragments

475   of phage DNA of ɸSA012 were not found in genome of phage resistance derivative, which

476   indicates that the bacteria likely did not use CRISPR/cas to defense the phage invasion. It is well

21

477 known that in many pathogenic bacteria, including *S. aureus*, many of the clones have lost

478 CRISPR/cas during evolution. As a result, *S. aureus* frequently exchange their genetic material

479 via phage-mediated horizontal gene transfer (Brussow et al. 2004; Lindsay 2010). Our study

480 provides deeper insights on how phage and its host can co-exist during co-culture and will help

481 guide future assessments of the advantages and disadvantages of phage therapy.

482 **Two *Myoviridae* Twort-like phages (φSA012 and φSA039) use different mechanisms to**

483 **infect *S. aureus***

484   *Myoviridae* Twort-like phages are well known for their wide host range and highly lytic

485 capabilities. Phages belonging to this genus have been considered promising candidates for

486 therapeutic use and as detection agents of bacterial contamination (Loessner et al. 1996).

487 Recently, Alves et al. (2014) showed that Twort-like phages were effective at reducing biofilm

488 formation by *S. aureus* strains. Our data showed that although the φSA012 and φSA039 genomes

489 share high similarity, these two phages infect *S. aureus* in different ways. φSA039 infectivity

490 towards SA003R11 and SA003R2 and its complemented mutant is lost or significantly lessened,

491 except in the instance of *S156* complementation. As a result, we conclude that φSA039 use *S156*

492 for its binding to *S. aureus* cell.

493   Comparison of φSA012 and φSA039 binding mechanism was performed in this study.

494 First, we found that both phages bind to WTA. We verified that the deletion mutant of *tagO* is

495 resistant to both phages. Second, we found that peptidoglycan from *S. aureus* was able to block

496 infection of φSA012 but not φSA039. It is reported that peptidoglycan can block infection of

497 *Siphoviridae* phages Coyette and Ghuysen (1968). Li et al. (2016) determined that φ11, one of

498 the best-studied *Siphovirus* es, requires O-acetylated peptidoglycan for adsorption. However, our

499 data in this study suggests that φSA012 does not require O-acetylated peptidoglycan for

22

500   adsorption (**Fig. 5B**). We assumed that ɸSA012 uses another component in the peptidoglycan

501   polymer. Finally, we confirm that *S156* which encodes β-N-acetyl glucosaminidase TarS, is

502   crucial for ɸSA039. This suggests that β-N-acetyl glucosamine in WTA acts as a receptor of

503   ɸSA039. Most *S. aureus* synthesize repeating unit of ribitol-phosphate (RboP)WTA with three

504   tailoring modification D-alanine, α-GlcNAc, and β-GlcNAc (Brown et al. 2013). The GlcNAc

505   moieties are attached to RboP by two independent enzymes namely α-GlcNAc   WTA

506   glycosyltransferase TarM (Xia et al. 2010), and β-GlcNAc WTA glycosyltransferase TarS

507   (Brown et al. 2012).

508   Most *S. aureus* phages target WTA and its GlcNAc moieties for adsorption. Notably, it

509   has been reported that *Siphoviridae* phages use α-GlcNAc moieties as receptors (Xia et al. 2011;

510   Li et al. 2016), while *Podoviridae* phages, such as ɸ44AHJD, ɸ66, and ɸP68, use β-GlcNAc

511   moieties (Li et al. 2015). In contrast, *Myoviridae* phages have been reported to simply require

512   WTA polymer, regardless of GlcNAc acetylation (Xia et al. 2011). It is not widely known that

513   phages belonging to the *Myoviridae* group also require GlcNAc moieties in WTA. Our study

514   shows that ɸSA039, a *Myoviridae* phage, with 96% similarity to phage ɸK, a well known

515   *Myoviridae* phage, requires β-GlcNAc in WTA for its binding. In addition, we also confirmed

516   that the t*arM* deletion mutant and its wild-type *S. aureus* have the same susceptibility to ɸSA039

517   (**Fig.5**). Hence, α-N-acetyl glucosamine of WTA is likely not essential for ɸSA039. As β-N-

518   acetyl glucosamine has been reported to serve as a binding site of PBP2a, the enzyme

519   responsible for β-lactam antibiotic resistance in *S. aureus* (Brown et al. 2012), the application of

520   ɸSA039 and β-lactam antibiotic may give a synergetic effect for the treatment of *S. aureus*

521   infection. We believe that this finding may give us novel insights into how closely related phages

522   in the group of *Myoviridae* differ in their host preference among *S. aureus* strains.

23

523 ***In silico* analysis of ϕSA039 and ϕSA012 genome shows potential viral protein that**

524 **contributes to different adsorption mechanism**

525   Several genes are missing in ϕSA012 and ϕSA039 (**Supplemental table S2**). In

526 particular, two terminal repeat regions (*orf195* and *orf200*) that exist in ϕSA012 genome are

527 missing in ϕSA039. Lobocka et al. (2012) reported that all genes located in the terminal

528 redundant region play a role in host takeover that is analogous to *B. subtilis* phage, SPO1, which

529 also possesses a terminal redundant region (Stewart et al. 1998). In addition, the gene of *orf81* of

530 ϕSA012, which contains a putative intron-encoded nuclease, and the gene of *orf39* of ϕSA012,

531 which encodes a DNA ligase are also missing in ϕSA039. Moreover, a partial deletion is found

532 in *orf96* of ϕSA039, a homolog of *orf99* of ϕSA012, which encodes tail morphogenetic protein.

533 As we described in a previous study, the mutant phage ϕSA012M20 carries a point mutation in

534 *orf99*, and subsequently accumulates two more point mutations to become the mutant phage

535 ϕSA012M38 (Takeuchi et al. 2016), which may indicate the importance of *orf99* in phage-host

536 interaction. Therefore, the different in *orf96* of ϕSA039 may contribute to the specificity

537 different.

538   In the tail or baseplate region of ϕSA039, compared to ϕSA012, several ORFs have been

539 shown to have low amino acid similarity (83–88%): i.e., *orf99*, *orf103*, and *orf104,* whereas

540 *orf103* and *orf104* are located in the unique region of the Twort-like tail/baseplate module (**Fig.**

541 **4**). During phage-host interaction, *orf103* of ϕSA012 had been reported to bind onto α-GlcNAc

542 of WTA, while *orf105* is likely bind onto the backbone of WTA. Since anti-ORF105 of ϕSA012

543 can neutralize of ϕSA039 infection, the binding activity of *orf102* of ϕSA039, a homolog of

544 *orf105* of ϕSA012, must be similar. Thus, in ϕSA039, *orf102* may bind onto backbone of WTA.

545 However, *orf100*, a homolog of *orf103* of ϕSA012, may bind to β-GlcNAc or another part of

24

546   WTA. Taken together, we suspect that ORFs of ɸSA039 located in the tail or baseplate region,

547   which has low amino acid similarity compared to ɸSA012 such as *orf100*, *orf101*, and *orf96,* are

548   likely to be the factors responsible for the observed differences in specificity between ɸSA012

549   and ɸSA039. However, further analysis of those potential genes might be necessary.

550

560

561   **Conflict of interest**

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

563

564   **Ethical approval**

565   This article does not contain any studies with human participants and animals performed by any

566   of the authors.

567

568

569   **Reference**

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776 **Figure legend**

777 **Figure 1 (A)** Spontaneous mutation in SA003R2; deletion of *tarS* and *scdA* gene, one point

778 mutation in *tagO*. **(B)** Spot test of φSA012M2 and φSA039 toward SA003R2 and its phage

779 resistance derivative. Adsorption assay of φSA012M2 **(C)** and φSA039 **(D)** toward SA003R2

780 and its phage resistance derivative. Spot test **(E)** and adsorption assay **(F)** of φSA012M11 toward

781 SA003R11 and its phage resistance derivative. Statistical significance was indicated by

782 *(P<0.05) or **(P<0.01).

783 **Figure 2** Percentage of total phosphate from extracted wall teichoic acid (WTA) (dark) and

784 whole-cell sugar (gray) relative to the wild-type strain SA003. Values are given as means ±

785 standard deviations (SD, n = 3). Statistical significance was indicated by *(P<0.05) or

786 **(P<0.01).

787 **Figure 3** Dose-dependent inhibition by peptidoglycan of phage φSA012 (black bar), phage

788 φSA039 (gray bar) and *E. coli* phage SP-21 (white bar). Data represent means ± standard

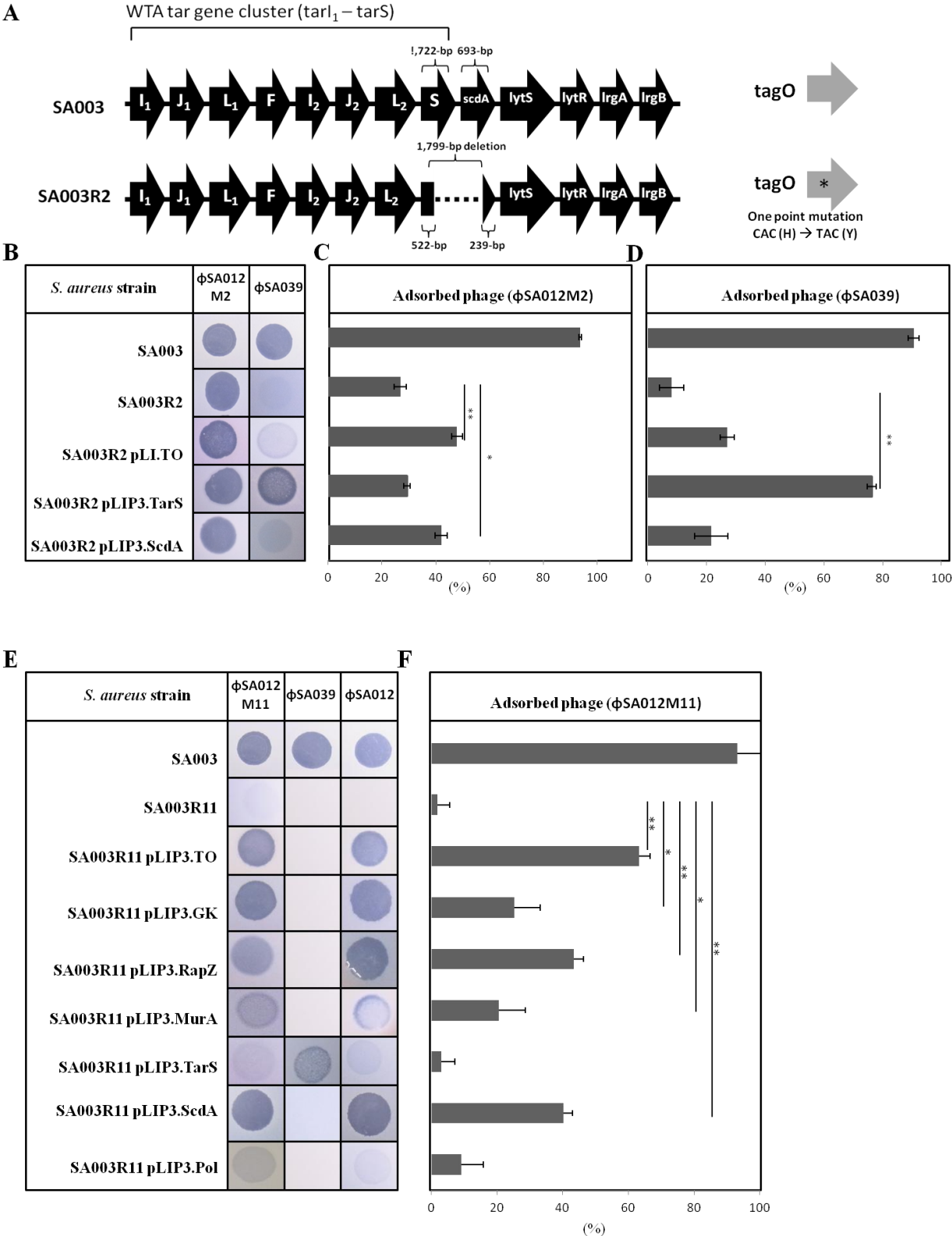789 deviations (SD, n = 3). Statistical significance was indicated by *(P<0.05) or **(P<0.01).

790 **Figure 4** Alignment of putative tail and baseplate region between φSA012 and φSA039.

791 **Figure 5** Infectivity of φSA012 and φSA039 towards RN4220, *tarM* deletion mutant

792 (RNΔTarM), *oatA*-deletion mutant (RN ΔOatA), *tagO*-deletion mutant (RNdTagO), and

793 complemented *tagO* (RNdTagO::pLIP3.TO). *tarS*-deletion mutant (SA003ΔTarS) and

794 complemented *tarS* (SA003ΔTarS::pLIP3.TarS) The infectivity assay is based on spot test **(A)**

795 and adsorption assay for φSA012 **(B)** and φSA039 **(C)**. Data represent means ± standard

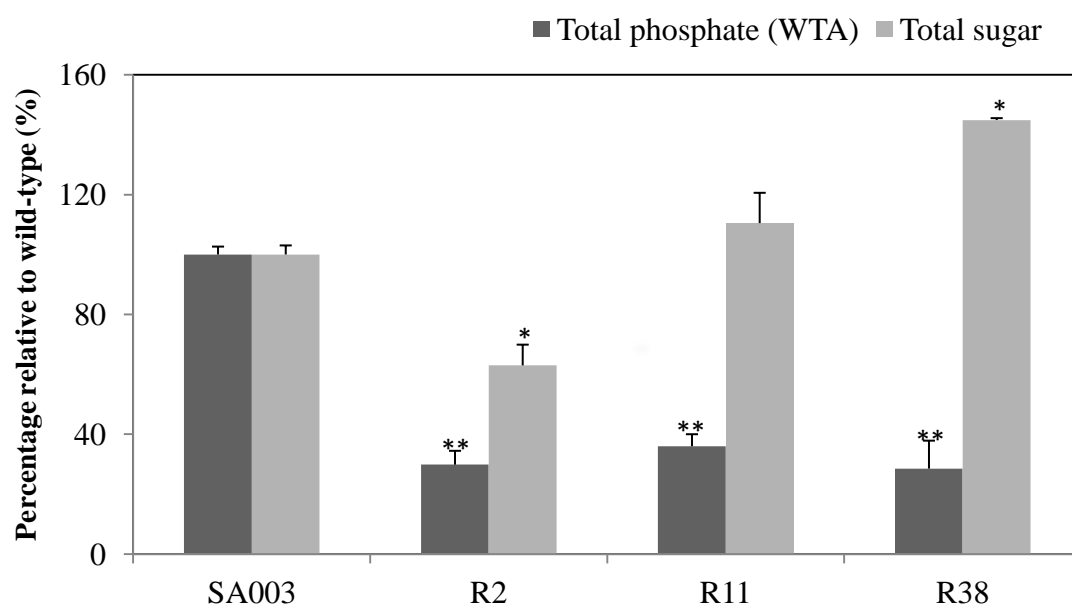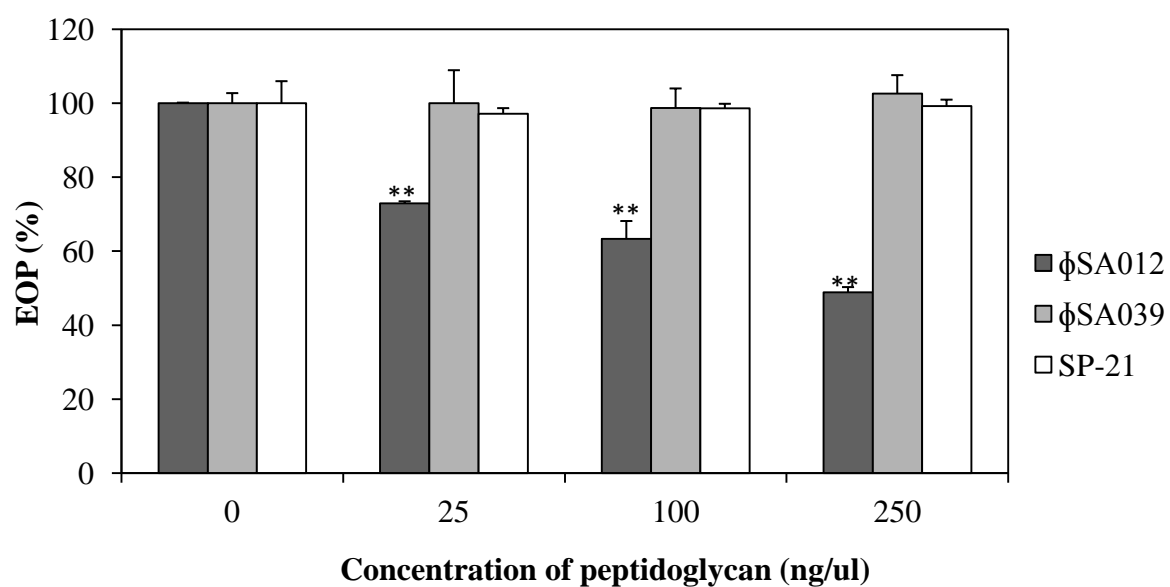796 deviations (SD, n = 3). Statistical significance was indicated by *(P<0.05) or **(P<0.01).

797

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5