HIJACKING THE CELL:
HOW BACTERIOPHAGE Sf6 USES *SHIGELLA FLEXNERI* OUTER MEMBRANE PROTEINS FOR INFECTION

By

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ABSTRACT

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HOW BACTERIOPHAGE Sf6 USES *SHIGELLA FLEXNERI*
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Viral infections cause problems worldwide and result in a multitude of human diseases ranging in severity from influenza to HIV. Most viruses infect their respective host cells by attaching to a receptor, ejecting their genomes, and replicating via the host cell machinery. The step of attachment and entry requires precise docking to a designated location on the surface of the cell followed by conformational changes that result in viral genome transfer into the cell. However, the molecular mechanisms that drive receptor binding have not been elucidated for the vast majority of viruses. Because viral protein structures and mechanisms are conserved across families of viruses, Sf6 and its host *Shigella flexneri*, may be used as a model system to provide insight into understanding viral attachment to host cells. Sf6 uses lipopolysaccharide (LPS) as a primary (1^{o}) receptor for an initial reversible, interaction, and it requires a secondary (2^{o}) irreversible receptor to commit to infection. Both outer membrane proteins A and C (OmpA and OmpC) may serve as secondary receptors for Sf6, although OmpA is slightly preferred. Here, we investigate how bacteriophage Sf6 utilizes OmpA and OmpC for infection. First, we identified that the surface loops of OmpA are important for Sf6 infection. Using a combination of *in vivo* and *in vitro* approaches including, but not limited to, phage plaque assays, site-directed mutagenesis, circular dichroism spectroscopy, and *in vitro* genome ejection assays we characterized which residues in the surface loops of OmpA

are responsible for productive Sf6 infection. We showed that individual amino acid substitutions have a range of effects implicating some locations in the loops as more important than others for infection. Next, we used BioLayer Interferometry (BLI), an optical biosensing technique, to determine binding affinities of Sf6 to OmpA and single substitution variants. We immobilized whole virions and determined the kinetic parameters of Sf6 to various OmpAs to be fast-on and slow-off. The binding affinity of Sf6 to *S. flexneri* OmpA is in the low nM range. We also show that Sf6 binds to five variant OmpAs and the resultant kinetic parameters vary only slightly. These kinetic data suggest that Sf6:Omp receptor recognition is not solely based on kinetics, but potentially on the ability of the Omp to induce the correct conformational changes in the virion which result in translocation of the DNA. Finally, we purified OmpC and using a limited proteolysis approach, we obtained trypsin resistant and functional trimeric OmpC. The resultant OmpC, in combination with LPS, causes Sf6 genome ejection *in vitro*, but at a lower efficiency and rate than with OmpA. Taken together, the data presented in this dissertation shed light on how Sf6 interacts with its secondary receptors, OmpA and OmpC, an important aspect of host recognition, and provide new insights into *Podoviridae* attachment.

This thesis is dedicated to my grandmother: Zofia Kurdziel.
Thank you for always supporting me and saying “Ucz się dziecko, ucz”, which to English translates as “Learn child, learn”.
Rest in peace grandma.

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KEY TO ABBREVIATIONS

AR2G	Amine reactive second generation
BLI	BioLayer Interferometry
CaMV	Cauliflower mosaic virus
CAR	Coxsackie adenovirus receptor
CD	Circular dichroism
CD4	Cluster of differentiation 4
CD46	Cluster of differentiation 46
CDC	Centers for disease control and prevention
DNA	Deoxyribonucleic acid
ds	Double stranded
HHV-6	Human herpes virus 6
HIV	Human immunodeficiency Virus
HSV	Herpes simplex virus
IBs	Inclusion bodies
LB	Lysogeny broth
LPS	Lipopolysaccharide
MERS-CoV	Middle East respiratory syndrome coronavirus
MP	Movement protein
Omp	Outer membrane protein
OmpA-$TM_{E.coli}$	Transmembrane domain of *E. coli* OmpA
OmpA-$TM_{S.flex}$	Transmembrane domain of *S. flexneri* OmpA

OmpA-TM$_{S.typh}$	Transmembrane domain of *S. typhimurium* OmpA
OmpA$_{E.coli}$	*E. coli* OmpA
OmpA$_{S.flex}$	*S. flexneri* OmpA
OmpA$_{S.typh}$	*S. typhimurium* OmpA
OmpCΔNT	OmpC lacking the N-terminal signal sequence
PFUs	Plaque forming units
PME	Pectin methylesterase
pOA$_{E.coli}$	Plasmid expressing *E. coli* OmpA
pOA$_{S.flex}$	Plasmid expressing *S. flexneri* OmpA
RNA	Ribonucleic acid
SARS-CoV	Severe acute respiratory syndrome coronavirus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	Single stranded
TM	Transmembrane
TMV	Tobacco mosaic virus
TNF/NGF	Tumor necrosis factor/nerve growth factor
TVCV	Turnip vein clearing virus
WT	Wild type

CHAPTER 1

Introduction

WHY STUDY VIRUSES?

Viruses are the most abundant entities in the biosphere with numbers estimated to exceed 10^{31}-10^{32} viral particles (1-3). Viruses have evolved to infect every domain of life: Bacteria, Archaea, and Eukarya (1, 4). The number of viruses exceeds the number of hosts by an order of magnitude (2) and as a consequence, organisms are likely constantly under attack by viruses. As such, viruses play profound roles in many areas, including human health, ecology, and structuring microbial diversity.

Viruses from over 20 different families either cause or are associated with diseases in humans (5). According to the Centers for Disease Control and Prevention (CDC), 5-20% of the United States population contracts the flu each year. These numbers translate to about 15-60 million people being infected with influenza. The estimated cost per year in direct medical expenses due to the flu alone is $10.4 billion, with an estimated number of deaths reaching up to 49,000 (CDC). Although much of virology has focused on studying viruses that infect and harm humans, viruses are also important in maintaining human gut microbiome health; recent work has shown that there are distinct viral profile differences in the guts of healthy individuals versus individuals with irritable bowel diseases, such as Crohn's disease (6).

In addition to playing important roles in humans, viruses play important ecological roles. In the oceans, the rate of viral infection is as high as 1×10^{23} infections per second (7). Even deep within the subsurface, it is hypothesized that viruses may play a role in altering biogeochemical cycles (8, 9). Viruses impact their host community structures through lysis of their cells which results in both top-down control and bottom-up control (8). Viruses control the population of hosts (top-down control) through the

lysis of cells; every day, about 20-40% of all bacterial cells in the ocean are lysed by viruses (7), which means that there is rapid turnover of organic matter (bottom-up control), thus contributing to recycling of nutrients. Other important effects viruses have, particularly on microbes, are mediating lateral gene transfer (10, 11).

Due to the vast number of viruses, their profound roles in human health, and their important ecological implications and roles in the biosphere, it is imperative to study viruses and understand how they infect their hosts. For a successful infection, virtually all viruses recognize their respective host cells and transfer their genetic information. This universally essential step amongst viruses has to be well-coordinated in order to ensure successful progeny formation, as premature genome ejection can negatively impact the future of the virus. Unfortunately, the important step of attachment to the host is not well understood for most viruses. Thus, is it crucial to understand how viruses solve the problem of recognition of host cells and how this process can be inhibited.

VIRUS: HOST RECOGNITION

The infection process of viruses is well-coordinated to ensure replication and progeny formation, as viruses are often considered to be obligate parasites and cannot reproduce on their own. A productive infection requires the following steps 1) attachment and entry into the host cell, 2) co-opting host cell machinery for the production of infectious virions, and 3) release of progeny.

All viruses contain nucleic acids, either in the form of single-stranded (ss) or double-stranded (ds) deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encapsulated in a protein coat. The nucleic acid, or the blueprints for the production of

more viruses, must be translocated from the capsid of the virus into the correct host for viral production. In order to do this, viruses must recognize their respective host cell. The step of attachment, followed by entry, is arguably the most important. It requires precise docking to a designated location on the surface of the cell followed by conformational changes in viral proteins which ultimately result with genome transfer into the cell. This is a universal phenomenon amongst all viruses and is highly regulated as premature genome release negatively impacts the future of the virus. Although there exist different methods of entry, including membrane fusion, endocytosis, or direct genome ejection after host recognition (12-14), some common methods and strategies of host recognition are employed, such as the utilization of receptor binding proteins (14).

Viruses that infect animal cells have evolved to use many cell surface proteins as receptors. For example, coxsackie adenovirus receptor (CAR), a protein that is found on the surface of many human and mouse cells, is recognized by several human viruses, including Coxsackie B viruses and adenoviruses (15, 16). Coxsackie B viruses are non-enveloped ssRNA viruses that belong to the picornavirus family (17) and adenoviruses are non-enveloped dsDNA viruses that belong to *Adenoviridae* (18). Despite differences in structures and nucleic acid composition, both of these viruses use the same receptor to co-opt the cell. In the absence of cluster of differentiation 4, CD4, a glycoprotein that is found on the surface of human immune cells, human immunodeficiency virus (HIV) is able to utilize CAR as a receptor (19, 20). Another cluster of differentiation protein, CD46, is utilized by the measles virus (21, 22), which is a ssRNA, enveloped virus. The same protein is used by human herpes virus 6, HHV-6 (23), a dsDNA virus, one of the

causative agents of *exanthema subitum*, also known as roseola (23, 24). Other herpes viruses, most notably herpes simplex 1 and 2 (HSV-1 and HSV-2), whose manifestations occur as oral and/or genital lesions (25), have been shown to use a member of the TNF/NGF (tumor necrosis factor/nerve growth factor) family (19, 25, 26). Thus, there exists similar host receptor binding protein usage between not only similar viruses, but also evolutionarily diverse viruses.

Viruses that infect plants often enter through a wound that has breached the plant cell wall and cell membrane (27). Although plant viruses do not initially enter host cells via active mechanisms, as the receptor binding ones described above, once inside the cell, the progeny viruses must spread from cell-to-cell (28, 29). Plant viruses have evolved a variety of mechanisms to exploit the plant to do this. One common strategy is to recognize the proteins on the surface of cells. For example, tobacco mosaic virus (TMV), like many other plant viruses, spreads to other cells using the plasmodesmata (30). However, to gain entry into a cell, TMV's movement protein (MP), a protein on the surface of the virus, requires interaction with the host cell pectin methylesterase, PME (31, 32). Deletion of the binding portion of the PME results in inactivation of TMV cell-to-cell movement (31). Other viruses that use PMEs to gain access to cells include turnip vein clearing virus (TVCV) and cauliflower mosaic virus (CaMV) (31). Even though initial entry of plant viruses is through wounds, the spread and continual propagation of many of these viruses is dependent upon the recognition of proteins expressed on the surfaces of the host cells.

The studies of viruses that infect Archaea, "archeoviruses", remain few, with only about 100 archeoviruses being studied compared to over 6,000 bacterial viruses (33,

34). In particular, insights into the receptor usage and entry of archeoviruses are lacking. However, the few published studies do suggest that host receptor binding proteins are used in the initial step of attachment by these viruses. For example, AFV1, a virus that infects the hyperthermophilic archaea *Acidianus,* has “claw-like” features at either end that use the tip of a pilus as a cell receptor (35). Another, more recently described virus SIRV2 has been shown to specifically recognize the tips of pilus-like filaments of its host: *Sulfolobus islandicus* (36). It has been hypothesized that other filamentous-linear archaeal viruses may also recognize and use the host pilus as a receptor (35). Work with *Acidianus* two-tailed virus ATV, which infects *Sulfulobus solfataricus,* has demonstrated that the virus has a specific interaction with $OppA_{Ss}$ (37), a glycoprotein present on the cell surface (38). Much more work is needed in field of archeoviruses to better understand their attachment and entry mechanisms. However initial studies support the usage of proteins present on the host surface as a recognition mechanism, similar to the phenomenon exhibited by viruses that infect Eukarya and Bacteria.

Much like the viruses described above, bacteriophage or “phage”, viruses that infect bacteria, have evolved to utilize a portion of the host cell as a receptor (14). *Bacillus subtilis* phage SPP1, in addition to teichoic acid, also requires recognition of membrane receptor protein YueB to irreversibly adsorb and commit to infection (39, 40). Like SPP1, *Lactococcus* phage c2 requires interaction with a membrane protein (41). Another Gram-positive phage that has been shown to require recognition of a membrane protein is *Bacillis anthracis* phage γ (42). Many bacteriophages that infect Gram-negative hosts, like many of their Gram-positive counterparts, also utilize

membrane proteins as receptors. Outer membrane proteins (Omps) of Gram-negative bacteria that are used as phage receptors include, but are not limited to, OmpA, OmpF, and LamB (43-47). A more thorough overview of Omps as phage receptors is provided later in the introduction. As illustrated, even though viruses infect across all domains, commonalities exist between diverse viruses and types of receptors recognized on the host.

BACTERIOPHAGES AS MODEL SYSTEMS TO STUDY VIRAL INFECTIONS

Although the number of viruses exceeds 10^{31} (1-3), structural similarities between viruses across kingdoms make it possible to utilize model systems of viruses and their respective hosts to study viral infection (1, 4). This discovery was first made by Michael Rossmann when he solved the first atomic structure of a human virus, rhinovirus, and noted that it was strikingly similar to a structure of a known icosahedral plant virus (48). A little over a decade later, Benson et al. showed that structural similarities exist between viruses that span across different domains (49). The authors show that the coat protein folds for both bacteriophage PRD1 and human adenovirus are architecturally similar. A few years later, the Young group solved the structure of an archaeal virus, STIV, and showed that it has a coat protein fold almost identical to that of bacteriophage PRD1 (50), thus showing that the “PRD1-like” protein fold spans all domains of life. It has since been hypothesized that only a handful of protein folds exist that form the virion, thus suggesting a possible common ancestor for viruses that infect hosts in different domains (1, 4).

There are many structural similarities between viruses. For example, the major capsid (1), scaffold (51), and portal proteins (52, 53) are conserved in dsDNA bacteriophages and HSV (54). The HSV portal protein was the first identified in a virus infecting eukaryotes and provided support for the hypothesis that the dsDNA phages and the herpes viruses share a common ancestor and that the mechanisms of DNA packaging are similar (52, 54). Furthermore, there also exists many structural and functional similarities with dsRNA viruses that belong to the families *Cystoviridae* (infect bacteria) and *Reoviridae* (infect plants, insects, and animals) (4, 55-58).

With respect to host recognition, there exists structural similarities between different viruses as well. Perhaps one of the best studied examples of this is that of the adenovirus cell-receptor binding protein, a protein part of the bacteriophage PRD1 receptor binding complex, and more recently, the tail-needle knob of bacteriophage Sf6 (59-62). Adenovirus recognizes and binds to its receptor CAR (15, 16) via a cell-receptor binding protein, a trimeric fiber with a globular knob at the end (62). These fiber head domains have been shown to have cell-type selective properties (63); thus, illustrating their importance in host cell recognition and attachment. The receptor binding complex of bacteriophage PRD1, located at each vertex, is made up of three proteins, whose overall structure resembles that of the adenovirus fiber with globular knob at the end (64). The tail-needle knob of bacteriophage S6 was hypothesized to interact with cell surface receptors, OmpA and OmpC (65). Although current work from the Parent lab (unpublished) has suggested that the knob of Sf6 is not the cell-receptor binding protein, it likely still plays a role in attachment and entry, perhaps by serving as a surface pressure sensor, like it is hypothesized that its cousin phage, P22, does (66,

67). Despite there being less than 10 % amino acid sequence similarity between the Sf6 tail-needle knob, PRD1 protein P5 (the protein that forms the knob domain of the receptor binding complex), and the adenovirus knob, many structural similarities exist (60). In addition to structural similarities, each of these proteins appears to play important roles in mediating recognition and attachment to host cells, although more experimentation is needed in the case of Sf6.

The phenomenon of low sequence identity at the amino acid level yet high degrees of structural similarity is a common theme seen throughout virology (1, 4). Therefore, these common viral structures and mechanisms make it possible to use model virus:host systems to study viral infection, particularly bacteriophages. What is learned from the model system can then be extrapolated to predict how other viruses with homologous protein folds infect their hosts. The model system studied in this dissertation is bacteriophage Sf6 and its host, *Shigella flexneri.*

THE MODEL SYSTEM: BACTERIOPHAGE Sf6 AND HOST *S. FLEXNERI*

Bacteriophage Sf6

The most abundant viruses are the tailed dsDNA phages (order *Caudovirales*) whose global population is estimated to exceed 10^{30} (68). Three families make up the order, *Siphoviridae,* long, non-contractile tailed phages, *Myoviridae*, long, contractile tailed phages, and *Podoviridae,* short-tailed phages. The temperate bacteriophage Sf6 belongs to the sub group of the “P22-like” phages in family *Podoviridae* (69), one of the least studied families in regards to phage-host interactions. Sf6 was originally isolated by Gemski in 1974 and shown to have a narrow host range as it can only infect *Shigella*

flexneri serotypes X or Y (70). Sf6 has a capsid diameter of 50 – 60 nM, has a short tail with a globular knob domain at the end, and has six tailspikes (71-75). This phage has been of particular interest to study due to its ability to alter the antigenic surface properties of its host when lysogenized (70-72), which in turn helps make the host a more successful pathogen. For all the studies described in this dissertation, Sf6 carries a mutation making the phage obligately lytic (71). Having a better understanding of how Sf6 interacts with its host, particularly using the clear plaque mutant, is pertinent to the development and potential use of Sf6 as an antimicrobial.

S. flexneri

The first *Shigella* was isolated in 1896 by Kiyoshi Shiga (76). *Shigella* are Gram-negative bacteria and the causative agents of shigellosis with some strains having a fatality of 10-15 %. There are over 165 million cases worldwide and transmission occurs via a fecal-oral spread, with ingestion of as few as 10 bacteria being sufficient to cause disease (77). In about 60 % of cases the most frequent isolate is *S. flexneri* (78, 79). According to the CDC, in the US alone there are 500,000 shigellosis cases a year and this trend has not changed significantly in the past 10 years. Moreover, recently between March-October of 2016 in Flint, MI there was an outbreak of *S. flexneri*, where close to 200 people contracted shigellosis according to the Michigan Department of Health & Human Services. Those with the highest risk of shigellosis include children and immunocompromised individuals, such those infected with HIV (78). Although the diarrhea associated with Shigellosis resolves in 5-7 days, there can be many complications due to the disease. Around 2 % of people develop post-infectious arthritis

which can last for months or even years (80). Other complications include sepsis, particularly in immunocompromised individuals, and in some cases seizures have been reported among young children with shigellosis (80). Due to the fact that Sf6 interaction with *S. flexneri* could result in a more dangerous pathogen there is great incentive to study these virus:host interactions.

Sf6 interaction with host S. flexneri

When Sf6 was first isolated by Gemski, initial studies showed that lipopolysaccharide (LPS) was important for infection; Sf6 could only infect a narrow range of *S. flexneri* strains depending on the structure of the O-antigen repeat units (70, 72). It had been recognized for a long time that the LPS of Gram-negative bacteria served as a receptor for many bacteriophages (81), thus for years it was thought that *S. flexneri* LPS served as a receptor for Sf6 which it recognized via its six trimeric tailspike proteins (82).

Decades later, comparisons between Sf6 and its cousin phage, P22, which infects *Salmonella typhimurium*, showed that the structural proteins between the phages are similar in size and amount (71). Interestingly, two unidentified proteins were found to be associated with Sf6, but not P22 (71). A few years later two groups identified these two protein bands by mass spectrometry, originally labeled as “?” (71), to be outer membrane proteins A and C (“OmpA” and “OmpC”) (73, 75). The Tang group hypothesized that OmpA and OmpC were host membrane proteins that were associated with the Sf6 virion and were important for structural integrity of the capsid (73). Parent et al. showed that OmpA and OmpC were not associated with the Sf6

capsid, but that these Omps co-purified with bacteriophage Sf6, even after CsCl purification, through outer membrane vesicles attached to the tail machinery (75); this phenomenon has not been observed in any of the other “P22-like” phages. Based on these data, Parent et al. were the first to hypothesize that Sf6, in addition to LPS, may require a secondary receptor to commit to infection, a role that may be fulfilled by Omps.

In 2012, the proposed model that existed for *Podoviridae* attachment was a three step model (83). In the first step, which is usually “reversible”, a virion binds to its primary receptor, LPS, via its tailspikes. The tailspikes cleave the polysaccharide through their endorhamnosidase activity, thus bringing the phage closer to the surface of the cell where it may now bind to a putative secondary receptor. This step is considered an “irreversible” interaction, as the virion has committed to infection and infectious virions cannot be recovered. Finally, interactions with the host receptors trigger conformational changes in the tail machinery that lead to these proteins rearranging and likely forming a channel to translocate the genetic information. Work with P22 showed that the LPS of the host is sufficient to trigger genome release *in vitro*, albeit slowly (66, 84). These data suggested that a secondary receptor may not be necessary for P22 to infect *Salmonella.* Yet, Sf6 co-purified with Omps (75), suggesting that Sf6 and P22 may differ in their host receptor usage. Moreover, the presence of Omps as secondary receptors for many phages has been reported (see below) thus, providing further support for the hypothesis that Omps may serve as secondary receptors for Sf6.

OUTER MEMBRANE PROTEINS AS BACTERIOPHAGE RECEPTORS

Outer membrane proteins are major constituents of the outer membranes of Gram-negative bacteria and play many important roles for bacterial cellular growth and survival. Omps are important for maintaining the membrane integrity of cells, bringing in nutrients, protection against harsh environments, osmoregulation, and have been shown to play a role in bacterial antibiotic resistance (85-89). Moreover, Omps are also important for bacterial adherence and invasion into human cells (90, 91). Moreover, several Omps, including OmpA, OmpC, and OmpF have been implicated as immunogenic targets (92, 93). Structurally, these homologous proteins are transmembrane β-barrels with differing pore diameters (85).

It is not surprising that bacteriophages have evolved to use Omps as receptors since Omps are some of the most abundant entities of the outer membrane and are easily accessible. Common Omps that are used by bacteriophages as receptors include OmpA (43-45, 94, 95), OmpC (45, 46, 95-100), OmpF (46, 101, 102), LamB (46, 47), TonB (103), FhuA (103, 104), OmpT (45), Tsx (105), and OmpX (95) (Table 1.1). Table 1.1 provides a comprehensive overview of Omps that are used as bacteriophage receptors and which phages use them. Likely due to the structural homology of Omps, many phages can evolve to recognize multiple Omps. For example, bacteriophage Ox2, which infects *E. coli*, can evolve to use OmpA, OmpC, or OmpX as a receptor (96, 106). Recently, a study has shown that under selective pressure in the lab phage λ can evolve to use OmpF, in the absence of LamB (107). With so many phages using Omps as receptors to gain access to host cells, it supports the hypothesis that Sf6 may use OmpA or OmpC as a receptor.

Table 1.1: Bacteriophage outer membrane protein receptors

Outer Membrane Protein	Species	Bacteriophage
OmpA	*Escherichia coli*	TuII* (108) K3 (109, 110) K4 (111) K5 (111) Ox2 (111) Ox3 (111) Ox4 (111) Ox5 (111) M1 (111) Ac3 (111)
	Shigella flexneri	Sf6 (65, 112)
OmpC	*Escherichia coli*	T4 (99) TuIb (109) Mel (109) PA2 (109) Hy2 (113) SS4 (113) TuIa^{+} (114) 434 (102) M1^{+} (45) Ox2^{+} (96) PP01 (98) SS1 (115)
	Salmonella	Gifsy-1 (97) Gifsy-2 (97) S16 (116)
	Shigella flexneri	Sf6 (65)
OmpF	*Escherichia coli*	T2 (101, 102) TuIa (108) TP1 (109) K20 (108) Ox2^{+} (106)
	Yersinia pestis	Yep-phi (117)
	Yersinia enterocolitica	TG1 (118) ϕR1-RT (118)
OmpP	*Escherichia coli*	Ox2^{+} (119)
OmpT	*Escherichia coli*	M1^{+} (45)
OmpX	*Escherichia coli*	Ox2^{+} (95)
LamB	*Escherichia coli*	TuIa^{+} (114) K10 (120) λ (47) SS1 (115) TuIb^{+} (46) Stx2ϕ-II (121)
Tsx	*Escherichia coli*	T6 (105) H3 (122)
FhuA (TonA)	*Escherichia coli*	T5 (104, 122) D (122) E21 (122) T1 (123) UC-1 (123) ϕ80 (123)
TonB	*Escherichia coli*	H8 (124)
	Salmonella	H8 (124)
	Xanthomonas campestris	ϕL7 (125, 126)
TolA	*Escherichia coli*	fd (127-129) f1 (127-129) M13 (127-129)
BtuB	*Escherichia coli*	BF23 (130) E15 (122) K6 (122) K8 (122) K11 (122) M3 (122) Ac4 (122)
FadL	*Escherichia coli*	T2^{+} (102, 131) Stx2ϕ-I (121) Stx2ϕ-II (121)
FepA	*Escherichia coli*	H8 (124)
	Salmonella	H8 (124)
TolC	*Escherichia coli*	TLS (132)
PhoE	*Escherichia coli*	TC45 (133) TC23 (133)
Ail	*Yersinia pestis*	Yep-phi (117)
NfrA	*Escherichia coli*	N4 (134)

$^{+}$Indicates a host range mutant

Sf6 USES *S. FLEXNERI* LPS AND OMPS AS RECEPTORS[1]

As of 2012, it was hypothesized that Sf6 recognizes *S. flexneri* LPS and may use OmpA and OmpC as secondary receptors (75). To test this hypothesis, single null *omp* strains of *S. flexneri* (75), *ompA*$^-$*, ompC*$^-$*,* and a double null strain (65), *ompA*$^-$*C*$^-$, were created. We showed that the plating efficiency of Sf6 drops ~10 fold on *ompA*$^-$*C*$^-$, yet the plating efficiency of Sf6 on the single knockout strains was comparable to that of Sf6 plated on the parent strain *S. flexneri* PE577 (135). Using one step growth curves to monitor the life cycle of Sf6 on various knockouts, we saw that the rates of phage adsorption to the knockouts were altered compared to the parent *S. flexneri* strain, with Sf6 infection rate on *ompA*$^-$ (where OmpC would be present for binding) dropping by an order of magnitude, whereas Sf6 infection rate on *ompC*$^-$ (where OmpA would be present for binding) was only slightly altered. Additionally, using cell survival assays to look at the ability of the phage to kill the various null strains, we saw that Sf6 is able to kill *ompC*$^-$ at an increased rate compared to *ompA*$^-$. These data showed that OmpA and OmpC influence Sf6 infection.

Work with other bacteriophages has shown that purified receptors are able to induce phage genome ejection *in vitro*. In addition to P22 (66, 84), groups have shown that phages λ, SPP1, and T7 eject their genomes *in vitro* in the presence of their purified receptors, *E. coli* LamB (136, 137), *B. subtilis* YueB (40), and *E. coli* LPS (138),

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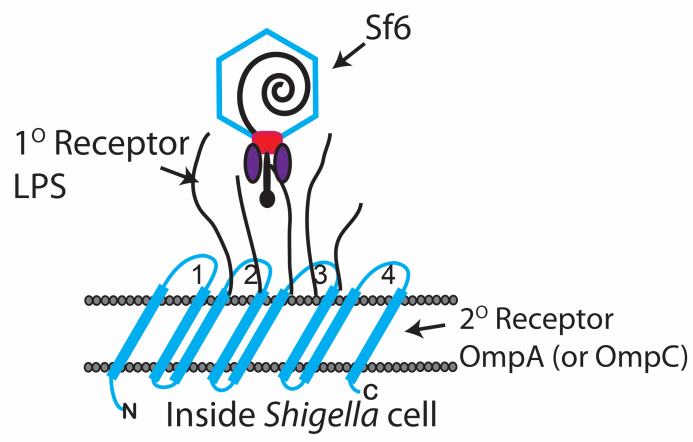
Modified from: [1] **Parent KN, Erb ML, Cardone G, Nguyen K, Gilcrease EB, Porcek NB, Pogliano J, Baker TS, Casjens SR.** 2014. OmpA and OmpC are critical host factors for bacteriophage Sf6 entry in *Shigella*. Mol Microbiol **92:**47-60.

This publication laid the groundwork for which my thesis project is based. As an author, my contributions to the publication were the expression, purification, and refolding of OmpA and the development of the *in vitro* Sf6 genome ejection assay.

respectively. Thus, we hypothesized that if OmpA and OmpC are the receptors for Sf6, we should see Sf6 genome ejection *in vitro* if we incubate Sf6 with an Omp. To test this hypothesis, we purified and refolded the transmembrane domain of OmpA (OmpA-TM) and purified *S. flexneri* LPS. We showed that in the presence of LPS and OmpA-TM, ~95% of Sf6 virions eject their genome; incubation with only one component (LPS or OmpA-TM) is not enough to induce genome ejection of Sf6. To measure Sf6 ejection rates, which we define as the observed rate of decrease in the overall number of infectious virions in the phage population over time, we tracked the number of intact virions remaining when exposed to LPS and OmpA-TM over time. In the presence of OmpA-TM and LPS, functional virions are lost within 10 minutes, which is a physiologically relevant timescale. Combined, these data illustrate a model where Sf6 infection requires both primary and secondary receptors. An initial and reversible interaction with LPS is followed by an irreversible interaction with a secondary receptor, OmpA or OmpC, although OmpA appears to be slightly preferred (Fig. 1.1).

Figure 1.1: Sf6 infection model



Attachment model for bacteriophage Sf6 illustrating the primary (1 °) receptor, LPS, and the secondary (2 °) receptor, OmpA (or OmpC).

OUTLINE OF DISSERTATION

Significance

How do charged macromolecules make it across a lipid membrane? This is a fundamental challenge that all viruses have been able to overcome, but little is known about how genomes of non-enveloped viruses enter their host cells. Most of these infect their respective host cells by attaching to a receptor, ejecting their genomes, and replicating via the host cell machinery. The infection process is not well understood in *Podoviridae*, however, which includes bacteriophage Sf6 that infects *Shigella flexneri.* Sf6 uses lipopolysaccharide (LPS) as a primary (1 ^{o}) receptor for an initial reversible, interaction, and it requires a secondary (2 ^{o}) irreversible receptor to commit to infection (83) (Fig. 1.1). *Shigella* outer membrane vesicles, which contain OmpA and OmpC, have previously been shown to co -purify with Sf6 virions (73, 75). We created an *ompA*$^{-}$*C*$^{-}$ null *Shigella* and found that this strain has increased resistance to Sf6 (65). We also showed that purified OmpA triggers *in vitro* genome ejection (65). Combined, these findings suggest that OmpA and OmpC serve as secondary receptors for Sf6, with OmpA being preferred over OmpC. Sf6 is a unique phage in that it can inherently utilize alternative receptors. *E. coli* OmpA and OmpC are both receptors for several bacteriophages. Analysis of *E. coli ompA* mutants showed an alteration in the ability of phage to infect the host cells, with >84% of 305 independently isolated mutations being in loops 2 and 3 (44, 94). Analysis of *E. coli ompC* mutants showed a decrease in the ability of phages to infect host cells, particularly alterations in loop 4 (113). However, the mechanisms of phage attachment have not been characterized biochemically or biophysically. My dissertation has addressed how Sf6 interacts with OmpA and OmpC,

a critical component necessary in DNA translocation, by answering the following questions: **(1)** which portions of OmpA are important for Sf6 attachment to the cell? **(2)** how do the changes in OmpA affect phage binding kinetics? and **(3)** is OmpC able to induce genome ejection of Sf6 *in vitro* at the same rate and efficiency as OmpA? Answering these questions has provided novel insights into the mechanisms that govern Sf6 host receptor binding and has allowed for a more complete image of the attachment process in Sf6.

Chapter 2: Key Residues of S. flexneri *OmpA Mediate Infection by Bacteriophage Sf6*

1) Which portions of OmpA are important for Sf6 attachment to the cell?

Hypothesis: We hypothesized that the surface loops of OmpA are essential for Sf6 infection; more specifically, we hypothesized that the residues that differ between *E. coli* and *S. flexneri* in the flexible portions of the surface loops are essential.

Summary: In chapter two, we identified that the surface loops of OmpA mediate *Shigella* infection. In addition, we characterized which residues in the surface loops are responsible for productive Sf6 infection using a combination of *in vivo* and *in vitro* approaches, including site-directed mutagenesis, phage plaque assays, and *in vitro* genome ejections.

Chapter 3: Whole Virion Biosensing: Kinetic Analysis of Bacteriophage Sf6 and Outer Membrane Protein A

2) How do the changes in OmpA affect phage binding kinetics?

Hypothesis: We hypothesized that OmpA variants that allow *S. flexneri* to become more resistant to phage attack will confer a decrease in binding affinity to Sf6.

Summary: In chapter three, we determined the kinetic parameters of Sf6 and variant OmpAs by using BioLayer Interferometry (BLI), an optical biosensing technique. We are the first to immobilize whole virions on the BLI platform. We show that all variant OmpAs bind Sf6 in the nM range and kinetics are fast-on and slow-off.

Chapter 4: Purified S. flexneri *OmpC Induces Sf6 Genome Ejection* in vitro

3) Is OmpC able to induce genome ejection of Sf6 *in vitro* at the same rate and efficiency as OmpA?

Hypothesis: We hypothesized that because OmpC is an alternative receptor, Sf6 genome ejection *in vitro* will be 1) not as efficient compared to OmpA and (or) 2) at a slower rate than that of OmpA.

Summary: In chapter four, we adapted a *Salmonella typhi* OmpC purification protocol to purify *S. flexneri* OmpC. We show that the resultant OmpC in combination with LPS causes Sf6 genome ejection *in vitro*, but at a lower efficiency and slower rate than OmpA.

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CHAPTER 2

Key Residues of *Shigella flexneri* OmpA Mediate Infection by Bacteriophage Sf6

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Minor edits have been made to this manuscript to conform to dissertation requirements.

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.

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 as well as 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 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 bacteriophage. 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 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 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 the subgroup of the “P22-like” phages in *Podoviridae* (27), which is one of the less well understood families in regards 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).

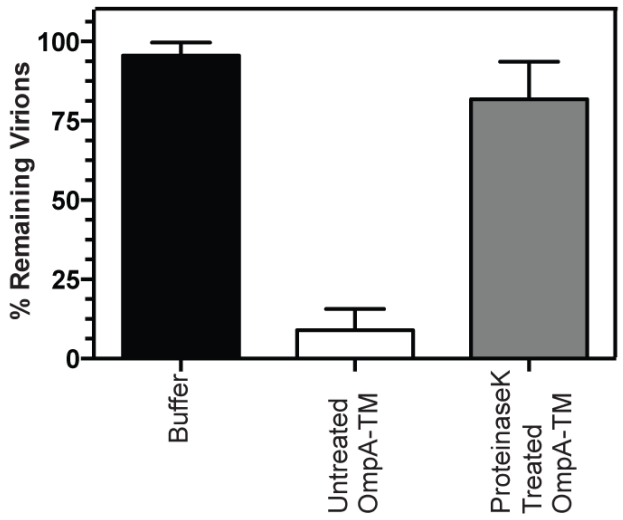
Many phages such as Sf6 are able to use more than one type of proteinaceous receptor for attachment, and they generally appear to have a preferred receptor. Since many porins (OmpA, OmpF, OmpC, FhuA, and LamB) have been identified as bacteriophage receptors (5-8, 20-22, 29), and these have homologous 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 this chapter 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 which confer 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 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 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 was then used in our *in vitro* experiments in combination with LPS and phage. Unlike undigested OmpA-TM, OmpA-TM treated with proteinase K is unable to trigger genome ejection of Sf6 (Fig. 2.1). Thus the loops of OmpA appear to be essential for Sf6 infection. To further probe which

Figure 2.1: *In vitro* genome ejection efficiency decreases with proteinase K treated OmpA-TM



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.

portion(s) 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. 2.2 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 at all temperatures (Fig. 2.2). 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 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 2.1) (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. 2.2). Therefore, we investigated if

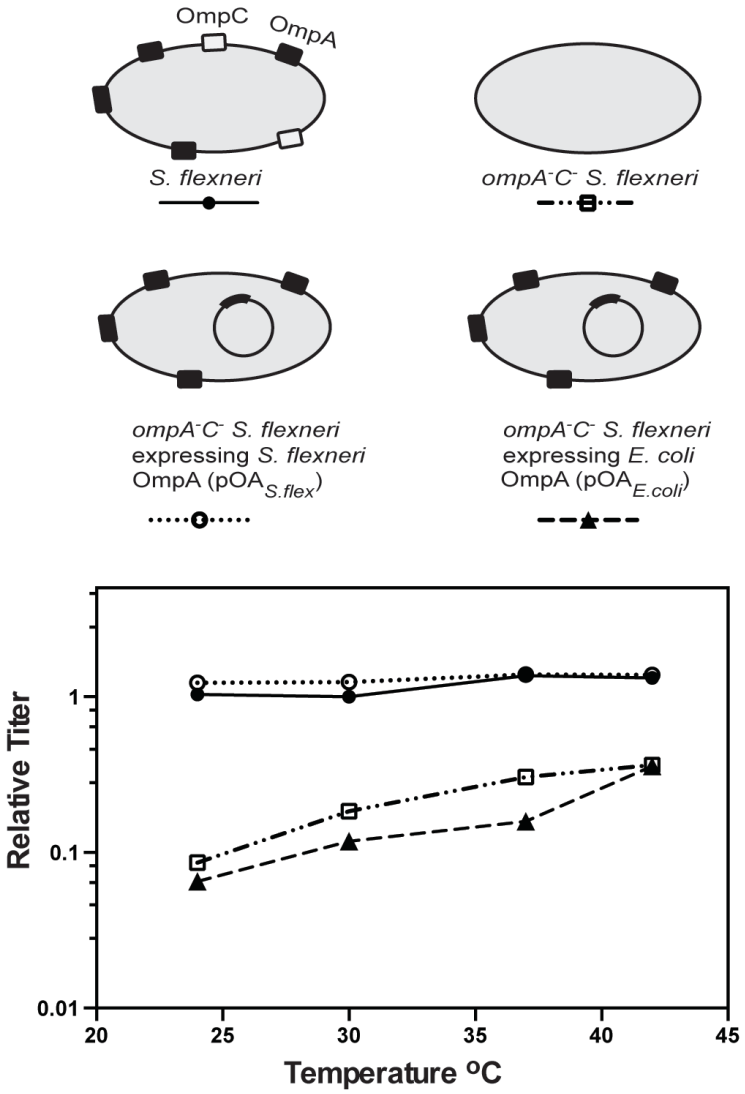
Table 2.1: Amino acid substitutions in the surface loops of OmpA

				OmpA Variants		
Loop Number	Residue* Number	*E. coli* A.A.	*S. flexneri* A.A.	No change in Resistance	Moderate Effect	Severe Effect
1	25	N	P	-	A, R	E^{+}
2	66	S	D	A, K	-	-
	67	V	N	-	-	A, E, H, R
	68	E	I	-	-	A, D, K, Q
3	108	S	A	-	E	R
	111	Y	P	-	A, E, R	-
	"insertion" (113, 114, 115, 116)	----	GASF	-	ΔGASF	-
4	155	H	N	R	A	E

* Residue numbering based on *S. flexneri* OmpA
+Variant did not grow on MacConkey agar

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. 2.3 are summarized here by their loss of function to serve as a receptor to Sf6 based on their relative titer.

Figure 2.2: 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.

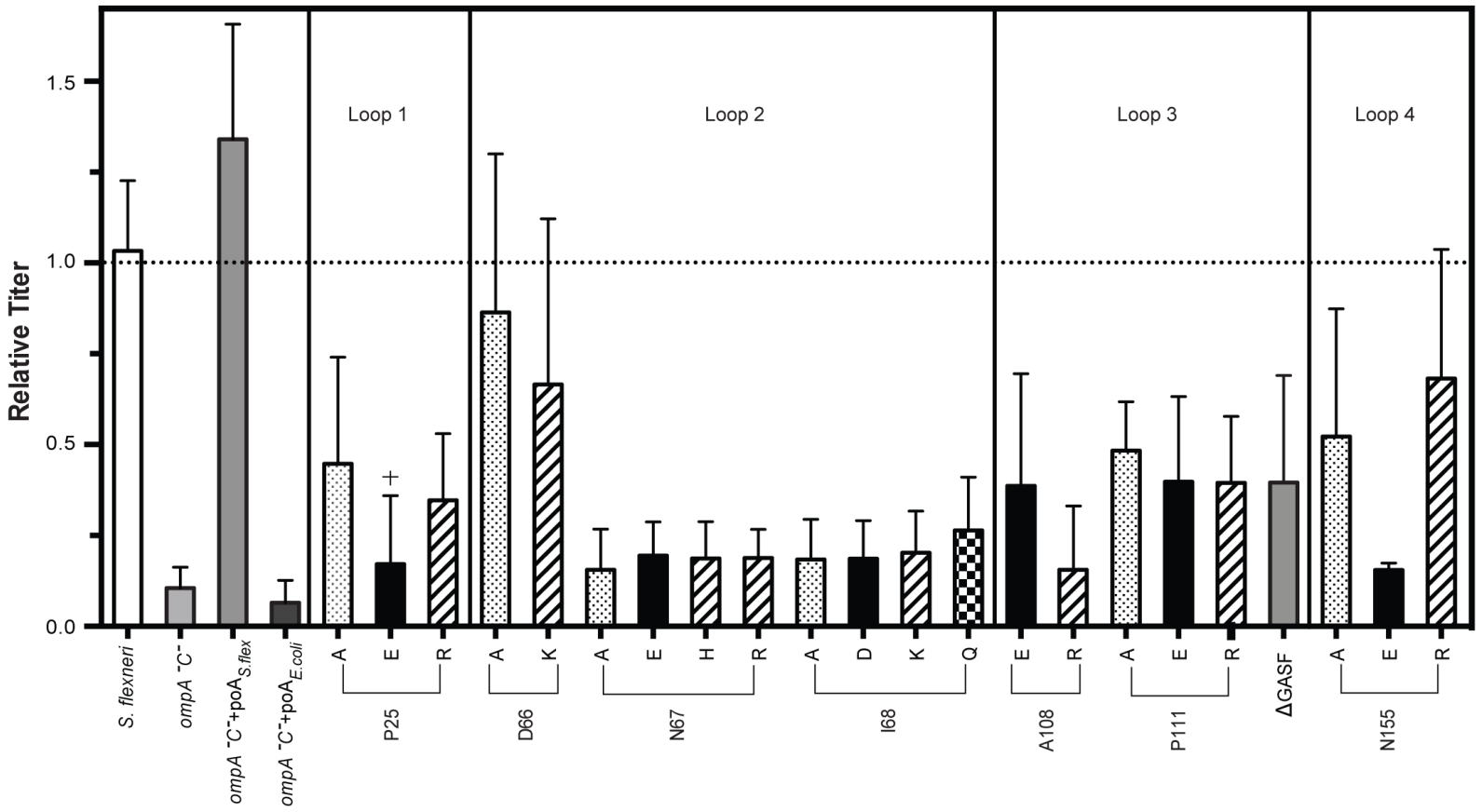
the differences between these two proteins play a role in mediating the inability of Sf6 to utilize the *E. coli* protein.

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 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 2.1). The Sf6 plating efficiency changes with some amino acid substitutions, but not others (Fig. 2.3). Complementation by three variants, D66A & D66K (loop 2) and N155R (loop 4), restore Sf6 plating efficiency of the *ompA*$^{-}$*C*$^{-}$ strain as well as the wild type gene: $ompA_{S.flex}$ (Fig. 2.3). Variants at two locations in loop 2 (N67 & I68) and one variant in loop 4 (N155E) had the lowest Sf6 plating efficiency, indicating the mutations confer a loss of function. All $OmpA_{S.flex}$ variants demonstrate the same relative phenotypes when plated at temperatures ranging 25 to 42 ˚C (data shown only for 25 ˚C for simplicity; Fig. 2.3).

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*, *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

Figure 2.3: Comparison of Sf6 infection efficiency on *ompA⁻C⁻ S. flexneri* expressing variant OmpAs



Relative titer of Sf6 on various strains (parent, *ompA⁻C⁻*, *ompA⁻C⁻* + pOA$_{E.coli}$, *ompA⁻C⁻* + pOA$_{S.flex}$,and *ompA⁻C⁻* + pOA$_{S.flex}$ expressing variant OmpA) at 25 ˚C. 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.

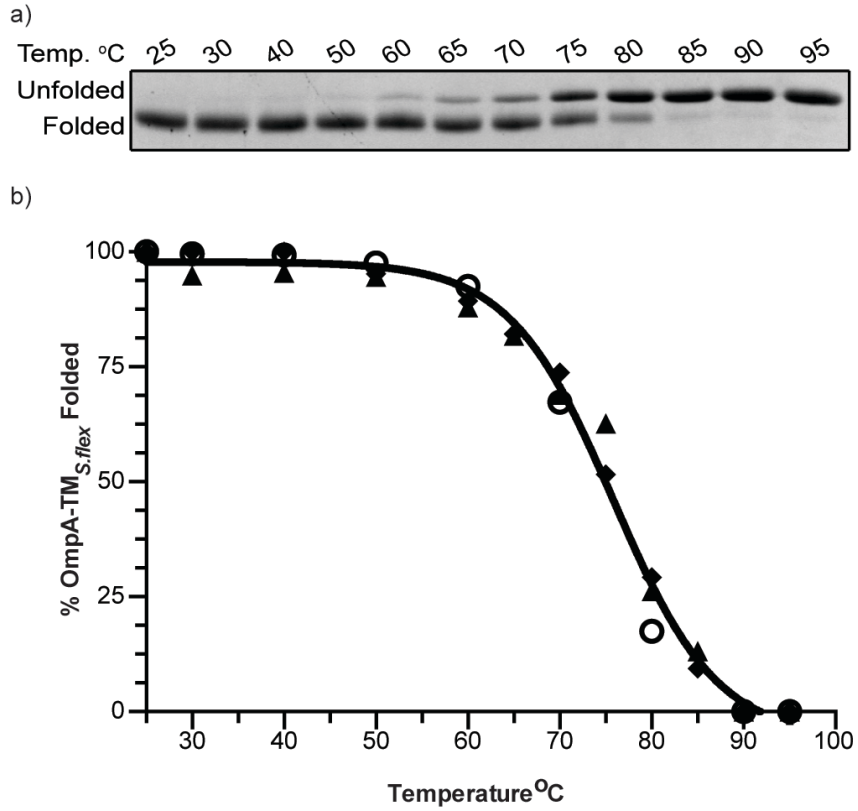
intact outer membrane integrity (37). With the exception of only P25E OmpA (designated as "+" in Fig. 2.3 and Table 2.1), all strains were able to grow on the MacConkey agar as efficiently as the *S. flexneri* parent strain (data not shown). As P25E OmpA was not incorporated correctly into the outer 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 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) as well as 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 Tm_{50}, 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 and determined the fraction of folded species by SDS-PAGE and gel densitometry since folded OmpA-TM migrates faster than unfolded OmpA-TM (Fig. 2.4A). The Tm_{50} for OmpA-$TM_{S.flex}$ was determined 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 reversible process. *E. coli* and the five selected variants of OmpA-$TM_{S.flex}$ had Tm_{50} values ranging between 73 and 76

Figure 2.4: WT *Shigella* OmpA-TM thermal stability



a) A representative 15% SDS gel stained with Coomassie blue 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 2.2: Tm_{50} 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
E. coli	75.8 ± 3.1

The calculated Tm_{50} 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. 2.4 for representative WT data.

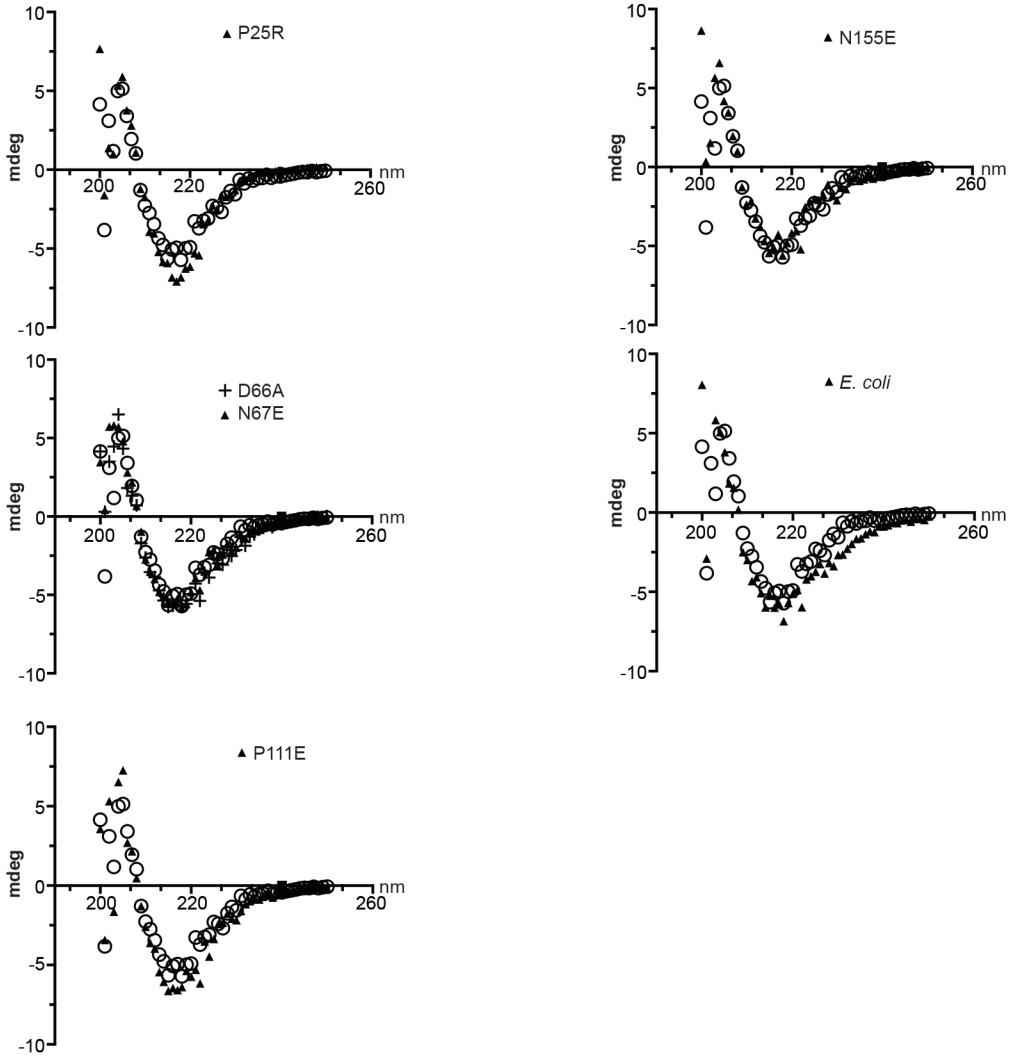
°C (Table 2.2), indicating that their relative stabilities are not significantly different from OmpA-TM$_{S.flex}$.

Second, to determine the effect, if any, of the amino acid substitutions on the secondary structure, we determined the variant OmpA-TM CD spectra and compared them to the OmpA-TM$_{S.flex}$ spectrum. Consistent with previously published data for *E. coli* OmpA (38-41), the CD spectrum of OmpA-TM$_{S.flex}$ predicts a β-barrel secondary structure (Fig. 2.5). The CD spectra of the variant and *E. coli* OmpA-TM proteins have no significant differences and are essentially identical to that of OmpA-TM$_{S.flex}$ (Fig. 2.5). 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. 2.3) 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 minutes (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 minutes at 37 °C. Incubation with OmpA-TM$_{S.flex}$ resulted in near-complete ejection, as previously reported (29), with only ~ 15% remaining virions (Fig. 2.6A). As expected, and based on our *in*

Figure 2.5: Circular dichroism spectra of selected OmpA-TMs

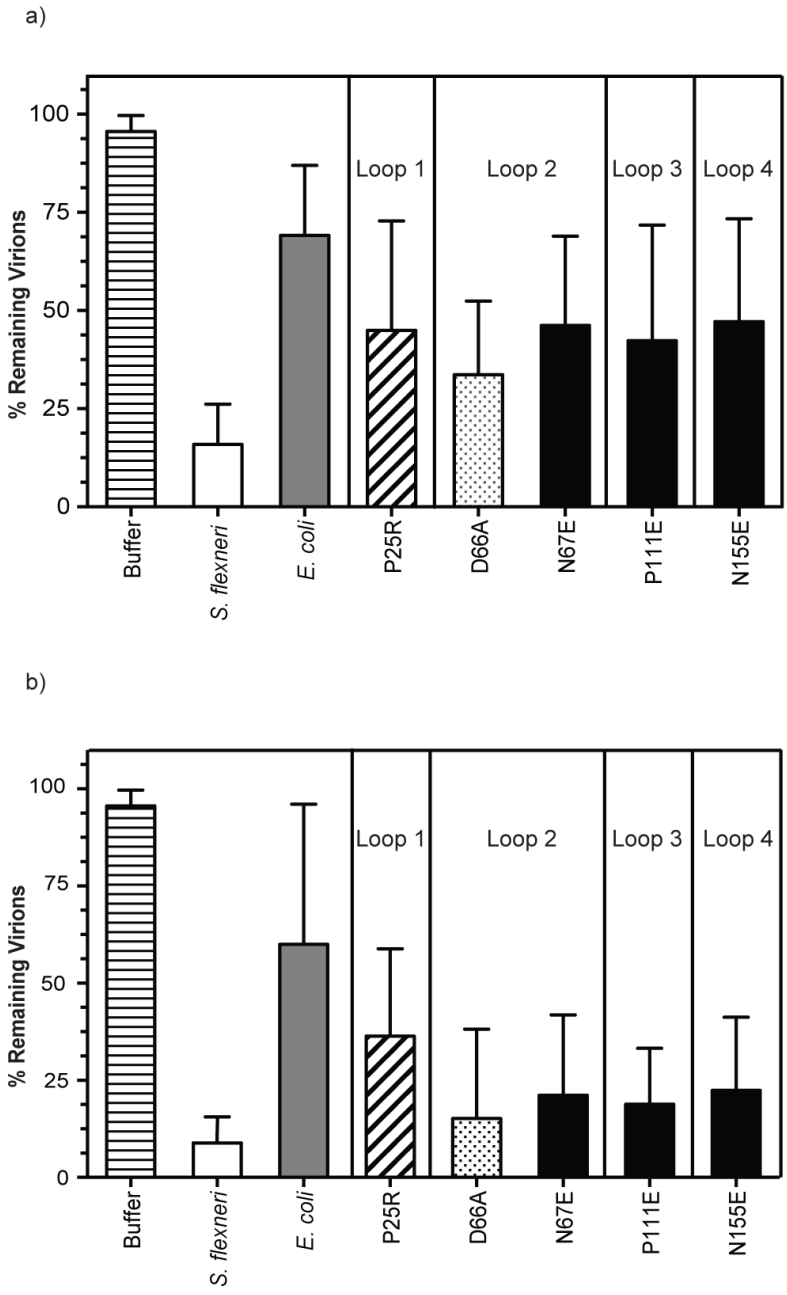


Representative CD spectra of OmpA-TM$_{E.coli}$, and OmpA-TM variants are shown. Open circles indicate the CD spectrum of OmpA-TM$_{S.flex}$ and are the same data shown in each panel.

vivo data (see Fig. 2.3), OmpA-TM$_{E.coli}$ *in vitro* was unable to efficiently induce genome ejection of Sf6 comparable to OmpA-TM$_{S.flex}$, with ~ 75% remaining virions after 10 minutes (Fig. 2.6A). 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$_{S.flex}$, the D66A variant does, on average, induce more genome ejection than the other versions of OmpA-TM (Fig. 2.6A), 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. 2.3) and *in vitro* (Fig. 2.6), 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$_{S.flex}$. Variant OmpA proteins are complemented from the same vector in our *in vivo* assays and are likely similar in abundance to OmpA$_{S.flex}$ and therefore readily available

Figure 2.6: 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 minutes (a) and 60 minutes (b) post mixing. Color-coding scheme is consistent with Fig. 2.3. Each data point is an average of at least five separate experiments; error bars signify one standard deviation.

for phage binding. However, there is still a decreased relative titer of Sf6 on these strains (Fig. 2.3). 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.

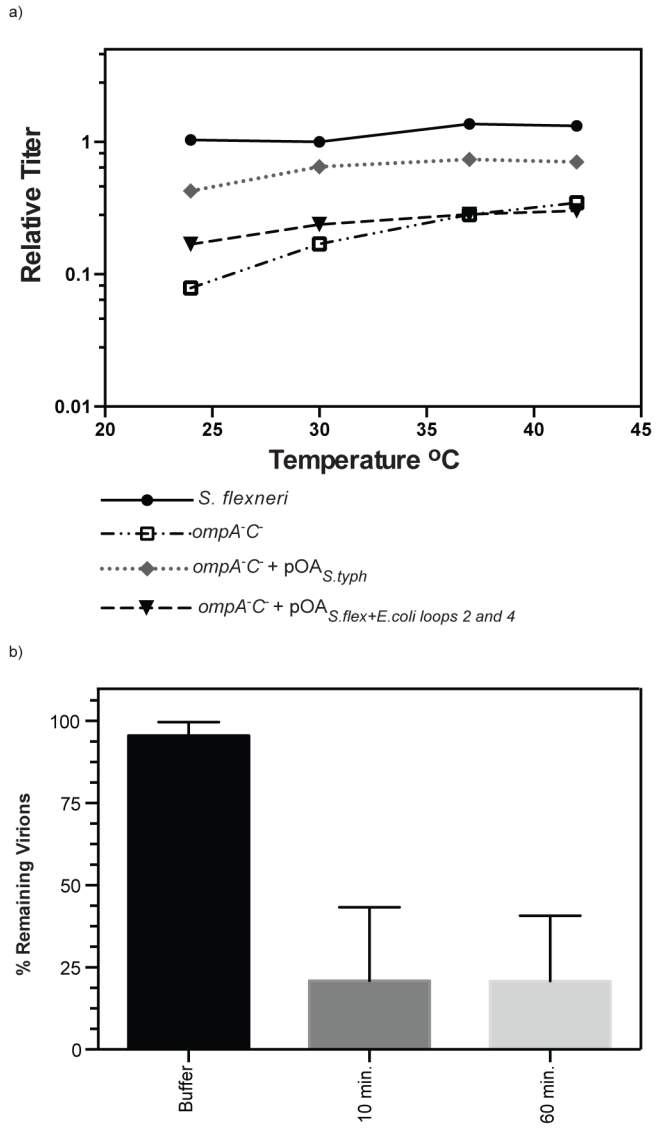
We therefore increased the incubation time to 60 minutes and measured the percent remaining PFUs under the conditions specified above. After 60 minutes incubation with OmpA-TM$_{E.coli}$, Sf6 is still unable to release its genome at the same level induced by OmpA-TM$_{S.flex}$ (Fig. 2.6B). However, we did see an increase in genome ejection with the other OmpA-TM variants starting to approach OmpA-TM$_{S.flex}$ levels. Lastly, after 60 minutes of incubation, the D66A variant induces genome ejection with efficiency similar to OmpA-TM$_{S.flex}$ (Fig. 2.6B).

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 2.7A).

Sf6 can tolerate several independent differences in loops 1 and 3 (Fig. 2.3). 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

Figure 2.7: 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. 2.2. B) Ejection efficiency of Sf6 incubated with parent *S. flexneri* LPS and OmpA-TM$_{S.\ typh}$ at 10 minutes and 60 minutes post mixing. Each data point is an average of at least five separate experiments; error bars signify one standard deviation.

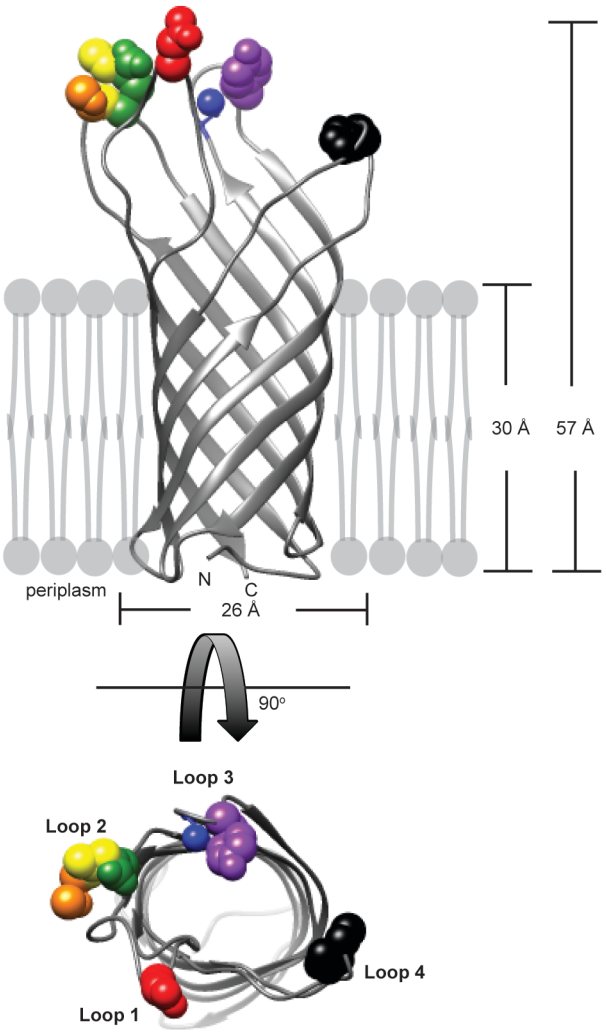
between *S. flexneri* and *S. typhimurium* OmpA include amino acid substitutions P25H and N27D in loop 1, A108S in loop 3, and the insertion “GASF” in loop 3 is GPST in *S. typhimurium* OmpA. However, these two proteins have identical sequences in loops 2 and 4. We therefore measured the ability of $OmpA_{S.typh}$ 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 restore the efficiency of infection of Sf6 close to that of $OmpA_{S.flex}$ (Fig. 2.7A). 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 (TM_{50}= 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 minutes. As expected, and consistent with our *in vivo* data (Fig. 2.7A), OmpA-$TM_{S.typh}$ is able to induce genome ejection of Sf6 at levels close to OmpA-$TM_{S.flex}$ *in vitro* (Fig. 2.7B). 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, 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 loops 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. 2.8), suggesting that these coliphages may not interact with the entire OmpA surface, but rather a preferential side of the protein. Additionally,

Figure 2.8: Sf6 interacts with entire OmpA surface



The crystal structure of *E. coli* OmpA (PDB: 1BXW (43)) 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 (44).

isolated mutations in the receptor for phage λ, LamB, that confer resistance to phage infection (45, 46) appear have a strong bias to a preferential side of LamB: when we modeled these amino acid substitutions into the LamB crystal structure (PDB: 1AF6 (47)), 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. 2.8). 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 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. 2.3) and the *in vitro* genome ejection data (Fig. 2.6). 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 the host to evolve changes in the loops of OmpA, to avoid Sf6 infection, OmpA has several other roles (48), including attachment to and invasion of eukaryotic cells (36, 49-54). 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 (49, 51, 52, 54). 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 (49). 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 *Shigella flexneri* pathogenesis. Not evolving resistance to phage infection is likely a trade-off to retaining pathogenicity, although this remains to be determined experimentally.

In this chapter, 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*$^{-}$*C*$^{-}$ *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 (LB) 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 (55). Phage were stored in phage buffer: 10mM tris, pH 7.6 and 10mM $MgCl_2$. *S. flexneri* strains include the parent strain PE577 (56) 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 minutes 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 below).

LPS extraction and in vitro *genome ejection experiments*

S. flexneri lipopolysaccharide (LPS) was extracted from the parent *S. flexneri* strain using a BulldogBio kit as 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 minutes 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 plaque forming units (PFUs) at each time point by the PFUs with buffer only added at t = 0 minutes. *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 minutes 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 25 - 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$_{S.flex}$, purified OmpA-TMs ranging in concentration 0.6 - 4 mg/mL were incubated at temperatures between 25 and 95 °C, run by 15% SDS-PAGE and 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 California USA, www.graphpad.com. 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 mm, scanning rate of 50 nm/min, and data integration time of 1 sec. 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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CHAPTER 3

Whole Virion Biosensing: Kinetic Analysis of Bacteriophage Sf6 and Outer Membrane Protein A

ABSTRACT

For a successful infection, most viruses must recognize their respective host cells. A common mechanism of host recognition by viruses is to utilize a portion of the host cell as a receptor. Bacteriophage Sf6, which infects *Shigella flexneri*, uses lipopolysaccharide as a primary receptor and requires a secondary receptor, a role fulfilled by outer membrane protein (Omp) A or C. Our previous work showed that specific residues in the loops of OmpA mediate Sf6 infection and result in a range of Sf6 infection efficiencies. To better understand Sf6 interactions with OmpA variants, we determined the kinetics of these interactions by BioLayer Interferometry, an optical biosensing technique used to measure the kinetic parameters of biomolecular interactions. Here, we successfully immobilized whole Sf6 virions and determined the binding constant of Sf6 to OmpA, and found it to be in the low nM range. Additionally, we show that Sf6 binds to five variant OmpAs and the resulting kinetic parameters vary only slightly. Based on these data, we propose a model in which Sf6:Omp receptor recognition is not only based on kinetics, but likely on the ability of the Omp to induce the correct conformational changes that result in infection.

INTRODUCTION

Virtually all viruses must translocate their genetic information into their respective host cells and replicate via the host cell machinery to produce progeny (1). The most abundant viruses known are the dsDNA bacteriophages, viruses that infect bacteria, with the global population estimated to be over 10^{30} (2), yet the molecular mechanisms that govern their host attachment are not completely understood. This essential step of host recognition must be well coordinated by the virus in order to ensure successful progeny formation, as premature genome ejection can negatively impact the future of the virus. One common mechanism of infection bacteriophages employ is to utilize a portion of the host cell as a receptor (3). For example, teichoic acid, peptidoglycan, and other components of Gram-positive bacteria have been shown to be receptors for many phages (4-9). Due to differences in cell wall composition, lipopolysaccharide (LPS) and proteins localized in the outer membrane of Gram-negative bacteria are also used as phage receptors (4). For instance, bacteriophage T7 recognizes the LPS of *Escherichia coli*, which leads to conformational changes in the tail machinery that result in T7 genome translocation into the bacterial cell (10). S16, a phage that has a broad host range and can infect many *Salmonella* species, recognizes outer membrane protein C (OmpC) (11). Other outer membrane proteins (Omps) are used as phage receptors for diverse phages and include, but are not limited to, OmpA, OmpF, and LamB (12-16).

Bacteriophage Sf6 is a short-tailed dsDNA virus that belongs to the subgroup of the “P22-like” phages in family *Podoviridae* that infects *Shigella flexneri* (strain PE577 (17)). Sf6 infection into *S. flexneri* is a two-step process; first, the phage recognizes the LPS on the surface of the cell via its tailspikes (18, 19). Second, the phage requires

interaction with a secondary protein receptor, an Omp, to commit to infection (20). Work with other bacteriophages has shown that host range mutants can utilize alternative receptors to gain access to hosts (14, 15, 21, 22). However, bacteriophage Sf6 seems to be unique in that it has the inherent ability to utilize more than one Omp as a secondary receptor (20). Sf6 preferentially uses OmpA, but can also use OmpC, and likely a third unidentified receptor (20). Our previous work showed that specific residues in the loops of OmpA mediate Sf6 infection and confer host range (18). Individual amino acid substitutions in OmpA resulted in a range of Sf6 infection efficiencies (18). In an effort to better understand Sf6 interactions with OmpA variants, we determined the kinetics of these interactions through the use of BioLayer Interferometry (BLI).

BLI is an optical biosensing technique used to measure the kinetic parameters of biomolecular interactions (23, 24). It works by immobilizing one binding partner, the ligand, to a sensor tip. The sensor tip with the immobilized ligand is then dipped into a sample that contains varying concentrations of the analyte, the binding partner. This technology works by analyzing the changes in the pattern of white light from optical layers on the sensor. Any interactions between the immobilized ligand and the analyte will cause a shift in the interference pattern that is then measured. This wavelength shift is a direct measurement of the change in molecules bound to the ligand on the sensor which is plotted against time. From these data, on and off rate constants can be determined.

Previously published kinetic analyses performed by BLI for viruses have used purified host receptors and studied interactions with purified viral receptor binding proteins (25-27); yet, no such studies exist for bacteriophages. Here, we successfully

immobilize intact Sf6 virions. To our knowledge, this represents the first study of whole virion immobilization completed on the Forté BLI platform. We determined the binding constant of Sf6 to OmpA, and found it is in the low nM range. Moreover, we show that Sf6 bound to five variant OmpAs and the resulting kinetic parameters vary only slightly. These results suggest that the altered infection efficiencies observed *in vivo* (18) are not solely dependent on the rate at which Sf6 interacts with OmpA.

RESULTS

Temperature does not significantly change kinetic parameters of Sf6 and OmpA-TM$_{S.flex}$

To our knowledge, to date, no published BLI studies have been conducted to investigate the kinetics of bacteriophages and their purified host receptors. Previously published kinetic analyses performed by BLI for animal viruses and their respective host receptors have shown that binding affinities are in the μM-pM range (25-27). Surface plasmon resonance (SPR), another optical biosensing technique, has also been used to study virus:host interactions. For example, Bonaparte et al. showed that the binding constant for Hendra virus attachment glycoprotein to its receptor, human ephrin-B2 is 1 nM (28). Another SPR study has shown that purified receptor binding proteins of human coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), and a bat coronavirus HKU4 can bind to human CD26 with K_Ds of 18.4 nM and 35.7 μM, respectively (29). Recently, Marti et al. have shown via SPR that the binding affinity of the long tail fiber of bacteriophage S16, the phage tail protein that mediates interaction with the host, and its host *Salmonella* is ~ 5 nM (11). Taking into account these reported literature values, we hypothesized that the binding affinity of Sf6 to *S. flexneri* OmpA would likely be in the nM range.

To test our hypothesis, we purified the transmembrane domain of *S. flexneri* OmpA (OmpA-TM$_{S.flex}$) (18). For all experiments described below, we measured the ability of OmpA-TMs to induce Sf6 genome ejection *in vitro* prior to performing BLI experiments, to confirm we had functional OmpA-TMs (18, 20) (data not shown). To ensure that sodium acetate, pH 4.0 buffer (a low pH buffer in which phage are not typically stored) had no effect on the phage, we monitored the titer of the phage stock over time and

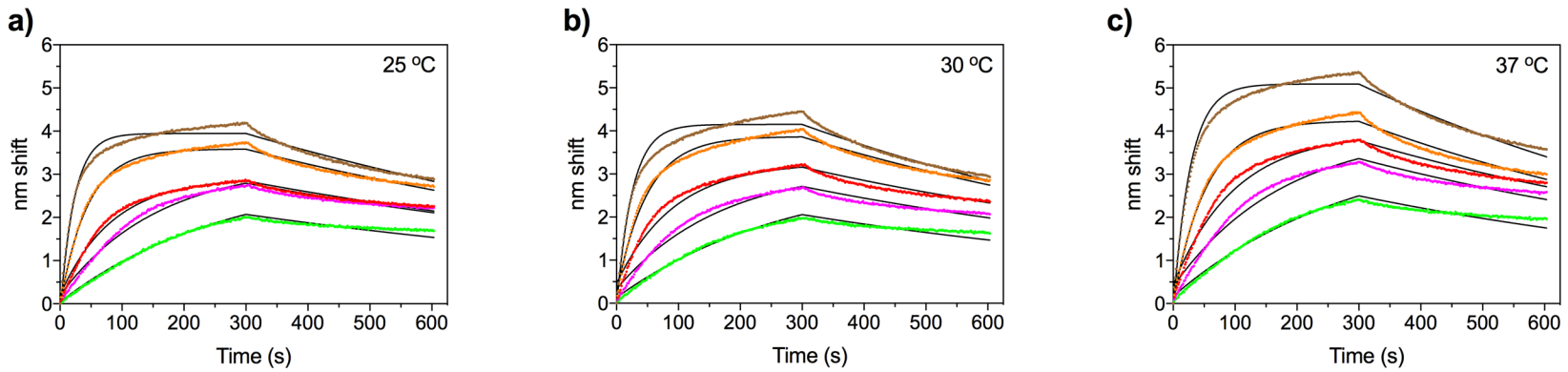
compared it to phage stored in phage dilution buffer, pH 7.6, and found no significant differences (data not shown). Moreover, we tested the ability of OmpA-$TM_{S.flex}$ to induce genome ejection of Sf6 stored in NaOAc, pH 4.0 buffer and found it to be similar to previously published results (18, 20) (data not shown). Therefore, the buffer likely has no effect on the ability of the phage to recognize its purified receptors (LPS and OmpA-$TM_{S.flex}$) to induce genome ejection *in vitro.* For all BLI experiments described herein, the ligand, Sf6 was immobilized on amine reactive (AR2G) sensors and OmpA-TM reconstituted into detergent micelles was used as the analyte.

To determine kinetic parameters of Sf6 and OmpA-$TM_{S.flex}$, we measured the change in interference patterns over time to generate sensorgrams at 25, 30, and 37 ^{o}C (Fig. 3.1, a-c). The generated data were fit in GraphPad prism to a global 1:1 association-then-dissociation model (Fig. 3.1, a-c). Calculated kinetic parameters are shown in Table 3.1. The analyte concentrations tested ranged from 1,000 nM to 62.5 nM. Consistent with our hypothesis and published results from other bacteriophage and host receptor biosensing work (11, 25-29), OmpA-$TM_{S.flex}$ bound Sf6 with nM affinity and varied only slightly in kinetic parameters with changing temperature (Table 3.1). Based on the calculated parameters in Table 3.1, OmpA-$TM_{S.flex}$ bound Sf6 with relatively fast-on and slow-off kinetics. Overall, these data suggest that temperature differences do not significantly affect Sf6 binding to OmpA.

Sf6 genome ejection efficiency is highest at 37 ^{o}C

We were surprised that the binding kinetics did not change with temperature as phage ejection can often be affected by temperature (8, 30-33).Therefore, we

Figure 3.1: BLI analysis of Sf6 and OmpA-TM$_{S.flex}$



BLI sensorgrams are shown for immobilized Sf6 and varying concentrations of OmpA-TM$_{S.flex}$ analyte 1,000 (brown), 500 (orange), 250 (red), 125 (magenta), and 62.5 (green) nM at (a) 25, (b) 30, and (c) 37 ^{o}C. Reference subtracted raw data are shown as points and global 1:1 association-then-dissociation non-linear fits are shown as solid black lines. Association and dissociation times were 300 s. Binding constants generated from a-c are shown in Table 3.1.

Table 3.1: Binding constants for Sf6 and OmpA-TM$_{S.flex}$

Temperature (°C)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
25	4.3×10^{4}	1.0×10^{-3}	23.3
30	3.8×10^{4}	1.2×10^{-3}	31.2
37	3.5×10^{4}	1.3×10^{-3}	36.4

Kinetic parameters for sensorgrams shown in Fig. 3.1 (a-c)

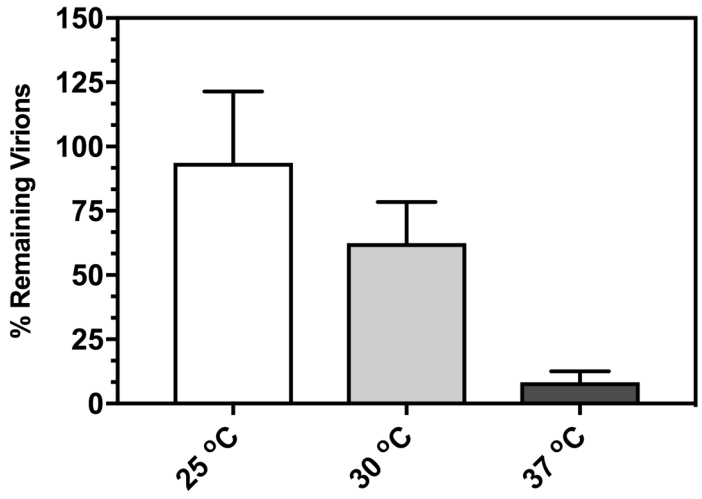
investigated if Sf6 ejection was affected by varying temperatures. We induced genome ejection *in vitro* using our standard assay (18, 20) and measured the efficiency of genome ejection at 25, 30, and 37 ^{o}C using plaque assays (Fig. 3.2). In this experiment, we incubated the reactions for 10 minutes, which is within the timeframe of our observed BLI association phases. Consistent with previously reported data, at 37 ^{o}C, the majority (>95%) of Sf6 virions have lost their genetic information at 10 minutes post initiation of ejection (Fig. 3.2) (18, 20). However, as temperature decreased, the observed genome ejection efficiency *in vitro* also decreased. For example, at 30 ^{o}C, ~ 40% of virions have lost their genetic information and only ~ 10% at 25 ^{o}C (Fig. 3.2). Therefore, all BLI experiments performed below were completed at 37 ^{o}C.

Sf6: OmpA-TM binding is one-state

As seen in Fig 3.1 a- c, a 1: 1 association then dissociation model appears to fit the data very well, except at higher concentrations, where a possible secondary component is visible. We hypothesized that this could be due to either 1) too high of an analyte concentration (experimental design) or 2) perhaps a one-state model is not the best to describe the interactions. Thus, we opted to collect BLI data at lower concentrations of analyte (OmpA-TM$_{S.flex}$), ranging between 250 nM to 7.8 nM, and to use a more statistically intensive method to determine the best model to describe the data.

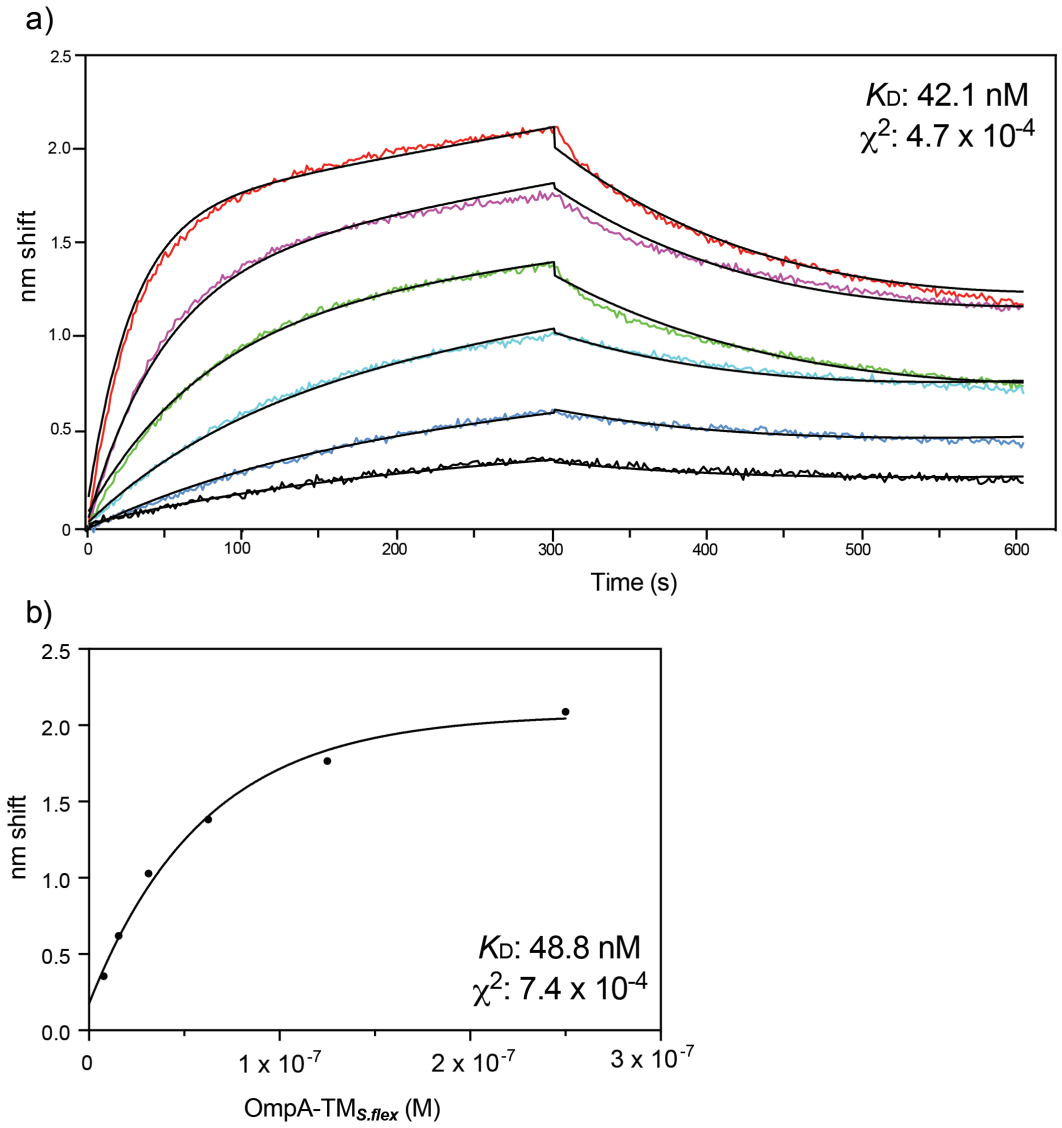
Using BiaEvaluation software and GraphPad Prism, we fit the data to several kinetic models and found that the best model was a 1:1 association-then-dissociation

Figure 3.2: Sf6 *in vitro* genome ejection efficiency with LPS and OmpA-$TM_{S.flex}$ at various temperatures



Ejection efficiency of Sf6 incubated at 25, 30, or 37 ^{o}C for 10 minutes with LPS + OmpA-$TM_{S.flex}$. "Percent remaining virions" was calculated as the number of PFUs remaining after incubation at each temperature divided by the number of PFUs when treated with buffer only. Each data point is an average of at least three separate experiments; error bars signify one standard deviation.

Figure 3.3: Kinetic analysis of Sf6 and OmpA-TM$_{S.flex}$



a) A BLI sensorgram is shown for immobilized Sf6 and varying concentrations of OmpA-TM$_{S.flex}$ analyte 250 (red), 125 (magenta), 62.5 (green), 31.25 (cyan blue), 15.6 (blue), and 7.8 (black) nM at 37 ^{o}C. Reference subtracted raw data are shown and global 1:1 association-then-dissociation non-linear fits with drifting baseline are shown as solid black lines. Association and dissociation times were 300 s. (b) Isotherm generated from data in a). Calculated K_D and χ^2 are shown.

Table 3.2: Binding constants for Sf6 and OmpA-TM$_{S.flex}$

k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)	χ^2
1.21×10^{5}	5.1×10^{-3}	42.1	4.77×10^{-4}

Kinetic parameters for sensorgram shown in Fig. 3.3 (a)

model with drifting baseline (Fig. 3.3 a). We also generated an isotherm plot from the data shown in Figure 3.3 a and showed that the K_D calculated from this method is similar to the one generated by the one-state model with drifting baseline, 48.8 nM compared to 42.1 nM, respectively (Fig. 3.3 and Table 3.2). The accuracy of the fits is described by χ^2 (34); the lower the χ^2, the better the model describes the fit of the data. In addition to χ^2, the one-state model was the only one to pass the *F* test (35). Thus, the potential secondary component that is visible at the higher concentrations of analyte (we saw this phenomenon for all OmpA-TM variants, data not shown), is minor at best, and probably reflective of some artifact rather than biology.

Recent work has compared different biosensor platforms and evaluated the strengths and weaknesses of each platform by looking at data consistency, comparability, and operational efficiency (34). This work showed that for data collected on a Forté platform, using the Octet RED384 BLI, drifts were observed in the data, particularly the dissociation curves. The authors suggested that this is likely due to sample evaporation over time in the plate, as the instrument is not a closed system. Although the Octet RED384 is a different platform than the one on which we collected data (Octet QK), similar instrument and experimental designs are utilized. Thus, it is likely that the slight deviation from a 1:1 association-then-dissociation model we see (Fig. 3.1 a-c), without incorporating a drifting baseline, is due to instrument and experimental design, although differences in calculated kinetic parameters between the two models are not significant (Table 3.1 and Table 3.2). Therefore, a one-state model fits well and we opted to use a global 1:1 association-then-dissociation model to calculate the kinetic parameters for the experiments described below.

Sf6 binds different OmpA-TMs at the same affinity as OmpA-TM$_{S.flex}$

In chapter 2, we showed that amino acid substitutions in OmpA resulted in altered infection efficiencies of Sf6 and altered *in vitro* genome ejection efficiencies (18). We hypothesized that these changes may be due to differences in binding affinities of the phage to its secondary receptor. Here, we purified *E. coli* OmpA-TM (OmpA-TM$_{E.coli}$) and four variant *Shigella* OmpA-TMs (D66A, N67E, P111E, and N155E) that displayed a range of phenotypes (18) and measured the kinetic parameters of Sf6 to these receptors.

We calculated kinetic parameters for OmpA-TM$_{E.coli}$ (Table 3.3) and *Shigella* OmpA-TM variants (Table 3.3) from BLI data collected at 37 ^{o}C. OmpA-TM$_{E.coli}$ and all *S.flexneri* OmpA-TM variants bound Sf6 with nM affinity and their kinetic parameters varied only slightly when compared to OmpA-TM$_{S.flex}$. The calculated K_Ds ranged between 6.86 and 65.4 nM. The calculated parameters were consistent with a fast-on and slow-off kinetics. Overall these data show that there are no large calculated kinetic differences in the rates at which Sf6 binds variant OmpA-TMs.

Table 3.3: Binding constants for Sf6 and various OmpA-TMs at 37 ^{O}C.

Protein	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
OmpA-TM$_{E.coli}$	8.7×10^{4}	2.2×10^{-3}	24.8
D66A	1.1×10^{5}	1.4×10^{-3}	12.9
N67E	5.2×10^{4}	3.4×10^{-3}	65.4
P111E	9.3×10^{5}	3.1×10^{-3}	32.8
N155E	9.9×10^{5}	6.8×10^{-4}	6.86

Kinetic parameters calculated from one-state association-then-dissociation fits.

DISCUSSION

In this chapter, to test our hypothesis that phenotypic differences seen *in vivo* (18) may due to differences in binding affinities of the phage to its secondary receptor, *S. flexneri* OmpA, we purified six versions of OmpA-TM (*S. flexneri, E. coli*, and *S. flexneri* variants D66A, N67E, P111E, and N155E). To determine the kinetic parameters of Sf6 and OmpA-TMs, we immobilized whole Sf6 virions on AR2G sensors and measured changes in the interference of white light using BLI to generate sensorgrams. Consistent with BLI and SPR studies published with purified phage proteins and host cells (11) or purified receptor proteins (36), we determined the binding affinity of Sf6 to OmpA-$TM_{S.flex}$ to be nM affinity (Fig. 3.1 and Table 3.1). Kinetics were fast – on and slow – off and fit a simple one – state model. Furthermore, OmpA-$TM_{E.coli}$ and *S. flexneri* OmpA-TM variants bound Sf6 with similar affinities and their calculated kinetic parameters varied only slightly when compared to OmpA-$TM_{S.flex}$ (Table 3.3).

The data presented here suggest that the previously reported differences in Sf6 infection efficiency we see *in vivo* and the differences in Sf6 genome ejection efficiency *in vitro* (18) are likely not based solely on kinetics. There are no significant kinetic differences between the various OmpA-TMs, so the ability of Sf6 to bind the Omps is not likely altered. Our data are similar to another study where the authors used SPR to examine the kinetics of purified bat coronavirus receptor binding proteins to human receptor CD26 (29). The authors show that amino acid substitutions in one of the binding partners (the coronavirus receptor binding protein) do not greatly affect the overall kinetic parameters. Their data support the idea that virus:host recognition is more complex and not dependent only upon binding affinities. In combination with the

work presented here, the coronavirus data suggest that this may potentially be a common theme throughout virology. So, what may be causing the changes in infection efficiencies we see? We hypothesize that it may have something to do with conformational changes in the phage proteins upon interaction with receptors.

The current working model for *Podoviridae* attachment has three steps (37). In the first, reversible, step a virion binds to LPS, the primary receptor, via its tailspikes. Cleavage of the LPS brings the phage closer to the surface of the cell where it may now interact with a putative secondary receptor; in the case of Sf6 this involves Omps (preferentially OmpA) (20). This step is referred to as an irreversible interaction (37). Third, in order to move the genetic information from the phage capsid into the cell, several conformational changes must occur. Hu et al. have shown that bacteriophages T4 (*Myoviridae*) (38) and T7 (*Podoviridae*) (39) undergo extensive structural remodeling during infection, particularly the tail machinery of the phages. We have previously shown that there are minor differences in Sf6 virion structure pre and post genome loss (20). Although limitations in the resolution make it difficult to discern precisely which proteins undergo changes, there do appear to be slight changes in parts of the tail machinery. We hypothesize that since amino acid substitutions in the surface exposed loops of OmpA do not affect the ability of the phage to bind the protein, the necessary conformational change(s) in the tail machinery to translocate genomic information are not induced correctly.

Based on these data, we propose a model in which Sf6:Omp receptor recognition is not solely based on kinetics, but likely on the ability of the Omp to induce the correct conformational changes (Fig. 5.1, Chapter 5). First, Sf6 interacts with LPS via its

tailspikes (20, 37, 40). Once Sf6 has cleaved LPS and is close enough to the surface of the cell it can interact with its secondary receptor, an Omp (20). Upon interaction with Omps by the tail machinery, a conformational change in the phage is likely triggered. Amino acid substitutions in the loops of OmpA may affect the ability of the phage to adopt the correct conformation that eventually results in the formation of a channel to translocate the DNA (37, 41-43). Although more work is necessary to discern a complete understanding of Sf6 (and *Podoviridae*) infection, the data presented here shed light on the kinetics of Sf6 interaction with a secondary receptor, OmpA, an important aspect of host recognition.

MATERIALS AND METHODS

Media and strains

Bacterial growth, plating experiments, and preparations of Sf6 phage stocks were all completed in Lysogeny broth (LB). Bacteriophage Sf6 (clear plaque mutant (44)) was propagated on *ompA*$^{-}$*C*$^{-}$ *S. flexneri* as previously described (20). Phage used for *in vitro* genome ejection experiments was stored in phage buffer (10 mM Tris, pH 7.6 and 10 mM $MgCl_2$) and phage used for BLI experiments was stored in NaOAc buffer (10 mM sodium acetate, pH 4.0 and 2 mM $MgCl_2$) (see below). *S. flexneri* strains used include parent strain PE577 (17) and *ompA*$^{-}$*C*$^{-}$ null *S. flexneri* (20). Variant OmpA-TMs were expressed from *E. coli* BL21/DE3/pLysS cells as previously described (18, 20).

LPS extraction and in vitro *genome ejections*

Using a BulldogBio kit, *S. flexneri* LPS was extracted from PE577, as previously described (20). Sf6 was incubated at 25, 30, or 37 °C with purified PE577 LPS (0.5 mg/mL) and OmpA-$TM_{S.flex}$ (0.05 mg/mL). The "percent remaining virions" was calculated by dividing the PFUs in each reaction by the PFUs with buffer only added. Plates were grown overnight at 30 °C on PE577.

BioLayer Interferometry

Kinetic analyses of variant OmpA-TMs binding to Sf6 phage were performed on a FortéBio (Menlo Park, CA) Octet QK using amine reactive sensors (AR2G) at 25, 30, or 37 ^{o}C. All volumes were 200 μL. A stock of Sf6 phage in NaOAc buffer at a titer of $1x10^{10}$ phage/mL was used for all experiments. For rapid immobilization of phage, the

AR2G sensors were wet and activated in 10 mM sulfo-NHS (N-hydroxysulfosuccinimide) and 400 mM EDC (1-ethyl-3-(3 dimethylaminopropyl)carbodiimide hydrochloride) for 300 seconds. Sensors were then dipped for 600 seconds in the phage stock followed by quenching in 1 M ethanolamine, pH 8.5 for 300 seconds. Baseline was established in 1.8 mM Triton X-100 over a period of 300 seconds. Sensors were then exposed to various OmpA-TM analytes (ranging between 1,000 nM and 7.8 nM) for 300 seconds to measure association. Dissociation was measured for 300 seconds by dipping the sensors into 1.8 mM Triton X-100. Data were reference subtracted. Nonspecific binding was measured by exposing a sensor without immobilized phage to the highest concentration of OmpA-TM$_{S.flex}$ and was found to be negligible. Data were fit using GraphPad Prism 7 for Mac OS X, GraphPad Software, La Jolla, CA, USA (www.graphpad.com) and BiaEvaluation Software. For most OmpA-TM variants, experiments were performed in triplicate; for OmpA-TM$_{S.flex}$, we performed >20 separate experiments, overall. The “global fits” were calculated from each set of experimental data, and overall there was relatively little binding variation between separate titrations.

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CHAPTER 4

Purified *Shigella flexneri* OmpC Induces Sf6 Genome Ejection *in vitro*

ABSTRACT

Outer membrane proteins (Omps), which are major constituents of the outer membrane of Gram negative bacteria, play important roles in bacterial growth and survival. Many bacteriophages, viruses that infect bacteria, have the inherent ability to utilize several types of Omps as an attachment mechanism. Bacteriophage Sf6, which infects *Shigella flexneri*, has been shown to use lipopolysaccharide as a primary receptor and also requires a secondary receptor, a role fulfilled by either OmpA or OmpC. Here, we adapted a *Salmonella typhi* OmpC purification protocol to purify *S. flexneri* OmpC. Using a limited proteolysis approach, we obtained trypsin resistant and functional trimeric OmpC. We show that the resultant OmpC in combination with lipopolysaccharide causes Sf6 genome ejection *in vitro*, but at a lower efficiency than OmpA. Overall, our data suggest a model in which Sf6 may have evolved to have differential interactions with Omps depending on the environmental conditions.

INTRODUCTION

Outer membrane proteins (Omps) are major constituents of the outer membranes of Gram negative bacteria and play many important roles in bacterial cellular growth and survival. Two of the most abundant Omps are OmpA and OmpC, which are present at >100,000 copies per cell (1-3). These homologous proteins are transmembrane β-barrels with differing pore diameters (1). They are important for maintaining the membrane integrity of cells, bringing in nutrients, protection against harsh environments, osmoregulation, and have been shown to play a role in bacterial antibiotic resistance (1, 3-6). Moreover, OmpA and OmpC have both been implicated as immunogenic targets (7, 8) and are important for bacterial adherence to and invasion into human cells (9, 10).

It is not surprising that some of the most abundant proteinaceous entities of the outer membrane are also used as receptors by bacteriophages. In addition to OmpA (11-17) and OmpC (13-15, 17-22), bacteriophages use many other Omps as receptors including: OmpF (22-24), LamB (22, 25), TonB (26), FhuA (26, 27), OmpT (14), Tsx (28), and OmpX (15). Likely due to the structural homology of Omps, phages either have the inherent ability, or can evolve to recognize, multiple Omps. For example, various isolated host range mutants of bacteriophage M1 can use OmpA or OmpC, and even OmpT, as a receptor to gain entrance into the host cell (14, 22). Bacteriophage Ox2 can evolve to use OmpA or OmpC (and OmpX) as a receptor (17, 29).

The model system in this study is bacteriophage Sf6, a short-tailed dsDNA virus that belongs to family *Podoviridae* and infects *Shigella flexneri* strain PE577 (30). Sf6 infection requires both primary and secondary receptors (13, 31). The primary receptor

is *S. flexneri* lipopolysaccharide (LPS) (13, 31). An initial and reversible interaction with LPS is followed by an irreversible interaction with a secondary receptor, an Omp (13). Sf6 has the inherent ability to use OmpA, OmpC, and potentially a third, as of yet unidentified, Omp (13).

In previous studies, phage adsorption to hosts with single *omp* knockouts was altered compared to the parent *S. flexneri* strain; Sf6 infection rates on *ompA*$^-$ decreased considerably, whereas Sf6 infection on *ompC*$^-$ was only slightly altered (13). Additionally, using a cell survival assay designed to measure the ability of Sf6 to kill the various *omp* null strains, we saw that Sf6 was able to kill *ompC*$^-$ hosts at increased efficiency compared to *ompA*$^-$(13). In the same study, we performed time lapse fluorescence microscopy to monitor the infection and genome translocation of Sf6 using the parent strain and three *omp* null knockouts: *ompA*$^-$, *ompC*$^-$, and *ompA*$^-$*C*$^-$. Interestingly, both single *omp* knockouts had similar times to infection. These times were later than the parent strain and earlier than the double *omp* null strain. Overall, our previous work with Sf6 demonstrated that OmpA and OmpC can function as secondary receptors, with OmpA being slightly preferred over OmpC (13). Although there are examples of viruses that utilize multiple receptors to gain entry into host cells (32, 33), there are few studies that fully investigate and characterize alternative receptor usage.

In this chapter we purify *S. flexneri* OmpC. In addition, we show that purified OmpC, in combination with LPS, is able to induce Sf6 genome ejection *in* vitro, but less efficiently than OmpA and LPS. The work presented here sheds light on alternative receptor usage in bacteriophage Sf6.

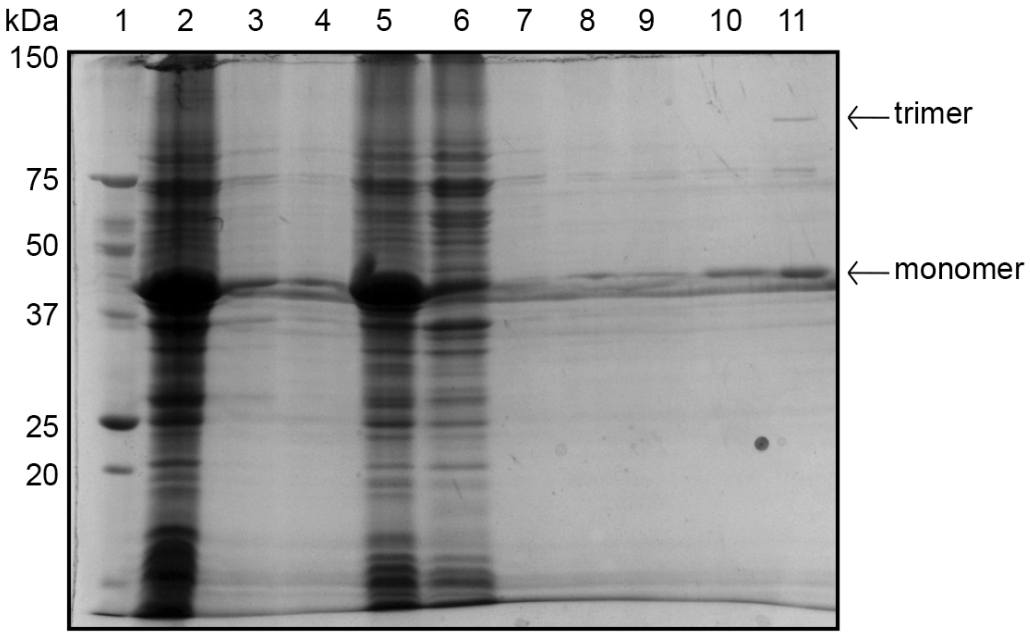
RESULTS

Purification and refolding of OmpCΔNT

OmpC is a receptor for many bacteriophages (13, 17-20, 34, 35) and several versions of OmpC have been previously purified, including those from *Escherichia coli* (36, 37), *Salmonella typhi* (38-40), and *Yersinia enterocolitica* (41). We adapted and modified one such approach (38) to overexpress and purify *S. flexneri* OmpC. As described for the purification of *Salmonella typhi* OmpC (38), we cloned and overexpressed *S. flexneri ompC* that encoded OmpC lacking the N terminal signal sequence (OmpCΔNT) from the PE577 strain (30). OmpCΔNT was then isolated as inclusions bodies (IBs) (Fig. 4.1). The IBs were solubilized with urea and unfolded OmpCΔNT was obtained (Fig. 4.1). Unfolded OmpCΔNT was refolded by rapid dilution as described for *Salmonella* OmpC (38). As shown in lane 11, only a small percentage of OmpCΔNT is a trimer; the majority remains unfolded (Fig. 4.1).

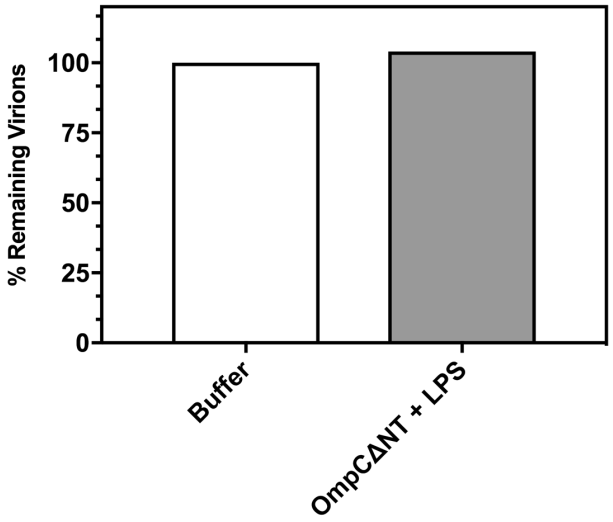
We further purified OmpCΔNT by anion exchange column chromatography to separate the two populations of refolded OmpCΔNT: the protease resistant trimer versus the metastable, protease sensitive trimer (38). Unlike *Salmonella* OmpC (38), purifying *Shigella* OmpCΔNT by anion exchange column chromatography did not yield functional, trimeric OmpCΔNT as tested by migration via gel electrophoresis (data not shown) and *in vitro* genome ejections (see below) (Fig. 4.2). Instead, we purified OmpCΔNT by trypsin digestion to obtain trypsin resistant trimeric OmpCΔNT, an alternative purification method suggested in Kumar and Krishnaswamy (38). After digestion, OmpCΔNT was concentrated using a centricon with a 100,000 Da cutoff and we determined OmpCΔNT folding by testing gel migration on a 12.5% SDS gel (Fig.

Figure 4.1: Purification of OmpCΔNT



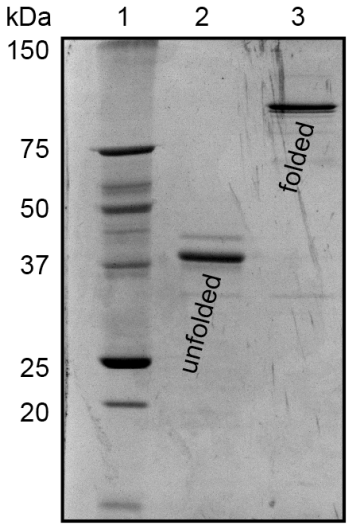
A 12.5% SDS gel, stained by Coomassie blue. Samples in all lanes were boiled at 95 ^{o}C for 5 minutes, except for lane 11. Lane 1, protein markers; 2, induced cells; 3, wash 1; 4, wash 2; 5, lysed cells; 6, wash 3; 7, wash 4; 8, wash 5; 9, wash 6; 10, unfolded OmpCΔNT; and 11, refolded OmpCΔNT.

Figure 4.2: Sf6 *in vitro* genome ejections with OmpCΔNT purified by anion exchange column chromatography



Ejection efficiency of Sf6 incubated at 37 ^{o}C for 10 minutes with LPS + OmpCΔNT. "Percent remaining virions" was calculated as the number of plaque forming units (PFUs) remaining after incubation with each variable divided by the number of PFUs when treated only with buffer. Only a representative data set is shown.

Figure 4.3: Folding assessment of trypsin-treated OmpCΔNT



A 12.5 % SDS gel, stained by Coomassie. Lane 1, protein marker; 2, OmpCΔNT (boiled at 95 ^{o}C for 5 minutes); 3, OmpCΔNT (not boiled). Lane 3 depicts folded, trimeric OmpCΔNT.

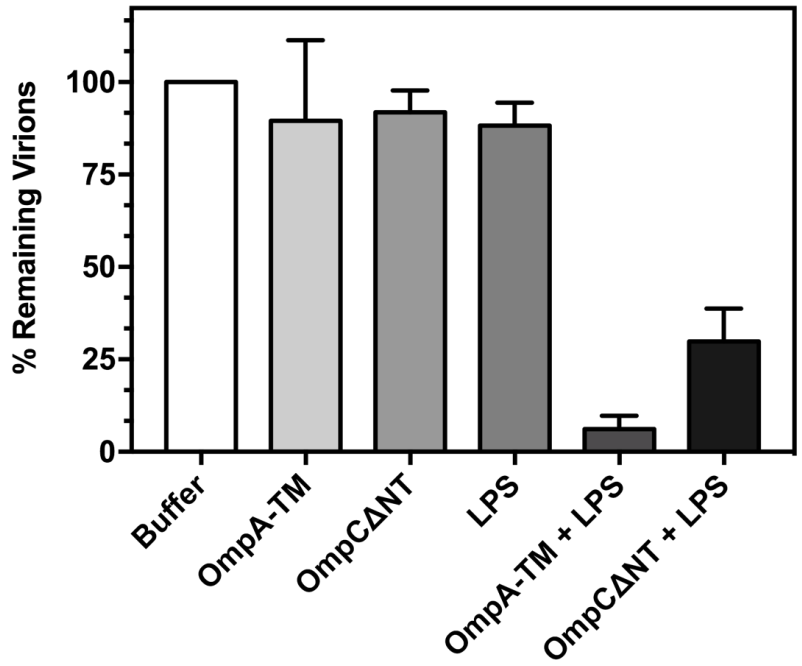
4.3). As depicted in figure 4.3, post trypsin digestion, OmpCΔNT is folded, exists as a trimer, and has a predicted molecular weight of 117 kDa (lane 3). The resultant OmpCΔNT was used for the assays described below.

OmpCΔNT induces Sf6 genome ejection in vitro

We used our previously developed *in vitro* genome ejection assay (13, 42) to test if OmpCΔNT in combination with purified host *S. flexneri* LPS induces genome ejection *in vitro.* We hypothesized that because OmpC is an alternative receptor, Sf6 genome ejection will be 1) not as efficient compared to OmpA and/or 2) at a slower rate than that of OmpA. For all *in vitro* genome ejection experiments described, genome ejection efficiency is measured using plaque assays to monitor the decrease in the number of infectious virions in a bulk population, thus we refer to Sf6 genome ejection rate as the observed rate of decrease in the overall number of infectious virions in the population over time.

Consistent with our previously published data (13), neither OmpA-TM (the transmembrane domain) or *S. flexneri* LPS alone trigger Sf6 genome ejection *in vitro.* In contrast, only a combination of OmpA-TM and LPS triggers ejection (Fig. 4.4). Here, Sf6 was incubated with OmpCΔNT alone or OmpCΔNT with LPS. Consistent with OmpA-TM, OmpCΔNT alone is not sufficient to induce genome ejection; Sf6 must be incubated with both OmpCΔNT and LPS for ejection to be triggered (Fig. 4.4). We determined the optimal concentrations of OmpA-TM and OmpCΔNT, where Sf6 genome ejection efficiency was highest, to use in the ejection assays by incubating Sf6 with a constant LPS concentration (previously determined to be optimal) and differing concentrations of

Figure 4.4: Sf6 *in vitro* genome ejection efficiency with LPS +/- Omps

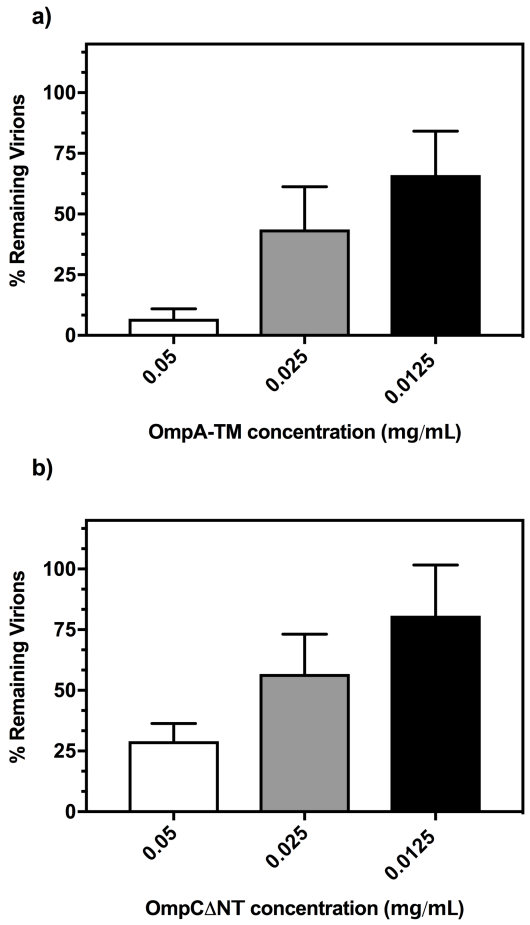


Ejection efficiency of Sf6 incubated at 37 ^{o}C for 10 minutes with: LPS, OmpA-TM, OmpCΔNT, LPS + OmpA-TM, or LPS + OmpCΔNT. “Percent remaining virions” was calculated as the number of PFUs remaining after incubation with each variable divided by the number of PFUs when treated only with buffer. Each data point is an average of at least three separate experiments; error bars signify one standard deviation.

either OmpA-TM or OmpCΔNT (Fig. 4.5). The observed optimal concentration of Omps was determined to be 0.05 mg/mL for both OmpA-TM and OmpCΔNT. Consistent with our first hypothesis, OmpCΔNT in combination with LPS is not as efficient at causing Sf6 genome ejection as OmpA-TM, with ~30% remaining virions compared to ~10% (Fig. 4.4, see discussion). Furthermore, increasing the OmpCΔNT concentration past 0.05 mg/mL did not increase genome ejection efficiency of Sf6 (data not shown).

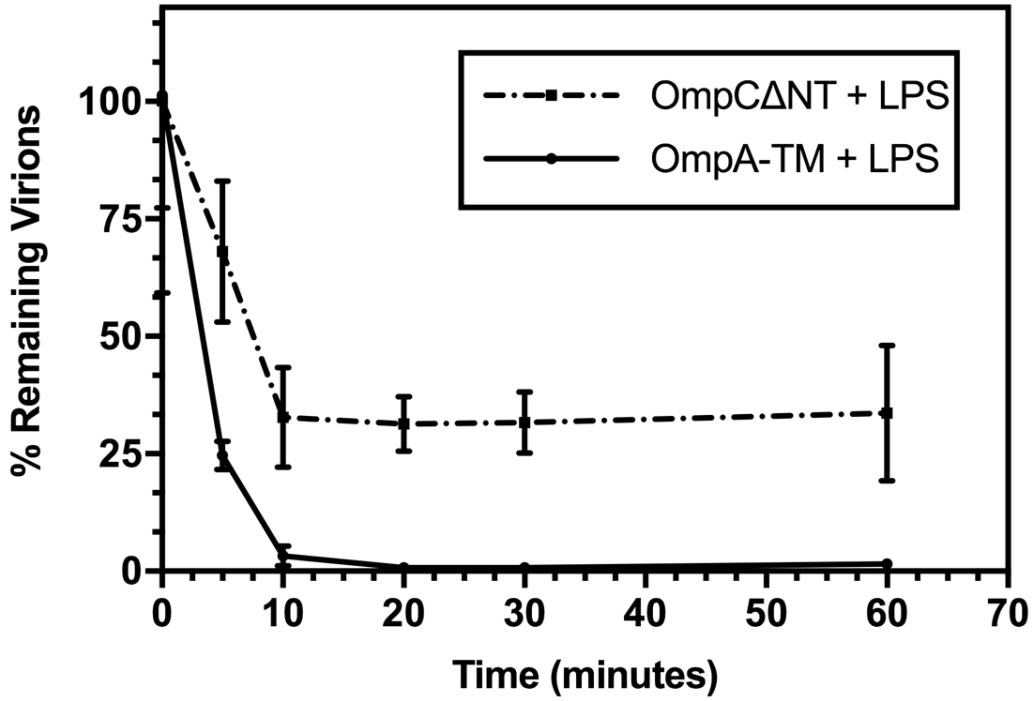
To measure if overall Sf6 ejection rate is slower in the presence of OmpCΔNT, we monitored virions by measuring the number of plaque forming units in the presence of LPS and Omps over time. Consistent with previous reports (13, 42), in the presence of OmpA-TM and LPS, functional virions are lost within 10 minutes, with ~10% of virions remaining (Fig. 4.6). In the presence of OmpA-TM or OmpCΔNT, the majority of Sf6 genome ejection occurs within 10 minutes and efficiency does not increase with increased incubation time. However, the initial rates of Sf6 ejection are slower in the presence of OmpCΔNT compared to OmpA-TM, as depicted by the different slopes in Figure 4.6. Overall, these data suggest that both rate and efficiency of Sf6 genome ejection is lower in the presence of OmpCΔNT, adding additional evidence to support the proposal that OmpA is slightly preferred over OmpC as a secondary receptor.

Figure 4.5: Sf6 *in vitro* genome ejection efficiency with LPS plus decreasing Omps



Ejection efficiency of Sf6 incubated at 37 ^{o}C for 10 minutes with: a) LPS + OmpA-TM or b) LPS + OmpCΔNT at decreasing concentrations. “Percent remaining virions” was calculated as the number of PFUs remaining after incubation with each variable divided by the number of PFUs when treated only with buffer. Each data point is an average of at least three separate experiments; error bars signify one standard deviation.

Figure 4.6: Sf6 *in vitro* genome ejection time course



Ejection efficiency of Sf6 incubated at 37 ^{o}C with: LPS + OmpA-TM, or LPS + OmpCΔNT. “Percent remaining virions” was calculated at each time point as the number of PFUs remaining after incubation with each variable divided by the number of PFUs when treated only with buffer at t = 0 minutes post incubation. Each data point is an average of at least three separate experiments; error bars signify one standard deviation. [1]

[1] Sf6 genome ejection efficiency in the presence of OmpA-TM and LPS is higher than previously published (13,42). This is likely due to improved protein sample purification methods which result in complete OmpA-TM protein folding.

DISCUSSION

In this chapter, we adapted a purification protocol from *S. typhi* OmpC (38) to purify *S. flexneri* OmpC. To further purify OmpCΔNT, we used a proteolysis approach to obtain trypsin resistant and functional trimeric OmpCΔNT (Fig. 4.3) as OmpCΔNT purified by anion exchange column chromatography did not induce Sf6 genome ejection (Fig. 4.2). This was likely due to the inability to obtain stable-trimeric OmpCΔNT using this method. Compared to OmpA-TM, trypsin resistant and functional trimeric OmpCΔNT is unable to induce genome ejection at the same efficiency (Fig. 4.4). As the concentration of OmpA-TM or OmpCΔNT is decreased, less genome ejection occurs, which makes sense as there is less receptor available for Sf6 binding. However, in all cases, genome ejection is more efficient in the presence of OmpA-TM than OmpCΔNT (Fig. 4.5). Not only is OmpCΔNT less efficient at inducing Sf6 ejection, but the initial rate of ejection is also slower than with OmpA-TM (Fig. 4.6). Therefore, in the presence of OmpCΔNT, both the rate and efficiency of Sf6 *in vitro* genome ejection are decreased.

On the cell surface, both OmpA and OmpC exist at >100,000 copies (1-3). So, why is OmpA the preferential secondary receptor over OmpC? The likelihood of Sf6 interacting with OmpA or OmpC is similar. We propose that this may have something to do with the regulation of Omps. OmpA is expressed constitutively and only downregulated once cells enter stationary phase (43). Sf6 only infects cells in exponential phase; therefore, this stationary phase decrease of OmpA would not affect infection. However, the expression of OmpC is tightly regulated in response to a variety of different parameters, including nutrient availability, osmolarity, and temperature (3). EnvZ/OmpR, a two-component system, regulates the expression of both OmpC and

OmpF, which are on the same regulon. With this mechanism, the total number of porin proteins present in the cell outer membrane remains relatively constant (3, 44), although the ratios of specific Omps will differ depending on environmental conditions. For example, OmpC is highly expressed during high osmolality, high nutrient availability, and higher temperatures, whereas OmpF is downregulated (3). In the opposite conditions, OmpF is upregulated and OmpC is downregulated. It is possible Sf6 has evolved to exploit and preferentially use an Omp that is not regulated by temperature/nutrient availability and whose expression levels are constitutive. Furthermore, we propose that with OmpC and OmpF being so closely regulated and given their sequence similarity (45), it is likely that the third, as of yet unidentified, receptor for Sf6 may be OmpF, although this is merely speculation and requires experimentation.

The *in vitro* data presented in this chapter add further support to our previous data, which suggest that OmpA is the preferred secondary receptor, whereas OmpC is an alternative receptor (13). In our previous studies, we see differences between the single *omp* knockouts at higher temperatures (37 ^{o}C in the single-step growth curves, and the cell survival assay), but virtually no differences at lower temperatures (25 ^{o}C in time lapse fluorescence microscopy experiments). Lower temperature and the differential expression of OmpC/OmpF may play a role. In *ompA*$^{-}$ *S. flexneri*, OmpC would be available for binding at the surface, however at lower temperatures, OmpC expression would be downregulated and OmpF would be present at the cell surface. In *ompC*$^{-}$ *S. flexneri*, OmpA and OmpF would both likely be present at the cell surface. We showed in chapter three that at room temperature (25 ^{o}C), OmpA-TM does not induce

Sf6 genome ejection *in vitro*. Therefore, perhaps in our *ompC*$^{-}$ knockout Sf6 may be interacting with OmpF. Overall, the data presented here in combination with our previous data suggest a model in which Sf6 may have evolved to have differential interactions with Omps depending on environmental conditions.

MATERIALS AND METHODS

Media and strains

Lysogeny broth (LB) was used for bacterial growth, plating experiments, and preparations of Sf6 phage stocks. Sf6 used in all experiments is a clear plaque mutant (46) and was propagated on *S. flexneri ompA*$^{-}$*C*$^{-}$ as previously described (13). Phage stocks were stored in phage buffer (10 mM Tris, pH 7.6 and 10 mM $MgCl_2$). *S. flexneri* strains used include the parent strain PE577 (30) and *ompA*$^{-}$*C*$^{-}$ (13). *E. coli* BL21/De3/pLysS cells were used for the overexpression of OmpA-TM and OmpCΔNT (see below).

Construct design

Genomic DNA from the parent *S. flexneri* was extracted and used for the construction of pOmpCΔNT. An overnight culture of PE577 was grown, shaking 200 rpm, and incubated at 37 °C. Cells were pelleted at 8,000 x g, 10 min, and pellets were resuspended in lysis buffer (0.6% SDS, 0.12 mg/mL Proteinase K, 1 mg/mL RNase1) made in TE buffer (10mM Tris, pH 8.0, 1mM EDTA, pH 8.0), and incubated at 37 °C for 1 hour. DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), twice with chloroform, and isolated by cold ethanol precipitation. The *ompC* gene was amplified from PE577 genomic DNA. Primers were designed to omit the N-terminal signal sequence and insert BamHI and HindIII cut sites at the 5' and 3' ends, respectively, using the forward primer 5' GCGCGGATCCGCTGAAGTTTACAACAAAGACGGCAAC 3' and reverse primer 5' GCGCAAGCTTTTAGAACTGGTAAACCAGACCCAGAGC 3'. The amplicon was then

cloned into BamHI and HindIII cut sites of pRSET_A_A185 vector (Life Technologies) for overexpression. This construct was subsequently named pOmpCΔNT and verified by Sanger sequencing at the Research Technology Support Facility at Michigan State University.

Overexpression of OmpCΔNT

E. coli BL21(DE3)pLysS cells were transformed with pOmpCΔNT. *E. coli* BL21(DE3)pLysS/pOmpCΔNT was grown overnight in LB with shaking (200 rpm) at 37 °C. Concentrated cells were stored in Buffer B (50 mM Tris, pH 8.5, 0.1 M NaCl, 2 mM EDTA) at - 80 °C until further processing.

Purification and refolding of OmpCΔNT

The purification and refolding of OmpCΔNT was adapted and modified from Kumar and Krishnaswamy (38). To isolate IBs, cells were thawed at 37 °C and washed three times with 0.8% NaCl. Cells were sonicated 6 x with 30 second bursts (99% amplitude) using a Sonics Vibra Cell. IBs were washed twice with Wash Buffer 1, (50 mM Tris, pH 8.5, 0.1 M NaCl, 2% Triton X-100), washed once with Wash Buffer 2 (50 mM Tris, pH 8.5, 0.1 M NaCl), and solubilized by shaking (200 rpm) at 37 °C overnight in IB Buffer (50 mM Tris,pH 8.5, 0.1 M NaCl, 4 M Urea). Solubilized IBs were centrifuged (8,000 x g, 15 min, 20 °C) to pellet debris. The supernatant (unfolded OmpCΔNT) was passed through a 0.2 μM filter. To refold OmpCΔNT, unfolded OmpCΔNT was added to Refolding Buffer (50 mM Tris, pH 8.5, 0.1 NaCl, 10% (v/v) glycerol, 0.2% (v/v) polyoxyethylene-9-laurylether($C_{12}E_9$)) at a 1:5 ratio and stirred at 4 °C overnight.

Trypsin digestion of OmpCΔNT

As described in (38), trypsin was added at a 1:400 ratio to OmpCΔNT and incubated at 37 °C for one hour. Following the protease digestion, OmpCΔNT was concentrated using a centricon (100 kDa cutoff). Purification and refolding of OmpCΔNT was confirmed by running samples on a 12.5% SDS gel. Gel densitometry (BIORAD Gel Doc XR +) was used to determine the concentration of OmpCΔNT.

Purification and refolding of OmpA-TM

S. flexneri OmpA-TM was purified and refolded as previously described (13, 42). Briefly, OmpA-TM was purified from IBs and refolded by nutation in 0.1% (1.8 mM) Triton X-100 at room temperature overnight. OmpA-TM was exhaustively dialyzed against 1.8 mM Triton X-100 to remove residual urea. Protein folding was confirmed by electrophoretic mobility via SDS-PAGE and protein concentration was determined by gel densitometry (BIORAD Gel Doc XR +).

LPS extraction and in vitro *genome ejections*

S. flexneri LPS was extracted from PE577 as previously described (13), using a BulldogBio kit. Sf6 was incubated at 37 °C with either purified LPS (0.5 mg/mL), OmpA-TM, OmpCΔNT, purified LPS and OmpA-TM, or purified LPS and OmpCΔNT. The final concentration of Omps was 0.05 mg/mL unless otherwise noted. The “percent remaining virions” was calculated by dividing the plaque forming units (PFUs) in each reaction by the PFUs with buffer only added. For the time course experiments, Sf6 phage were incubated with LPS and Omps (OmpA-TM or OmpCΔNT) as described

above, and an aliquot was taken at each time point (0, 5, 10, 20, 30, 60 minutes). The “percent remaining virions” was calculated by dividing the PFUs at each time point by the PFUs with only buffer added at t = 0. Plates were grown overnight at 30 °C on PE577.

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CHAPTER 5

Summary and Conclusions

BACKGROUND

Many viruses have the ability to utilize alternative proteinaceous receptors as an attachment mechanism to infect cells. This essential and virtually universal step of host recognition has to be well coordinated by the virus in order to ensure successful progeny formation, as premature genome ejection can negatively impact the future of the virus. Yet, the molecular mechanisms that drive receptor binding have not been determined for most viruses, which infect every domain of life (1, 2). Moreover, viruses amongst these domains have only a handful of common protein folds that form the virion (1, 2). Thus, common viral structures make it possible to utilize model systems of viruses and their respective hosts to study viral infection. Using bacteriophage Sf6 and its host *Shigella flexneri* as a model, we previously have shown that Sf6 uses the host lipopolysaccharide (LPS) as a primary receptor and requires a secondary receptor, a role that is fulfilled by outer membrane protein A (OmpA), although other Omps, such as OmpC, may suffice (3). The work presented here has addressed the following questions: **1)** Which portions of OmpA are important for Sf6 attachment to the cell? **2)** how do the changes in OmpA affect phage binding kinetics? and **3)** is OmpC able to induce genome ejection of Sf6 *in vitro* at the same rate and efficiency as OmpA? By answering these questions my thesis has provided a more complete image of the attachment process in Sf6. The key findings from each chapter are highlighted below.

SUMMARY

Chapter 2: Key Residues of S. flexneri OmpA Mediate Infection by Bacteriophage Sf6

1) Which portions of OmpA are important for Sf6 attachment to the cell?

In chapter two, using *in vitro* experiments that monitor the loss of infectivity of mature virions, and therefore, imply genome ejection, we demonstrated that OmpA that has had its surface loops cleaved by proteinase K treatment is no longer able to induce genome ejection *in vitro*. These data suggest that the loops are essential for Sf6 infection. Furthermore, using a plasmid complementation system, we determined which portions of the surface loops are important. We showed that *S. flexneri* OmpA expressed *in trans* is able to restore the infection efficiency of Sf6 on a null *ompA*$^{-}$*C*$^{-}$ *S. flexneri* strain; yet, *E. coli* OmpA is unable to restore Sf6 infection efficiency. By systematically changing both the size and charge of the residues that differ between *E. coli* and *S. flexneri* OmpA, we found that the infection efficiency of Sf6 changed with some amino acid substitutions, but not others, particularly those located in loops 2 or 4 had the lowest Sf6 infection efficiency. Using our *in vitro* genome ejection system, we showed that variants which demonstrated a loss of function to serve as a receptor for Sf6 *in vivo*, as seen by a decrease in Sf6 infection efficiency, had a decreased efficiency to trigger Sf6 genome ejection *in vitro*. Data from chapter 2 suggest that overall amino acid substitutions are less deleterious in loops 1 and 3 of *S. flexneri* OmpA compared to loops 2 and 4. However, in no case were we able to completely block Sf6 infection, supporting general phage plasticity for receptor usage. These data suggest that Omps play a crucial role in the infection process of phages and provided new insights in the phage field about *Podoviridae* attachment.

Chapter 3: Whole Virion Biosensing: Kinetic Analysis of Bacteriophage Sf6 and Outer Membrane Protein A

2) How do the changes in OmpA affect phage binding kinetics?

In chapter three, using BioLayer Interferometry (BLI) we determined the kinetic parameters between Sf6 and purified OmpA-TM. We found that Sf6 binds *S. flexneri* OmpA-TM with nM affinity, with fast-on and slow-off kinetics. Next, we investigated if amino acid substitutions in OmpA also affect phage binding kinetics. Our data show that Sf6 binds these variant OmpA-TMs with similar affinities as *S. flexneri* OmpA, albeit differences were observed in infection efficiencies and in *in vitro* genome ejection efficiencies (chapter 2). In all, these data suggest that virus:host recognition is more complex and not dependent upon just binding affinities, but perhaps on the ability of the receptor to induce the correct conformational change to allow genome ejection into the host cell.

Chapter 4: Purified S. flexneri OmpC Induces Sf6 Genome Ejection in vitro

3) Is OmpC able to induce genome ejection of Sf6 *in vitro* at the same rate and efficiency as OmpA?

In chapter four, we purified and refolded *S. flexneri* OmpC. We used a proteolysis approach to obtain trypsin resistant and functional OmpC. Using our *in vitro* genome ejection system, we showed that OmpC is less efficient at inducing Sf6 ejection compared to OmpA. In addition, the initial rate of ejection is slower than with OmpA. The *in vitro* data presented in chapter four add further support to our previous data which suggest that OmpA is the preferred secondary receptor, whereas OmpC is an

alternative receptor (3). Overall, the data presented in this chapter in combination with our previous data suggest a model in which Sf6 may have evolved to have differential interaction with Omps depending on environmental conditions.

CONCLUSIONS AND FUTURE DIRECTIONS

The composite of the work presented in my dissertation in conjunction with studies from other phage:host systems has allowed for a more complete image of the Sf6 infection. Based on these data, we propose the following improved Sf6 model (Fig. 5.1). First, Sf6 interacts with LPS via its tailspikes (3-5). Recent work with P22, cousin phage to Sf6, and *Bacillus* phage ϕ29 has shown that these phages interact with the cell at an angle initially (6, 7); it is likely that this is the case for Sf6 as well. Moreover, work has shown not all tailspikes or tail fibers attach to the cell at the same time. For example, bacteriophage P22 needs at least three of six total tailspikes for a successful infection (8, 9). Cryo-electron tomography studies with bacteriophage T4 show that depending on the stage of infection, differing numbers of tail fibers are attached to the host (10). Sf6 has a total of six trimeric tailspikes and it is likely that initial cell interaction is made by a few tailspikes. Although, this is merely speculation based on studies with other phages and requires further experimentation in our system.

Once Sf6 has cleaved LPS and is close enough to the surface of the cell it can interact with its secondary receptor, an Omp (3). We previously showed that OmpA and OmpC, and likely a third (unidentified) Omp, serve as the secondary receptors for Sf6, with OmpA being slightly preferred to OmpC (3). Data from chapter 4 add support to this hypothesis since compared to OmpA, OmpC does not induce Sf6 genome ejection *in*

vitro at the same rate or efficiency. Moreover, based on sequence similarity of OmpF to OmpC, we propose that OmpF may also serve as a receptor for Sf6 and that Sf6 may have evolved to use different Omps as receptors depending on the environment, as the regulation of many Omps is linked to environmental conditions (11, 12). However, much of the work studying the regulation of Omps has been completed with *E. coli*. Although, *E. coli* and *S. flexneri* are similar, with sequence divergence being ~ 1.5 % (13), and it is likely that *S. flexneri* regulates production of its Omps in a similar manner, this has yet to be shown experimentally. Future work includes studying *S. flexneri* at different temperatures (and other variables) and investigating which Omps are expressed and looking at which Omps co-purify with Sf6, as outer membrane vesicles containing Omps have been shown to co-purify with Sf6 (3, 14). These experiments will shed light on not only Omp regulation in *S. flexneri*, but may also help identify other secondary receptors that Sf6 uses to gain access to the host.

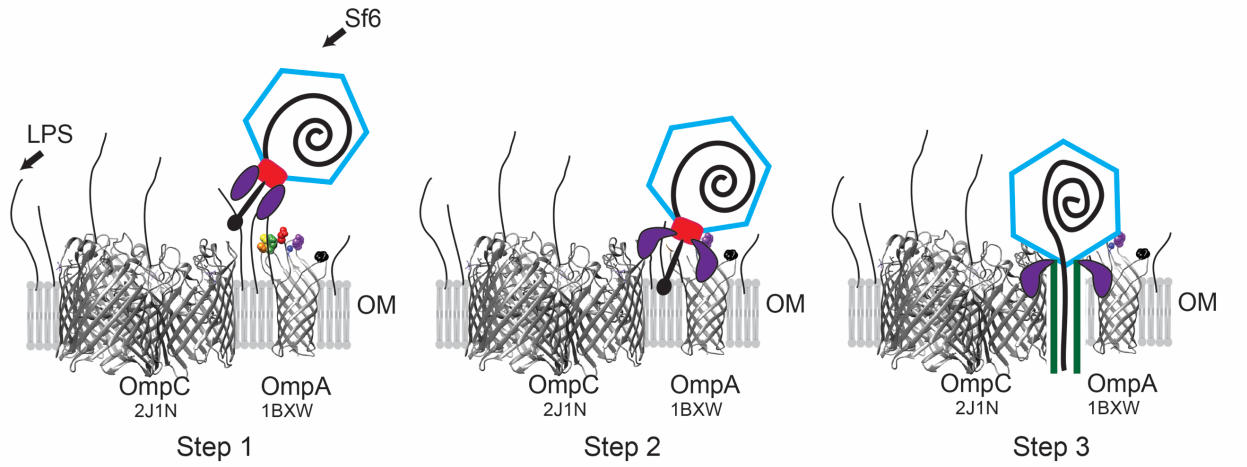
Data from chapter 3 show that affinity towards OmpA is in the nM range and amino acid substitutions in the loops of OmpA do not change Sf6 affinity. We hypothesize that since Sf6 can use either OmpA or OmpC (3), and OmpA variants do not change kinetic parameters, Sf6 likely has a similar affinity to OmpC, although this remains to be tested. Kinetics, although an important aspect of host receptor recognition, do not explain the differences seen in Sf6 infection and in *in vitro* genome ejection efficiencies (Chapter 2). It has been postulated before that interactions with host receptors lead to conformational changes and rearrangement of phage tail proteins to form a channel to move nucleic acids into the host (4). Recent work in the field provides support for this hypothesis. For example, Hu et al. has shown that binding of

the T4 long tail fibers with host receptors results in conformational changes in the baseplate which leads to movement of the short tail fibers and contraction of the tail sheath (10). Cryo-electron tomography has shown that bacteriophage T7 virions undergo structural remodeling during infection (15). Receptor binding by T7 results in the tail fibers moving and in the insertion of an extended tail into the cell membrane. We previously showed, using cryo-electron tomography, that “full” and “empty” Sf6 virions have slight changes in the tail machinery structure; although due to resolution limits, it is difficult to discern the extent of the virion remodeling (3).

Based on our work and data from other phage studies in the literature, we propose that Omps are responsible for mediating conformational changes in the tail machinery of the phage that are necessary for genome translocation. A likely target for the portion of Sf6 that interacts with OmpA (and other Omps) is the tailspike of Sf6 (N. Hubbs & Parent Lab, unpublished data). Therefore, it is possible that amino acid substitutions in the loops of OmpA affect the correct “induced fit” of Sf6, which results in the altered infection efficiencies we observe (16). In addition to conformational changes in the tailspikes upon interaction with Omps, there may be a second conformational change due to the “sensor protein”, the tail needle (17, 18). Alternatively, these events may be coupled or occur simultaneously. For example, it is possible that interaction with OmpA (or OmpC) triggers a small conformational change in the tailspikes which is then exacerbated by the tail needle, as the N-terminus of the tail needle sits inside the phage and directly interacts with the tailspikes (18). Or, it is possible that changes in the tailspikes induce the tail needle to adapt its post-ejection extended conformation (18), helping to form the channel necessary for DNA movement. The exact order of these

events remains to be determined. Ultimately, however, these events lead to formation of a channel where the phage DNA can be ejected into the host cell (4, 19). Future work includes investigating the roles that Sf6 interaction with receptors have on the structure of the virion and determining the likely conformational changes that are induced by Omps. Although the overall understanding of the infection process of Sf6 is still incomplete, this dissertation has helped address this process, particularity by characterizing the interactions of Sf6 and OmpA and C, an important aspect of host recognition.

Figure 5.1: Proposed Sf6 infection model



Schematic of steps in Sf6 attachment (adapted from (4)). Step 1: A virion, likely coming in at an angle, binds to the primary surface receptor: lipopolysaccharide (LPS). Step 2: The tailspikes (purple) hydrolyze the LPS bringing the virion closer to the outer membrane (OM) surface, where it can now interact with the secondary receptor: OmpA (PDB: 1BXW) or OmpC (PDB: 2J1N). The crystal structures of *E. coli* OmpC and OmpA are depicted as ribbon diagrams with substituted OmpA amino acids shown as spheres: red, P25; orange, D66; yellow, N67; green I68; blue, A108; violet, P111; and black N155, using UCSF Chimera (20). Interaction with the secondary receptor likely results in conformational changes in the tail machinery. Step 3: dsDNA likely enters the cell through a channel formed by the tail and the ejection proteins.

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