Bacteriophage Sf6 host range mutant that infects *Shigella flexneri* serotype 2a<sub>2</sub> strains

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One sentence summary: Isolation of bacteriophage Sf6c mutant that infects Shigella flexneri serotype 2a<sub>2</sub> strain, the most prevalent serotype that causes shigellosis

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Abstract

*Shigella flexneri* serotype 2a<sub>2</sub> (II:9;10) is the most prevalent strain in causing bacillary dysentery in developing countries. Chemical modifications such as glucosylation, O-acetylation, and phosphoethanolamine modifications of lipopolysaccharide (LPS) O antigen (Oag) contribute to the emergence of various serotypes. Sf6 is a *Shigella*-specific bacteriophage that infects only a limited range of *S. flexneri* serotypes [X, Y]. LPS Oag is the primary receptor for bacteriophage Sf6 where it uses its tailspike protein (TSP) in binding and hydrolysing LPS Oaggs. Sf6TSP has recently been shown to be capable of hydrolysing the LPS Oag of Type II strains, albeit modestly. Phage therapy has regained attention in recent years as an alternative therapeutic approach. Therefore, this study aimed to expand the host range of Sf6 to the prevalent *S. flexneri* serotype 2a<sub>2</sub> strain. We discovered a new lytic Sf6 host range mutant that is capable of infecting S. flexneri serotype 2a<sub>2</sub> and identified residues in Sf6TSP that may potentially be involved in binding and hydrolysing serotype 2a<sub>2</sub> LPS Oag. This work increased the limited *Shigella*-specific bacteriophage collection and may be useful in the future for phage therapy and/or biocontrolling of *S. flexneri* in contaminated food and water.

Keywords: bacteriophage, Sf6, *Shigella flexneri*, serotype 2a, host range mutant, tailspike protein

Introduction

*Shigella flexneri* is one of the most dominant strains in causing shigellosis in developing countries (Livio et al. 2014). Serotype 2a (29%), 3a (14%), and 1a (9%) are the three most prevalent serotypes in Asian countries (von Seidlein et al. 2006, Ye et al. 2010). Shigellosis is a global burden that has an annual morbidity rate of ~269 million cases and ~210000 of mortality cases (Khalil et al. 2018). World Health Organisation has listed *Shigella* as a priority organism as it possesses great threat to human health (Taconcelli et al. 2018).

All *S. flexneri* serotypes except serotype 6 share the same O antigen (Oag) polysaccharide backbone that is composed of one N-acetylgalacosaamine (GlcNAC) residue and three L-rhamnose (Rha<sup>1</sup>-Rha<sup>II</sup>-Rha<sup>III</sup>) residues (Kenne et al. 1977). This forms the O-ag group O-factor Y. Various chemical modifications such as glucosylation, O-acetylation and phosphoethanolamine modification on different sugars increase Oag diversity and serotypes. *Shigella flexneri* serotypes are defined by a series of type and group O-factors (Boyd 1938, Carlin et al. 1989). Type O-factors are defined by Roman numerals (I, II, III, IV, V, VI, and VII) while group O-factors are defined by Arabic numerals (3, 4, 6, 7, 8, 9, and 10). Type O-factors are defined within one serotype (e.g. serotype 2a and 2b share the type O-factor II) while group O-factors are shared among different serotypes (e.g. group factor 7,8 is found in serotype 2b, 3a, 5b, and X) (Carlin et al. 1989). The group O-factor 3,4 is defined as a backbone epitope and is shared by serotype 2a, 3b, 4a, 5a, and Y (Carlin and Lindberg 1987, Perereplov et al. 2012). The serotype Y strain has the antigenic formula of (-3,4) because it does not have type O-factor.

*S. flexneri* 2457T (2a<sub>2</sub>) has two known Oag modifications, glucosylation on Rha<sup>1</sup> (confers type O-factor II), and O-acetylations on Rha<sup>II</sup> at position 3 or 4 (confers group O-factor 9) and on GlcNAC at position 6 (confers group O-factor 10), which are mediated by the *gtrIIBA* operon, *oacB*, and *oacD* genes, respectively (Kubler-Kielb et al. 2007, Perereplov et al. 2009, Sun et al. 2014, Wang et al. 2014). Therefore, the antigenic formula of *S. flexneri* 2457T is designated as (II:9;10). Both *gtrIIBA* operon and *oacD* gene are carried by the serotype-converting bacteriophage SFII prophage and are inserted between *proA* and *adrA* (Knirel et al. 2015). The *oacB* gene is carried by a transposon-like mobile element which is flanked by integrase and insertion sequences and is located upstream of *adrA* (Wang et al. 2014). Gene deletion of *oacD* only, *oacB* only or both *oacD* and *oacB* in *S. flexneri* 2457T (2a<sub>2</sub>) gave rise to serotype 2a<sub>1</sub> (II:9), 2a<sub>1</sub> (II:10), and 2a<sub>1</sub> (II:3,4), respectively, where serotype 2a<sub>1</sub> and 2a<sub>2</sub> are resistant to bacteriophage Sf6 (Teh et al. 2020).

Bacteriophage Sf6 is a short-tailed dsDNA virus that belongs to the family of *Podoviridae*. Sf6 is a temperate bacteriophage and is part of the subgroup ‘P22-like’ phages because the morphology of Sf6 is very similar to P22 (Csisjars and Thuman-Commike 2011). Sf6 is known to infect serotype X and Y of *S. flexneri* strains, converting them into serotype 3a and 3b, respectively (Clark et al. 1991). Our recent study showed that Sf6 is also capable of infect-
ing *S. flexneri* serotype 2a (II:3,4) and 2a1 (II:10) strains lacking the oacB gene, converting them into serotype 3b (III:6-3) and 3b1 (III:6-10), respectively (Teh et al. 2020). Sf6 recognise specific LPS Oag via its tailspike protein (SF6TSP). SF6TSP is an endorhamnosidase enzyme that binds reversibly to LPS Oag (primary receptor) (Kang et al. 2016) which facilitates the cleavage of Oag repeat units between the 1,3-α-linkage of two rhamnose residues, releasing an octasaccharide product (Lindberg et al. 1978, Chua et al. 1999). Hydrolysis of Oag repeat units brings Sf6 closer to the bacterial surface. Sf6 then binds irreversibly to OmpA or OmpC (secondary receptor) on the outer membrane (Parent et al. 2014).

SF6TSP exists as a trimer (Muller et al. 2008) and consists of two domains, the highly conserved N-terminal domain (114 residues) that forms the capsid binding domain and the variable C-terminal domain (509 residues) that is responsible in recognising and cleaving the receptor into fragments (Steinbacher et al. 1997, Chua et al. 1999, Casjens et al. 2004). The enzymatic active sites (residues E366 and D399) of SF6TSP are located on two different subunits of a trimer (Muller et al. 2008). Hence, a trimeric SF6TSP contains three independent elongated glycan binding sites for LPS Oag fragments (Kunstmann et al. 2020). SF6TSP has been shown to interact with LPS Oag of serotype 2a weakly (Kunstmann et al. 2018) and we have recently shown that SF6TSP is capable of modestly cleaving LPS Oag repeat units of serotype 2a1, 2a2, 2a3, and 2a strains (Teh et al. 2020).

To date, there are no Shigella vaccines generally available, and the emergence of multi-antibiotics resistance strains have necessitated a need to seek alternative methods of treatment. Phage therapy has regained attention in recent years to treat diseases caused by multi-antibiotic resistance bacteria. This study aims to isolate a Sf6 host range mutant that is capable of infecting the most prevalent *S. flexneri* strain.

A new Sf6 host range mutant that infects *S. flexneri* serotype 2a2, was isolated in this study. Amino acid residues that are potentially involved in binding and hydrolysing LPS Oag of serotype 2a strains were also identified in this study. Our study contributes to understanding of the interaction between bacteriophage SF6 and *S. flexneri* serotype 2a.

## Materials and methods

### Bacterial strains and bacteriophage

The strains used in this study are listed in Table 1. SF6c is a clear plaque derivative of SF6 and was propagated on *S. flexneri* serotype Y P/E577 (Morona et al. 1994).

### Growth media and growth conditions

All strains used in this study were routinely grown in Lysogeny Broth (LB) medium. Bacterial cultures were cultured for 18 h, diluted 1:20 and grown for 4 h with aeration at 37°C.

### Bacteriophage plaque assay and spot assay

The bacteriophage plaque assay was performed by mixing 100 μL of bacteriophage (10^8 of an approximately 1 x 10^11 pfu mL^-1 stock) with equal amount of bacterial culture and incubated for 4 min at RT followed by 1 min incubation at 45°C. Then, 3 mL of soft agar (0.75% [w/v] LB agar, prewarmed at 45°C) was added into the bacterial/phage mixture and immediately overlayed onto a 25 mL LB agar plate. The plate was incubated at 37°C for 18 h.

For the bacteriophage spot assay, 15 μL of bacterial culture was mixed with 0.35 mL of soft agar (0.75% [w/v] LB agar, prewarmed at 45°C) and immediately overlayed onto a 3 mL LB agar in a 12-well tray. Once the soft agar overlay was dried, 5 μL of phage (undiluted phage stock) was spotted onto the overlay and let dry. The plate was incubated at 37°C for 18 h.

### DNA manipulation

The *gp14* gene (encodes TSP) of SF6(2a)c was PCR amplified and subsequently sequenced with primers MY228 Up tsp F (5′-cttgg-ccaaacaccaggaac 3′) and MY229 Down tsp R (5′-gttaagaTcgggtactg-3′) that target upstream and downstream of *gp14*, respectively. DNA sequence was analysed by Australian Genome Research Facility (Adelaide, Australia).

### Expansion of SF6c host range mutant

To isolate a new host range mutant that form clear plaques on *S. flexneri* serotype 2a2, SF6c was first propagated in co-culture of *S. flexneri* serotype 2a1, 2a2, 2a3, and 2a strains (Teh et al. 2018). To isolate a new host range mutant that form clear plaques on *S. flexneri* serotype 2a2, Sf6c was first propagated in co-culture of *S. flexneri* serotype 2a1, 2a2, 2a3, and 2a strains (Teh et al. 2020).

### Preparation of LPS samples and LPS analysis

The equivalent of 1 x 10^8 bacteria were centrifuged (2200 x g, 1 min) and resuspended in 50 μL lysis buffer, heated at 100°C for 10 min, cooled and treated with 2.5 μg mL^-1 Proteinase K

### Table 1. Strain used in this study.

| Strain | Relevant characteristics | Reference |
|--------|-------------------------|-----------|
| 2457T  | Virulence plasmid positive S. flexneri serotype 2a | Lab collection |
| PE577  | S. flexneri serotype Y | Lab collection |
| RMA2159 | Virulence plasmid-cured S. flexneri serotype 2a | Lab collection |
| RMA4266 | S. flexneri serotype 2b (PE565) | Lab collection |
| RMA4267 | S. flexneri serotype 4a (PE566) | Lab collection |
| RMA4269 | S. flexneri serotype 3b (PE645) | Lab collection |
| RMA4270 | S. flexneri serotype 2b (PE824) | Lab collection |
| RMA4271 | S. flexneri serotype 3a (PE843) | Lab collection |
| RMA4272 | S. flexneri serotype 4b (PE572) | Lab collection |
| RMA4274 | S. flexneri serotype 1a (PE839) | Lab collection |
| RMA4275 | S. flexneri serotype 1b (PE840) | Lab collection |
| RMA4278 | S. flexneri serotype 5a (PE856) | Lab collection |
| RMA4335 | S. flexneri serotype X (PE576) | Lab collection |
| MYRM1069 | S. flexneri 2457T ΔoacB serotype 2a1 | Teh et al. 2020 |
| MYRM1071 | S. flexneri 2457T ΔoacD AgrIIBA serotype Y | Teh et al. 2020 |
| MYRM1091 | S. flexneri 2457T ΔoacD serotype 2a1 | Teh et al. 2020 |
| MYRM1124 | S. flexneri 2457T ΔoacD ΔoacB serotype 2a | Teh et al. 2020 |
| MYRM1134 | S. flexneri 2457T ΔgtriIBA ΔoacB serotype Y | Teh et al. 2020 |
| MYRM1136 | S. flexneri 2457T ΔgtriIBA serotype Y2 | Teh et al. 2020 |
| MYRM1138 | S. flexneri 2457T ΔAllDEBAΔH serotype Y | Teh et al. 2020 |
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Preparation of formalin-fixed bacteria

The equivalent of $1 \times 10^9$ bacteria were centrifuged (2200 $\times$ g, 1 min), washed once with PBS and fixed with 100 $\mu$L of 1% (v/v) formalin in PBS at RT for 45 min. The formalin-fixed bacteria were washed once with PBS and resuspended in 100 $\mu$L PBS.

LPS hydrolysis assay by bacteriophage

The formalin-fixed bacteria ($1 \times 10^9$ bacteria mL$^{-1}$) were centrifuged (2200 $\times$ g, 1 min), resuspended in 100 $\mu$L of bacteriophage ($1 \times 10^{11}$ pfu mL$^{-1}$) and incubated at 37$^\circ$C for 45 min. The treated bacteria were washed twice with Milli-Q water and prepared LPS samples as described above.

Result and discussion

Sf6 expanded its host range to serotype 2a$_2$ and 2a$_3$

Sf6c is a derivative of Sf6 that forms clear plaques (lytic) on S. flexneri serotype Y (Morona et al. 1994). We and others have recently shown that Sf6TSP has some weak interaction with LPS Oag of serotype 2a strains (Kunstmann et al. 2018) and Sf6TSP is capable of hydrolysing LPS Oag of all serotype 2a strains (Teh et al. 2020). Therefore, we hypothesised that propagation of Sf6c in a S. flexneri co-culture that contained PE577 (serotype Y; susceptible to Sf6c) and RMA2159 (serotype 2a$_2$; resistant to Sf6c) would allow Sf6c to evolve and improve its interaction with serotype 2a strains. RMA2159 was used as it is a virulence plasmid-cured S. flexneri strain and was safer to handle during phage propagation. The process of isolating Sf6c host range mutant was described in the Methods. Sf6c that couldn’t plaque on RMA2159 (2a$_2$) initially started forming opaque plaques after few rounds of propagation in co-cultures (data not shown) and eventually formed clear plaques on RMA2159 after eight rounds of propagation in co-cultures. We named the newly isolated host range mutant of Sf6c as ‘Sf6(2a)c’.

Sf6(2a)c plaque assay results showed that Sf6(2a)c was capable of infecting PE577 (Y), RMA2159 (2a$_2$), and all isogenic serotype variant strains of 2457T, such as $\Delta$All$\alpha$inBA-Y, $\Delta$acD $\Delta$gtrIIBA-Y, $\Delta$gtrIIBA $\Delta$acD (Y$_3$), $\Delta$acB (2a$_1$), $\Delta$acD $\Delta$acB (2a) and $\Delta$acD (2a$_2$) strains (Fig. 1). Serotype 2a$_2$ and 2a$_3$ strains that had previously been shown to be resistant to Sf6c infection were now susceptible to Sf6(2a)c (Teh et al. 2020). This indicated that Sf6(2a)c had evolved and capable of infecting S. flexneri serotype 2a strains that were resistant to Sf6c.

Sf6(2a)c retained its ability to infect serotype Y and X

We found that Sf6(2a)c was still capable of infecting serotype Y strains. We then investigated if Sf6(2a)c was still able to infect serotype X (like Sf6c) and/or had expanded its host range to other serotypes. A spot assay was performed on various serotypes strains (Y, X, 1a, 1b, 2a$_2$, 2b, 3a, 3b, 5a, 5b, 4a, and 4b) (Fig. 2). Sf6(2a)c only formed clear spots on the serotype Y strain, and hence Sf6(2a)c retained its ability to infect serotype X. Surprisingly, Sf6(2a)c formed an opaque spot on serotype 2b strain (RMA4270), indicating that the growth of serotype 2b strain was affected (Fig. 2). However, Sf6(2a)c could not form plaques on the serotype 2b strain (data not shown), suggesting that Sf6(2a)c has extremely weak interaction with serotype 2b strain and is only able to affect....

Figure 1. Sensitivity of serotype Y and 2a S. flexneri strains to the new host range mutant Sf6(2a)c. Bacteriophage Sf6(2a)c plaque assay. About 100 $\mu$L of overnight cultures of S. flexneri were incubated with 100 $\mu$L Sf6(2a)c ($10^{-8}$) prior to mixed with 3 mL of soft LB agar and overlayed onto 25 mL LB agar plates. The plates were incubated at 37$^\circ$C, 18 h. Serotypes of each strain are indicated in parentheses. Scale bar indicates 10 mm.

Figure 2. Sf6(2a)c spot assay on S. flexneri strains with different serotypes. Fifteen microliters of overnight cultures of S. flexneri were mixed with 350 $\mu$L of soft LB agar, overlayed onto 3 mL LB agar in a 12-well tray and let dry. About 5 $\mu$L of undiluted Sf6(2a)c phage stock was then spotted onto the soft agar. The tray was incubated at 37$^\circ$C for 18 h. Serotypes of each strain are indicated in parentheses.
the growth of serotype 2b at high titre. The serotype 2b LPS Oag has glucosylation on both RhaI and RhaII residues, while serotype 2a2 and 2a3 LPS Oags have both glucosylation and O-acetylation on RhaI and RhaII, respectively (Teh et al. 2020). A recent study reported that O-acetylation or glucosylation on RhaII had a similar effect on Oag helical conformation (Hlozek et al. 2020). Therefore, Sf6(2a)c which has the ability to infect 2a2 and 2a3 strains may then be able to interact with the LPS Oag of serotype 2b, albeit weakly.

**Sf6(2a)c tailspike protein has mutations that correlate to infection of serotype 2a2 and 2a3 strains**

TSP (encoded by gp14) is the protein that mediates binding of Sf6 to LPS Oag of S. flexneri. Therefore, we hypothesised that Sf6(2a)c has mutated residues within the binding groove of TSP in order to increase its binding affinity to LPS Oag of serotype 2a2 and 2a3 strains. Sequencing of the Sf6(2a)c gp14 gene revealed three amino acids change: Q325L, A426G and N508T. Coincidentally, these amino acids mutations are in close proximity to the enzyme’s active site residues (E366 D399), where both Q325L and A426G are located within the glycan binding groove while N508T is located below the groove (Fig. 3). In a trimeric TSP, Q325L is located on the same subunit as E366, while A426G and N508T are located on another subunit like D399 (Fig. 3). A recent MD simulations study reported that two Sf6TSP E366A D399A variants (V204C and S246C) that are in close proximity to the Oag binding site, had a slight increase in binding affinity to serotype Y LPS Oag (Kunstmann et al. 2020). This indicated that Sf6TSP can be engineered to increase its binding affinity to LPS Oag. In Sf6(2a)c, its TSP has mutated as a result of adaptation to improve its interaction with LPS Oag of serotype 2a2 and 2a3. The adaptive mutational alterations in the tsp gene arose spontaneously, and the sequences are not present in the genomes of the host strains.

**Sf6(2a)cTSP has improved serotype 2a LPS Oag hydrolysis**

We have previously shown that while Sf6TSP can hydrolyse serotype 2a2 LPS Oag, it does so modestly, in a dose-dependent manner (Teh et al. 2020). We then investigated if the Sf6(2a)cTSP was able to hydrolyse serotype Y (PE577) and 2a2 (RMA2159) LPS Oags. We initially attempted to overexpress and purify Sf6(2a)cTSP for use in LPS hydrolysis assays but were unsuccessful due to low yield and stability. Therefore, LPS hydrolysis assays were performed on formalin-fixed and washed S. flexneri strains. LPS samples were then subjected to SDS-PAGE and LPS silver staining as described in the Methods. The untreated and bacteriophage-treated LPS Oag repeat units 11–17 were measured by densitometry using ImageLab 6.1 and the results were presented as percentage of LPS hydrolysis/reduction.

As expected, Sf6c-mediated LPS hydrolysis of PE577 (Y) and RMA2159 (2a2) were > 98% and > 27%, respectively (Fig. 4, Lane 2 and 4, respectively). This is consistent with our previously reported data by using Sf6TSP protein (Teh et al. 2020). This is also similar for Sf6(2a)c-mediated LPS hydrolysis where serotype Y LPS Oag had > 92% hydrolysis (Fig. 4, Lane 6). Excitingly, there was a significant increase in Sf6(2a)c-mediated LPS hydrolysis of RMA2159 (> 68%), in comparison to Sf6c-mediated LPS hydrolysis (> 27%) of the same strain (Fig. 4, Lane 8 and 4, respectively). This suggested that the amino acids mutations within the Sf6(2a)cTSP protein enhanced LPS hydrolysis of serotype 2a LPS Oags and subsequently allowing infection of 2a strains by Sf6(2a)c.
phage. Notably, Sf6(2a)c-mediated LPS hydrolysis on RMA2159 was incomplete and this is consistent with our previous finding that Sf6TSP was not able to achieve complete hydrolysis on LPS Oag of \textit{S. flexneri} 2457T-derived strains (Teh et al. 2020). The data also suggested that partial hydrolysis of LPS Oags is sufficient for Sf6(2a)c to infect a serotype 2a strain.

**Conclusion**

A new host range mutant of Sf6c that infects the most prevalent serotype (2a2) was successfully isolated in this study. We showed that Sf6(2a)cTSP had improved LPS hydrolysis ability on serotype 2a strain and infection. The isolation of Sf6(2a)c contributes to our understanding of the interaction between Sf6TSP and its Oag receptor. Sf6(2a)c may be useful for phage therapy or biocontrolling of \textit{S. flexneri} in contaminated food or water.

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