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Expression of Foreign Genes in the Pseudomonas Bacteriophage Pf3

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Expression of Foreign Genes in the *Pseudomonas* Bacteriophage Pf3

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioinformatics at Virginia Commonwealth University.

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List of Abbreviations

EGFP. . . . . Enhanced Green Fluorescent Protein

M13-G. . . . . .Phage M13 Engineered to Express EGFP

MCP. . . . . Major Coat Protein

modMCP. . . . . .Modified Major Coat Protein

Pf1-G. . . . . Phage Pf1 Engineered to Express EGFP

Pf3-G10. . . . . Phage Pf3 Engineered to Express EGFP

Pf3-G10-SH. . . . . .Phage Pf3 Containing modMCP and Engineered to Express EGFP

Pf3-SM1(4)-SH. . . . . .Phage Pf3 Containing modMCP and Engineered to Express SM1(4)

SM1. . . . . .Salivary and Midgut Peptide 1

SM1(4) . . . . . .Gene Cassette Containing 4 Tandem Copies of SM1 Separated by Spacers

Abstract

EXPRESSION OF FOREIGN GENES IN THE *Pseudomonas* BACTERIOPHAGE Pf3

By Krystin Virginia Weathers, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioinformatics at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Major Director: Dr. Luiz Shozo Ozaki, Associate Professor, Center for the Study of Biological Complexity

Filamentous bacteriophages were engineered to express foreign genes with the ultimate purpose of displaying transmission control anti-malarial peptides as in phage display. It was hypothesized that expression of foreign genes would be possible using the phage's promoters. This hypothesis was tested by assuming that promoters for the phage major coat protein (MCP) gene would also promote the expression of any foreign gene inserted downstream of the MCP gene. As proof of principle, the bacteriophages Pf3, Pf1, and M13 were engineered in this way to successfully synthesize Enhanced Green Fluorescent Protein (EGFP). Type 88 phage display on the EGFP recombinant Pf3 was attempted by fusing a second copy of its MCP gene to the existing EGFP gene. This resulted in a phage display Pf3 replacement vector which was then used to construct a phage for displaying an anti-malarial peptide.

1. Introduction

1.1 Bacteriophages

There are an estimated 10^{31} different bacteriophages (phages) on earth (Breitbart, 2005). Phages are the largest group of biological entities found in the world (Ackermann, 2003). Even though phages outnumber all other entities on earth, relatively little is known about most of them. There is a tremendous potential for scientific advancement through the use of phages in several fields including biomedical research and nanotechnology (Rakonjac, 2011; Dang, 2011; Chandler, 2011).

As Ackermann cited, there are thirteen different families of phages (Table 1, Figure 1, Ackermann, 2003).

Table 1: Bacteriophages and their distinguishing features*

Family Name	Distinguishing Features
Microviridae	Polyhedral shape; Single-stranded, circular DNA
Myoviridae	Contractile tail; Double-stranded, linear DNA
Siphoviridae	Long, non-contractile tail; Double-stranded linear DNA
Podoviridae	Short, non-contractile tail; Double-stranded linear DNA
Corticoviridae	Complex capsid; lipids; Double-stranded, circular DNA
Tectiviridae	Double-stranded, linear DNA; Internal lipoprotein vesicle
Lipothrixviridae	Envelope; lipids; Double-stranded, linear DNA
Plasmaviridae	Envelope; Lipids; No capsid; Double-stranded, circular DNA
Rudiviridae	Helical rods; Double-stranded, lineasr DNA
Fuselloviridae	Spindle-shaped; no capsid; Double-stranded, circular DNA
Inoviridae	Rods or filaments; Single-stranded, circular DNA
Leviviridae	Single-stranded, linear RNA
Cystoviridae	Double-stranded, linear RNA

* Ackermann, 2003

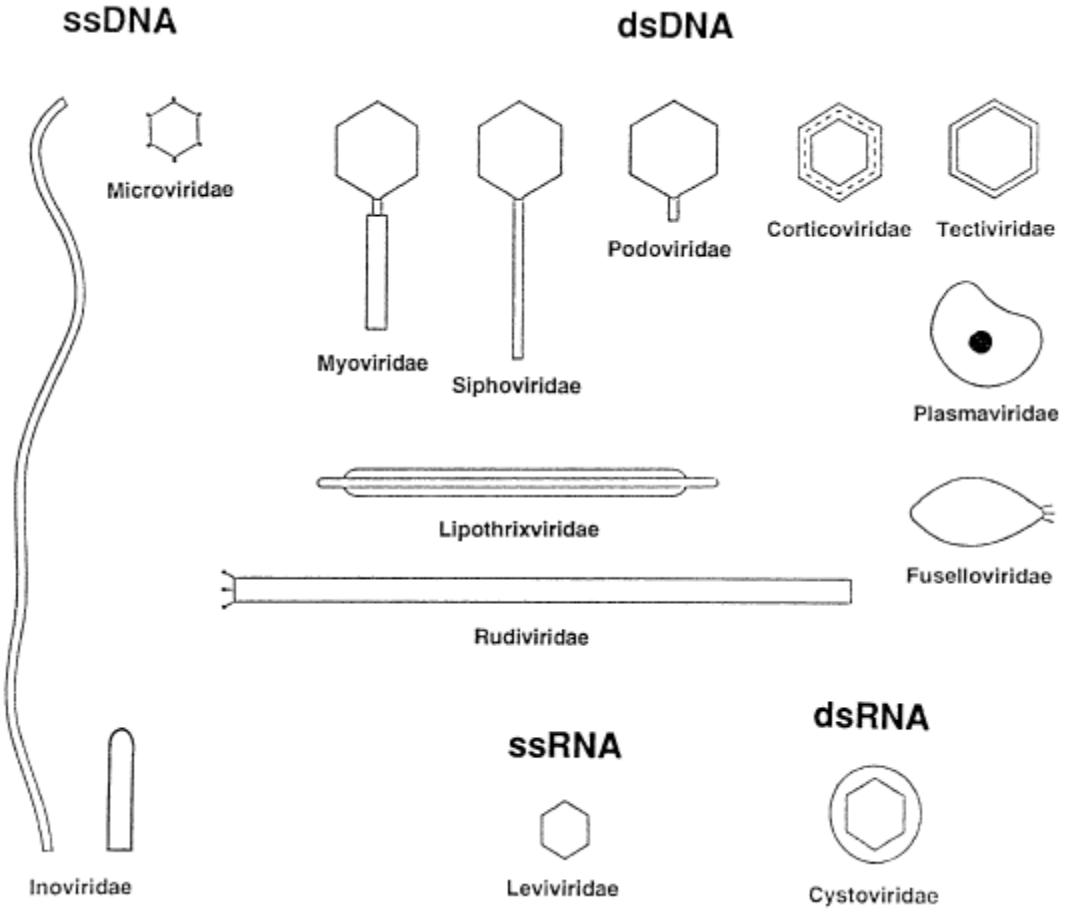

Figure 1: Morphologies of bacteriophages. (Reprinted with permission from Ackermann, H.W. (2003). Bacteriophage observations and evolution. Research in Microbiology, 154, 245-251. Copyright 2003 Elsevier).

Phages in each family have different morphological and genomic characteristics. For example, all *Siphoviridae* have long non-contractile tails and a double-stranded DNA genome, while the *Leviviridae* lack tails, and have ssRNA genomes surrounded by a simple capsid. Phage genomes may be either DNA or RNA and may be single stranded or double stranded. All phages use the replication system of their host cell; some completely take over the host and eventually cause cell lysis while others are able to replicate without severely disrupting the host cell. Some phages are able to integrate into the host bacterial cell genome where they may propagate or remain dormant until triggered by some environmental factor. This study will focus on the *Inoviridae,* which are filamentous phages with a single-stranded DNA genome.

The genomes of all phages from the family Inoviridae are composed of circular, single-stranded DNA which, once inside the host cell, is converted into double stranded DNA, known as the replicative form, and replicates by a rolling circle mechanism (Gilbert and Dressler, 1968). The genera *Plectrovirus* and *Inovirus* include filamentous phages, thusly named for their long, rod like shape. The Inoviridae lack genes encoding proteins that lyse their host bacteria. Because they replicate without killing their host, filamentous phages may be produced and extruded by the host cell indefinitely (Paschke, 2006). Some filamentous phages integrate into the host genome and become temperate while others not (Rakonjac, 2011). The majority of hosts for filamentous phages are Gram negative bacteria but there are two known filamentous phages with Gram positive bacterial hosts (Rakonjac, 2011). Filamentous phage hosts display a pilus, which is how the phage initially interacts with the bacteria (Iannolo, 1995; Russel, 1997). In filamentous phage M13, 1) the N-terminal domain of protein III at the end of the phage attaches to the F pilus, 2) the pilus retracts to the bacterial cell drawing the phage in, 3) it interacts with a secondary receptor and then 4) it is allowed to enter the cell with the N-terminal domain of

protein III entering first (Smith, 1997). As the phage enters the cell, the coat proteins encasing the phage genome are removed (Russel, 1997). Upon completion of phage genome replication, a copy of the DNA is encased by the phage's coat proteins as it passes through the cell membrane exiting the cell; the first portion of the phage to emerge from the cell is the packaging signal end (Russel, 1997).

The best studied group of filamentous phages is the Ff group which includes M13, fd and f1; the following discussion of genome contents and phage structure is based on this group (Russel, 1997; Rakonjac 2011). The DNA of a filamentous phage codes for the production of ten to eleven proteins (Figure 2).

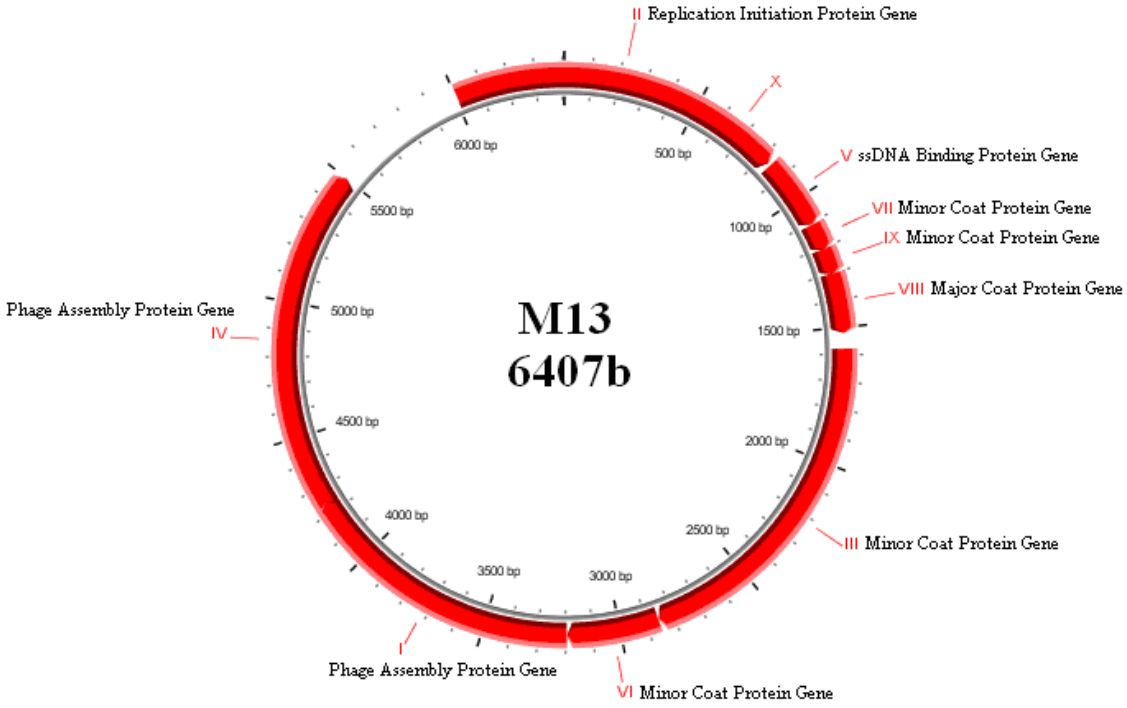

Figure 2: Genome map of model filamentous phage M13. Each gene is indicated by the red circular arrows and the gene number is indicated in roman numerals. Genome map created using CGView.

Of these, 5 are assembled into the phage coat. The major coat protein, encoded by gene VIII, covers the body of the phage while the ends are covered by minor coat proteins, encoded by genes III, VI, VII, and IX (Rakonjac, 2011). Genes II, V, and X, are responsible for DNA synthesis while genes I and IV are responsible for phage assembly (Russel, 1997). Filamentous phages are structured such that their genomes are encapsidated in proteins. There are approximately 2700 copies of the major coat protein surrounding the single-stranded phage DNA (Wang, 2010). Approximately 5 copies of each of the minor coat proteins can be found on the phage coat; 5 copies of protein IV and 5 copies of protein III on one end and 5 copies of protein VII and 5 copies of protein IX on the other end (Wang, 2010). Proteins VII and IX are essential for phage particle assembly while proteins III and IV are necessary for stability and infectivity. More specifically, protein III is responsible for interaction with the bacterial host F pilus as well as removing the phage coat and allowing the genome access to the host cell's cytoplasm (Russel, 1997). There are several ways in which the phage may exit its host cell. In some filamentous phages, protein IV, which is homologous to host secretins, may form a complex in the membrane to create a gated channel for the phage to exit through (Iannolo, 1995). Alternatively, as in phage CTXΦ, the phage may use the host cell secretins but phage production is very low in these systems, indicating a lower efficiency in using host secretins (Rakonjac, 2011).

1.2 Phage Display

Smith first proposed creating a "fusion phage" in which a foreign peptide was fused to protein III of filamentous phage f1 (Smith, 1985). The widely used technology of phage display arose from this. In phage display, a foreign peptide is fused to a phage coat protein, usually protein III or VIII and a spacer, or hinge, may be used to distance the foreign peptide from the phage (Smith, 1997; Kim, 2004). While proteins III and VIII are the most frequently used, any

phage coat protein may be used to link a foreign peptide for phage display. The peptides are situated on the N-terminal side of either protein III or VIII so that they are exposed on the surface of the phage as opposed to being internalized (Smith, 1997). There are 20 amino acids of protein VIII naturally exposed on the surface of the phage, which provide a large target for attaching a foreign peptide (Kim, 2004). The expressed foreign peptide generally acts as if it were not attached to the phage coat because of its accessibility to solvent and any spacers or hinges that are used (Smith, 1997).

There are six popular models of phage display that are theoretically possible: Type 3, Type 33, Type 3+3, Type 8, Type 88, and Type 8+8 as shown in Figure 3 (Smith, 1997).

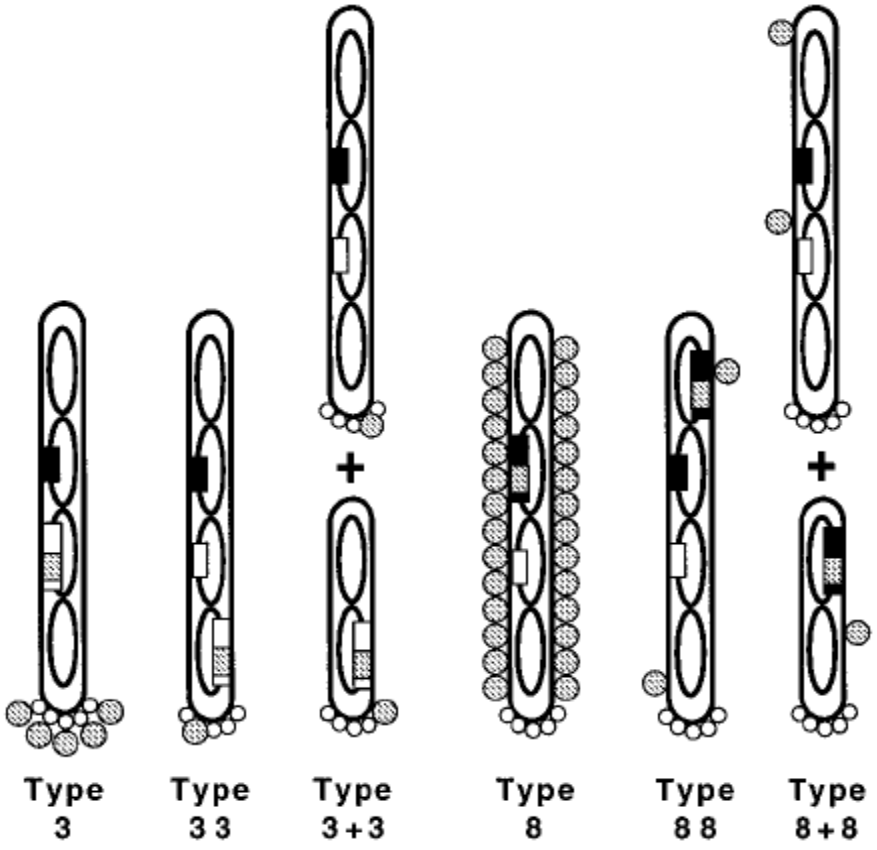

Figure 3: Types of phage display. The black boxes in the genome indicate the location of gene 8 or derivatives of gene 8 while the white boxes indicate the location of gene 3 or gene 3 derivatives. The grayed box indicates the location of the foreign peptide in each construct. The grayed spheres indicate the location of the foreign gene on the phage coat. (Reprinted with permission from Smith, G.P. and Petrenko, V.A. (1997). Phage Display. Chemistry Review, 97, 391-410. Copyright 1997 American Chemical Society).

In Type 3 and Type 8 phage display, the DNA sequence for the foreign peptide is directly fused to the one and only copy of the given coat protein gene, protein III or protein VIII respectively, and thereby should be expressed on every copy of that coat protein on the phage (Smith, 1997). These two types of phage display are rarely viable because the foreign peptide disrupts the ability of the coat proteins to align correctly around the phage DNA. Only small peptides of fewer than 8 amino acids may be used in this type of phage display (Smith, 1997). In Type 33 and Type 88 phage display, a second copy of the given coat protein gene is inserted into the genome of the phage and that second copy is fused to the foreign peptide (Smith, 1997). In this type of system the phage itself produces both the wild type coat protein as well as the fusion coat protein. Upon phage assembly a few of the fusion coat proteins may be incorporated on to the phage coat without disrupting the coat protein alignment and thereby producing viable phages; this dual-expression system allows for the use of large foreign peptides (Smith, 1997). Type 3+3 and Type 8+8 systems require two genetic elements; the phage contains the wild type coat protein, this entity is usually called the "helper phage". An accompanying plasmid contains the coding sequence for the phage's coat protein gene fused to the foreign peptide gene, this is usually called the "phagemid" (Smith, 1997). Within Type 3+3 and Type 8+8 the same principle of having the foreign peptide on only a few copies of the phage coat applies and creates a viable phage. The requirement of two genetic elements increases the difficulty of engineering a Type 88 or Type 33 phage display system but is the most commonly used type of phage display (Smith, 1997). In order to use type 8+8 or type 3+3 phage display, a plasmid must be characterized for the host bacteria. In types 33 and 3+3 it is common to only have one or fewer copies of the foreign peptide displayed (or expressed) on each phage, since gene III is a minor protein and present in just a few copies per phage. The number of foreign peptides exposed in

types 88 and 8+8 depends upon the size of the foreign peptide (Wang, 2010). Smaller peptides tend to be expressed in higher numbers because the space on the phage coat is limited and higher numbers of small peptides are able to fit together.

There are a wide range of applications for phage display that use the same concept of fusing a foreign peptide to a phage coat protein to explore the molecular interactions of the foreign peptide. These applications include the detection of monoclonal antibodies (Wang, 2010) and the study of molecular interactions (Lanzillotti, 2007). To study molecular interactions, the peptide on the phage coat mimics a ligand to probe receptors (Lanzillotti, 2007). Additionally, phage display is the basis for the creation of peptide and protein libraries (Wang, 2010) to enable the screening of a large number of molecules in an interaction assay. Host-parasite interactions may also be discovered and characterized through phage display (Lanzillotti, 2007). The biggest advantage of phage display is the ability to recover the phages and identify the peptide that is attached (Wang, 2010). Alternatively, a sample may be probed with free peptides which can not be recovered in large quantities.

Our specific interest is to use phage display to study ligand-receptor interactions of a host-pathogen system. One of the most extensively researched host-pathogen interactions is of the malaria parasite, *Plasmodium*, with its mosquito host. Within the mosquito host, the parasite's lifecycle can be interrupted by blocking the molecular interaction between the parasite and the mosquito tissue. Phage infecting bacteria residing in the mosquito gut may provide an opportunity to use phage display as a novel anti-malarial approach. The following sections will describe the disease and preliminary work to create a phage display system to block transmission of Plasmodium.

1.3 Malaria: The Disease and the Parasite

Malaria dates back over four thousand years in recorded history and has had a large influence on shaping human history and populations (CDC, 2010). Each year approximately 1500 cases of malaria are diagnosed in the United States; most of these cases occur in travelers and immigrants from areas where malaria is present (CDC, 2010). There is also the possibility of native mosquitoes becoming infected by travelers from malaria endemic regions and then transmitting the infection to residents (CDC, 2010). Even though malaria has been officially eradicated in the United States, the three main mosquito vectors, *Anopheles quadrimaculatus, Anopheles freeborni,* and *Anopheles albimans,* are still present meaning there is still a risk of a malaria outbreak (CDC, 2010). While malaria does not pose a significant and immediate health risk in the United States, it is still a large problem in many countries.

Approximately half of the world's population lives in areas which put them at risk to contract malaria (CDC, 2010). Over a million people are killed by the disease worldwide each year (CDC, 2010). Malaria is the second most deadly disease in Africa; second only to HIV/AIDS (CDC, 2010). Sub-Saharan Africa is most strongly affected by the disease; in the year 2008, there were approximately 200 million cases of malaria worldwide with approximately one million people dying of the disease in sub-Saharan Africa, the majority of them children (CDC, 2010).

Malaria is caused by parasites of the genus *Plasmodium*. There are over one hundred known species of *Plasmodium.* These species are thought to be host specific, and can infect assorted animals ranging from reptiles to birds to mammals including humans (CDC, 2010). Of the numerous species of *Plasmodium*, only five are known to infect humans; those are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and

Plasmodium knowlesi (CDC, 2010). Of the human malarias, *P. falciparum* is the most deadly, killing approximately one million people each year; however, *P. vivax* is the most prevalent (CDC, 2010).

The lifecycle of *Plasmodium* entails infection of an invertebrate as well as a vertebrate host (Figure 4; CDC, 2010).

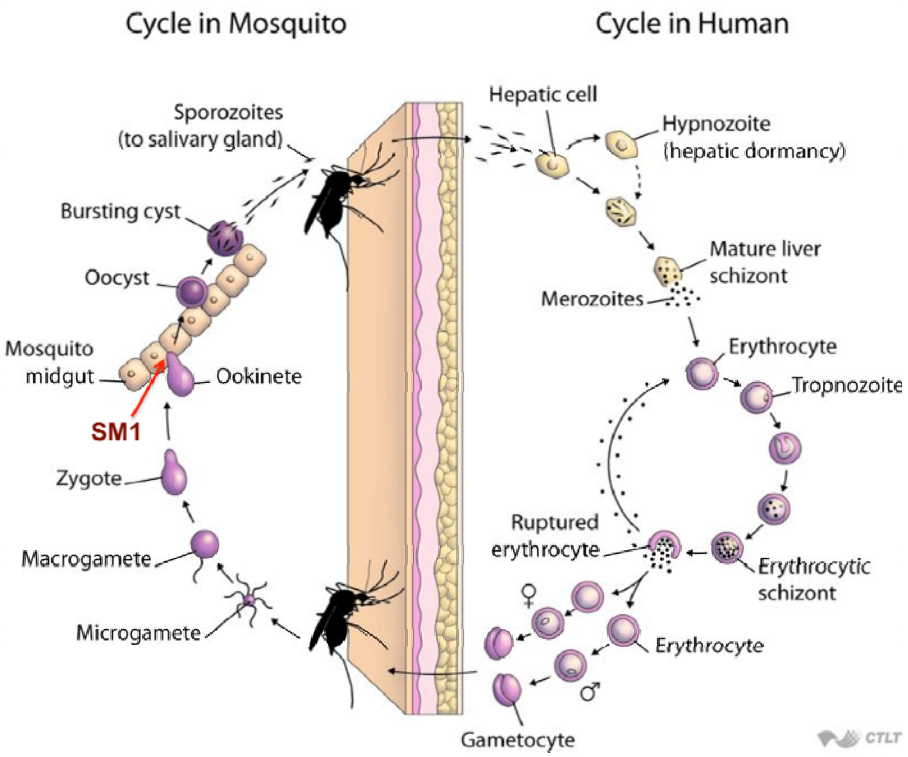

Figure 4: The lifecycle of the malaria parasite. Here the lifecycle of the parasite can be seen in both the invertebrate host, the mosquito, and the vertebrate host, in this case, a human. The development from ookinete to oocyst can be hindered by the SM1 peptide. The peptide blocks the ookinete interaction with a receptor in the mosquito midgut essential for its differentiation into an oocyst. "Life cycle of the malaria parasite" from Epidemiology of Infectious Diseases. Available at: http://ocw.jhsph.edu. Copyright © Johns Hopkins Bloomberg School of Public Health. Creative Commons BY-NC-SA.

Female mosquitoes serve as the invertebrate host of *Plasmodium* (CDC, 2010). Even though there are approximately four hundred and thirty *Anopheles* species, only thirty to forty species are able to transmit mammalian malaria parasites (CDC, 2010). *Plasmodium* gametocytes are found in the bloodstream of infected mammalian hosts; the gametocytes are transferred to Anopheline mosquitoes during a blood meal which is needed for egg production (CDC, 2010). In the mosquito mid-gut the two forms of gametocytes, macro- and microgametocytes, fuse to form the zygote, which develops into the ookinete. The ookinete traverses the midgut epithelium forming the oocysts. Upon maturation, the oocyst ruptures thereby releasing sporozoites which then migrate to the salivary glands (CDC, 2010). These sporozoites are then injected with the mosquito's saliva into the next human blood meal host (CDC, 2010). Once inside the newly infected human host, *Plasmodium* travels to the liver where it develops into a schizont. The schizont ruptures, allowing between thirty thousand and forty thousand merozoites to enter the blood stream and parasitize the red blood cells of the human host. These merozoites develop into gametocytes, perpetuating the life cycle of the parasite (CDC, 2010). Targets for blocking parasite development are at the critical steps in the life cycle such as the step where the parasite interacts with the epithelium in the mosquito midgut (red arrow, Figure 3).

1.4 Transmission Controls of Malaria

One avenue for controlling the spread of malaria is through the control of parasite transmission, either by directly affecting the insect or through genetic engineering of the insect, rendering it unsuitable for the parasite. Control options in which the insect is directly affected include killing the insect with insecticides, bed nets impregnated with insecticides, the use of attractants and traps, and controlling the larval habitat. These methods are not always possible or

effective. For instance a country needs to be able to afford the control option and the people of the country need to be willing to use the control option. Additionally, many countries lack the infrastructure to educate their people about transmission control methods. Also of consideration is resistance to insecticides arising from frequent, high-volume use (Hemingway, 2000).

Two current genetic models for hindering the *Plasmodium* parasite include transgenesis, in which the mosquito host is manipulated, and paratransgenesis, in which the bacteria inhabiting the mosquito host are altered. Both of these methods pose the similar issue of releasing a genetically altered, living organism into the environment. Transgenesis models have not been only created for malaria; other vector borne diseases have been targeted as well. A few transgenesis models for malaria have been created which show great promise. One transgenesis model was created using a blocking protein that reduces oocyst formation by 81.6% on average. Another model promoting increased amounts of a naturally occurring peptide reduced oocyst formation by 80% on average (Ito et al., 2002; Abraham et al., 2005). However, one challenge with existing transgenic mosquito models is reduced fitness such that the transgenic mosquitoes cannot survive in the wild (Abraham et al., 2005; Enserink, 2002).

Paratransgenesis is an appealing alternative to transgenesis because the varying mosquito midgut microflora typically provide several bacterial species to work with. Researchers have discovered a different profile of species of bacteria in each mosquito midgut; Straif et al. found the same majority bacterial genus in only 3% of studied *Anopheles* mosquitoes while Gonzalez-Ceron found that the majority of mosquitoes had no cultivatable bacterial inhabitants (Straif et al., 1998; Gonzalez-Ceron et al., 2003). This means that in order to create a widespread paratransgenesis model to fight mosquito borne diseases, a bacterial strain may need to be adapted to live in the mosquito gut prior to any genetic modifications (Riehle et al., 2005). It is

also of note that the number of bacteria increase exponentially when the mosquito takes a blood meal; this may or may not assist with the efficiency of a paratransgenic model (Riehle et al. 2005). Another consideration in the development of a paratransgenic model in mosquitoes is that only one bacterial genus, *Asaia*, is passed from generation to generation meaning that any paratransgenesis model would need to target each generation of adult mosquitoes (Favia, 2008; Riehle et al., 2005).

While transgenesis and paratransgenesis are both actively researched in the lab; there is no consensus on how they can be implemented in the natural world (James, 2011). The Cartagena treaty, adopted in 2003 by 168 countries, is an agreement on the handling, transport, and use of living modified organisms; however, transgenic and paratransgenic organisms are not thoroughly covered in the treaty (Secretariat, 2000). It is safe to say that safety standards as stringent as those required for vaccine trials will be required if not even more restrictive due to the potential effects on human inhabitants of test areas who have not agreed to participate in the study (Enserink, 2002). Most researchers would agree that preliminary cage tests need to be conducted but choosing locations for any field tests will prove difficult as they should occur in an area from which it is difficult for the mosquitoes to escape (Enserink, 2002). Any field test will need to be handled with utmost care and community awareness so as not to create health hazards and/or bad publicity leading to public opposition of field tests and the inability to use any new genetic tools that are being created around the world (Subbaraman, 2011).

Researchers have discovered a peptide that is capable of hindering the development of *Plasmodium* in the mosquito host by competing with the parasite for binding to the microvilli of the mosquito midgut. Salivary gland and midgut peptide 1 (SM1) was described by Ghosh et. al. to potentially competitively bind to the same ligand on the mosquito midgut lumen as the malaria

parasite *Plasmodium berghei* in mice (Ghosh et. al., 2001). This twelve amino acid peptide strongly inhibits the parasite from entering the salivary glands and crossing the midgut epithelia (Ghosh et. al., 2001).

1.5 Combining Phage Display and Vector Borne Disease Control

The intention here is to develop a novel system to deliver the SM1 peptide to the mosquito gut to inhibit the life cycle of the parasite in the mosquito. The use of phages and phage display technology is proposed in a method we call *phagotransgenesis*. This thesis describes preliminary work to develop a phage display system using only one genetic element to aid in the prevention of the transmission of the malaria parasite and expand that technology to aid in the prevention of the transmission of other insect borne parasites.

Here it is hypothesized that any filamentous phage and its host can be used for foreign peptide production and expression with only one genetic element. Furthermore it is hypothesized that a foreign gene inserted downstream of the major coat protein in filamentous phages will be expressed by the same promoter. Because the major coat protein gene is highly expressed it is assumed that the inserted foreign gene will also be highly expressed.

Due to the ultimate goal of using phages as in phage display to block the development of the malaria parasite in the anopheline mosquito, a bacteria found to be a natural inhabitant of the mosquito gut was selected. Through an extensive literature review a list of bacteria found to be naturally occurring in the gut of anopheline mosquitoes was compiled (Table 2).

Table 2: Bacteria naturally found in the mosquito midgut

Mosquito	Bacteria	Reference
Anopheles Albimanus (Mexico)	*Serratia marcescens, Enterobacter cloacae,Enterobacter amnigenus 2, Enterobacter sp., and Serratia sp.*	GONZALEZ-CERON et al., J. Med. Entomol. 40(3): 371Ð374 (2003)
Anopheles darlingi (South America)	*Aeromonas, Pantoea and* ***Pseudomonas****, Enterobacter hormaechei-cluster*	TERENIUS et al., Marinotti J. Med. Entomol. 45(1): 172Ð175 (2008)
Anopheles gambiae, *Anopheles funestus* (Africa)	*Nocardia corynebacterioides, Acidovorax temperans, Mycoplasma wenyoniid, Stenotrophomonas maltophilia, Stenotrophomonas maltophilia, Stenotrophomonas sp., Spiroplasma sp., Paenibacillus sp., Anaplasma ovis, Ehrlichia sp.. Aeromonas hydrophila, Aeromonas sp.*	Lindh, Olle Terenius, and Ingrid Faye Appl Environ Microbiol. 2005 November; 71(11): 7217–7223.
Culex quinquefasciatus	*Acinetobacter junii, Ac. calcoaceticus, Aeromonas culicicola, Bacillus thuringiensis, Microbacterium oxydans, Pantoea agglomerans,* ***Pseudomonas aeruginosa****, Staphylococcus epidermidis, Stenotrophomonas maltophila*	PIDIYAR,Am. J. Trop. Med. Hyg., 70(6), 2004, pp. 597–603

*Shown in red are the bacteria species selected for the present work.

From this list, *Pseudomonas aeruginosa* was selected because it is well characterized and is a host to well characterized filamentous bacteriophages. It was important that the selected bacteriophages have a small genome to make genetic manipulations possible. *Pseudomonas aeruginosa* strain PAO1 phage Pf3 is a highly characterized and studied filamentous phage which has a known genome of 5833 nucleotides.

As a proof of principle, *Pseudomonas aeruginosa* strain PAO1 specific filamentous phage Pf3 was first used to express EGFP, a reporter gene. The gene coding for EGFP was placed immediately downstream of gene VIII, the major coat protein gene. After inserting the EGFP gene into the Pf3 genome, creation of a type 88 phage display system using EGFP in Pf3 was attempted. Any phage coat protein may be used for phage display, however gene VIII, the major coat protein, is the most popular. The major coat protein was used here due to the high number of copies of the protein required to create a phage particle. As previously described, three types of phage display may be accomplished using the major coat protein: Type 8, Type 8+8, and Type 88. Type 88 was chosen because Type 88 typically produces a viable phage particle with a coat comprised of mostly wild type major coat protein and a few fused coat proteins. In this construct, a second copy of the major coat protein gene is fused to a foreign peptide gene and inserted into the wild type phage genome.

Subsequent to genetic manipulations of phage Pf3 to insert the EGFP gene, similar genetic manipulations were conducted to create a phage Pf3 that expressed the SM1 gene. This Pf3-SM1 construct is believed to express SM1 fused to a second copy of the Pf3 major coat protein such that it is on the phage coat as in Type 88 phage display. As an extension of this approach, EGFP expression systems similar to the initial Pf3-EGFP construct were created in

Pseudomonas bacteriophage Pf1 and *Escherichia coli* bacteriophage M13 such that the EGFP gene was not fused to the major coat protein.

2. Materials and Methods

2.1 Genetic Engineering of bacteriophage Pf3 to Express the Enhanced Green Fluorescent Protein (EGFP) Gene

2.1.1 PCR Amplification

Due to the small genome size of phage Pf3, the entire genome can be amplified using polymerase chain reaction (PCR). We amplified the whole genome of bacteriophage Pf3 using proofreading polymerase (Exact Polymerase, 5 Prime, Gaithersburg, MD) by PCR using the following program: 95 °C 5min (94 °C 30sec, 58 °C 30sec, 72 °C 14min)x35 14 °C forever. Oligonucleotide primers were designed such that PCR resulted in the linearization of the Pf3 phage circular DNA with the opening point of the circular DNA between gene VIII and the transcription termination signal of the phage genome (Table 3 and Figure 5).

Table 3: Oligonucleotides for the genetic engineering of bacteriophage Pf3 with the EGFP gene

Primer Name	Primer Sequence
[a]For amplification of the Pf3 genome	
Pf3MCP50upSpeI	AGACCAACTAGTGCCAAAAGCCCAAGGACGGATC
Pf3MCP50dwBglII	CGACCAGATCTCTCAATCGTTATAAGGGGGCTTCG
[b]For amplification of the EFGP gene	
EGFPFXbaI	ACATCTAGAAAGGAGATATATACATGAGTAAAGGAGAAGAACTTTTC
EGFPFRBamHI	CCTGGATCCTTATTTGTATAGTTCATCCATGC
[c]For sequencing around the insertion site	
Pf3-8_seq2	TGGGCTGAACGTTCAGATTTGC
Pf350R_seq	GAGCAACAATCAAACGACCCT
[d]For amplification of the Pf3-G10 genome	
G10upBHI	ACCGGATCCTTTGTATAGTTCATCCATGCCATG
Pf3MCP50dwSpeI	CACACTAGTCTCAATCGTTATAAGGGGGCTTC
[e]For assembling and amplifying the Pf3 modMCP gene	
modMCPF	ATG CAA TCC GTG ATT ACC GAT GTG ACG GGC CAA CTG ACA GCT GTG CAA GCT GAT ATC ACC ACC ATT GGT GGT GCC ATT ATT GTT
modMCPstopc	TCA AAA GAA TTG CGC TTT GAT CCA ACG AAT ACC CAG CAC AAC GGC GGC CAG AAC AAT AAT GGC ACC ACC AAT GGT GGT GAT ATC
8hingeBgIIF	CCTAGATCTGGTGGAGGCTCAGGCGGAATGCAATCCGTGATTACCGATG
8XbaIR	CACTCTAGATCAAAAGAATTGCGCTTTGATCC

[a]Oligonucleotide primers for linearizing the Pf3 phage genome and inserting the EGFP gene amplified with oligonucleotide primers EGFPFXbaI and EGFPFRBamHI[b].
[c]Oligonucleotide primers for amplification and sequencing of the regions around the insertion site.
[d]Oligonucleotide primers for linearizing the Pf3-G10 phage genome and inserting the modMCP gene assembled with oligonucleotide primers modMCPF and modMCPstopc and amplified with 8hingeBgIIF and 8XbaIR [e].

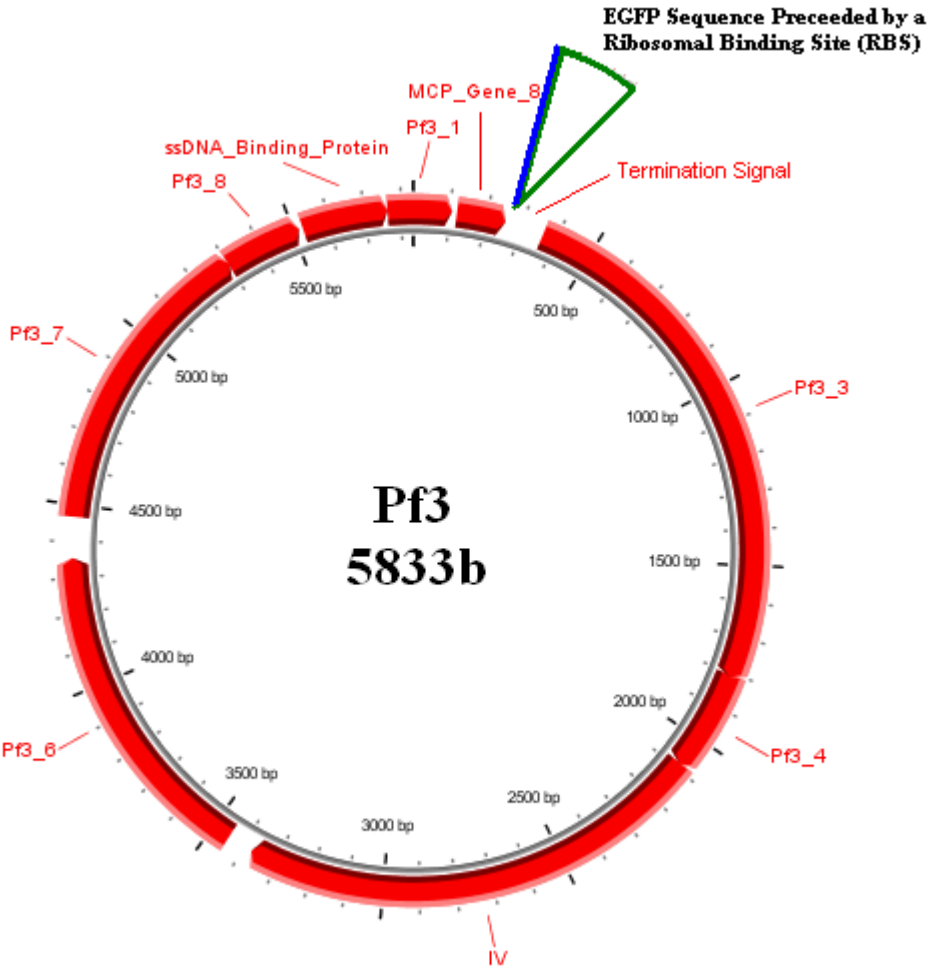

Figure 5:Insertion site of EGFP in the Pf3 genome. EGFP gene was inserted between gene VIII which codes for the Major Coat Protein of Pf3 and the phage transcription termination signal. The EGFP gene was immediately preceded by a ribosomal binding site. Genome map created using CGView.

Restriction enzyme recognition sequences were added to each oligonucleotide primer such that the resulting linear DNA had a Spe I site on one end and a Bgl II site on the other end (Figure 6).

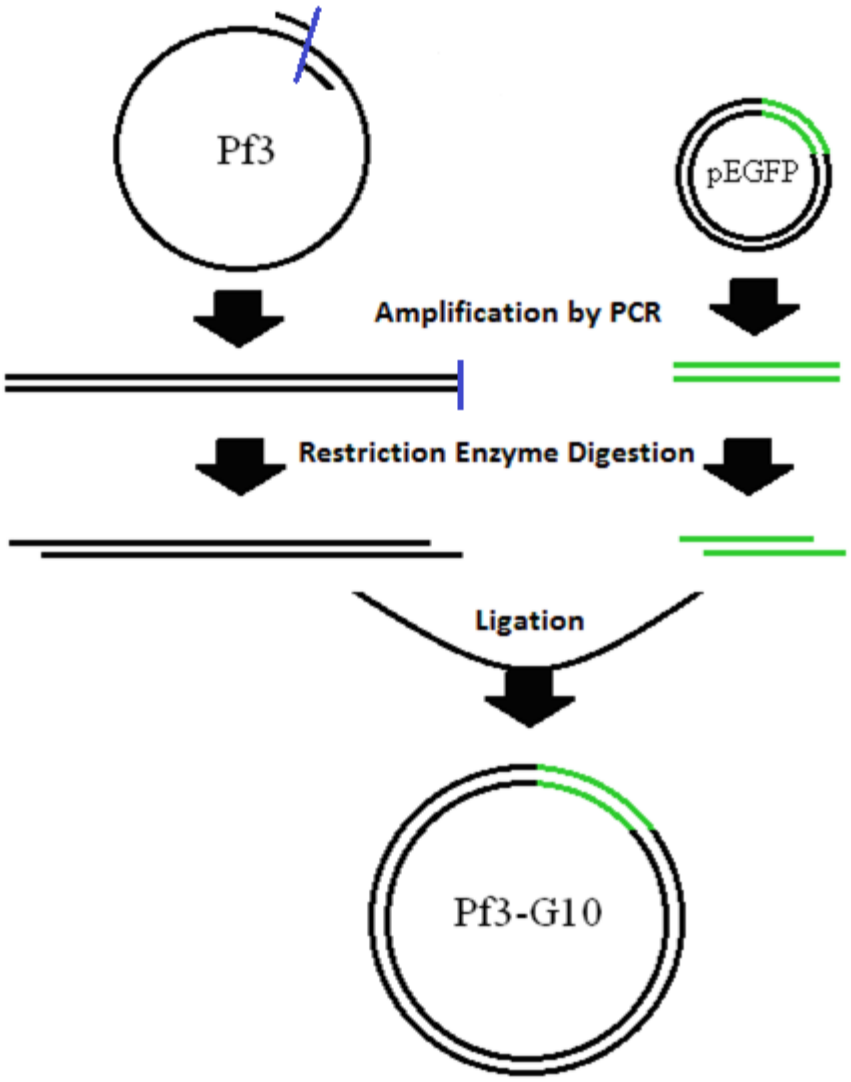

Figure 6: Steps for constructing the recombinant phage Pf3-G10. The genome of bacteriophage Pf3 is amplified by PCR and digested with restriction enzymes. The oligonucleotide primers used to linearize Pf3 are shown by the small black lines leading away from the opening site indicated by the blue line. The EGFP gene is amplified from a plasmid and digested with restriction enzymes. The two digested fragments are then ligated together to form a double stranded recombinant molecule.

The EGFP gene was amplified by PCR with a proofreading enzyme (Exact Polymerase, 5 Prime, Gaithersburg, MD). Oligonucleotide primers for the reaction were designed such that the entire gene was replicated (Table 3). Restriction enzyme recognition sites were added to each primer such that the result of the PCR was double-stranded, linear DNA with a restriction enzyme recognition site on each end (Figure 4). Additionally on the forward priming oligonucleotide, the nucleotide sequence of a known ribosomal binding site (RBS) of several genes of *Pseudomonas* was included (Avichezer, 1992; Shine and Dalgarno, 1975).

2.1.2 Purification of PCR Products

The PCR products were purified using the magnetic beads AMPure® XP (Ampure, Beckman Coulter Genomics, Danvers, MA) according to the manufacturer's protocol. Briefly, 90 μl of the magnetic bead suspension are added per 50 μl of PCR mixture, homogenized and left for 5 minutes at room temperature. A magnetic base is used to separate the magnetic beads containing the DNA from the liquid. After the samples had been on the magnetic base long enough for the beads to form a firmly aggregated pellet on the tube wall, approximately 5 minutes, the liquid is carefully removed. Two hundred microliters of 70% ethanol are used to rinse the pellet twice and the tube was allowed to dry in a 37 °C air incubator. Once dry the tube is removed from the magnetic holder and the magnetic beads were suspended in 20 μl of water to elute the bound DNA. The suspension is allowed to sit at room temperature for 5 minutes then placed back on the magnetic base until the beads forme a firmly aggregated pellet on the tube wall (approximately 5 minutes). The liquid containing the DNA was recovered and stored at – 20 °C until use.

2.1.3 Restriction Enzyme Digestion of Purified PCR Products

The Pf3 DNA PCR product was digested overnight in a 37 °C water bath with the restriction enzymes using the suggested double digestion manufacturer's buffers (New England Biolabs, Inc., Ipswich, MA). Different restriction enzymes were selected to promote correct orientation of an inserted gene into the opening point of the Pf3 genome. This strategy was used throughout the entire project.

The EGFP-containing PCR product was digested overnight in a 37 °C water bath with the restriction enzymes Xba I and Bam HI (New England Biolabs, Inc., Ipswich, MA). Different restriction enzyme sites compatible with those used in Pf3 were selected for the EGFP gene so not to recreate these restriction enzyme sites after ligation. This was done so that the same restriction enzyme sites could be re-introduced in future engineering without the concern of cutting the DNA at sites other than the desired ones.

2.1.4. Ligation of the Digested PCR Products

Digested Pf3 and the PCR amplified EGFP DNA were mixed at the ratio of 1 Pf3 to 5 EGFP DNA molecules and ligated using T4 DNA ligase (New England Biolabs, Inc., Ipswich, MA) in a volume of 20 μl. The reaction was left at room temperature overnight. The ligated DNA was purified with magnetic beads as previously described above and used for transfection of *P. aeruginosa* PAO1 competent cells.

2.2 Transfection of *Pseudomonas. aeruginosa* PAO1 with the Ligated DNA

2.2.1 Competent Cell Preparation

The method used for transfection was electroporation. Electro-competent *P. aeruginosa* PAO1 cells were prepared as described by Choi et al (2005). Briefly, PAO1 cells were inoculated in 5 ml of LB broth supplemented with 50 ug/ml of kanamycin and grown overnight.

One and a half milliliters of the overnight culture was centrifuged at room temperature for 4 minutes at 17,000xg to pellet the cells. The supernatant was removed, the cells re-suspended in 100 μl of 10% sucrose then re-pelleted. This procedure was repeated three times at room temperature. The final cell pellet was re-suspended in 40 μl of ten percent sucrose. The electro-competent cells were stored on ice or used immediately.

2.2.2 Transfection

Four microliters of ligated Pf3-EGFP DNA were added to 40 μl of freshly prepared competent cells. The sample was transferred into an electroporation cuvette with a 2 mm gap (Fisher Scientific, Pittsburg, PA) and electroporated using the following electroporator settings: 200 Ohms resistance, 25 μFD capacity, and 2.5 set volts with 2.51 actual volts and a time constant of 3.9 seconds (Biorad Gene Pulser II, Hercules, CA). Immediately after electroporation, one milliliter of super optimal broth with catabolite repression (SOC; Hanahan, 1983) was added to the cell suspension, and two different aliquots were plated, 100 μl and 300 μl, on LB broth agar plates containing 50 μg/ml of kanamycin with 3 ml of 0.75% top agar. To the 100 μl cell aliquot, 200 μl of fresh PAO1 cell culture was added prior to plating to insure a bacterial lawn with proper density. The plates with the transfected PAO1 cells were incubated at room temperature overnight.

2.3 DNA Sequencing of Engineered Phage Pf3 Expressing EGFP

2.3.1 Screening of Recombinant Phages by Fluorescence Visualization

Transfected PAO1 cells expressing EGFP were visualized in a gel imager (Alpha Imager, Alpha Innotech, Santa Clara, CA) equipped with a 488 nm wavelength filter on the UV light source. EGFP fluorescence was recorded using a camera with an emission filter of 500 nm wavelength. Fluorescing phage plaques were isolated and further characterized.

2.3.2 Amplification of Recombinant Pf3 at the EGFP Insertion Site and DNA Sequencing

To confirm insertion of the EGFP gene into the Pf3 genome, a segment of the recombinant phage genome containing the EGFP gene was amplified by PCR using Hotmaster Taq DNA polymerase (5 Prime, Gaithersburg, MD). PCR primers used for the amplification are shown in Table 2; one anneals upstream of the EGFP gene insertion site while the other anneals downstream of the same site such that an amplified fragment contains the EGFP gene as well as the segments of the Pf3 genome on either side of the inserted gene. Amplified fragments from the screened recombinant phages were visualized by agarose gel electrophoresis and compared to lambda DNA cut with the restriction enzyme Hind III (New England Biolabs, Inc., Ipswich, MA) to estimate the lengths of the amplified fragments. Fragments showing the expected length were submitted the VCU Nucleic Acid Research Facilities (NARF) for sequencing using the Sanger method (Figure 7).

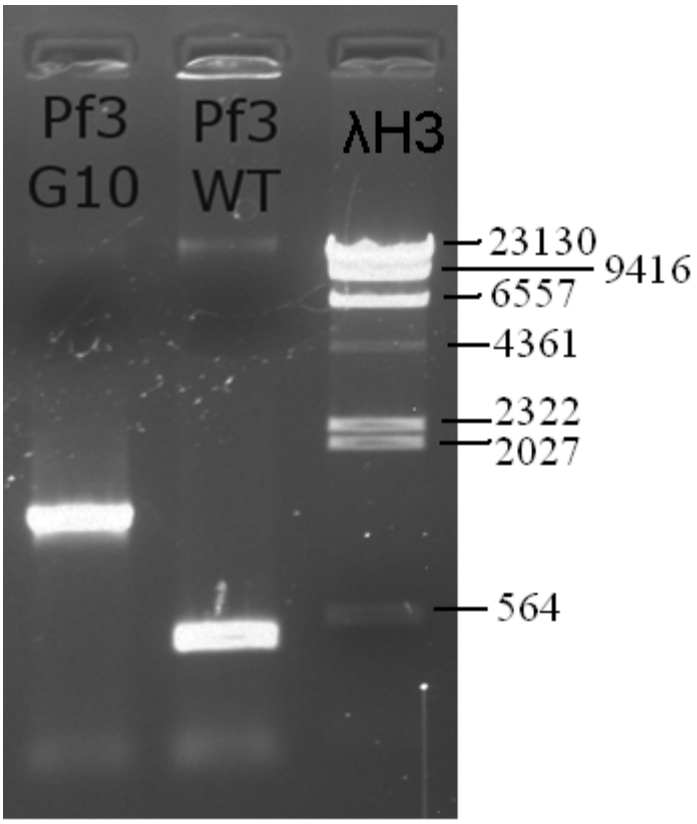

Figure 7: Amplification of recombinant phage Pf3-G10 and wild type Pf3 genomes. The phage constructs are marked and compared to lambda DNA cut with the Hind III restriction enzyme. Markers on the right indicate the nucleotide size of each band in the size marker.

2.4 Construction of Type 88 Phage Display in Phage Pf3 with EGFP

2.4.1 Recombinant Phage DNA Manipulation

The genome of the Pf3 phage engineered to express EGFP (Pf3-G10) was amplified by PCR with primers shown in Table 2 so that it was linearized with the EGFP gene on one end and the phage transcription termination signal on the other end (Velocity DNA Polymerase, Bioline, Taunton, MA). The modified major coat protein gene (modMCP) was produced by annealing oligonucleotides and then amplified from the annealed oligonucleotides using primers with restriction enzyme recognition sites (Table 2). This was not an exact replica of the MCP gene already present in the Pf3 genome; approximately every 6^{th} codon was modified for a total of seven modified nucleotides. Due to synonymous codon usage, the amino acid sequence was not modified (Figure 8). Based on Pierce, this created a more stable phage genome than having two identical copies of the MCP because it decreased the likelihood of genetic deletions in the phage resulting from homologous recombination (Pierce, 1989).

Pf3 MCP gene

ATGCAATCCGTGATTACTGATGTGACAGGCCAACTGACAGCGGTGCAAGCTGATATCACTACCA
TTGGTGGTGCTATTATTGTTCTGGCCGCTGTTGTGCTGGGTATTCGCTGGATCAAAGCGCAATT
CTTTTGA

Pf3 modMCP gene

ATGCAATCCGTGATTACCGATGTGACGGGCCAACTGACAGCTGTGCAAGCTGATATCACCACCA
TTGGTGGTGCCATTATTGTTCTGGCCGCCGTTGTGCTGGGTATTCGTTGGATCAAAGCGCAATT
CTTTTGA

Amino-acid sequence of Pf3 MCP = modMCP

MQSVITDVTGQLTAVQADITTIGGAIIVLAAVVLGIRWIKAQFF*

Figure 8: Changes of bases in the Pf3 MCP gene sequence for the synthesis of the modMCP gene. The 7 bases changed in modMCP relative to the wild type MCP gene are shown red. These changes did not affect the amino-acid sequence of modMCP.

2.4.2 PCR Amplification Digestion with Restriction Enzymes

The amplified Pf3-G10 genome was digested with restriction enzymes Bam HI and Spe I while the amplified modMCP gene was digested with Bgl II and Xba I (New England Biolabs, Inc., Ipswich, MA). Magnetic beads were used along with a magnetic base to concentrate and purify the PCR product digestions as previously described (Ampure, Beckman Coulter Genomics, Danvers, MA).

2.4.3 Ligation

Digested Pf3-G10 DNA and modMCP DNA were mixed at a ratio of 1 Pf3-G10 to 5 modMCP DNA molecules and ligated using T4 DNA ligase and the manufacturer's buffers (New England Biolabs, Inc., Ipswich, MA) in a volume of 20 μl. The reaction was left at room temperature overnight. The ligated DNA was purified with magnetic beads as previously described above and used for transfection of PAO1 competent cells. Here a modified copy of the Pf3 major coat protein was inserted so that it was hinged to the EGFP gene.

Competent cell preparation, transfection, and verification of expression were conducted as previously described in 2.2 and 2.3. The resulting recombinant phage was named Pf3-G10-SH (Figure 9).

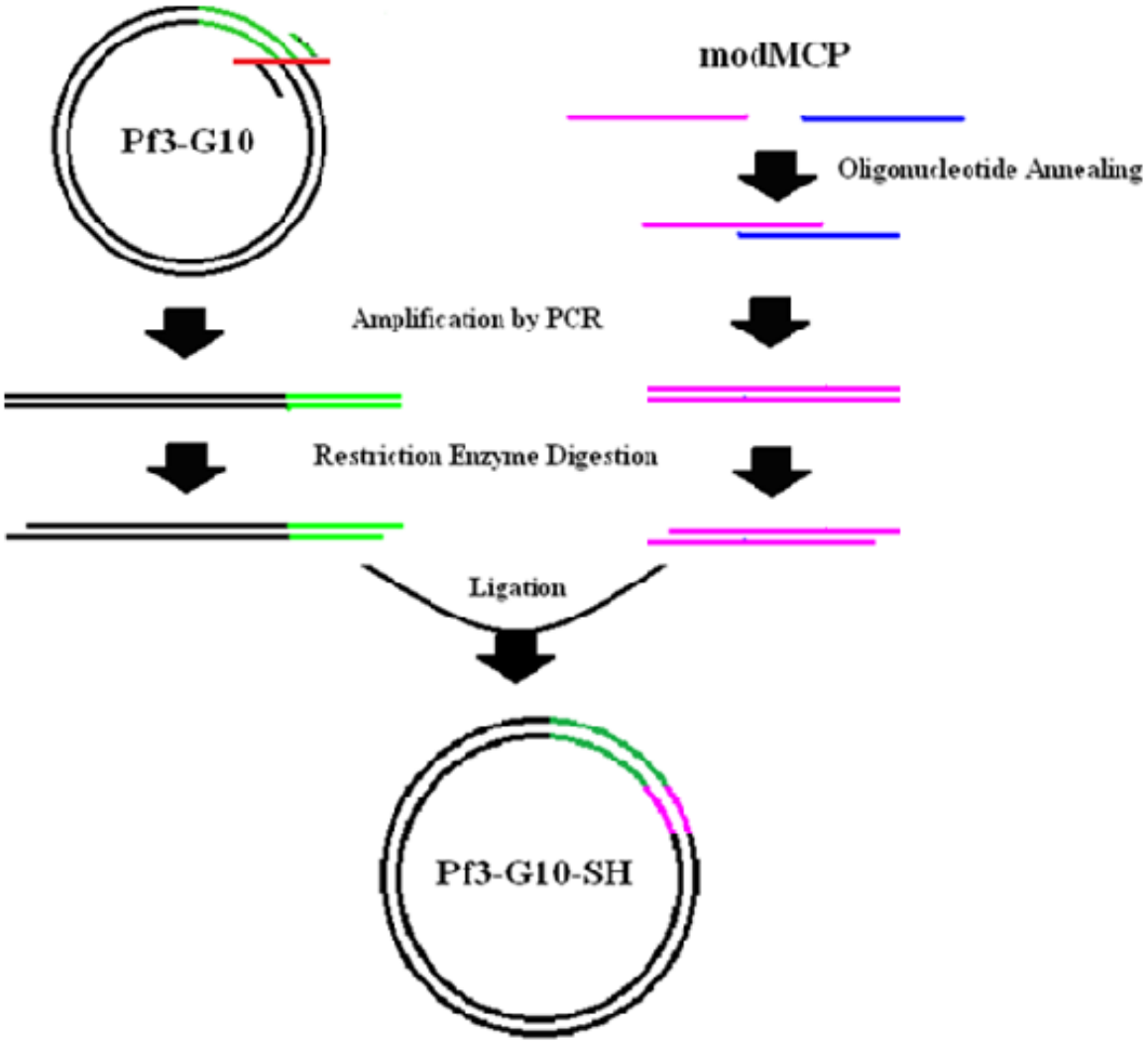

Figure 9: Steps for constructing the recombinant phage Pf3-G10-SH. The genome of bacteriophage Pf3-G10 was amplified and digested with restriction enzymes. The point of linearization is indicated by the red line an d the oligonucleotide primers are indicated by the small green line and the small black line moving away from the point of linearization. The modified MCP gene was created by annealing two oligonucleotides together and then amplified by PCR and digested with restriction enzymes. The two digested fragments were then ligated together to form a double stranded recombinant phage.

2.5 Non-denaturing SDS-PAGE

Cultures of uninfected PAO1, wild type Pf3 infected PAO1 cells, Pf3-G10 infected PAO1 cells, and Pf3-G10-SH infected PAO1 cells were grown for 18 hours in LB supplemented with 50 μg/ml of kanamycin with shaking at room temperature. The cells from 1.5 ml of culture were harvested by centrifugation and the supernatant was removed to a fresh tube. The cell fraction was treated with 1.5 ml BugBuster Protein Extraction Reagent (EMD Chemicals, Gibbstown, NJ) which lysed the cells without denaturing proteins. Each sample from the lysed cell pellet and from the supernatant was passed through a .22 μm syringe filter and then concentrated using 10K column filters (Millipore, Billerica, MA). Samples were loaded on to a pre-cast 12% polyacrylamide gel (Mini-Protean TGX, Biorad, Hercules, CA) after being combined with Native Sample Buffer (Biorad, Hercules, CA). The gel was run for 30 minutes at 220V and samples expressing EGFP were visualized in the gel imager equipped with a 488 nm wavelength filter on the UV light source. EGFP fluorescence was recorded using a camera with an emission filter of 500 nm wavelength.

2.6 Construction of Pf3-SM1(4)

2.6.1 SM1(4) DNA Amplification

The SM1(4) cassette consists four tandem copies of the SM1 peptide coding sequence and was amplified from the plasmid PDB48(SM1)4, kindly provided by Dr. Sibao Wang from Johns Hopkins School of Public Health. Oligonucleotide primers SM1-4FATGblunt and SM1-4RBHI were used to amplify the SM1(4) cassette (Table 4).

Table 4: Oligonucleotides for the genetic engineering of bacteriophage Pf3-G10-SH with SM1(4) gene cassette

Primer Name	Primer Sequence
[a]For amplification of Pf3-G10-SH genome and replacement of EGFP gene	
G10EGFPBH1dwn	ACCGGATCCAGTAAAGGAGAAGAACTTTTCAC
G10RBSup	CATGTATATATCTCCTTTCTAGTGCC
[b]For amplifying SM1(4) gene cassette	
SM1-4FATGblunt	ATGGCTAGCCCCTGTCAGCG
SM1-4RBHI	CCCAGGTCCCGGGGGTGGATCCGT
[c]For sequencing around the insertion site	
Pf3-8_seq2	TGGGCTGAACGTTCAGATTTGC
Pf350R_seq	GAGCAACAATCAAACGACCCT

[a]Oligonucleotide primers for linearizing Pf3-G10-SH phage genome and replacing the EGFP gene with the SM1(4) gene cassette amplified with oligonucleotide primers SM1-4FATGblunt and SM1-4RBHI[b].
[c]Oligonucleotide primers for amplification and sequencing of the regions around the insertion site.

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2.6.2 Ligation, Transfection, and Testing Putative Recombinant Phages

The amplified SM1(4) cassette was ligated into Pf3-G10-SH linearized by PCR with oligonucleotide primers listed in Table 4. The PF3-SM1(4) ligated DNA was transfected into competent PAO1 cells as described previously in 2.2 and 2.3 (Figure 10).

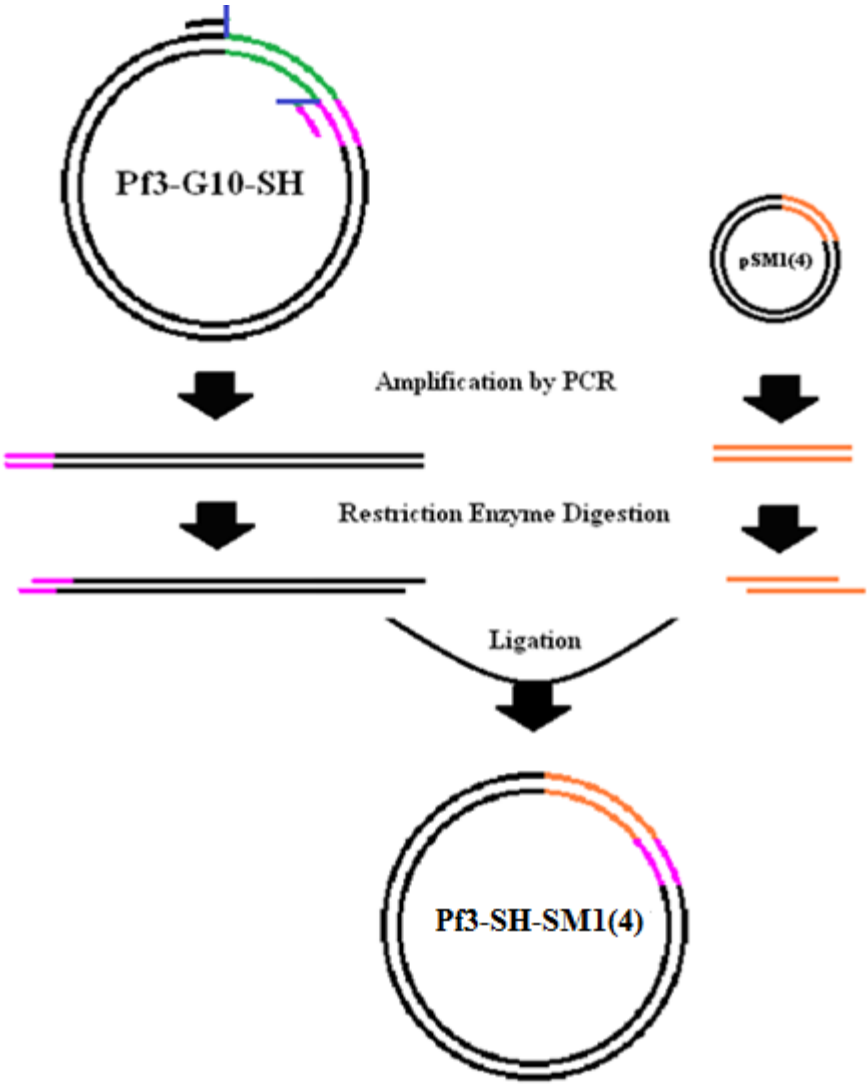

Figure 10: Steps for constructing ~~a~~ the recombinant phage Pf3-SH-SM1(4). The genome of bacteriophage Pf3-G10-SH is amplified and digested with restriction enzymes. The primers are indicated by the small black line and small pink line moving away from the EGFP gene. The SM1(4) cassette was amplified from plasmid PDB48(SM1)4. The two digested fragments were then ligated together to form a double stranded recombinant phage.

2.7 Construction of Pf1-G and M13-G

Manipulations of phages Pf1 and M13 were conducted as previously described to construct phage Pf3-G10. The EGFP oligonucleotides used for Pf1 and M13 were the same as those used for phage Pf3. Oligonucleotide primers were unique for each phage; those for phage Pf1 can be found in Table 5 and those for phage M13 can be found in Table 6.

Table 5: Oligonucleotides for the genetic engineering of bacteriophage Pf1 with the EGFP gene

Primer Name	Primer Sequence
[a]For amplification of the Pf1 genome	
Pf1MCP5upSpeI	AGACCAACTAGTCACACCGACCAGAGCACCCG
Pf1MCP5dwnBglII	CGACCAGATCTGTTGGGGGCGTTCTTCGCCG
[b]For amplification of the EFGP gene	
EGFPFXbaI	ACATCTAGAAAGGAGATATATACATGAGTAAAGGAGAAGAACTTTTC
EGFPFRBamHI	CCTGGATCCTTATTTGTATAGTTCATCCATGC
[c]For sequencing around the insertion site	
Pf18R_seq	CGGTGAACTGTCCATTGAGC
Pf1MCPF_seq	CCTCGTTCCGCAACCTGT

[a]Oligonucleotide primers for linearizing the Pf1 phage genome and inserting the EGFP gene amplified with oligonucleotide primers EGFPFXbaI and EGFPFRBamHI[b].
[c]Oligonucleotide primers for amplification and sequencing of the regions around the insertion site.

Table 6: Oligonucleotides for the genetic engineering of bacteriophage M13 with the EGFP gene

Primer Name	Primer Sequence
[a]For amplification of the M13 genome	
M138dwnSpeI	CAACTAGTCTTTTGGAGCCTTTTTTTTTGGAGATTTTC
M138upBclI	CGACCTGATCAGAGCCTTTAATTGTATCGGTTTATCAG
[b]For amplification of the EFGP gene	
EGFPFXbaI	ACATCTAGAAAGGAGATATATACATGAGTAAAGGAGAAGAACTTTTC
EGFPFRBamHI	CCTGGATCCTTATTTGTATAGTTCATCCATGC
[c]For sequencing around the insertion site	
M138dwn_seq	GCGCAACTATCGGTATCAAGC
M138up_seq	TCAACAGTTTCAGCGGAGTGAG

[a]Oligonucleotide primers for linearizing the Pf1 phage genome and inserting the EGFP gene amplified with oligonucleotide primers EGFPFXbaI and EGFPFRBamHI[b].
[c]Oligonucleotide primers for amplification and sequencing of the regions around the insertion site.

2.8 Identification of Promoter Sequences

An online promoter prediction program (Neural Network Promoter Prediction, Berkeley Drosophila Genome Project) was used to predict promoters in the genomes of Pf3, Pf1, and M13 in order to predict gene expression in these phages.

3. Results

3.1 Insertion of EGFP Gene into the Pf3 Genome (Pf3-G10)

PCR amplification was conducted on potentially recombinant phages with oligonucleotides that amplified a segment of the phage genome containing the EGFP gene. The PCR product was analyzed by agarose gel electrophoresis and the results are shown in Figure 7. Recombinant phages that produced PCR products about 1300 bases long, the expected length of the EGFP gene combined with flanking Pf3 gene segments, were sequenced (Figure 11).

```
Pf3-G10   AAGCGCAATTCTTTTGATCCGTCCTTGGGCTTTTGGCACTAGAAAGGAGATATATACATGAGTAAAGGAGAAGAACTTTTCACTGG
Pf3 MCP   AAGCGCAATTCTTTTGA
RBS                                                AAGGAGATATATAC
EGFP                                                              ATGAGTAAAGGAGAAGAACTTTTCACTGG
```

Figure 11: The DNA sequence of the EGFP gene insertion site in Pf3-G10. The 3' end of the original Pf3 MCP gene is shown in the second line. The ribosomal binding site (RBS) sequence and its position in relation to the ATG of the EGFP gene is shown in the third line.

A copy of the EGFP gene was successfully inserted into the wild type Pf3 genome to produce a recombinant phage named Pf3-G10. This includes the ribosomal binding site (Shine and Dalgarno, 1975) as characterized in *Pseudomonas* (Avichezer, 1992).

3.2 Expression of EGFP Gene by Phage Pf3

EGFP fluorescence was observed on phage plaques on bacterial lawns, and in liquid bacterial cultures (Figures 12). The plaque morphology of the recombinant phage is similar to that of the wild type Pf3 and is shown in Figure 12 (A and B). Under the fluorescent microscope cells infected with pF3-G10 is readily seem

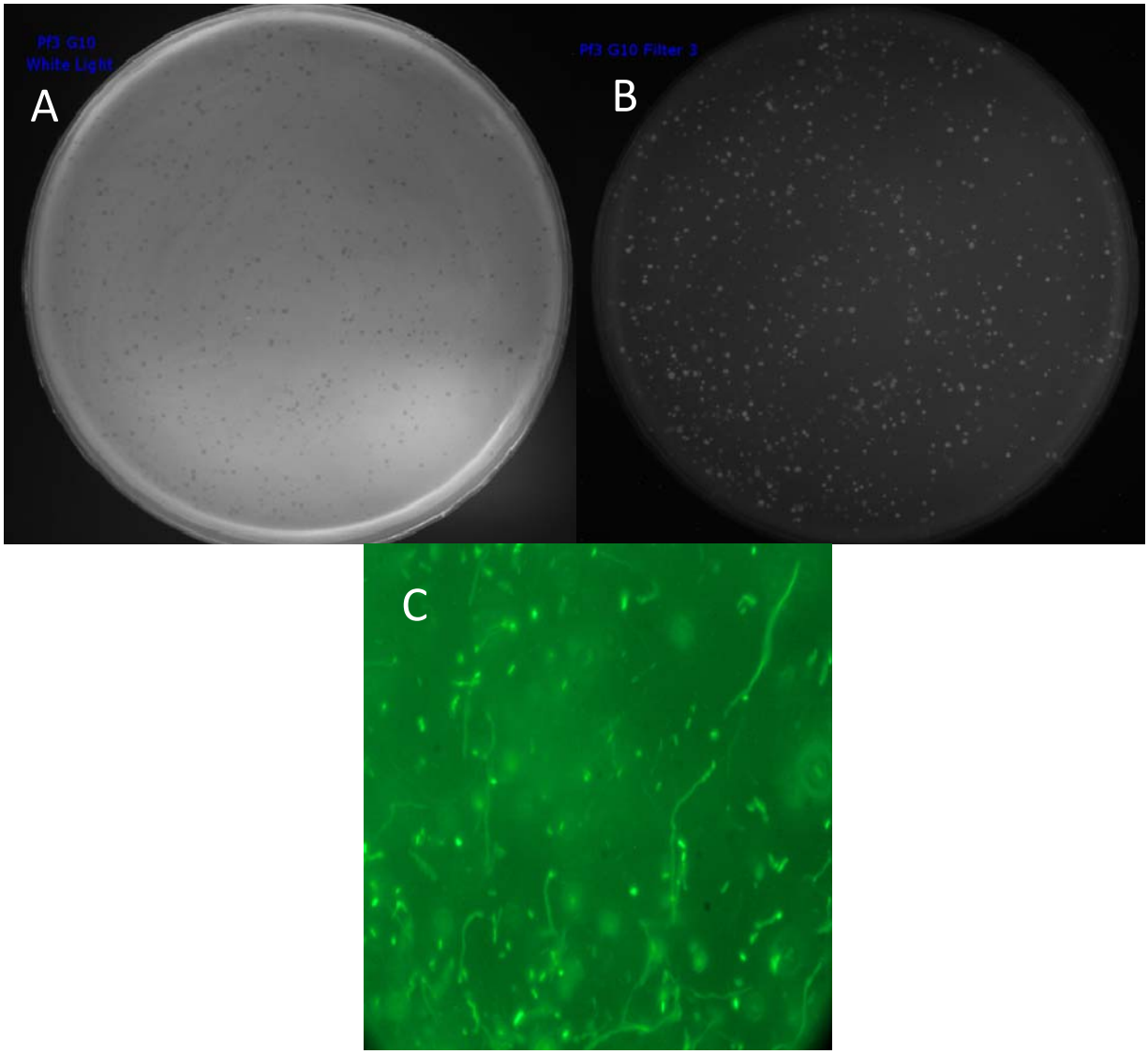

Figure 12: *Pseudomonas aeruginosa* bacteria infected with Pf3 engineered with the EGFP gene (Pf3-G10). A) Colonies of G10 infected cells viewed under white light. B) Colonies of G10 infected cells viewed under UV light through a filter of 488 nm. C) Liquid culture of phage infected cells (100X) under the microscope with filter of 488 nm, photo courtesy of Najla Matos of the facilities of Instituto de Pesquisas em Doenças Tropicais (IPEPATRO), Porto Velho, RO, Brazil.

3.3 Creating Type 88 Phage Display in Pf3

3.3.1 Synthesis of Modified Major Coat Protein (modMCP) Gene

To create modified Pf3 major coat protein, two partially overlapping, complimentary oligonucleotides were synthesized and annealed. The nucleotide sequence of the major coat protein was modified while the amino acid sequence remained the same. This was done because work by Pierce shows an increased possibility of genetic deletions of a second exact copy of a gene inserted into a bacteriophage (Pierce, 1989). This fragment was then amplified by PCR and analyzed by agarose gel electrophoresis (Figure 13).

Figure 13. Synthesis of the Pf3 modified major coat protein. A) Oligonucleotides modMCPF and modMCPStopC, shown in red, were annealed to create a nucleotide sequence with overhanging ends. The consensus sequence of the two anneal oligonucleotides is shown in green. B) The annealed oligonucleotides, shown in green with a purple line indicated a break in the sequence, were then amplified by PCR using oligonucleotides 8XbaIR and 8hingeBgIIF, shown in blue, which contained restriction enzyme recognition sites.

3.3.2 Insertion of modMCP into Pf3-G10 Genome (Pf3-G10-SH)

After transfection with the Pf3-G10 genome with the inserted modified MCP, potentially recombinant phages were amplified by PCR and sequenced. From sequence analysis it can be seen that the modified MCP gene has been successfully inserted into the previously engineered phage Pf3-G10. This phage, called Pf3-G10-SH for self-helper, could also be viewed as glowing plaques on a lawn of PAO1 cells. It can be seen that EGFP is being expressed on the lawn of PAO1 where there are plaques (Figure 14).

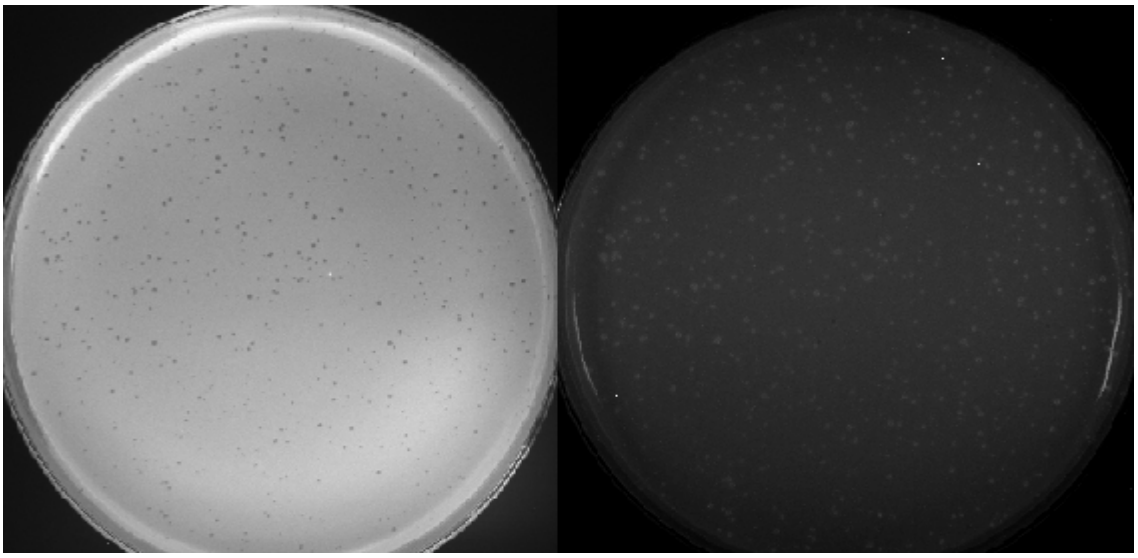

Figure 14: *Pseudomonas aeruginosa* bacteria infected with Pf3-G10 engineered with a modified MCP gene (Pf3-G10-SH) A) Colonies of Pf3-G10-SH infected cells viewed under white light. B) Colonies of SH infected cells viewed under UV light.

3.4 Fate of the EGFP in PAO1 Infected with Pf3-G10 and Pf3-G10-SH .

Due to the ultimate goal of developing a phagotransgenesis model to combat the malaria parasite, it is important to know the location of the expressed foreign peptide. For applications in disease research it is imperative that the foreign peptide be released from the cell so that it may come in contact with the extracellular environment. To analyze the location of the expressed foreign protein, a non-denaturing protein gel of both the supernatant and cell fractions of 18 hour culture of uninfected PAO1 cells, PAO1 cells infected with wild type Pf3, PAO1 cells infected with Pf3-G10 and PAO1 cells infected with Pf3-G10-SH was conducted (Figure 15).

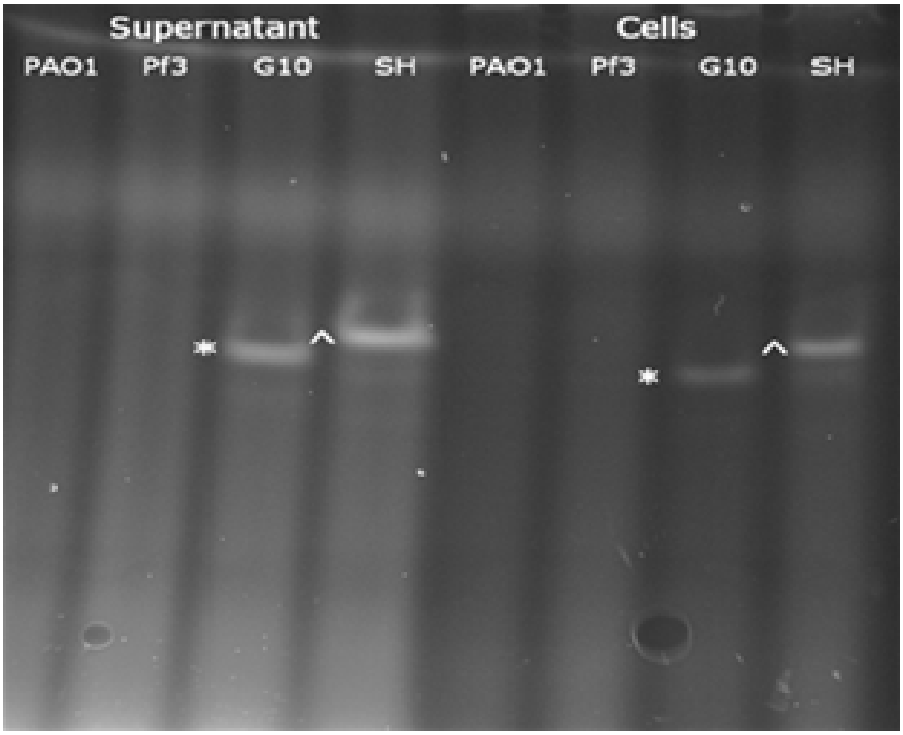

Figure 15. Non-denaturing Protein Gel of Uninfected PAO1 cells, PAO1 Cells Infected with Wild Type Pf3, PAO1 Cells Infected with Pf3-G10, PAO1 Cells Infected with Pf3-G10-SH, and Their Respective Supernatants photographed under UV light with a black and white camera. There are clearly no bands in either of the PAO1 cell column or in the PAO1 cell supernatant column. The Pf3 infected PAO1 cells show no bands in either the cell or supernatant columns. Both clones Pf3-G10(*) and Pf3-G10-SH(^) contain a copy of the EGFP gene and have visible bands shown on the gel in both the cell fraction columns and the supernatant fraction columns. This indicates that not only is EGFP being produced by the cell, it is located outside of the cell.

The cell fraction which would contain any foreign protein that had not been secreted from the cells was separated by filtration from the supernatant fraction which contained any proteins secreted from the cells. Fluorescing bands of EGFP protein can be seen to indicate the presence of the EGFP protein in the supernatant of both the Pf3-G10 and the Pf3-G10-SH as well as in the cell fraction of both Pf3-G10 and Pf3-G10-SH. The Pf3-G10-SH lane indicates a band of protein that migrated slower than the band produced by Pf3-G10 which indicates that the EGFP protein produced by Pf3-G10-SH infected PAO1 is larger than that produced by Pf3-G10 infected PAO1 due to additional amino acids that were fused downstream of the EGFP gene in an attempt to create a fusion protein.

3.5 Sequence Analysis of Pf3-G10-SH

Upon further analysis, it was discovered that even though the fully modified MCP was successfully inserted into engineered phage Pf3-G10, the modified MCP is not in the same reading frame as the EGFP gene and therefore EGFP is not fused to modMCP protein.

3.6 Insertion Replacement of SM1 Gene into Pf3-G10-SH

Even though creation of Type 88 phage display using bacteriophage Pf3 was unsuccessful with EGFP, the engineered phage Pf3-G10-SH may be used as a vector for inserting other foreign peptides into the Pf3 genome. The Pf3-G10-SH genome was processed as described above and the SM1(4) cassette was successfully inserted (Figure 16).

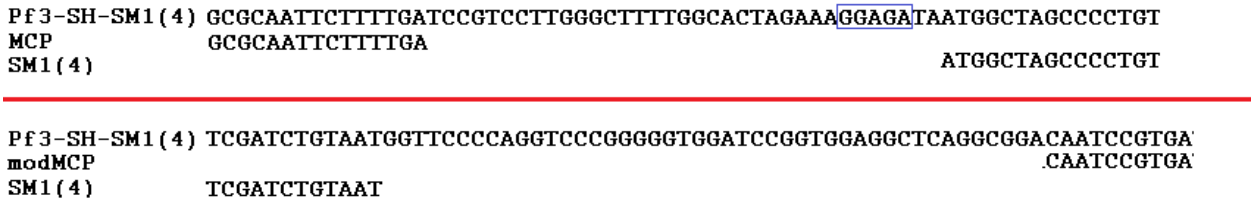

Figure 16: Insertion site of the SM1(4) gene cassette. Above the red line, the Pf3-SH-SM1(4) construct sequence can be seen to contain the end of the Pf3 MCP gene and the beginning of the SM1(4) gene cassette as well as the RBS shown in the blue box. Below the red line, the Pf3-SH-SM1(4) construct sequence can be seen to contain the end of the SM1(4) gene cassette as well as the beginning of the modMCP gene. The SM1(4) gene cassette is in the same reading frame as the modMCP gene.

3.7 EGFP Gene Expression in Phages Pf1 and M13

Based upon similar gene organization, the same methods were used to construct analogous recombinant phages in filamentous phage Pf1 of *P. aeuriginosa* strain PAK and filamentous phage M13 of *E. coli* (Figure 17).

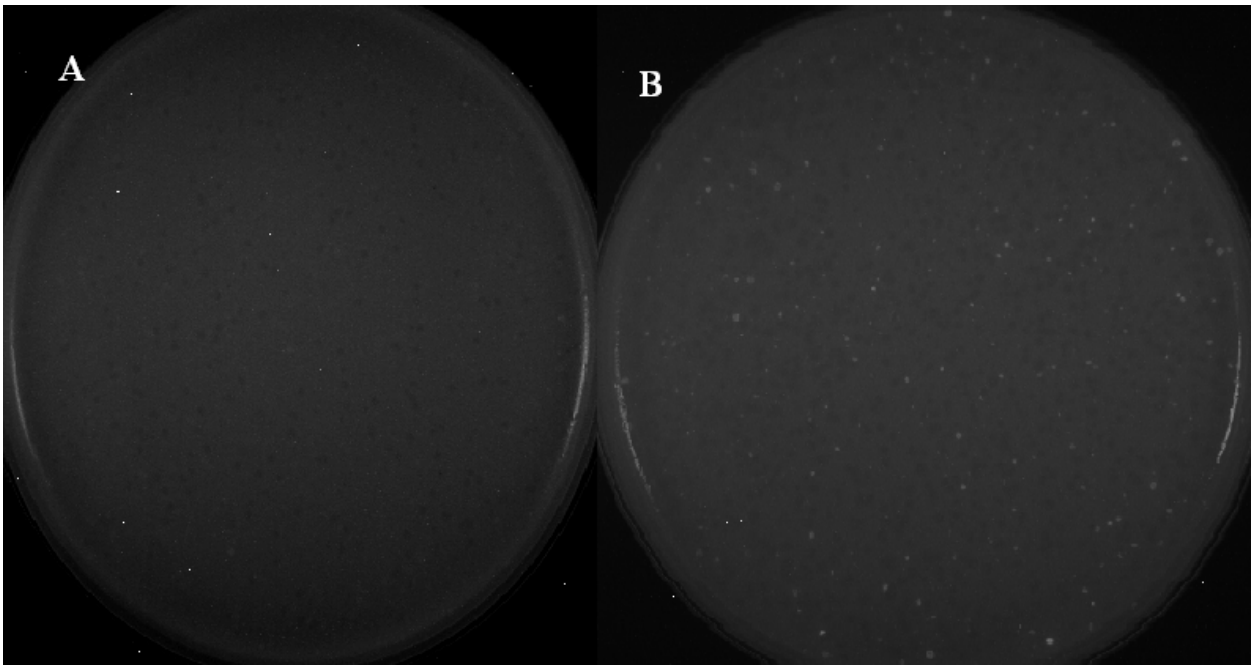

Figure 17. M13 and Pf1 phages engineered with the EGFP gene. A) Plaques of M13-G phage visualized by excitation UV light through a 488nm wavelength filter and emission light captured through a camera equipped with a 500nm wavelength filter. B) Plaques of Pf1-G infected cells visualized by excitation UV light through a 488nm wavelength filter and emission light captured through a camera equipped with a 500nm wavelength filter.

3.8 Promoter Analysis in Phages Pf3, Pf1, and M13

With the goal of identifying putative promoters, the Berkeley Drosophila Genome Project's Neural Network Promoter Prediction tool was used to predict potential promoters within the genomes of phages Pf3, Pf1, and, M13. Because phages use host cell machinery for transcription of their genome, prokaryotic promoter regions were searched for. A score is provided for predicted promoters, and the score indicates the percentage of all promoters recognized at that threshold, as well as the number of false positives. All promoters analyzed produced a minimum score of .85 indicating that 60% of all promoters were indicated with a false positive of 0.4% and a correlation coefficient of 0.72. In the three analyzed phages, it can be seen that known or putative single stranded DNA binding protein is transcribed in frame with and immediately precedes the major coat protein. This is logical since both the single stranded binding protein and the major coat protein are highly expressed. Analysis of these three phages for potential promoters reveals at least one promoter occurring immediately prior to the start of the single stranded binding protein gene in each phage with two promoters prior to the start of the single stranded binding protein gene in phages Pf1 and M13. Multiple putative promoters can be seen in all presented genome maps (Figure 18).

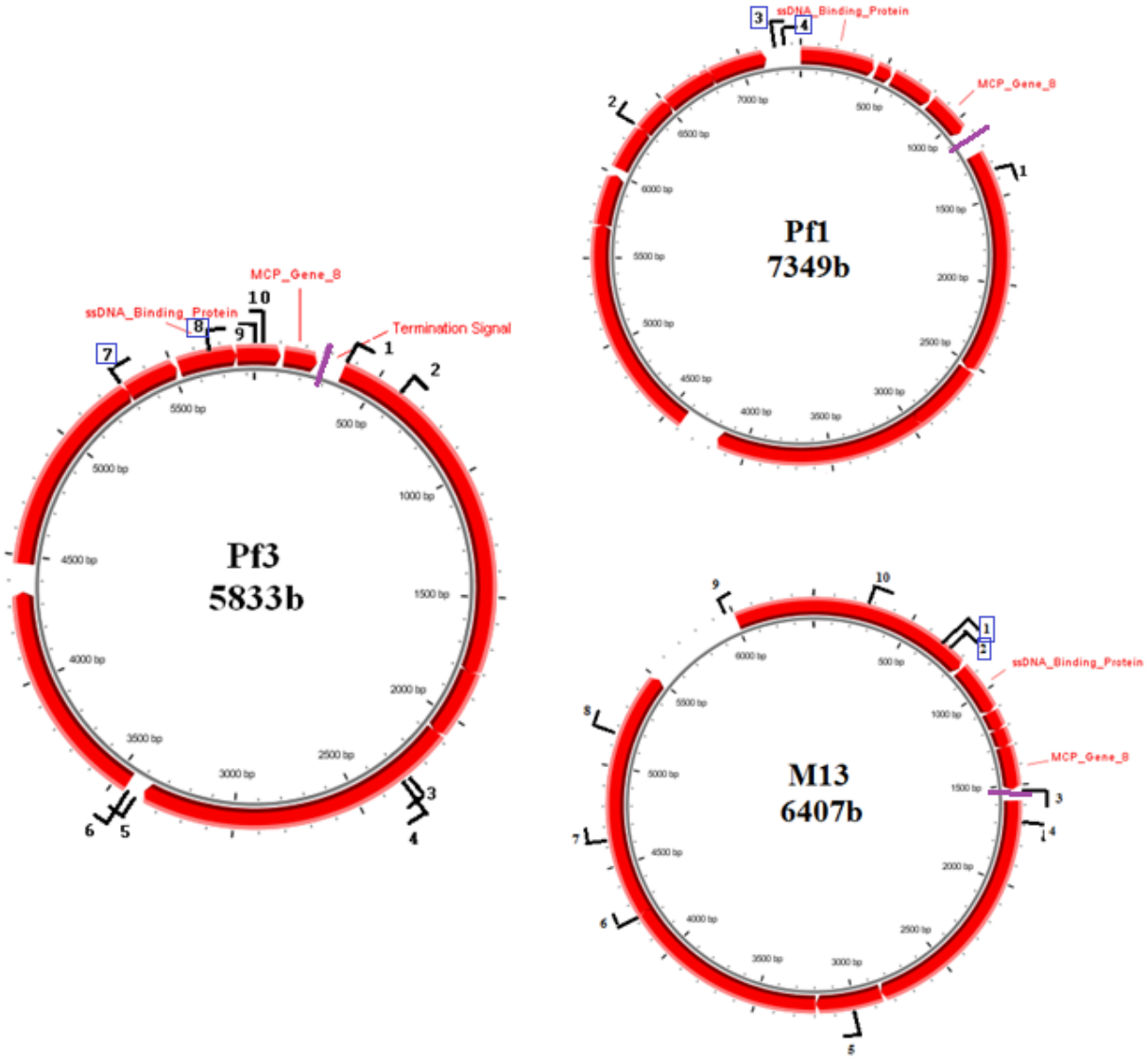

Figure 18: Alignment of bacteriophage genomes with their respective genes and putative promoters. For clarity, only the single stranded binding protein (SSBP) is indicated as well as the major coat protein (MCP) genes, and the two putative promoters located immediately upstream of the SSBP gene are highlighted in a blue box. The EGFP gene insertion site in each of the three phage genomes is marked by a purple line. Genome maps created using CGView.

4. Discussion

4.1 EGFP Gene Expression in phages Pf3, Pf1, and M13

Here it is shown that the EGFP gene can be inserted downstream of the major coat protein of phage Pf3, Pf1, or M13 and be expressed under the control of the major coat protein promoter. Since most filamentous phages have a comparable gene organization to the three systems that have been constructed, this suggests that any filamentous phage can be used to construct a similar system. Hence, there may be unlimited potential for this technology. Furthermore, this expression system could be used in any environment that filamentous phages are found because the foreign peptide is solely dependent upon expression from the phage's promoters.

Additional characterization of the construct would need to be conducted along with protein size based experiments in order to determine the range of foreign peptides that may be expressed in this manner. There is no reason to believe that two fused proteins could not be expressed in this fashion leading to the hypothesis that a foreign gene being fused to a phage gene could be expressed to create a Type 88 phage display system.

4.2 Type 88 Phage Display in Pf3 with the EGFP gene

For the ultimate goal of using phages as in phage display to block the transmission of the malaria parasite it is imperative that the foreign peptide be secreted from the host cell. This is of such great importance because the foreign peptide must be able to compete with the malaria

parasite for interaction with receptors on the midgut epithelia of the mosquito. Creating a Type 88 phage display system in which the foreign peptide is expressed on the phage coat would be an ideal system for the foreign peptide to be secreted from the host cell. Type 88 phage display also meets the need of using only one genetic element. Construction of Type 88 phage display using phage Pf3 was first attempted using the EGFP gene.

Attempts to construct a phage display system with the EGFP gene on the phage coat of Pf3 were unsuccessful because the inserted copy of the EGFP gene was not in frame with the inserted modified major coat protein. The inability to create an appropriate construct may be due to problems caused by proofreading polymerase used during PCR. The enzyme that was used disrupts the ends of the DNA strands and may not produce the intended product; this was discovered well after the experiments had already been conducted and could have easily caused a few bases to be deleted resulting in the EGFP gene and the modMCP gene being in different reading frames. Additional characterization of this engineered phage needs to be conducted and attempts to put the EGFP gene in frame with the modified MCP gene are underway but have been unsuccessful up to this point.

The success here is in the creation of a replacement vector that can be used for insertion of other foreign peptides into the genome of Pf3 such that they are fused to the modified major coat protein and thereby displayed on the phage coat as in phage display. One differentiating point between this Type 88 system and traditional phage display is the use of a promoter region. In traditional M13 phage display, a promoter region is inserted into the phage genome so that the foreign gene is expressed (Sidhu, 2001). Here, the created system does not require a promoter to be inserted prior to the foreign gene in order for there to be expression. This is likely due to the location in the bacteriophage genome where the foreign gene fused to the modified major coat

protein is inserted. The major coat protein is a highly expressed, phage-coded protein and here the inserted gene fusion is placed immediately after the major coat protein and before the transcription termination signal.

4.3 Type 88 Phage Display in Pf3 with the SM1 gene

The constructed Pf3 replacement vector was used to insert a gene cassette containing four copies of the SM1 gene into the Pf3 genome so that they were in frame with the modified major coat protein. Four tandem copies of SM1 (SM1(4)) were used because it Ito has already shown that the blocking effect of the protein increased with the number of copies of the protein gene that were being expressed but that the increase in blocking is insignificant after the fourth copy (Ito, 2002).

This construct was successfully obtained but optimization is required. As seen in Figure 15, all of the needed pieces are present in the construct; however, there are only two bases between the ribosomal binding site and the beginning of the SM1(4) cassette. This was not intentional and, again, is most likely a consequence of the proofreading polymerase which was used in the PCR amplifications. There is no reason to believe that the SM1(4) cassette is not being expressed and no reason to believe that it is not fused to the modified major coat protein; however, optimization of the construct to introduce additional bases between the ribosomal binding site and the beginning of the SM1(4) cassette would be ideal. Typically in *Pseudomonas* the ribosomal binding site is found 6 to 10 nucleotides prior to the beginning of a gene (Avichezer, 1992, Blumer, 1999; Olins, 1988). Using the same spacing would be ideal when constructing a recombinant phage to be used in *Pseudomonas*. Expression would likely be increased with appropriate placement of the ribosomal binding site as Olins reported a 340-fold increase in gene expression when using a ribosomal binding site (Olins, 1988).

4.4 Expressed Foreign Peptide Secretion

As seen in Figure 14, the expressed EGFP, as well as the EGFP fused with additional amino acids, is being secreted from the cell. The method of secretion is unknown and characterization is needed. There are several ways that filamentous phages may be secreted from host cells, and the expressed EGFP is likely to use one of these methods.

The EGFP may be simply using the host encoded secretion channel as seen in the phage CTXΦ which uses the same host encoded channel as cholera toxin for secretion (Rakonjac, 2011). Alternatively, the EGFP may also be using a phage encoded secretion channel. In either case the EGFP is being secreted from the host cell as desired; it is imperative that expressed foreign peptides be secreted from the cell so that they may interact with the extracellular environment. In presented case of malaria, the SM1 peptide must be secreted from the cell so that it can competitively bind to the same ligand as the malaria parasite. Secretion may be a distinct property of EGFP and may not be observed when other foreign proteins are inserted into the Pf3 genome. Further characterization of the secretion system that is acting in the Pf3-G10 and Pf3-G10-SH constructs needs to be conducted so that this system may be applied to other foreign proteins. The method of secretion may dictate how this type of system can be used in the future.

4.5 Promoter Sequence Analysis

There is at least one putative promoter occurring immediately prior to the start of the single stranded DNA binding protein gene in each of the three phages that were analyzed. This promoter would act to promote transcription of both the single stranded binding protein as well as the major coat protein and, in the Pf3, Pf1, and M13 constructs presented here, the foreign

EGFP gene that was inserted into the phage genome. There were also promoters predicted in the opposite direction of gene transcription which may play a role in expression control.

Polycistronic gene transcription is highly likely because filamentous bacteriophage genes are spaced in tandem, without room for individual promoters and there were not promoters predicted prior to each gene in the phage genomes.

In prokaryotes, there is a promoter region 10 bases upstream from the transcription start site and a similar structure has been described in bacteriophages (Gershenzon, 2005; Olins, 1988). This is consistent with the predicted promoter sequences in the genomes of phages Pf3, Pf1, and M13. Here, there were promoters predicted inside of genes which further supports the idea that the phage genes are organized into operons to control expression levels. Highly expressed genes such as the single stranded DNA binding protein and the major coat protein appear to be organized into an operon while the minor coat proteins appear to be organized into another. When inserting a foreign gene consideration should be given to the operon the gene is inserted into. Here, high expression of the foreign gene was desired so placing it within a highly expressed operon was helpful.

4.6 Future Uses

Based on the results, the future implications of foreign gene expression using the systems similar to those created here are tremendous. Clearly there is a potential application in controlling the transmission of the malaria parasite. While the SM1 protein blocks the development of the malaria parasite found to infect mice, once a similar protein is found that block the parasite that infects humans, a phage system that expresses that protein may be easily constructed following the same protocol used here.

Other vector borne diseases may also be blocked using this type of system. For instance, the BMAP-18 peptide, a truncated version of the bovine myeloid antimicrobial peptide – 27 (BMAP-27) is has been reported to block the trypanosomatid parasites that cause Chagas disease (Haines, 2009). Any filamentous phage-host pair found to naturally occur in the host for the trypanosomatid parasite may be engineered to express BMAP-18, using the same technique used here, thereby blocking the parasite. Release of a genetically altered phage is more favorable than release of a genetically modified insect or bacteria because phages are unable to propagate outside of their host bacteria meaning that phagotransgenesis is more favorable than transgenesis or paratransgenesis in blocking vector borne parasites.

4.7 Conclusions

Here a foreign gene has been successfully expressed in bacteriophages Pf3, Pf1, and M13 using the phage's naturally occurring promoters. This was then applied to create a Type 88 phage display system to control the spread of the malaria parasite. The Type 88 phage display system met the biggest requirement of a malaria parasite blocking system; one genetic element. While this phage display system has been designed for use in the control of the malaria parasite, alternative applications abound.

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