

Key Residues of *S. flexneri* OmpA Mediate Infection by Bacteriophage Sf6

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Abstract

Many viruses, including bacteriophage, have the inherent ability to utilize several types of proteinaceous receptors as an attachment mechanism to infect cells, yet the molecular mechanisms that drive receptor binding have not been elucidated. Using bacteriophage Sf6 and its host, *Shigella flexneri*, we investigated how Sf6 utilizes outer membrane protein A (OmpA) for infection. Specifically, we identified that surface loops of OmpA mediate *Shigella* infection. We further characterized which residues in the surface loops are responsible for Sf6 binding and productive infection using a combination of *in vivo* and *in vitro* approaches including site-directed mutagenesis, phage plaque assays, circular dichroism spectroscopy, and *in vitro* genome ejection assays. Our data indicate that Sf6 can productively interact with other bacterial OmpAs as long as they share homology in loops 2 and 4, suggesting that these loops may determine host specificity. Our data provide a model in which Sf6 interacts with OmpA using the surface of the protein and new insights into viral attachment through binding to membrane protein receptors.

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Introduction

Viruses infect every domain of life. For a successful infection, all known viruses must transfer their genomic information into their hosts [1] and can employ different strategies to accomplish this. One common strategy is to utilize a portion of the respective host cell as a receptor, at a site suitable for entry. Understanding the binding events that occur between the host cell and these viruses is critical in order to develop methods to circumvent infection.

Although viruses can have extremely diverse life cycles, there are several commonalities. For example, many archaeal, eukaryotic, and bacterial viruses require proteinaceous receptors on the host surface used for attachment [2–12]. In addition, many of these viruses demonstrate plasticity in their binding mechanisms [3,13]. Throughout the evolutionary arms race, hosts can develop resistance to viral infection and viruses face extinction if they can no longer gain entry into their continually evolving

hosts. The innate ability to utilize more than one type of receptor and the ability to evolve easily to utilize novel receptors can allow viruses to circumvent host resistance and may be essential for the continued pathogenicity of a given virus.

Work with eukaryotic viruses has shed some light on multiple receptor usage. Studies with herpes simplex virus (HSV) have revealed that different HSV serotypes encode distinct glycoproteins that are required for attachment [14]. HSV, additionally, has the ability to utilize different cell proteins as receptors, thus allowing it to infect a broader range of host cells [10,13]. The human immunodeficiency virus has also evolved to use its single envelope glycoprotein to gain entry into different cell types [13]. Even in the absence of its primary receptor, CD4, some human immunodeficiency virus isolates are still able to infect cells [15], and studies have shown that Fusin/CXCR4 can serve as an alternative cell receptor [16]. Moreover, in the case of Adenovirus, not only is the native virus able to utilize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [3] in the absence of its preferred receptor,

CAR [4], but also the fiber head domains of the virus have cell-type selective properties [17].

In addition to the inherent capability for binding multiple receptors, viruses can also gain access to different receptor types as clearly demonstrated with studies of bacteriophage. Various *Escherichia coli* outer membrane proteins (Omps) function as receptors for many bacteriophages. For example, bacteriophage T2 has the ability to use two different Omps as receptors: OmpF and FadL [18,19]. Moreover, bacteriophage Ox2 [20] can evolve to utilize OmpA, OmpC, and OmpF as receptors [21]. Studies with Ox2 have shown that the phage tail fibers/adhesins are a major determinant for the Omp specificity. Under selective pressure in the laboratory, phage λ evolved to infect its host *E. coli* through a novel pathway; rather than using its preferred receptor, LamB, λ acquired several mutations in its recognition protein J that allowed infection through a novel receptor, OmpF [22]. Combined, these data illustrate that viruses can evolve receptor plasticity as a strategy to circumvent host resistance and it implies that receptor plasticity is an inherent trait of viral evolution.

Viruses, although extremely diverse in their morphogenetic pathways, generally use only a handful of common protein folds to form infectious virions. For example, for dsDNA (double-stranded DNA) phage and HSV, the major capsid [23], scaffolding [24], and portal [25,26] proteins are conserved. This high level of structural homology makes it possible to utilize model systems to study general strategies for viral infection. The model system chosen for this study is bacteriophage Sf6 and its host, *Shigella flexneri*. Sf6 is a short-tailed dsDNA virus that belongs to the subgroup of the “P22-like” phages in *Podoviridae* [27], which is one of the less well understood families in regard to phage–host interaction [28]. Sf6 infection requires binding to both primary and secondary receptors [29]. Lipopolysaccharide (LPS) serves as primary receptor in an initial and reversible interaction [30], followed by an irreversible interaction, with a secondary receptor, which is an Omp [29]. Our previous work demonstrated that OmpA is the preferred secondary receptor for Sf6, yet OmpC can serve as an alternate [29].

Many phages such as Sf6 are able to use more than one type of proteinaceous receptor for attachment, and they generally appear to have a preferred receptor. Since many porins (OmpA, OmpF, OmpC, FhuA, and LamB) have been identified as bacteriophage receptors [5–8,20–22,29], and these have homologous structures [31], we can predict that analogous regions within these Omps might be globally important for phage infection. However, there are few published studies that delineate molecular mechanisms governing phage attachment to these receptors and none to date involving a member of the P22-like phages. In the present study,

we identified via site-directed mutagenesis coupled with *in vivo* phage biology and biochemical assays specific residues of OmpA that are critical to mediate Sf6 infection and confer host range. Our data provide new insights into *Podoviridae* attachment through binding to protein receptors.

Results

S. flexneri OmpA extracellular loops are important for Sf6 infection

E. coli OmpA is a receptor for several bacteriophages. Analysis of *E. coli* strains isolated after developing resistance to over 15 different strains of coliphages shows that mutations conferring resistance are localized to the four OmpA surface loops [32,33]. Our previous work showed that *S. flexneri* OmpA acts as the preferred secondary receptor for Sf6 [29]. Since *E. coli* and *S. flexneri* OmpA are highly similar (sequence identity of 99.6% [34]), we hypothesized that the surface loops of *S. flexneri* OmpA may also play a role in mediating Sf6 infection. *In vitro* experiments can monitor loss of infectivity from mature Sf6 virions (and thus implies genome ejection) using purified *S. flexneri* LPS and the OmpA transmembrane domain, “OmpA-TM_{*S.flex*}” (see Materials and Methods) [29]. We adopted this approach to determine if the surface loops of OmpA were crucial for triggering Sf6 genome ejection. Since it has been demonstrated that LPS alone is unable to trigger Sf6 genome release [29], any observed changes would result from altered OmpA

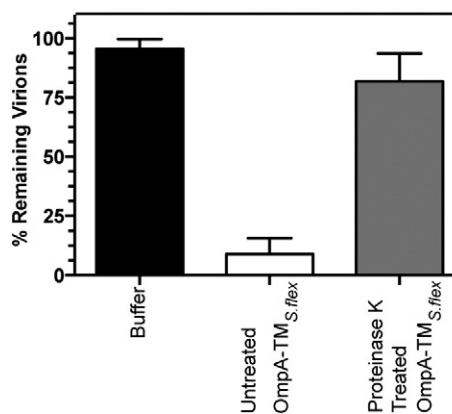


Fig. 1. *In vitro* genome ejection efficiency decreases with proteinase K treated OmpA-TM_{*S.flex*}. The “% remaining virions” was calculated as the number of PFUs remaining after incubation with *S. flexneri* LPS and OmpA-TM_{*S.flex*} (untreated and proteinase K treated) divided by the number of PFUs when incubated with only buffer. Each data point is an average of at least three separate experiments; error bars signify one standard deviation.

ability to serve as a receptor. We used a limited proteolysis approach as proteinase K, subtilisin, and trypsin have all had their cleavage sites thoroughly mapped to OmpA surface loops [35]. Here, OmpA-TM_{S.flex} was incubated with proteinase K, which has cleavage sites in all four loops [35]. Cleavage was confirmed by SDS-PAGE (data not shown). Digested OmpA-TM_{S.flex} was then used in our *in vitro* experiments in combination with LPS and phage. Unlike undigested OmpA-TM_{S.flex}, OmpA-TM_{S.flex} treated with proteinase K is unable to trigger genome ejection of Sf6 (Fig. 1). Thus, the loops of OmpA appear to be essential for Sf6 infection. To further probe which portions of these four surface loops are important, we developed a plasmid complementation system to screen full-length OmpA constructs *in vivo*.

Previously, we have shown that the relative titer of Sf6 propagated on *ompA*^{-C-} null *S. flexneri* drops

~10-fold compared with Sf6 grown on the parent *S. flexneri* strain and that, of these two gene deletions, *ompA*⁻ demonstrated the largest effect on Sf6 infection [29]. Therefore, expression of OmpA *in trans* (referred to as “OmpA_{S.flex}”) in the *ompA*^{-C-} background should restore the ability of Sf6 to efficiently infect these cells. Full-length *S. flexneri* OmpA was expressed from plasmid “pOA_{S.flex}” in the null *ompA*^{-C-} background (see Materials and Methods and Fig. 2, schematic). This construct has been shown to restore protein levels and incorporation of OmpA_{S.flex} into the outer membrane to that of the parent *S. flexneri* strain [36]. We compared infection of Sf6 at temperatures ranging from 25 to 42 °C on three strains of *S. flexneri*: parent strain, *ompA*^{-C-}, and *ompA*^{-C-} + pOA_{S.flex}. Expression of OmpA_{S.flex} *in trans* is able to restore the efficiency of infection of Sf6 in the *ompA*^{-C-} background to that of the parent strain, as seen by a relative titer ~1

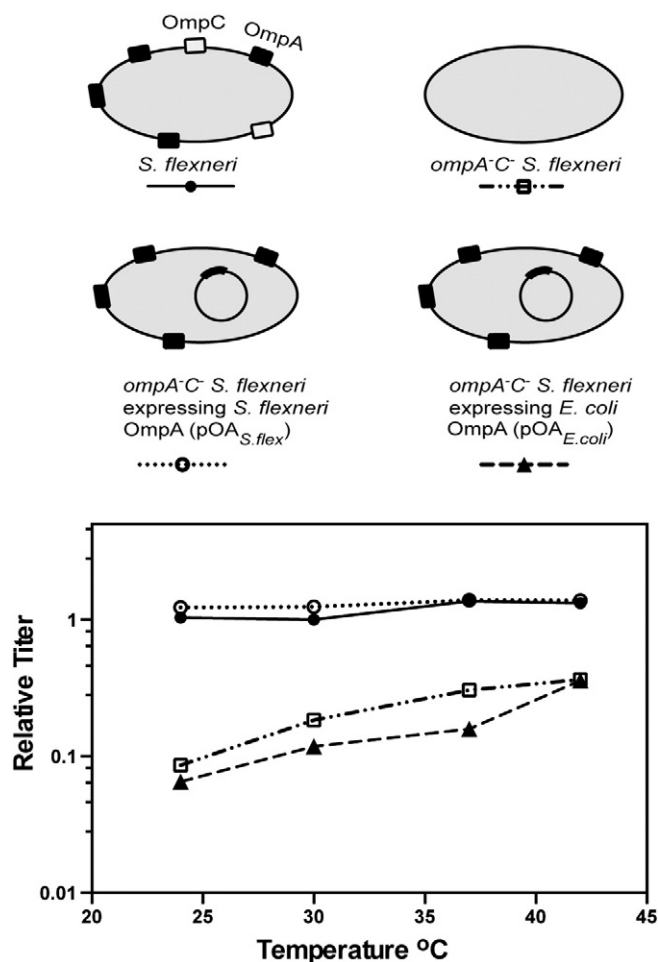


Fig. 2. Relative titer of Sf6 is restored on *ompA*^{-C-} *S. flexneri* expressing *S. flexneri*, but not *E. coli* OmpA. A schematic of the complementation system is shown. Relative titer of Sf6 was calculated by dividing the PFUs on each *S. flexneri* strain (parent, *ompA*^{-C-}, *ompA*^{-C-} + pOA_{S.flex}, and *ompA*^{-C-} + pOA_{E.coli}) at each temperature by the number of PFUs on the parent *Shigella* strain PE577 at a permissive temperature (30 °C). Each data point is an average of at least five separate experiments.

at all temperatures (Fig. 2). Therefore, OmpA_{S.flex} is both necessary and sufficient to restore infection efficiency of Sf6 in *ompA*⁻*C*⁻ *S. flexneri*.

E. coli and *S. flexneri* OmpA have high level of sequence identity, with only seven residue differences and a four-amino-acid insertion in the surface loops, in an area that is accessible to phage (Table 1) [34]. We therefore investigated whether *E. coli* OmpA expression *in trans* (“OmpA_{E.coli}”) was also able to restore the ability of Sf6 to infect *ompA*⁻*C*⁻ *S. flexneri*. Unlike OmpA_{S.flex}, OmpA_{E.coli} was unable to restore Sf6 infection levels in the null *ompA*⁻*C*⁻ *S. flexneri* background (Fig. 2). Therefore, we investigated if the differences between these two proteins play a role in mediating the inability of Sf6 to utilize the *E. coli* protein.

Amino acid substitutions in the loops of OmpA decrease Sf6 infection efficiency

To address which of the residues that differ between *E. coli* and *S. flexneri* OmpA were responsible for the observed phenotype, we systematically changed both the size and the charge of each by site-directed mutagenesis (see Materials and Methods). We measured the relative titer by plating Sf6 on *ompA*⁻*C*⁻ *S. flexneri* complemented with these 22 different versions of OmpA (Table 1). The Sf6 plating efficiency changes with some amino acid substitutions, but not others (Fig. 3). Complementation by three variants, D66A and D66K (loop 2) and N155R (loop 4), restore Sf6 plating efficiency of the *ompA*⁻*C*⁻ strain and the wild-type (WT) gene: *ompA*_{S.flex} (Fig. 3). Variants at two locations in loop 2 (N67 and I68) and one variant in loop 4 (N155E) had the lowest Sf6 plating efficiency, indicating that these mutations confer a loss of function. All OmpA_{S.flex} variants demonstrate the same relative phenotypes when plated at temperatures ranging from 25 to

42 °C (data shown only for 25 °C for simplicity; Fig. 3).

The observed loss of function of OmpA variants to serve as a receptor for Sf6 could have several mechanistic explanations. Amino acid alterations in the loops of OmpA may interfere with (1) the ability of the phage to bind OmpA, (2) folding of OmpA and therefore function, or (3) incorporation of OmpA into the outer membrane. First, to test whether the variant OmpAs incorporated correctly *in vivo*, we plated the parent strain *S. flexneri* and *ompA*⁻*C*⁻ and *ompA*⁻*C*⁻ strains expressing the 22 variant OmpAs on MacConkey agar, a bile-salt-rich medium that selects for Gram-negative bacteria with intact outer membrane integrity [37]. With the exception of only P25E OmpA (designated as “+” in Fig. 3 and “b” in Table 1), all strains were able to grow on the MacConkey agar as efficiently as the *S. flexneri* parent strain (data not shown). As P25E OmpA was not incorporated correctly into the outer membrane, it was excluded from further analysis.

Amino acid substitutions in OmpA-TM loops do not affect protein stability or folding

In order to more quantitatively determine the effect of these amino acid substitutions, we purified seven versions of OmpA-TM for biochemical characterization (below and the next section). In addition to *Shigella* and *E. coli* OmpA-TMs, we purified one representative variant per each surface loop that had some loss of function for Sf6 infection (P25R, N67E, P111E, and N155E) and one variant that showed no change in Sf6 infection (D66A).

First, to compare the relative stability, we used a heat titration assay to calculate the TM₅₀, which is defined as the temperature where 50% of the protein species is folded. We incubated these purified proteins at temperatures ranging from 25 to 95 °C

Table 1. Amino acid substitutions in the surface loops of OmpA.

Loop number	Residue ^a number	<i>E. coli</i> amino acid	<i>S. flexneri</i> amino acid	OmpA variants		
				No change in resistance	Moderate effect	Severe effect
1	25	N	P	—	A, R	E ^b
2	66	S	D	A, K	—	—
	67	V	N	—	—	A, E, H, R
3	68	E	I	—	—	A, D, K, Q
	108	S	A	—	E	R
	111	Y	P	—	A, E, R	—
4	“insertion” (113, 114, 115, 116)	—	GASF	—	ΔGASF	—
	155	H	N	R	A	E

The amino acids differing between *E. coli* and *S. flexneri* OmpA flexible loops are organized by loop and residue number. The OmpA variants shown in Fig. 3 are summarized here by their loss of function to serve as a receptor to Sf6 based on their relative titer.

^a Residue numbering based on *S. flexneri* OmpA.

^b Variant did not grow on MacConkey agar.

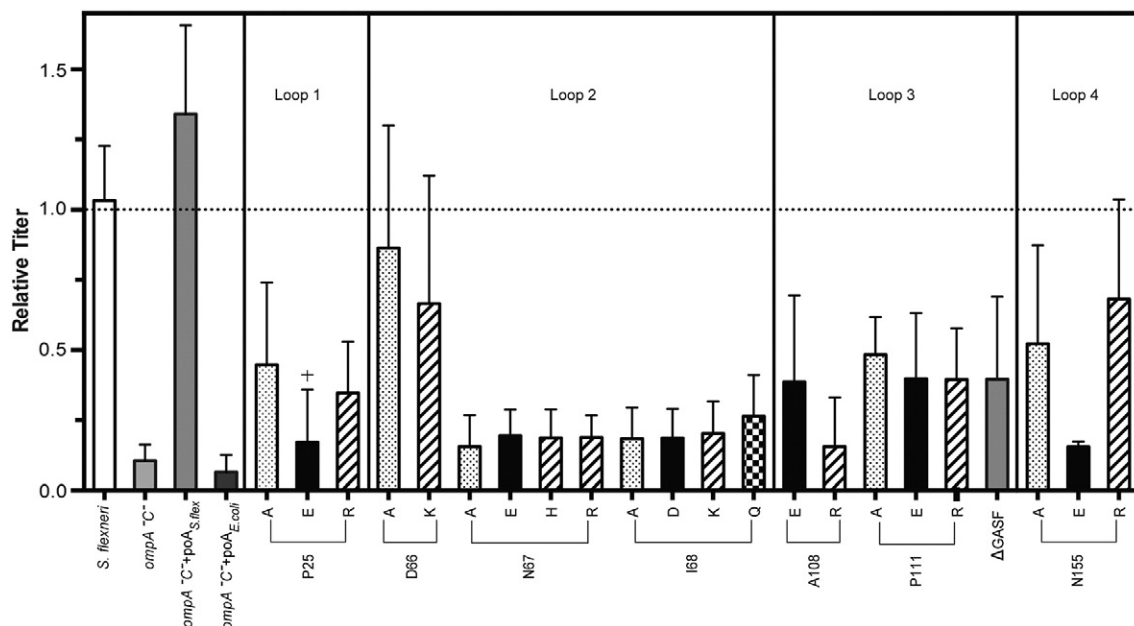


Fig. 3. Comparison of Sf6 infection efficiency on *ompA*^{-/-} *S. flexneri* expressing variant OmpAs. Relative titer of Sf6 on various strains at 25 °C (parent, *ompA*^{-/-}, *ompA*^{-/-} + pOA_{*E.coli*}, *ompA*^{-/-} + pOA_{*S.flex*}, and *ompA*^{-/-} + pOA_{*S.flex*} expressing variant OmpAs). Amino acids with similar properties are shown with the same color-coding scheme. Each data point is an average of at least five separate experiments; error bars signify one standard deviation.

and determined the fraction of folded species by SDS-PAGE and gel densitometry since folded OmpA-TM migrates faster than unfolded OmpA-TM (Fig. 4a). The TM₅₀ for OmpA-TM_{*S.flex*} was deter-

mined to be 75.5 °C. In addition, OmpA-TM_{*S.flex*} that had been boiled and then allowed to refold triggered Sf6 genome ejection efficiently (data not shown), indicating that the refolding of this protein is a

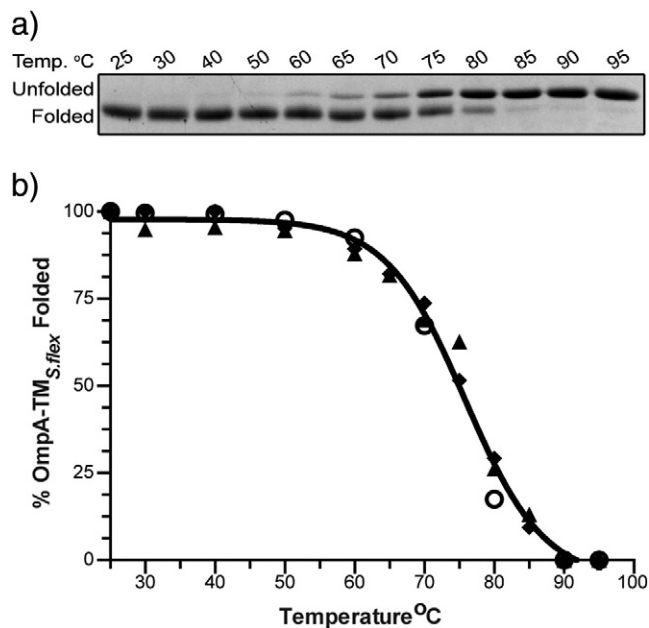


Fig. 4. WT *Shigella* OmpA-TM thermal stability. (a) A representative 15% SDS gel stained with Coomassie of OmpA-TM_{*S.flex*} after incubation at increasing temperatures. (b) Percent OmpA-TM_{*S.flex*} folded species as a function of temperature. Open circles, triangles, and diamonds each represent individual data sets.

Table 2. TM₅₀ values of purified OmpA-TMs.

Protein Variant	TM ₅₀ (°C)
WT	75.5 ± 1.9
P25R	76.2 ± 2.4
D66A	75.7 ± 1.7
N67E	74.4 ± 2.2
P111E	75.6 ± 1.9
N155E	73.0 ± 1.8
<i>E. coli</i>	75.8 ± 3.1

Table shows the calculated TM₅₀ for OmpA-TM (*S. flexneri*, *E. coli*, and *S. flexneri* variant OmpA-TMs) after heat titration and analysis of percent folded protein at increasing temperatures. See Fig. 4 for representative WT data.

reversible process. *E. coli* and the five selected variants of OmpA-TM_{*S. flexneri*} had TM₅₀ values ranging between 73 and 76 °C (Table 2), indicating that their relative stabilities are not significantly different from OmpA-TM_{*S. flexneri*}.

Second, to determine the effect, if any, of the amino acid substitutions on the secondary structure, we determined the variant OmpA-TM circular dichroism (CD) spectra and compared them to the OmpA-TM_{*S. flexneri*} spectrum. Consistent with previously published data for *E. coli* OmpA [38–41], the CD spectrum of OmpA-TM_{*S. flexneri*} predicts a β-barrel secondary structure (Fig. 5). The CD spectra of the variant and *E. coli* OmpA-TM proteins have no significant differences and are essentially identical with that of OmpA-TM_{*S. flexneri*} (Fig. 5). Therefore, it is likely that amino acid substitutions in the extracellular loops of OmpA-TM do not affect the overall protein structure.

Some amino acid substitutions in OmpA-TM surface-exposed loops reduce Sf6 genome ejection efficiency *in vitro*

To test if OmpA variants that demonstrated a loss of function to serve as a receptor for Sf6 *in vivo* (Fig. 3) also have decreased efficiency to trigger genome ejection *in vitro*, we incubated Sf6 with purified *S. flexneri* LPS combined with our purified OmpA-TM proteins. Previously, we showed that the physiological rate for Sf6 genome ejection is less than 10 min [29]. To determine whether Sf6 genome ejection efficiency is affected by these various OmpA-TMs, we calculated the percent remaining plaque-forming units (PFUs) after incubation for 10 min at 37 °C. Incubation with OmpA-TM_{*S. flexneri*} resulted in near-complete ejection, as previously reported [29], with only ~15% remaining virions (Fig. 6a). As expected, and based on our *in vivo* data (see Fig. 3), OmpA-TM_{*E. coli*} *in vitro* was unable to efficiently induce genome ejection of Sf6 comparable to OmpA-TM_{*S. flexneri*}, with ~75% remaining

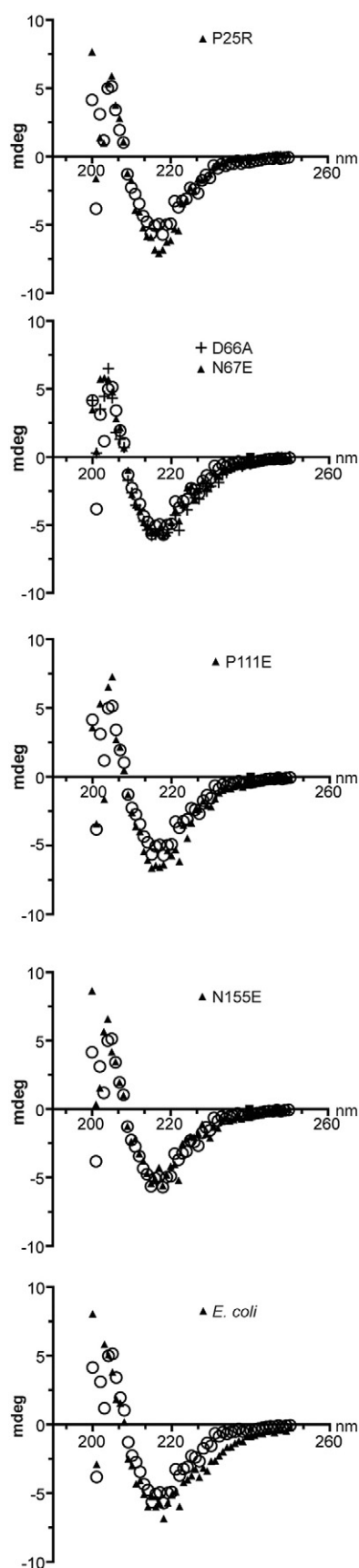
virions after 10 min (Fig. 6a). Furthermore, with those OmpA-TM variants that corresponded to lower Sf6 infection *in vivo* (P25R, N67E, P111E, and N155E), we also saw a reduction in the level of *in vitro* genome ejection, with an average of ~45% remaining virions. Additionally, although not as efficient as OmpA-TM_{*S. flexneri*}, the D66A variant does induce more genome ejection than the other versions of OmpA-TM (Fig. 6a), consistent with our *in vivo* data. It is important to note that, in these experiments, variations in OmpA sequence do not completely obliterate the plating efficiency of Sf6. As our previous work [29] suggests, there is likely a third receptor present at low copy number that Sf6 can use, albeit poorly, to gain entry (see Discussion for more in-depth discussion on this point).

Taken together, reduced efficiency for ejection *in vivo* (Fig. 3) and *in vitro* (Fig. 6) likely correlates with a decrease in binding affinity of the phage to its secondary receptor. Infection *in vivo* on the *ompA*[−] *C*[−] null strain could be less efficient due to either (1) the phage utilizing a third, as of yet unidentified receptor, which may have much lower abundance on the cell surface or (2) the binding efficiency to a third receptor is significantly decreased based on molecular differences. Our *in vivo* complementation system expresses OmpA using its native promoter, and this construct has been shown to produce physiologically relevant concentrations of OmpA [36] and our *in vitro* experiments use identical concentrations of each variant protein relative to OmpA-TM_{*S. flexneri*}. Variant OmpA proteins are complemented from the same vector in our *in vivo* assays and are likely similar in abundance to OmpA_{*S. flexneri*} and therefore readily available for phage binding. However, there is still a decreased relative titer of Sf6 on these strains (Fig. 3). Our data suggest that Sf6 does not interact efficiently with these proteins. Therefore, we might expect to see an increase in genome ejection efficiency in our *in vitro* system if we incubate the phage with these variant receptors for an extended period of time, allowing a greater probability of productive interaction.

We therefore increased the incubation time to 60 min and measured the percent remaining PFUs under the conditions specified above. After 60 min of incubation with OmpA-TM_{*E. coli*}, Sf6 is still unable to release its genome at the same level induced by OmpA-TM_{*S. flexneri*} (Fig. 6b). However, we did see an increase in genome ejection with the other OmpA-TM variants starting to approach OmpA-TM_{*S. flexneri*} levels. Lastly, after 60 min of incubation, the D66A variant induces genome ejection with efficiency similar to OmpA-TM_{*S. flexneri*} (Fig. 6b).

OmpA loops 2 and 4 are the most critical for mediating Sf6 host specificity

Combined, our data suggest that OmpA loops 2 and 4 are the most critical for Sf6 being able to



productively interact with *Shigella* but not *E. coli* OmpA. Therefore, we made a hybrid construct of full-length OmpA_{S.flex} that has the *E. coli* sequence in both loops 2 and 4. In our *in vivo* plating efficiency experiments, this hybrid is non-functional (Fig. 7a).

Sf6 can tolerate several independent differences in loops 1 and 3 (Fig. 3). Therefore, we would anticipate that Sf6 could productively interact with other bacterial OmpAs as long as they share homology in loops 2 and 4 to *S. flexneri* OmpA. One candidate of interest is *Salmonella typhimurium* OmpA (“OmpA_{S.typh}”). Differences between *S. flexneri* and *S. typhimurium* OmpA include amino acid substitutions P25H and N27D in loop 1 and A108S in loop 3, and the insertion “GASF” in loop 3 is GPST in *S. typhimurium* OmpA. However, these two proteins have identical sequences in loops 2 and 4. We therefore measured the ability of OmpA_{S.typh} to complement Sf6 infection in our *in vivo* complementation system. As expected, expression of OmpA_{S.typh} in the null *ompA*⁻*C*⁻ *S. flexneri* background shows a gain of function and is able to restore the efficiency of infection of Sf6 close to that of OmpA_{S.flex} (Fig. 7a). This strain is able to grow as efficiently on MacConkey agar as the parent *S. flexneri* strain, indicating that OmpA_{S.typh} is localized and incorporated correctly (data not shown). We also expressed and purified OmpA-TM_{S.typh} and found that it is stable (TM₅₀ = 76.5 ± 3.6 °C) and has a CD spectrum indistinguishable from OmpA-TM_{S.flex} (data not shown). We assessed the ability of OmpA-TM_{S.typh} to trigger Sf6 genome ejection *in vitro* by calculating the percent remaining virions after incubation for 10 and 60 min. As expected, and consistent with our *in vivo* data (Fig. 7a), OmpA-TM_{S.typh} is able to induce genome ejection of Sf6 at levels close to OmpA-TM_{S.flex} *in vitro* (Fig. 7b). These data further support the idea that loops 2 and 4 of *S. flexneri* OmpA mediate Sf6 interaction and host specificity.

Discussion

Many viruses have the inherent ability to use more than one type of proteinaceous receptor for attachment, with one receptor type being preferred. In this work, we identified which portions of *S. flexneri* OmpA, the preferred secondary receptor for Sf6 [29], mediate phage infection and confer host range. We created several OmpA variants through site-directed mutagenesis and investigated their ability to alter Sf6 infection of *S. flexneri*. Here, we have shown that Sf6 interacts with the surface loops of OmpA. Moreover, individual substitutions have a range of effects,

Fig. 5. CD spectra of selected OmpA-TMs. Representative CD spectra of OmpA-TM_{E.coli} and OmpA-TM variants are shown. Open circles indicate the CD spectrum of OmpA-TM_{S.flex} and are the same data shown in each panel.

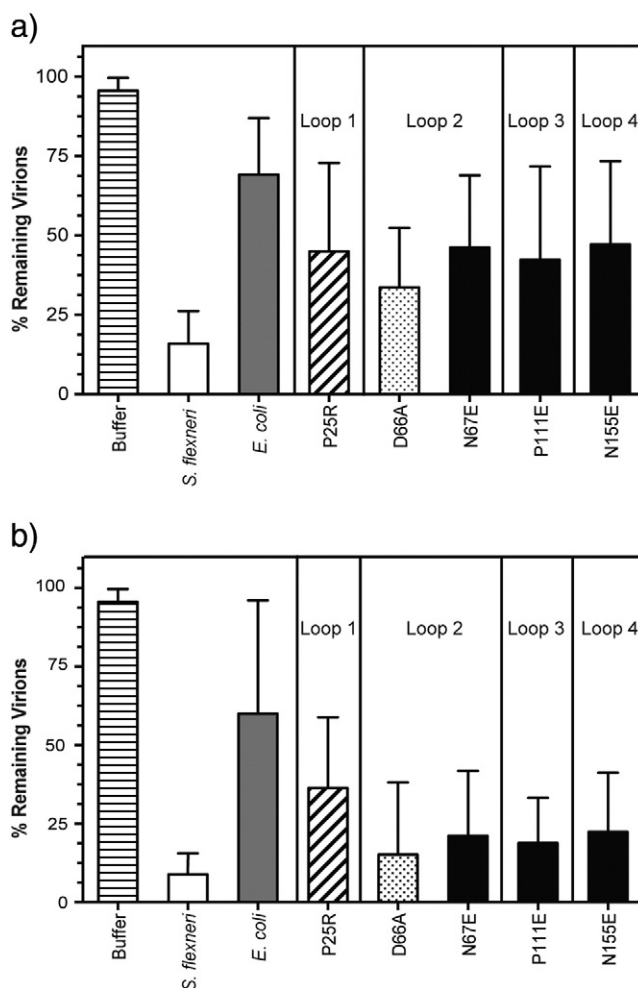


Fig. 6. Sf6 *in vitro* genome ejection efficiency with LPS and variant OmpA-TMs. Ejection efficiency of Sf6 incubated with *S. flexneri* LPS and OmpA-TM (*S. flexneri*, *E. coli*, and variant OmpA-TMs) at 10 min (a) and 60 min (b) post-mixing. Color-coding scheme is consistent with Fig. 3. Each data point is an average of at least five separate experiments; error bars signify one standard deviation.

implicating some locations in the loops as more important than others for infection. However, in no case were we able to completely block Sf6 infection. These data support general phage plasticity for receptor usage. If Sf6 has indeed adapted to use OmpA, OmpC, and a third, as of yet unidentified receptor, as our previous work suggests [29], it is unlikely that a single amino acid substitution in OmpA would completely obliterate infection *in vivo*.

Sf6 may interact with the surface loops of OmpA in one of two ways. (1) Sf6 may interact preferentially with one specific portion of the protein or (2) the phage may interact with the protein surface as a whole. Studies with T5 and FhuA have proposed that phage T5 interacts preferentially with only a portion of the Omp surface [42]. Work with many different coliphages has shown that these phage do not tolerate amino acid mutations in loop 2 or 3 of *E. coli* OmpA, since >84% of 305 independently isolated

mutations in OmpA from phage-resistant cells are found in these loops [32,33]. Loops 2 and 3 are adjacent (Fig. 8), suggesting that these coliphages may not interact with the entire OmpA surface but rather a preferential side of the protein. Additionally, isolated mutations in the receptor for phage λ , LamB, that confer resistance to phage infection [43,44] appear have a strong bias to a preferential side of LamB: when we modeled these amino acid substitutions into the LamB crystal structure (PDB ID: 1AF6 [45]), the substitutions were localized to neighboring loops.

Amino acids that confer resistance to Sf6 infection are located at flexible portions of the loops of OmpA (Fig. 8). If, like the coliphages, Sf6 were to also interact with a preferential portion of OmpA, such as a particular side, we would expect amino acid substitutions that allow resistance to phage infection to have a bias to a single loop or two neighboring

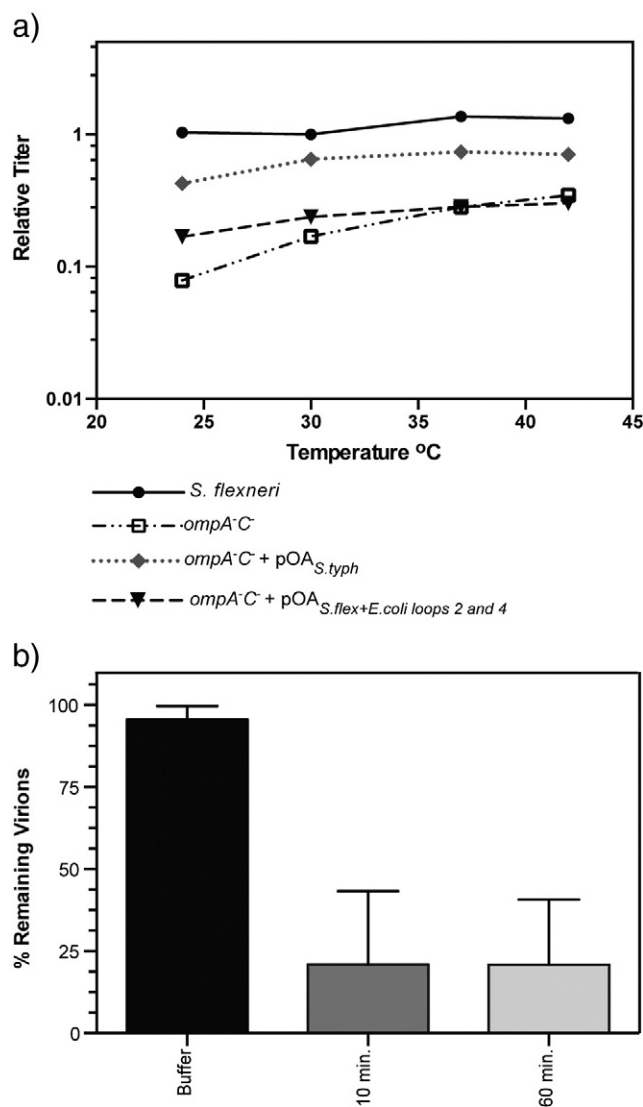


Fig. 7. Sf6 can productively interact with *S. typhimurium* OmpA. (a) The relative titer is the number of PFUs of Sf6 on each *S. flexneri* strain (parent, *ompA*⁻*C*⁻, *ompA*⁻*C*⁻ + pOA_{*S.typh*}, *ompA*⁻*C*⁻ + pOA_{*S.flex+E.coli*} loops 2 and 4) at each temperature divided by the number of PFUs on the parent strain at the permissive temperature (30 °C). Data shown for Sf6 on *ompA*⁻*C*⁻ and the parent strain is the same as in Fig. 2 each data point is the average of at least three independent experiments. (b) Ejection efficiency of Sf6 incubated with parent *S. flexneri* LPS and OmpA-TM_{*S.typh*} at 10 min and 60 min post-mixing. Each data point is an average of at least five separate experiments; error bars signify one standard deviation.

loops. Our data suggest that, overall, amino acid substitutions are less deleterious in loops 1 and 3 of *S. flexneri* OmpA compared to loops 2 and 4, as seen by both differences in the relative plating efficiency (Fig. 3) and the *in vitro* genome ejection data (Fig. 6). However, some substitutions in loops 1 and 3 do have a slight decrease in infection efficiency (P25R: loop 1 and A108R: loop 3, as examples). Therefore, we hypothesize that, unlike the coliphages, Sf6 can interact with the entire surface of OmpA, rather than with a preferential side of the receptor. This may be a fundamental difference in binding profiles as

seen by phage with long flexible *Siphoviridae* tails and phage with the short, stubby tails of *Podoviridae*. More experimental evidence is needed to determine if this is a global mode of binding for members of *Podoviridae*.

Theoretically, to evolutionarily avoid phage infection, mutations within bacterial cells would be selected for that decrease infection. Therefore, one might expect to see mutations in the loops of Omps that lead to decreased binding affinity and therefore a corresponding decrease in phage infection. However, this phenomenon is not necessarily always observed in nature. Although it may be beneficial to

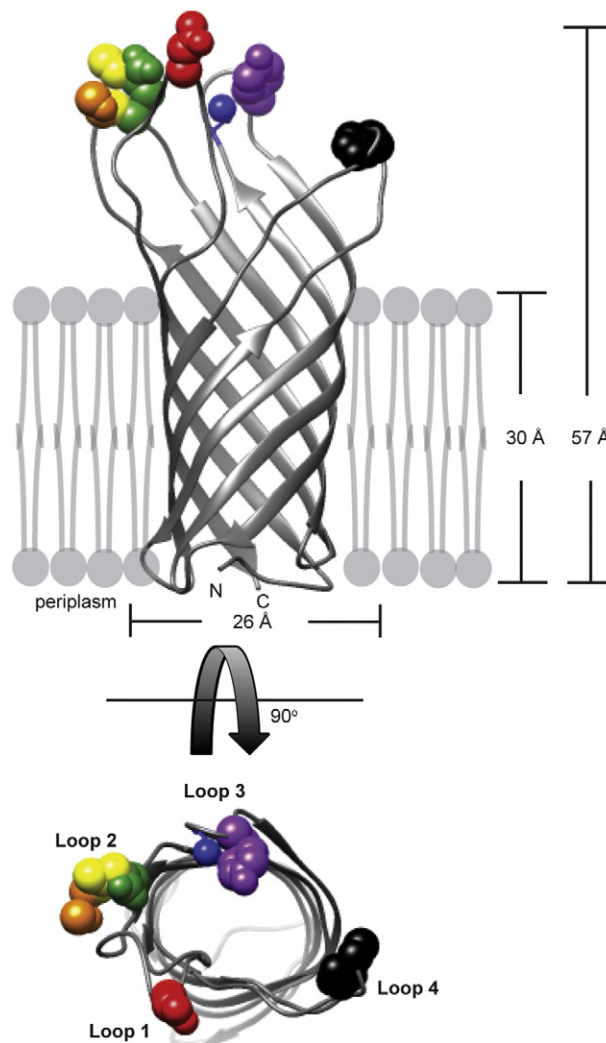


Fig. 8. Sf6 interacts with entire OmpA surface. The crystal structure of *E. coli* OmpA (PDB ID: 1BXW [55]) is depicted as a ribbon diagram with substituted amino acids shown as spheres: red, P25; orange, D66; yellow, N67; green, I68; blue, A108; violet, P111; and black, N155 using UCSF Chimera [56].

the host to evolve changes in the loops of OmpA, to avoid Sf6 infection, OmpA has several other roles [46], including attachment and invasion of eukaryotic cells [36,47–52]. Work with meningitic *E. coli* OmpA has implicated the surface loops as important for the pathogenesis of *E. coli*, fulfilling roles such as attachment, survival, and cell–cell spread [47,49,50,52]. Moreover, loop 2 of *E. coli* OmpA appears to have several overlapping roles, as alterations in this loop affect several key virulence factors of *E. coli*: attachment, intracellular survival, and invasiveness [47]. Therefore, although mutations in the surface loops of OmpA may lead to an increase in resistance to phage infection, the ability of *S. flexneri* to invade eukaryotic cells may be decreased, thereby decreasing the bacterial pathogenicity. This point is merely speculation as little experimental evidence is currently

available on the specific role of OmpA surface loops in *S. flexneri* pathogenesis. Not evolving resistance to phage infection is likely a trade-off to retain pathogenicity, although this remains to be determined experimentally.

In the present study, we showed that the surface loops of OmpA mediate phage Sf6 infection of *S. flexneri*. Coupling site-directed mutagenesis and *in vivo* phage biology allowed us to delineate which portions of the surface loops interact favorably with Sf6. Our data suggest that some amino acid substitutions in the loops decrease phage infection efficiency. By complementing the *ompA*[−] *S. flexneri* strain with *S. typhimurium* OmpA, we found that Sf6 could productively interact with other bacterial OmpAs as long as they share homology in loops 2 and 4, thus suggesting that host specificity may be

determined by these loops. We propose a model in which Sf6 interacts with OmpA on the whole surface rather than only on a preferential side of the protein, unlike what other known phages do with their respective Omp receptors. Our data provide new insights into *Podoviridae* attachment through binding to membrane protein receptors.

Materials and Methods

Media and strains

Lysogeny Broth was used for bacterial growth, most plating experiments, and preparations of Sf6 phage stocks. MacConkey agar (BD Difco) was used to select for bacteria with intact outer membranes [37]. Sf6 phage used in all experiments carries a mutation making the phage obligately lytic and was prepared as previously described [53]. Phage were stored in phage buffer: 10 mM Tris (pH 7.6) and 10 mM MgCl₂. *S. flexneri* strains include the parent strain PE577 [54] and *ompA*^{-C-} [29]. Plasmids expressing *S. flexneri* (“pOA_{S.flex}” [36]), *S. typhimurium* (“pOA_{S.typh}”), and *E. coli* OmpA (“pOA_{E.c}”) were transformed into *ompA*^{-C-} *S. flexneri*. Similar to pOA_{S.flex}, *E. coli* OmpA was constitutively expressed off pACYC184 plasmid (Cam^r) with its native promoter and was generated by Dr. Alexander Chang and kindly provided by Dr. Nemani Prasadarao. For the purification of *S. flexneri*, *E. coli*, and *S. typhimurium* OmpA-TM, the transmembrane domain (residues 1–175 for *S. flexneri* OmpA-TM [29], residues 1–171 for *E. coli* OmpA-TM, and residues 1–175 for *S. typhimurium* OmpA-TM) with a 6-histidine tag on the N-terminus was subcloned into a pRSET_A vector (Invitrogen) (Amp^r) and expressed in *E. coli* BL21 (DE3)pLysS. All vectors encoding OmpA variants (either as the full-length protein or as OmpA-TM) were generated through single or serial rounds of QuikChange site-directed mutagenesis using pOA_{S.flex} (for *in vivo* complementation experiments) or “pNBP01” (for protein purification) [29] as the starting template. For all constructs generated in this study, sequences were verified using Sanger sequencing at the Research Technology Support Facility at Michigan State University.

Purification and refolding of variant OmpA-TMs

OmpA-TM variants were purified and refolded as previously described [29]. Briefly, OmpA-TM was refolded by nutation in 0.1% (1.8 mM) of Triton X-100 at room temperature overnight. Protein folding was confirmed by electrophoretic mobility via SDS-PAGE. Refolded OmpA-TMs were exhaustively dialyzed against Triton X-100 (1.8 mM) to remove residual urea.

Proteinase K treatment

Proteinase K (Roche) and folded WT OmpA-TM were incubated at 37 °C for 15 min at a 1:5 ratio. Loop cleavage was confirmed by SDS-PAGE and digested OmpA was then used for some *in vitro* genome ejection experiments (as described in the next section).

LPS extraction and *in vitro* genome ejection experiments

S. flexneri LPS was extracted from the parent *S. flexneri* strain using a BulldogBio kit as previously described [29]. Sf6 was incubated at 37 °C with purified LPS at 0.25 mg/mL and OmpA-TM at 0.15 mg/mL. Aliquots were taken 10 and 60 min post-addition of phage, serially diluted, and plated on the parent *S. flexneri* strain; plates were incubated at 30 °C. “Percent remaining virions” was calculated by dividing the PFUs at each time point by the PFUs with buffer only added at *t* = 0 min. *In vitro* genome ejections with “boiled and refolded” OmpA-TM_{S.flex} were set up as described above after OmpA-TM_{S.flex} was boiled for 5 min at 95 °C and allowed to refold overnight.

Measuring the relative titer of Sf6 on *S. flexneri*

Sf6 was plated on various *S. flexneri* strains (the parent strain PE577, *ompA*^{-C-}, *ompA*^{-C-} + pOA_{S.flex}, *ompA*^{-C-} + pOA_{E.coli}, *ompA*^{-C-} + pOA_{S.typh} or *ompA*^{-C-} + pOA_{S.flex} expressing variant OmpAs) at temperatures ranging from 25 to 42 °C. The relative titer was calculated by dividing the resultant PFUs on each strain and at each temperature by the PFUs on the *S. flexneri* parent strain at the permissive temperature, 30 °C.

Thermal stability of variant OmpA-TMs

To measure the stability of variant OmpA-TM relative to OmpA-TM_{S.flex}, we incubated purified OmpA-TMs ranging in concentration 0.6–4 mg/mL at temperatures between 25 and 95 °C, run by 15% SDS-PAGE, and then stained by Coomassie. Gel densitometry (BIORAD Gel Doc XR+) was used to determine percent folding at each temperature. Data were plotted and fit with a sigmoidal curve using GraphPad Prism version 6.0 for Mac OS X, GraphPad Software, La Jolla, CA, USA[†]. Data for determining thermal stability were collected in triplicate for each OmpA-TM protein.

Circular dichroism

Far UV CD spectra were taken with a JASCO J-815 CD spectrometer (JASCO Analytical Instruments, Easton, MD) in a 1 mm (Starna cells quartz) cuvette at 25 °C. Spectra were recorded from 200 to 250 nm with a bandwidth of 1.0 nm, scanning rate of 50 nm/min, and data integration time of 1 s. Ten scans were averaged for each sample. Protein concentration was normalized to OmpA-TM_{S.flex} by SDS-PAGE and gel densitometry prior to CD. Three technical replicates of CD spectra were collected for each protein type.

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†www.graphpad.com.

Abbreviations used:

PFU, plaque-forming unit; LPS, lipopolysaccharide; Omp, outer membrane protein; HSV, herpes simplex virus; WT, wild type.

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