Adsorption of Staphylococcus viruses S13’ and S24-1 on Staphylococcus aureus strains with different glycosidic linkage patterns of wall teichoic acids

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Abstract

The group of phages belonging to the family Podoviridae, genus P68virus, including Staphylococcus viruses S13’ and S24-1, are important because of their benefits in phage therapy against Staphylococcus aureus infections. The O-glycosidic linkage patterns of wall teichoic acids (WTAs) in S. aureus cell walls seem to be important for adsorption of this phage group. In this study, the adsorption of Staphylococcus viruses S13’ and S24-1 to S. aureus was examined using strains with modified WTA glycosidic linkage patterns. We found that the β-O-N-acetylglucosamine of WTAs was essential for S13’ adsorption, while N-acetylglucosamine, regardless of the α- and β-O-glycosidic linkages of the WTAs, was essential for S24-1 adsorption. Next, examining the binding activities of their receptor-binding proteins (RBPs) to cell walls with different WTA glycosidic patterns, the β-O-N-acetylglucosamine of the WTAs was essential for S13’ RBP binding, while N-acetylglucosamine, regardless of the α- and β-O-glycosidic linkages of the WTAs, was essential for S24-1 RBP binding. Therefore, the results of the RBP binding assays were consistent with those of the phage adsorption assays. Bioinformatic analysis suggested that the RBPs of Staphylococcus viruses S13’ and S24-1 were structurally similar to the RBPs of phage phi11 of the family Siphoviridae. Phylogenetic analysis of the RBPs indicated that two phylogenetic subclusters in the family Podoviridae were related to the glycosidic linkage patterns required for phage adsorption, possibly mediated by RBPs. We hope that this study will encourage the future development of therapeutic phages.

INTRODUCTION

Bacteriophage (phage) therapy, in which phages are applied as a bioagent to eliminate target bacteria, is predicted to be reintroduced. Phage therapy has been used in Eastern Europe since the beginning of phage discovery [1, 2], and because of the recent emergence of multidrug-resistant bacteria, its use has been revisited [1, 2]. Recent developments have proposed the application of preadapted phages with improved adsorption to increase their therapeutic effects and reduce the risk of the emergence of phage-resistant bacteria [3–5]. However, the mechanism of phage adsorption is not fully understood. Therefore, detailed studies of phage adsorption are required.

Staphylococcus aureus is an opportunistic pathogen, and drug-resistant S. aureus strains threaten immunocompromised patients in hospitals and healthy individuals in the community [6, 7]. The S. aureus phages belonging to the family Podoviridae, subfamily Picovirinae, genus P68virus, formerly named genus Ahjlikevirus, have been isolated and studied for phage therapy [8–17]. Phages belonging to this taxonomic group have a head 50 nm in diameter and a short tail 30 nm in length [8, 11]. Their double-stranded DNA genome is approximately 16–19 kbp in length [8]. We have recently isolated Staphylococcus viruses S13’ and S24-1 belonging to the genus P68virus, and have identified one of their receptor-binding proteins (RBPs) [18]. Although Staphylococcus viruses S13’ and S24-1 were isolated in the same geographical region, their genomes are different [11, 18].

The wall teichoic acids (WTAs) of S. aureus are composed of a repeating unit of ribitol phosphate, in which hydroxyls of ribitol phosphate are substituted with D-alanine, α-O-N-acetylglucosamine (α-O-GlcNAc) and β-O-GlcNAc, and a disaccharide-based linkage unit covalently attached to the N-acetylmuramic acid of peptidoglycan (Fig. 1) [19, 20]. The
biosynthesis of WTAs has been studied in detail [19]. The TagO protein is an enzyme that is essential for WTA biosynthesis, and the TarM and TarS proteins add O-GlcNAc to the ribitol phosphate repeats of WTA with α- and β-linkages, respectively. S. aureus strains with a deficiency in WTA and in α- and/or β-O-GlcNAc of WTAs were constructed based on this knowledge of the WTA biosynthesis pathway [19, 20]. Phage infectivity and adsorption were studied using these genetically modified bacterial strains [21–24]. The WTA glycosylation pattern of S. aureus, particularly the β-O-GlcNAc of WTAs, has recently been shown to be important for infection and adsorption of phages belonging to the genus Pe8virus [22]. The RBP sequences of Staphylococcus viruses S13’ and S24-1 differ slightly from each other [18], and absorption of S13’ and S24-1 was hypothesized to be mediated by RBPs which recognize different molecules of WTA. In this study, we analysed the WTA glycosylation patterns required for adsorption of the genus Pe8virus together with the genetic variation of the phage, using the interaction of Staphylococcus viruses S13’ and S24-1 and their RBPs with genetically modified S. aureus.

**RESULTS AND DISCUSSION**

**Infectivity and adsorption of Staphylococcus viruses S13’ and S24-1 to bacterial strains with different patterns of WTA glycosidic linkage**

To study the relationship of phage lytic activity and adsorption with different glycosidic linkage patterns of WTA (i.e. α-O-GlcNAc and/or β-O-GlcNAc) in S. aureus, streak tests and adsorption assays were performed on the genetically modified strains. The genetically modified strains included gene-knockout strains deficient in tagO or tarM and/or tarS genes and their complemented strains, which were prepared from S. aureus strain RN4220 (Table 1) [25–27]. Because Staphylococcus viruses S13’ and S24-1 formed very small and faint plaques on strain RN4220 and its genetically modified strains, the lytic activity (i.e. plaque formation or lysis-from-without) of the Staphylococcus viruses S13’ and S24-1 on these strains was examined using streak tests, which enabled not only the examination of the phage lytic pattern such as lysis-from-within, lysis-from-without and no lysis, but also semiquantitative examination of phage infection efficiency. Second, to assay adsorption, the concentrations of the unbound phages in the culture supernatant of strain RN4220 and its genetically modified derivatives were measured by plaque assay using strain SA27 as an indicator host, after incubating the phages with these bacteria. *Staphylococcus* viruses S13’ and S24-1 form clear visible plaques on strain SA27.

The lytic activity and adsorption of S13’ on the genetically modified S. aureus strains were examined (Table 2). No lysis was observed in strains T174, T803, T807 and T842, while lysis was observed in the other strains tested. Moreover, phage adsorption was examined by analysing the percentages of unbound phage (i.e. mean±standard deviation). The levels of unbound S13’ for bacterial strains T174, T803, T807 and T842 were 103.2±3.0, 90.0±14.5, 114.3±16.3 and 86.1±11.5 %, respectively, which are significantly higher than those in RN4220 (2.5±0.4 %; P<0.01). The levels of the unbound phage for the other strains, T111, T790, T817, T813 and T844, were 1.7±0.4, 2.3±0.4, 2.8±0.6, 1.7±0.3 and 1.9±0.4 %, respectively, which are not significantly different to those in RN4220 (i.e. 2.5±0.4 %). Thus, the β-O-GlcNAc of WTA is required for S13’ adsorption, which was considered to influence its subsequent lytic activity.

Next, the lytic activity of S24-1 was examined (Table 2). When the lytic activity was tested on the genetically modified S. aureus, no lysis was observed in strains T174 and T807, while lysis was observed in the other strains (Table 2). Moreover, S24-1 adsorption was examined as described above for S13’. The levels of unbound S24-1 for bacterial strains T174 and T807 were 80.5±0.9 and 49.1±3.1 %, respectively, which are significantly higher than those for RN4220 (16.7±3.4 %; P<0.01). The levels of unbound phage for strains T111, T790, T817, T803, T813, T842 and T844 were 10.7±1.2, 10.1±6.3, 10.5±2.5, 8.8±1.3, 8.5±3.7, 18.6±2.4 and 11.9±1.2 %, respectively, which are not significantly different to those in RN4220. Thus, it is possible that not only α- and β-O-glycosidic linkages of WTA but also other components of the WTA...
GlcNAc, N-acetylglucosamine; WTA, wall teichoic acid.

**Table 1. Staphylococcus aureus strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Description†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>Parental strain</td>
<td>Parental strain</td>
<td></td>
</tr>
<tr>
<td>T174</td>
<td>RN4220 ΔtagO::erm</td>
<td>Deficiency of WTA, because of the gene knockout</td>
<td>[25]</td>
</tr>
<tr>
<td>T790</td>
<td>RN4220 Δapla::phleo ΔtarM::erm</td>
<td>Deficiency of α-O-GlcNAc of WTA and protein A, because of the gene knockouts</td>
<td>[26]</td>
</tr>
<tr>
<td>T803</td>
<td>RN4220 Δapla::phleo ΔtarS::km</td>
<td>Deficiency of β-O-GlcNAc of WTA and protein A, because of the gene knockouts</td>
<td>[26]</td>
</tr>
<tr>
<td>T807</td>
<td>RN4220 Δapla::phleo ΔtarM::erm ΔtarS::km</td>
<td>Deficiency of both α-O-GlcNAc and β-O-GlcNAc of WTA and protein A, because of the gene knockouts</td>
<td>[26]</td>
</tr>
<tr>
<td>T111</td>
<td>RN4220 ΔtagO::erm/pStagO</td>
<td>Deficiency of WTA, because of the gene knockouts; supplementation of WTA by plasmid pStagO</td>
<td>[27]</td>
</tr>
<tr>
<td>T817</td>
<td>RN4220 Δapla::phleo ΔtarM::erm ΔtarS::km/psTagM</td>
<td>Deficiency of α-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of α-O-GlcNAc of WTA by plasmid pStagM</td>
<td>This study</td>
</tr>
<tr>
<td>T813</td>
<td>RN4220 ΔtarS::km</td>
<td>Deficiency of β-O-GlcNAc of WTA and protein A, because of the gene knockouts; β-O-GlcNAc supplemented by plasmid pStarS</td>
<td>This study</td>
</tr>
<tr>
<td>T842</td>
<td>RN4220 ΔtarM::erm/Δapla::phleo/psStarM</td>
<td>Deficiency of α-O-GlcNAc and β-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of α-O-GlcNAc by plasmid pStarM</td>
<td>[26]</td>
</tr>
<tr>
<td>T844</td>
<td>RN4220 ΔtarM::erm/Δapla::phleo/psStarS</td>
<td>Deficiency of α-O-GlcNAc and β-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of β-O-GlcNAc by plasmid pStarS</td>
<td>[26]</td>
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Gene-knockout strains supplemented with complementary plasmid

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Description†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T111</td>
<td>RN4220 ΔtagO::erm/pStagO</td>
<td>Deficiency of WTA, because of the gene knockouts; supplementation of WTA by plasmid pStagO</td>
<td>[27]</td>
</tr>
<tr>
<td>T817</td>
<td>RN4220 Δapla::phleo ΔtarM::erm ΔtarS::km/psTagM</td>
<td>Deficiency of α-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of α-O-GlcNAc of WTA by plasmid pStagM</td>
<td>This study</td>
</tr>
<tr>
<td>T813</td>
<td>RN4220 ΔtarS::km</td>
<td>Deficiency of β-O-GlcNAc of WTA and protein A, because of the gene knockouts; β-O-GlcNAc supplemented by plasmid pStarS</td>
<td>This study</td>
</tr>
<tr>
<td>T842</td>
<td>RN4220 ΔtarM::erm/Δapla::phleo/psStarM</td>
<td>Deficiency of α-O-GlcNAc and β-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of α-O-GlcNAc by plasmid pStarM</td>
<td>[26]</td>
</tr>
<tr>
<td>T844</td>
<td>RN4220 ΔtarM::erm/Δapla::phleo/psStarS</td>
<td>Deficiency of α-O-GlcNAc and β-O-GlcNAc of WTA and protein A, because of the gene knockouts; supplementation of β-O-GlcNAc by plasmid pStarS</td>
<td>[26]</td>
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GlcNAc, N-acetylglucosamine; WTA, wall teichoic acid.

*erm, erythromycin; phleo, phleomycin; km, kanamycin.

**Table 2. Phage lytic activity of Staphylococcus viruses S13’ and S24-1**

| Bacterial strain | Brief description | S13’ | Lytic activity* | Unadsorbed phage in adsorption assay (%)† | Mean | sd | S24-1 | Lytic activity* | Unadsorbed phage in adsorption assay (%)† | Mean | sd
<table>
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<tbody>
<tr>
<td>RN4220</td>
<td>Wild-type strain</td>
<td>○</td>
<td>2.5</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>16.7</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>T174</td>
<td>Deficiency of WTA</td>
<td>×</td>
<td>103.2</td>
<td>3.0 S</td>
<td></td>
<td></td>
<td></td>
<td>×</td>
<td>80.5</td>
<td>0.9</td>
<td>S</td>
</tr>
<tr>
<td>T111</td>
<td>Complementation of WTA in strain T174</td>
<td>○</td>
<td>1.7</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>10.7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>T790</td>
<td>Deficiency of α-O-GlcNAc of WTA</td>
<td>○</td>
<td>2.3</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>10.1</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>T817</td>
<td>Complementation of α-O-GlcNAc of WTA in strain T790</td>
<td>○</td>
<td>2.8</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>10.5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>T803</td>
<td>Deficiency of β-O-GlcNAc of WTA</td>
<td>×</td>
<td>90.0</td>
<td>14.5 S</td>
<td></td>
<td></td>
<td></td>
<td>×</td>
<td>9.8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>T813</td>
<td>Complementation of β-O-GlcNAc of WTA in strain T790</td>
<td>○</td>
<td>1.7</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>8.5</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>T807</td>
<td>Deficiency of α-O-GlcNAc and β-O-GlcNAc of WTA</td>
<td>×</td>
<td>114.3</td>
<td>16.3 S</td>
<td></td>
<td></td>
<td></td>
<td>×</td>
<td>49.1</td>
<td>3.1</td>
<td>S</td>
</tr>
<tr>
<td>T842</td>
<td>Complementation of α-O-GlcNAc of WTA in strain T807</td>
<td>○</td>
<td>86.1</td>
<td>11.5 S</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>18.6</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>T844</td>
<td>Complementation of β-O-GlcNAc of WTA in strain T807</td>
<td>○</td>
<td>1.9</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td>○</td>
<td>11.9</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

* ×, no lysis; O, lysis.
† The phage quantity remaining in the supernatant of the RN4220 cell treatment compared with that of the other strain by Student’s t-test, and significance is indicated by ‘S’ (P<0.01).

are important for S24-1 adsorption, and α- and β-O-glycosidic linkages of WTA was considered to influence its subsequent lysis. Therefore, absorption of *Staphylococcus* viruses S13’ and S24-1 seemed to require recognition of different receptor molecules, particularly different linkage patterns of O-GlcNAc. Binding abilities of RBPsof *Staphylococcus* viruses S13’ and S24-1 to bacterial strains with different patterns of WTA glycosidic linkage

One of the RBPs of S24-1 has been identified as open reading frame (ORF) 16, which is designated ORF16_S24-1 in this study [18]. Corresponding putative gene products, which are
designated ‘gene product_phage name’, can be found in other phages belonging to the genus *P68virus*. For example, the corresponding ORF to ORF16_S24-1 in S13’ was designated ORF16_S13’. The analysis of ORF16_S13’ by *BLAST* showed a high similarity to ORF16_S24-1 (98.9 % query coverage, 0.0 E-value and 69.8 % identity). Recombinant RBPs of *Staphylococcus* viruses S13’ and S24-1 were produced in an *Escherichia coli* protein-expression system. Purified RBPs were prepared for this study and designated rORF16_S13’ and rORF16_S24-1, respectively (Fig. 2a). The binding activities of rORF16_S13’ and rORF16_S24-1 to the cell walls of the genetically modified RN4220 strains were examined.

After mixing rORF16_S13’ or rORF16_S24-1 protein with the cell walls of genetically modified RN4220 strains and separation of the cell walls and the supernatants by centrifugation, the proteins bound to the cell wall and those in the supernatant were detected by Western blot (Fig. 2b). As a control experiment, cell wall suspensions of strains T174 and T111 were tested. The rORF16_S13’ and rORF16_S24-1 proteins were detected on the cell walls of strain T111 but not on those of strain T174 (Fig. 2c). Therefore, both rORF16_S13’ and rORF16_S24-1 proteins appeared to bind to the cell walls containing WTA, meaning that this assay system could be applied to the evaluation of the binding abilities of rORF16_S13’ and rORF16_S24-1 proteins to a suspension of cell walls with modified WTA glycosylation (Fig. 2c). First, the binding activity of the rORF16_S13’ protein was examined. The rORF16_S13’ protein was detected in the supernatant of strains T803, T807 and T842, while it was not detected in the supernatant of strains T790, T817, T813 and T844. Therefore, the rORF16_S13’ protein did not seem to bind to cell walls with WTA deficient in β-O-GlcNAc. However, rORF16_S24-1 protein was detected in both the cell wall and supernatant of strain T807, while it was not detected in the supernatant of strains T790, T817, T803, T813, T842 and T844. Therefore, the rORF16_S24-1 protein did not seem to bind to WTA molecules deficient in α- and β-O-GlcNAc.

![Fig. 2. Assay of binding of the RBPs using the recombinant RBPs, rORF16_S13’ and rORF16_S24-1. (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) image of the purified rORF16_S13’ and rORF16_S24-1. Three micrograms of the purified rORF16_S13’ and rORF16_S24-1, together with the molecular weight standard (XL-Ladder Broad; APRO Life Science Institute), were electrophoresed on a 12.5 % SDS-PAGE gel. Proteins were stained by Coomassie Brilliant Blue R-250. The left, middle, and right lanes labelled with ‘M’, ‘rORF16_S13’, and ‘rORF16_S24-1’ indicate the electrophoretic profiles of the molecular standard, the purified rORF16_S13’, and the purified rORF16_S24-1, respectively. (b) Experimental scheme of the binding assay using the RBP. Five minutes after mixing the RBPs with the cell walls, the supernatant and cell walls were separated by centrifugation. The RBPs were detected by Western blot. (c) Binding assay using purified rORF16_S13’ and rORF16_S24-1. Results of the Western blots using ORF16_S13’ and ORF16_S24-1 proteins are shown on the top and bottom, respectively. The detected bands are shown.](image-url)
When these results were compared with those for phage infectivity and adsorption, the binding abilities of the rORF16_S13’ and rORF16_S24-1 proteins seemed to reflect the adsorption specificity of Staphylococcus viruses S13’ and S24-1, respectively. Therefore, the ORF16_S13’ and ORF16_S24-1 proteins, which recognized different glycosidic linkages of WTA, were considered to be major RBPs of Staphylococcus viruses S13’ and S24-1.

Bioinformatic analysis of RBPs of Staphylococcus viruses S13’ and S24-1

The protein function and structure of ORF16_S13’ and ORF16_S24-1 were analysed using HHpred and Phyre2 (Tables S1 and S2, available in the online Supplementary Material) [28, 29]. The ORF16_S13’ and ORF16_S24-1 proteins were predicted to be structurally similar to the GP45 of Staphylococcus phage phi11 belonging to family Siphoviridae, genus Phietavirus (GP45_phi11 protein), which has been identified as a major RBP [24, 30]. Comparison of the ORF16_S13’ and ORF16_S24-1 proteins with the GP45_phi11 protein using BLASTP identified sequence similarities (72.0% query coverage, 5e-43 E-value and 28.4% identity; 72.0% query coverage, 2e-46 E-value and 29.6% identity, respectively).

Structural studies of the GP45_phi11 protein have identified two features: a metal-binding site and a potential GlcNAc-binding pocket [24, 30]. Protein sequence alignment of GP45_phi11 with ORF16_S13’ and ORF16_S24-1 (Fig. 3) compared the amino acids of the GP45_phi11 protein which are important for its metal-binding site and the potential GlcNAc binding pocket with those of ORF16_S13’ and ORF16_S24-1 proteins. His-42 and His-50 of the GP45_phi11 protein have been shown to be essential for Fe³⁺ binding [30], and ORF16_S13’ and ORF16_S24-1 proteins had the same amino acids, Ser-53 and Arg-61, at the sites corresponding to His-42 and His-50, respectively, of the GP45_phi11 protein.

Met-164, Gln-165, Thr-211, Met-329 and Gln-330 of the GP45_phi11 protein are assumed to be required for GlcNAc molecule binding. Four of these five amino acids were conserved in the ORF16_S13’ and ORF16_S24-1 proteins, including the polar Gln-165, Thr-211 and Gln-330, and the apolar Met-329 present in the GP45_phi11 protein. The corresponding amino acids in the ORF16_S13’ and ORF16_S24-1 proteins were Gln-174, Thr-222, Met-331 and Gln-332, respectively. However, one of the five amino acid residues required for GlcNAc binding, the apolar Met-164 of the GP45_phi11 protein, was not conserved in the ORF16_S13’ and ORF16_S24-1 proteins. The ORF16_S13’ and ORF16_S24-1 proteins had Asn-173 at the residue corresponding to Met-164 in the GP45_phi11 protein.

We also investigated the differences between ORF16_S13’ and ORF16_S24-1 proteins. Unfortunately, because the amino acid residues corresponding to the key amino acids were the same in the ORF16_S13’ and ORF16_S24-1 proteins, we were unable to postulate the protein moiety of the ORF16_S13’ and ORF16_S24-1 proteins responsible for binding to WTA.

Phylogenetic analysis of RBPs of staphylococcal podophages

The RBPs and major virion proteinsof Staphylococcus viruses S13’ and S24-1 were phylogenetically compared with the corresponding proteins in other phages belonging to the genus P68virus (i.e. 66, 44AHJD, P68, GRCS, Psa3, SCH1, SLPW, BP39 and SAP-2), together with those in phages belonging to family Siphoviridae, genus Phietavirus (i.e. phi11, phiMR25 and 80alpha) (Fig. 4). In phylogenetic trees based on both RBPs and major virion proteins, there were two distinct phylogenetic clusters of staphylococcal phages: a cluster including the family Podoviridae, genus P68virus and one including the family Siphoviridae, genus Phietavirus. In the phylogenetic tree based on the RBPs, there seemed to be two subclusters in the family Podoviridae (one including S24-1 and BP39 and the other including the rest of the phages); this was not seen in the phylogenetic tree based on the MCPs.

We then examined the glycosidic linkage patterns required for phage adsorption. There are two types of glycosidic linkage patterns necessary for phage adsorption in these phages: one group required GlcNAc regardless of α- and β-O-glycosidic linkages and the other group required β-O-GlcNAc [22, 24, 31]. In this study, to add information to this analysis we included phiMR25, which is a member of family Siphoviridae, genus Phietavirus and has been shown to require the GlcNAc of WTA regardless of α- and β-O-glycosidic linkage for its adsorption (Fig. S1) [32]. The family Siphoviridae and the subcluster of the family Podoviridae including S24-1 and BP39 seemed to require the GlcNAc of WTA regardless of α- and β-O-glycosidic linkages for their adsorption, while the other subcluster of the family Podoviridae seemed to require the β-O-GlcNAc of WTA.

Because phage genomes are considered to have evolved by genetic drift [33], the RBPs in the subclusters of the family Podoviridae were considered to have evolved divergently. However, it is not clear why different viral families such as Podoviridae and Siphoviridae, which are considered to have evolved separately, recognize similar molecules as a receptor for phage adsorption [34, 35]. There are two hypotheses to explain these observations: convergent evolution and horizontal gene transfer [36]. Considering the above-mentioned protein sequence similarities of the ORF16_S13’ and ORF16_S24-1 proteins to GP45_phi11, the latter hypothesis is more plausible than the former.

Development of therapeutic phage against S. aureus infections using RBPs

There are three major taxonomical groups of staphylococcal phages: those belonging to the family Myoviridae, genus Twortvirus, those belonging to the family Podoviridae, genus P68virus, and those belonging to the family Siphoviridae [8, 35]. Phages belonging to family Podoviridae and family Myoviridae, genus Twortvirus, are virulent, and
**Fig. 3.** Protein sequence alignments of ORF16_S13’, ORF16_S24-1 and GP45_phi11. On the bottom of the sequence alignments, the level of the amino acid match is shown. The asterisk and dot indicate 100% match and two-thirds match, respectively. The amino acid residues of GP45_phi11 for Fe\(^{3+}\) binding (i.e. His-42 and His-50) and the corresponding amino acid of the ORF16_S13’ and ORF16_S24-1 are highlighted in grey. The polar amino acids essential for GlcNac binding in GP45_phi11 (i.e. Gln-165, Thr-211 and Gln-330) and their corresponding amino acids in the ORF16_S13’ and ORF16_S24-1 proteins are boxed in black lines. The apolar amino acids essential for the GlcNac binding in GP45_phi11 (i.e. Met-164 and Met-329) and their corresponding amino acids in the other proteins are boxed in dotted black lines.


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therefore are considered to be appropriate for therapeutic purposes [1]. Because insufficient understanding of the requirements for adsorption and the identity of the RBPs of therapeutic phages may lead to the use of inappropriate phages and impair their therapeutic effects, further studies of the adsorption and RBPs of phages belonging to both family Podoviridae and family Myoviridae, genus Twortvirus are required before they can be used against S. aureus infections.

The phages belonging to the P68virus genus cannot adsorb to and infect all S. aureus strains because the S. aureus strains have different WTA glycosylation patterns [22, 37]. This study implied that the RBP type was important for selection of particular phages belonging to the genus P68virus. To understand the differences in the RBPs of phages belonging to the genus P68virus (i.e. ORF16_ S13’ and ORF16_ S24-1 types), the structural basis of the binding of these RBPs should be studied in more detail. Moreover, the RBPs of the phage group belonging to the family Myoviridae, genus Twortvirus have not been identified. Recent studies of staphylococcal phages belonging to the genus Twortvirus suggested that there may be at least two receptor-binding molecules [23, 38]. A cryoelectron microscopic study of 812, a member of the genus Twortvirus, recently indicated two possible (inner and outer) RBPs [39], ORF125 and ORF127 of 812 (ORF125_812 and ORF127_812, respectively). Based on HHpred analysis, the N-terminal to middle section of the ORF127_812 protein was assumed to contain the C-terminal portion of a GP45_phi11 protein domain (Table S1), while ORF125_812 did not. The RBPs of Listeria phage A511, which is a member of the family Myoviridae, subfamily Spounavirinae, have been purified and identified [40], suggesting that the potential RBPs of the S. aureus phages belonging to family Myoviridae, genus Twortvirus will be purified and studied in the future.

More efficient adsorption of a phage produced using the technology of evolutionary biology increases its therapeutic effects and reduces the chance of the emergence of phage-resistant bacteria [3, 4]. Phages with modified adsorption devices have also been manufactured using synthetic biology [41–43]. Therefore, we hope that phages belonging to both genera P68virus and Twortvirus with greatly increased adsorption efficiency will be developed using the
technologies of evolutionary and synthetic biology, and combined into a cocktail to achieve greater safety and stronger therapeutic effects for next-generation phage therapy.

**METHODS**

**Phage, bacteria, culture, culture media and reagents**

*Staphylococcus* viruses S13′ and S24-1, isolated in Kochi, Japan, were used in this study [11, 18]. The genetically modified *S. aureus* strains described in Table 1, which were prepared based on strain RN4220, were donated by Dr Kenji Kurokawa [25–27]. The genetically modified strains T817 and T813 and the plasmid pStarM, and the strain T803 and the plasmid pStarS, respectively (Table 1) [26]. The plasmids pStarM and pStarS were prepared by cloning the tarM and tarS genes into pND50 plasmids, respectively [44]. *S. aureus* strain SA27 was used as an indicator host for plaque assays [45]. The plasmids pCold II S13′ and T813 were constructed from the strain T790 and the plasmid pS plasmid pS were prepared by cloning the *S. aureus* genes into pND50 plasmids, respectively [44]. The genetically modified strains T817 and T803, isolated in Kochi, Japan, were used in this study [11, 18]. The genetically modified strains T817 and T803 were transformed into competent *E. coli* NiCo21 (DE3) (New England Biolabs), and the protein was overexpressed in 250 ml LB broth, according to the manufacturer’s instructions. The bacterial culture was centrifuged (8000 g, 10 min, 4 °C), and the bacterial pellet was prepared.

After sonication of the bacterial pellet in 25 ml of lysis solution (100 mM sodium phosphate, 300 mM NaCl, pH 7.8) and centrifugation (8000 g, 10 min, 4 °C), the cell lysate was incubated with cobalt-loaded resin (ProteNova; overnight, 4 °C). The non-specifically bound proteins were washed out with lysis solution and wash solution supplemented with 5 mM imidazole. The eluate was then collected after the application of the wash solution supplemented with 350 mM imidazole. After dialysis of the eluate against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, 1.8 mM KH₂PO₄, pH 7.2), the protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were quantified using Bradford reagent (Sigma-Aldrich). The experiments were performed in triplicate.

**Bacterial cell walls**

The bacterial cell walls were prepared as described elsewhere [18]. Briefly, the bacterial overnight culture was heated in 4 °C overnight (121 °C, 15 min). The crude bacterial cell walls were pelleted by centrifugation (6000 g, 5 min, room temperature), and the pellets were washed five times with PBS using the same centrifugation conditions. Finally, the crude cell wall pellets were suspended in PBS to an optical density at 600 nm (OD₆₀₀) of 0.3.

**Overexpression and purification of the recombinant RBP**

The plasmid pCold II S13′_ORF16 was prepared by the method described elsewhere [18]. Briefly, phage genomic DNA was prepared, and the coding sequence of the gene was amplified by polymerase chain reaction (PCR) with the primer set S13′_ORF16_F, 5′-CGGTACCCGGATCGG TACCAGCATATAATGAAAACGTATTTAATTTT-3′; S13′_ORF16_R, 5′-CGAATTAGGATCATAGTT TATTCTTTCTAAACCAATCGATT-3′, using phage genomic DNA as a template. The DNA fragment was cloned into the cloning vector pUC19 using an In-Fusion HD Cloning Kit (Takara Bio), and the accurately amplified DNA fragments were then cloned into the expression vector pCold II (Takara Bio). The plasmid pCold II S13′_ORF16 was used in this study, as described elsewhere [18]. The plasmids were transformed into competent *E. coli* NiCo21 (DE3) (New England Biolabs), and the protein was overexpressed in 250 ml LB broth, according to the manufacturer’s instructions. The bacterial culture was centrifuged (8000 g, 10 min, 4 °C), and the bacterial pellet was prepared.

After sonication of the bacterial pellet in 25 ml of lysis solution (100 mM sodium phosphate, 300 mM NaCl, pH 7.8) and centrifugation (8000 g, 10 min, 4 °C), the cell lysate was incubated with cobalt-loaded resin (ProteNova; overnight, 4 °C). The non-specifically bound proteins were washed out with lysis solution and wash solution (50 mM sodium phosphate, 300 mM NaCl, pH 7.8) and wash solution supplemented with 5 mM imidazole. The eluate was then collected after the application of the wash solution supplemented with 350 mM imidazole. After dialysis of the eluate against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, 1.8 mM KH₂PO₄, pH 7.2), the protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were quantified using Bradford reagent (Sigma-Aldrich). The experiments were performed in triplicate.

**Examination of recombinant RBP binding activity by Western blotting**

After pelleting 500 µl of the bacterial cell wall, the cell wall pellet was suspended in 200 µl of the phage proteins diluted to 20 µg ml⁻¹. After incubation (37 °C, 10 min), the cell wall suspension was centrifuged (12,000 g, 5 min), and the supernatant was collected. One volume of 3 × Laemmli sample buffer (3 % SDS, 37.5 % glycerol, 0.015 % bromophenol blue, 15 % β-mercaptoethanol, 100 mM Tris-HCl, pH 6.8) was added to two volumes of the supernatant. The cell walls were washed with PBS twice, and 100 µl of 1 × Laemmli sample buffer (1 % SDS, 12.5 % glycerol, 0.005 % bromophenol blue, 5 % β-mercaptoethanol, 33.3 mM Tris-HCl, pH 6.8) was added. After heating (95 °C, 5 min), both samples
were centrifuged (12 000 g, 5 min). Both supernatant and cell wall samples, together with XL-Ladder Broad (APRO Life Science Institute) protein molecular weight markers, were subjected to SDS-PAGE. After separation using a 12.5 % SDS-PAGE gel, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond; 0.2 µm PVDF; GE Healthcare). The blotted membrane was blocked with 3 % skimmed milk in PBS, with 0.05 % Tween 20 (PBS-T), at 4 °C overnight, and washed with PBS-T (3 × 10 min). The membrane was incubated with anti-6-His-tag mouse antibody conjugated with horseradish peroxidase (Anti-His-tag mAb-HRP-Direct; MBL), diluted 1:5000 with 3 % skimmed milk in PBS-T, for 1 h at room temperature. The membranes were washed (3 × 10 min) in PBS-T. Immunoblot signals were detected using an ECL start Western blotting detection system (GE Healthcare), and visualized using Image Quanti LAS 4000 mini (GE Healthcare). The experiments were performed in triplicate.

Bioinformatic analysis

The protein sequences were analysed using homology detection and structure prediction by HMM–HMM comparison (HHpred; https://toolkit.tuebingen.mpg.de/#tools/hhpred) and using protein homology/analogy recognition engine v2.0 (Phyre2; http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [28, 29].

The protein sequence alignments were made using CLUSTALW and phylogeny inference by the neighbour-joining method, using MEGA (http://www.megasoftware.net/) [47].

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


