To the Graduate Council:

I am submitting herewith a thesis written by Michael R. Jackson entitled "Phage Display of R67 Dihydrofolate Reductase." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in biochemistry, cellular, and molecular biology.

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Phage Display Of R67 Dihydrofolate Reductase

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Degree
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Michael R. Jackson
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Dedicated to my family and two very special friends, Brian and Ashleigh.

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Abstract

Two clones have been constructed for the phage display of active R67 dihydrofolate reductase (DHFR). These clones will be used in future experiments for affinity selection of mutants with increased catalytic efficiency. R67 dihydrofolate reductase is an R-plasmid-encoded enzyme that confers clinical resistance to the antibacterial drug trimethoprim. The enzyme shows no sequence or structural homology to the *Escherichia coli* chromosomal DHFRs. R67 DHFR is a homotetramer with an active site pore that traverses through the length of the protein. The protein possesses exact 222 symmetry. The symmetry element allows for binding of one DHF or one NADPH molecule in each half-pore site although they are bound differently. Crystal structures with bound folate and $NADP^+$ are not available for evaluation of R67 DHFR's hydride transfer reaction. Also, mutations in the active site inevitably produce four mutations per enzyme; this further complicates studies of its mechanism. Phage display of R67 DHFR will allow for selection of randomly mutated variants possessing increased catalytic properties that could be the result of a diverged active site or addition of a general acid. It is not likely that the phage display of the 78 amino acid monomer of R67 DHFR can work because of complications associated with the formation of the homotetramer on the surface of virions. Therefore, a quadruple R67 DHFR was used where four monomers are covalently linked and folds into an active enzyme fused to either gene III or gene VIII phage coat protein. This is the first case of a fusion involving a tandem array. The first step leading to the phage display of the quadruple R67 DHFR was to construct two gene fusion constructs that contained the single gene copy of R67 DHFR linked in frame to the gene III or gene VIII of M13 bacteriophage. Then these

two constructs were used as templates for engineering the two fusions containing the quadruple R67 DHFR fused to the gene III phage coat protein or to the gene VIII phage coat protein for phage display. These two fusions take advantage of the different display environments that each system has to offer. All four constructs conferred trimethoprim resistance upon host *E. coli.* Purified phage indicated the quadruple R67 DHFR was displayed as an active enzyme. However, altered K_m values were noticed. No exact assessment can be made for the altered K_m values observed and the low activity measured. The altered properties of the fusion product do allow for a general trimethoprim resistance selection. A more efficient selection will use a bisubstrate analog in future experiments.

Table of Contents

Chapter		Page
I.	Introduction	1
II.	Materials and Methods	15
III.	Results	25
IV.	Discussion	37
List of References		46
Appendix		50
Vita		68

Figures Page

1. Images of the monomer structure of R67 DHFR depicting residues in the active site (A) and the association of two monomers into a dimer (B).....52

2. Two depictions of the homotetramer of R67 DHFR.....53

3. A drawing representing the mosaic display of R67 DHFR on the surface of the M13 phage using the gene VIII system (left) and gene III system (right).....54

4. The sequence of synthetic R67 DHFR.....55

5. The 250 bp PCR product for the double mutant R67 sequence with an Nsi I site at the 5' end and an Apa I site at the 3' end 56

6. Construction of the wlz1pIII phagemid.....57

7. A 1.2% agarose gel used to screen for the double mutant gene VIII phagemid with an R67 gene insert 58

8. Construction of the blz1pVIII phagemid.....59

9. Construction of the MRJpVIII and MRJpIII phagemids (four gene copies of R67 DHFR sequence linked in frame to the phage gene coat protein sequences).....60

10. Double restriction enzyme digest of the four constructs with either single copy or four copy R67 DHFR sequence inserted into the gene VIII and gene III phagemids.....61

11. Colony lifts detecting the four fusion proteins using the antibody for R67 DHFR.....62

12. The protein analysis of phage particles with the attached fusion protein after CsCl density purification.....63

13. Some of the bisubstrate compounds that our collaborator Bruce Hathaway proposes to synthesize 64

Tables

Page

I. Presence of DHFR activity during phage purification.....66

II. Relative velocities of samples containing phage with the active quadruple R67 DHFR displayed on its surface. Samples assayed have been purified using CsCl density centrifugation.....67

List of Abbreviations

Abs_{340}	absorbance at 340 nm
Amp	ampicillin
Asp	aspartate
bp	base pair
blz1pVIII	phagemid with the single R67 gene sequence linked to the gene VIII protein sequence
CsCl	cesium chloride
DHF	dihydrofolate
DHFR	dihydrofolate reductase
E. coli	*Escherichia coli*
EDTA	ehtylenediaminetetraacetic acid
GST	gutathione S transferase
hCG	human chorionic gonadotropin hormone
His	histidine
MES	2-[N-Morpholino]ethanesulfonic acid
MRJpIII	phagemid with the four gene copy of R67 linked to the gene III protein sequence
MRJpVIII	phagemid with the four gene copy of R67 linked to the gene VIII protein sequence
NADPH	nicotinamide adenine dinucleotide phosphate
OmpA	signal peptide sequence used for membrane insertion
PEG	polyethylene glycol
PCR	polymerase chain reaction
stII	signal peptide sequence from the *Enterococcal* bacterial species used for membrane insertion
THF	tetrahydrofolate
TMP	trimethoprim
wlz1pIII	phagemid with the single R67 gene sequence linked to the gene III protein sequence
wt	wild type

Chapter I

Introduction

One goal of protein engineering is to design new enzymes with different properties and catalytic activities, and phage display allows for a selection of those desired rare enzyme products. The phage display technique mimics natural selection and is a sensitive method to find rare variants because of the linkage between replication and recognition. Libraries of randomly mutated gene sequences can be linked to their expressed product and screened on the basis of a researcher's criteria. The method has been extensively employed with smaller peptide sequences and antibody fragments, yet phage display is beginning to be used for the display of active enzymes. The possibilities of finding new enzymes with different binding and catalytic properties would provide information on the structure and function relationships, and this goal is the reasoning behind the application of phage display of R67 dihydrofolate reductase (for a review of phage display see Smith and Petrinko (1) and Kay et al (2)).

Overview of R67 DHFR

Dihydrofolate reductase (DHFR) catalyzes the hydride transfer reaction from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to 7,8-dihydrofolate (DHF), releasing the product 5,6,7,8-tetrahydrofolate (THF) and oxidized cofactor, $NADP^+$. THF is an essential intermediate for cellular metabolism and growth,

particularly in the synthesis of deoxythymidylate (dTMP), which is used in subsequent DNA synthesis and replication (3). Inhibition of DHFR is therefore lethal to rapidly dividing cells, and DHFR is a target for antitumor and antibacterial drugs.

The antibacterial drug, trimethoprim (TMP), was found to bind selectively to the chromosomal DHFR of bacteria (4) and proven to be an effective antibacterial agent. However, resistance to TMP eventually arose by several mechanisms including cell wall impermeability, overproduction of the chromosomal enzyme, mutants of the thymidylate synthetase enzyme, but mainly from the production of plasmid-encoded forms of DHFR (4-9). The type II R67 DHFR (or now classified as dfrB1, dfrB2, and dfrB3 (10)) is one of the plasmid-encoded types conferring resistance to TMP in bacteria, but it is unusual in that it is not homologous in sequence and structure to the chromosomal DHFR (4,11). R67 DHFR may have originated from a non-specific oxidoreductase (12) or it could be a model of a primitive enzyme (11). R67 DHFR is also the least susceptible to TMP pressure compared to the other common types I and III DHFR's (or now dfrA1 and dfrA3) that are homologous to the chromosomal enzyme (11). These characteristics promoted interest into the study of R67 DHFR.

The structure of R67 DHFR was solved as an apoenzyme and with 2 bound folate molecules (11). The 78 amino acid monomer (M_r ~8,400) forms a compact β-barrel composed of five antiparallel β-strands that are connected by three short loops and one 3_{10}-helix turn (figure 1a) (see appendix). The longest strand has a bulge near its midpoint that bends it by 90° and distorts the monomer into two orthogonal β-sheets. The structure

of the monomer is similar to that observed in SH3 domains (11). Exterior surfaces of three β–sheet strands from one monomer form an intersubunit β-barrel with the three same strands from another monomer. This monomer-monomer interface forms the inactive dimer that predominates at pH 5 (figure 1b). At higher pH values, the dimers can associate into an active homotetramer, forming an active site pore with exact 222 symmetry (figure 2) (11,13,14).

R67 DHFR reversibly dissociates from an active tetramer to two inactive dimers with an approximate pK_a of 6.8 (13,15). Residues have been studied in our lab that contribute to the stability of the homotetramer. The association of the homotetramer partly involves hydrogen bond interactions between a His (His 62 and its symmetry related residues) from one monomer with a buried Ser (Ser 59) from the other monomer at the dimer-dimer interface. Upon protonation of His 62 and its symmetry related residues, the interface is destabilized. There are no other hydrogen bond acceptors in the vicinity of Ser 59 and its symmetry related residues in the pocket at the dimer-dimer interface, and the proximity of the protonated imidazole ring is unstable in the hydrophobic pocket (13,14). The homotetramer is also stabilized by interactions involving W38 and K32 and their symmetry related residues, as W38F and K32M mutants are predominantly dimeric in solution (16,Hicks, S. and Howell, E.E., personal communication).

The crystal structure of R67 DHFR has provided information mostly about its folded structure and association. However, there is no structure of the R67 DHFR with

both substrate and cofactor bound. This would provide further information about R67's function and mechanism of catalysis. However, some data has been accumulated by studies of mutated residues potentially involved with binding and catalysis. One result reflects the cumulative effects of mutations involved with the 222 symmetry as one mutation in the gene reflects four mutations per active site. The 222 symmetry element not only presents the challenge in crystallizing the enzyme with a bound folate and $NADP^+$ molecule but also with the analysis of single mutations within R67 for the interpretation of its effects on the mechanism of binding and catalysis.

Unusual binding stoichiometries are observed in the active site pore formed by the homotetramer (17). The active site is 25 Å in length and has an hourglass shape. Presumably the hydride transfer reaction occurs at the central constriction. The 222 symmetry gives the enzyme a potential of four binding sites, but steric constraints only allow for each half pore to bind either one DHF or one NADPH molecule. With the symmetry element the binding mechanism is complex. The enzyme has two nonproductive binding modes where two DHF or two NADPH molecules can bind, or it can bind one DHF and one NADPH to form the productive ternary complex. In addition, positive cooperativity is seen with the binding of two DHF molecules and negative cooperativity with the binding of two NADPH molecules. Therefore, the formation of the productive ternary complex is preferentially accomplished by NADPH binding first. This inhibits another NADPH molecule from binding because of the observed negative cooperativity between two NADPH molecules, but bound NADPH must impart a favorable interaction for DHF to bind in the other half pore site as positive cooperativity

is observed between NADPH and folate. The symmetry element and cooperativity between bound ligands are essential factors for shifting the equilibrium towards the formation of the productive ternary complex. (11,17,18).

Some information from our studies is starting to be accumulated into the mechanism of hydride transfer for the R67 DHFR enzyme. The reaction catalyzed by R67 DHFR likely occurs near the center of the pore. Interligand Overhauser effects have recently shown that the bound NADPH assumes a syn conformation between the nicotinamide and ribose rings (19). After DHF binds, a stereo specific Re (or A side) hydride transfer occurs between C4 of NADPH and C6 of DHF (19,20). So far, only a few residues have been studied that may be involved with this reaction. For example, the Q67 residue and its symmetry related residues play a major role near where the hydride transfer process likely occurs. They form the roof and ceiling at the center of the pore in the active site (11). They are presumably partially responsible for juxtaposition of the nicotinamide ring of NADPH and the pteridine ring of DHF (18,19). Recent studies suggest that the rings partially overlap, perhaps explaining how positive cooperativity between molecules occur (18,19,21). Work is still being done to elucidate the transition state for this reaction as well as the role of specific amino acids in transition state stabilization.

DHF must be protonated at the N5 position before it can accept a hydride ion from NADPH. Studies in our lab show that R67 DHFR may utilize a proton from solution. pH profiles of R67 DHFR show an increase in catalytic rate with decreasing pH

up until there is a protein concentration dependent decrease in activity due to the dissociation of active homotetramer to inactive dimers. The lower pH increases the concentration of the protonated DHF species (15).

The protonation of DHF is of interest because the method by which R67 DHFR acquires a proton from solution is undetermined and different from its *E. coli* chromosomal counterpart. The Asp 27 residue in *E. coli* chromosomal DHFR has been proposed to act as a proton donor in the active site. As DHF binds, the pK_a of the N5 is perturbed from 2.5 to 6.5 (22,23). It is suggested that the elevated pK_a results from the formation of the enol tautomer of the substrate's pterin ring. The Asp 27 residue is buried in a hydrophobic environment. It likely becomes protonated because a negative charge is unfavorable with bound DHF in the pocket (23). The activity of this enzyme greatly decreases with this residue removed. The mutant D27N shows a predominant loss in catalytic rate (24), yet the pH profile of this mutant shows that this step can be bypassed by lowering the pH and increasing the protonated DHF species. DHF acquires a proton from solution in this case. This is probably what occurs with R67 DHFR as it does not contain a proton donor and may utilize a proton from solution to form bound protonated DHF. In support of this, studies with the H62C mutant of R67 DHFR that stabilizes the active tetrameric species shows increased activity as the pH is decreased to pH 5, and this behavior is similar to the D27S mutant of *E. coli* chromosomal DHFR. It would be interesting to determine the mechanism by which R67 DHFR protonates DHF and to see if phage display of R67 DHFR can introduce a residue acting as a proton donor similar to how the Asp 27 residues functions in *E. coli* chromosomal DHFR.

Site directed mutations have been constructed to identify residues that may be involved in stabilizing the ground state and/or transition state in the active site. Mutations in the amino acid sequence of R67 DHFR, however, reflect four mutations in the homotetramer. It is challenging to understand the effects of mutants generated because of the symmetry element. One of the residues, Q67, was proposed from the crystal structure with bound folate and docking studies to play a significant role in the active site (11,18,19). Studies with the Q67H mutant R67 DHFR have shown a significant increase in binding affinity for both ligands as well as a decrease in catalysis (18,25). The His imidazole moiety may form ring stacking interactions that tighten binding with both substrate and cofactor, shifting the binding equilibrium towards the nonproductive complexes. Docking studies have provided some explanation for the effects of the mutations seen. For example, our lab's docking studies with Q67 predict that different symmetry related Q67 residues make several contacts with both substrate and cofactor. Q67 from the B and D subunits makes contacts with the pteridine ring of DHF and Q67 from the A and C subunits makes contacts with the nicotinamide ring of NADPH (21). The symmetry element imparts a dual role for these residues (a double duty) in the active site (18,21). Other residues mutated in the active site have been shown to weaken binding such as I68L, M and Y69F mutants. They are found to be important for ground state and transition state stabilization for both ligands (18). It would be interesting to determine the effects of a single mutation in the homotetramer because most site directed mutants tested have decreased activities or catalytic efficiencies as a result of substrate or cofactor inhibition or weaker transition state binding.

Clearly the R67 DHFR enzyme has an entirely different approach to catalyze the reaction compared to the *E. coli* chromosomal DHFR. The chromosomal enzyme is expressed as a monomer (MW~18000). It catalyzes the hydride transfer reaction much more efficiently and is considered to be a maximally evolved enzyme (26,27). It has separate binding sites for DHF and NADPH. As mentioned above, an Asp 27 residue acts as a proton donor greatly increasing the catalytic rate. Protonation of DHF occurs followed by acceptance of a hydride from NADPH. The rate limiting step is the release of the product, THF (26). The enzyme's catalytic rate is near diffusion control. In summary, *E. coli* chromosomal DHFR enzyme utilizes specific binding sites for each ligand, and it facilitates protonation of the substrate upon binding – a key component for the enhanced rate of catalysis

In contrast, R67 DHFR is hypothesized to be a primitive enzyme that has arisen due to the clinical use of trimethoprim. R67 DHFR does not have a proton donor and simply may associate the ligands in a close spatial orientation (an endo or eclipsed geometry) for the reaction to proceed. The 222 symmetry element has advantages. Residues on one subunit can make contacts with either DHF or NADPH, depending on which one binds in the half pore site. As mentioned before, residues play a dual role for ligand binding (18,21). Although the symmetry allows for a sufficient rate of catalysis, it is still considered a very inefficient enzyme due to the presence of non-productive complexes in equilibrium with the productive ternary complex. What may be required to increase the catalytic efficiency of this enzyme is divergence of its active site. The phage

display method may allow for R67 DHFR to mimic the evolution that occurred for *E. coli* chromosomal DHFR.

Overview of the phage display technique for R67 DHFR

Phage display of R67 DHFR has two purposes. The technique may allow us to mimic the evolutionary process by selection of mutations that possibly diverge the active site to allow specific binding of each ligand as well as introduce a mechanism or residue that protonates bound DHF. In the event of such a mutation, an increased rate of catalysis gives us a method for selection. In addition to mimicking evolution, phage display can provide an alternative method for studying asymmetric mutations. These variants can then be studied for information into the exact processes of binding, cooperativity, and hydride transfer for this enzyme.

There are only a few cases applying phage display techniques to enzymes and their mechanism of action (28-30). Their method selects for variants or new enzymes with altered catalytic properties that bind with higher affinity to a transition state or inhibitory analog tethered to a solid support matrix (usually beads). Several rounds of selection are used to cull and amplify populations containing the desired catalytic properties. This is the direction we propose to undertake with phage display of R67 DHFR even though no transition state analog is yet available.

A review article by Smith and Petrinko (1) points out that selection efficiency is determined by the methods used and what types of variants are selected from a library. A selection strategy for R67 DHFR phage display has been implemented, but the method described may be considered general and not as effective compared to affinity selection. In the case of β–lactamase phage display, a suicide inhibitor was used to select for active enzymes (29,30). This molecule was already well characterized as an inhibitor of the enzyme. Active enzymes displayed on the phage bind to the inhibitor with high affinity. A transition state molecule was used for selecting mutants of the human GST A1-1 enzyme possessing altered catalytic properties corresponding to the ability to catalyze S_nAr reactions (28). These cases show that an analog molecule is more likely to select for specific variants defined by the structure of the molecule.

A transition state analog is not yet available for the DHFR reaction. Also, there are many questions as to which transition state analog would be most appropriate in a bisubstrate reaction. For example, a transition state molecule resembling the cofactor is likely not to select variants with a proton donor. If the molecule resembles DHF, there is the question of how much affinity the enzyme would have for it, because of the positive cooperativity associated with DHF binding. Would two molecules of the analog bind with tighter affinity than one and would this select for a two DHF molecule complex? Is the molecule stable? Therefore, it may be more feasible to select initially with a bisubstrate analog. In our case such a molecule would have structural components of the nicotinamide ring of NADPH and the pteridine ring of DHF linked together, allowing binding in the central constriction similar to how the substrate and cofactor rings bind in

spatial orientation with respect to each other in the productive ternary complex. Several possibilities exist and a collaboration with Bruce Hathaway at SE Missouri State University is underway to synthesize some of these molecules.

Since the selection application using a bisubstrate molecule is under investigation, an alternative approach uses the activity associated with the expression of the fusion product which can confer TMP resistance in cells. This can act as a positive selection method for active variants grown in minimal media. It is possible that a variant with higher activity can be selected as a result of conferring higher TMP resistance. This may not be as efficient as selecting phage with an analog molecule, but overall the method can select for a broader range of variants including those containing a diverged active site and possibly with a proton donor. A variant containing a diverged active site can be imagined where half the pore preferentially binds DHF and the other half preferentially binds NADPH. This may ultimately allow crystallization of R67 DHFR with bound substrate and cofactor.

The phage display technique applied in this study fuses the R67 DHFR gene to the minor (gene III) and major (gene VIII) phage coat protein genes. R67 DHFR is expressed as a monomer that associates into a homotetramer as described above. It is not likely that the display of the fused R67 DHFR monomer will display an active, folded protein or that the monomer fusion will be able to associate on the phage surface to form a homotetramer. Formation of a homotetramer seems unlikely on the surface of a phage virion. Therefore, a synthetic R67 DHFR gene sequence has been used (31) to construct

a four copy tandem array expressing an active monomer with similar binding and catalytic properties to the homotetramer (32). This sequence has been linked in-frame to the sequence of the minor coat gene III and major coat gene VIII of the filamentous bacteriophage.

Display of the R67 DHFR enzyme has been explored as fusions to two phage coat components (or two types of phage display systems), both as a mosaic type display (figure 3). In a mosaic display system, the fusion is expressed on a plasmid that contains two origins of replication – one for the plasmid and one for viral replication. The plasmid or phagemid also contains a selective marker. The phagemid alone cannot be packaged as a viral particle; therefore, cells containing the phagemid are infected with helper phage. The expression of the fusion product and the wild type gene for the phage coat protein from the helper phage genome both become incorporated into the protein coat of the virion particle secreted from the cell. This type of display produces virions that have dispersed fusion protein on the surface (a mosaic). For each virion particle there are five copies of the gene III protein and approximately 2500 copies of the gene VIII protein composing its protein coat. The mosaic display expresses one to two copies of the fusion product for the gene III system and tens to hundreds of the fusion product for the gene VIII system. The gene III mosaic type of display has been predominantly used to successfully display larger proteins and enzymes while the gene VIII fusion most frequently has been used to display peptide sequences (1,2). Exploring the gene VIII system provides an advantage of higher copy number for our displayed R67 DHFR enzyme. A homogenous display system would not work for such a large enzyme. This

type of system would have every copy of the fusion product incorporated on the coat surface. This cannot work for the gene VIII protein, because of steric constraints that would disrupt the protein fold. Only a maximum of six amino acid residues can be displayed for this type of system (1,2). The gene III system can accommodate larger peptides, but it is likely to interfere with the function of gene III – replication and infection – again supporting the use of a mosaic display system (1,2,33).

This thesis is presented to determine whether the gene III and/or gene VIII phage fusions with R67 DHFR are active and displayed. Both gene fusions have been constructed to determine if one or both can efficiently display an active enzyme on the phage coat surface and if so, to compare the advantages for each system of display. Construction of the fusion gene has also been optimized for the hypermutagenesis strategies that would generate the necessary library of variants. The quadruplicated gene sequence of R67 DHFR has been mutagenized to allow for gene III and gene VIII fusions. These fusion displays of R67 DHFR have been tested for activity and assayed by partially purifying and detecting the fusion product on immunoblots. Methods of selection have been tested including a method that may identify revertants under higher TMP stress. A similar process has been previously used in selecting revertants of the D27S mutant *E. coli* chromosomal DHFR. Revertants were obtained that had increased catalytic efficiency due to second site mutations including F137S, F153S and I155N mutations (34,35). The selection protocols explored, however, do not include the affinity selection with an analog molecule tethered to a solid support. This method would potentially allow screening of the desired R67 DHFR variants having altered catalytic

activities and could result in mutants possessing enhanced efficiency. The alternative methods presented still select for the enhanced efficiency, but they are a more generalized approach.

Chapter II

Materials and Methods

Construction and characterization of the fusion gene for both phagemid expression systems

Two phagemids were received as gifts to be used for the cloning of the R67 DHFR gene for phage display – a gene VIII and gene III fusion construct. A 5,358 bp plasmid (phGH-M13gIII) from James Wells (33) contained the phage display construct for fusion with the M13 gene III phage coat protein. In this phagemid, the human chorionic gonadotropin hormone (hCG) sequence was linked in-frame at the 5' end with the stII membrane insertion and secretion sequence and was linked in-frame at the 3' end with the gene III sequence which expresses the minor phage coat protein that forms the head of the virion responsible for reinfection. The gene sequence of the M13 gene III was truncated and represents the carboxyl-terminal domain. The N-terminal domain responsible for virion attachment to the F' pili of bacteria was removed. Transcription of the fusion protein was under control of the lac promoter/operator sequence. A 3108 bp plasmid from David Baker (36) contained the phage display construct for fusion with the M13 gene VIII major phage coat protein. In this phagemid, a sequence of the 62-residue B1 IgG binding domain of protein L was linked in-frame at the 5' end with the ompA membrane insertion and secretion sequence and was linked in-frame at the 3' end with the gene VIII sequence which expresses the major phage coat protein forming the body of

the virion. Transcription of the fusion protein was under control of the Tac (trp/lac) promoter. Both phagemids contain origins of replication for plasmids and for phage particle replication. The latter was used upon super infection with helper phage. Both phagemids also contain an ampicillin (Amp) selective marker.

Mutagenesis of the sequence of R67 for cloning into the gene III phagemid

Primers were designed to add the Nsi I restriction site at the 5' end of the R67 DHFR sequence in pUC18 and the Apa I restriction site at the 3' end of the sequence for cloning into the gene III phagemid. The primer NSIIDHFR (5'**AT GCA** TCC ATG ATC AGG AGC TCT AAT GAA G3') and APAIDHFR (5'**GGG CCC** GTT GAT GCG TTC AAG CGC C3') annealed at opposite ends of the sequence onto complementary template strands. PCR reactions for mutating the R67 DHFR sequence were done using the Thermocycler from BioRad. Contents of the reaction with a total volume of 100µl included 1ng/ml template DNA; 0.3 µM of each primer; and the concentrations for the dNTP's, buffer, and *PFu* enzyme as describe in the protocol from Stratagene® for PCR reactions using *PFu* polymerase. Amplification was done using 38 cycles. The first cycle was at 94°C for 5 minutes. The next cycle raised the temperature to 94°C for 35 seconds, lowered it to 65°C for 30 seconds, and then raised it to 70.5°C for 1 minute repeating the cycle 33 times. The final cycles set the temperature to 72°C for 7 minutes and finally at 4°C.

Mutagenesis of the gene VIII vector for insertion of the R67 DHFR sequence

Two sets of complementary primers were designed as outlined in the Quickchange kit from Stratagene® to change the Nco I restriction site to Nsi I and the Mlu I site to an Apa I site in the gene VIII phagemid. The first set used were primers NCOINSIIA (5'CAGGCGGCCC**ATGCAT**GAAGTAACAAT3') and NCOINSIIB (3'GTCCGCCGGG**TACGTA**CTTCATTGTTAG5') followed by the second set of primers, MLUIAPAIA (5'GGAGGAAAC**GGGCCC**GCTGAGGGTGACG3') and MLUIAPAIB (3'CCTCCTTTG**CCCGGG**CGACTCCCACTGC5'). PCR reactions for mutating the gene VIII phagemid were done according to the protocol outlined in Stratagene's Quickchange™ PCR reaction kit. Two reactions amplifying the phagemid with each mutation were done consecutively. The second set of primers was added to the reaction after the first set of reactions was completed.

Cloning, DNA analysis, and cells utilized for transformation

Cloning and DNA analysis were performed using standard methods described in Sambrook (37) as well as following the information provided with the purchased reagents for DNA preparation, enzymatic digestion, DNA extraction (Millipore), ligation, and dephosphorylation. Gel electrophoresis of the DNA samples was done using either a horizontal 1.2% agarose gel in modified TAE buffer (40 mM Tris-acetate, pH 8.0, 0.1

mM EDTA) or a vertical 6% polyacrylamide gel in TBE buffer (Tris, Borate, EDTA) staining with ethidium bromide.

E. coli cells were transformed by chemical methods ($CaCl_2$) as described in Sambrook (37). Colonies were grown on YT plates with (20 μg/ml trimethoprim). For selection and cloning of the tandem array of R67 DHFR, ElectroMax™ STBL4™ *E. coli* cells to be transformed by electroporation were prepared as follows: 200ml of STBL 4 *E. coli* were grown in LB media at 30°C to 0.6 OD_{550}. Cells were pelleted at 6K rpm by centrifugation and placed on ice. They were then resuspended in a 1:1 ratio of YT with no NaCl added and ddH_2O. Cells were re-pelleted at 6K rpm for five minutes at 4°C and washed twice in a volume of sterile 10% glycerol. Finally, cells were resuspended in 400 μl 10% glycerol to be aliquoted and stored at –80°C. Electroporation was done by adding 1μl of a DNA sample to 35μl of prepared bacteria on ice in a 1cm Gene Pulser® cuvette from BioRad. Pulse parameters applied a 1.45 kV charge with a 25μF capacitance and low (100 ohm) ohm resistance using a Gene Pulser ™ electroporator attached with a Pulse Controller from BioRad. After the pulse, cells were grown in SOC media at 30°C for recovery and then spread on YT or 2xYT plates with 20 μg/ml TMP (or with 200μg/ml Amp if transformation efficiency was low). Colonies were selected for screening their phagemid DNA. The phagemid DNA was recovered using DNA prep kits from Stratagene®. Then double digestion reactions with Nsi I and Apa I were done using one buffer. This was followed by either agarose or polyacrylamide gels analysis of the DNA bands generated from the digest reaction. Verification of all sequences were done with primers outside the R67 DHFR insert sequence as well as outside the gene III and

gene VIII sequences. The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit from Perkin Elmer was utilized by the University of Tennessee Sequencing Facility.

Colony Lifts

JM107 or STBL 4 *E. coli* cells containing the constructed phagemids were used in the colony lifts. An antibody to the R67 DHFR enzyme (previously generated in our lab by Bob Nicols (38)) was used for detecting the fusion product, and an anti-fd bacteriophage antibody purchased from Sigma was used for detection of phage particles secreted from the cell via the antibody binding to the gene III and gene VIII phage coat proteins. JM107 or STBL4 cells with no plasmid and those containing a pUC8 vector constitutively expressing the R67 DHFR enzyme were used for controls. STBL4 *E. coli* (*mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *gal*$^{-}$ *sup*E44 λ^{-} *thi*-1 *gyr*A96 *rel*A1 Δ(*lac-pro*AB)/F'*pro*AB^{+} *lac*I^{q}ZΔM15 Tn*10* Tet^{r}) was used for the expression of the quadruple R67 DHFR-gene VIII/III protein fusion. JM107 and STBL4 *E. coli* were both used for the expression of the R67 DHFR monomer-gene VIII/III protein fusion. For the colony lifts, cells were grown in YT media with 200μg/ml Amp at 30°C for STBL 4 or 37°C for JM107. Cells were grown to stationary phase which was about 6 hours for JM107 and 12 hours for STBL4. Cell cultures at this phase were then infected with a stock of M13K07 helper phage and incubated for 1-2 hours for JM107 or 3-4 hours for STBL 4. Cultures infected with the helper phage were streaked onto 2XYT plates with 20μg/ml TMP. Plates incubated 6-12 hours for the JM107 cultures at 37°C or overnight for the STBL 4 cultures at 30°C. Next,

colonies were overlaid with a nitrocellulose membrane for 1 minute. Then the membrane was washed in ddH_2O. Any cells adhering to the membrane were gently removed with a squirt bottle of ddH_2O. Membranes were blocked for 1 hour with a 10% non-fat dry milk solution in PBS. The next step was a wash (3X) with PBS/tween and then an incubation with anti R67 (1:600) or anti–fd (1:5000) antibodies in PBS with 0.05% goat serum (Fisher) for 2 hours. The filter was washed again and incubated with goat anti-rabbit conjugated to horseradish peroxidase (Fisher) in PBS/tween containing 0.05% goat serum for 1 hour at 37°C. After a final wash, the fusion product and phage particles were detected using a substrate solution (6 ml of 4-chloro-1-naphthol, 12 µl of 20% H2O2, and 30ml PBS) for the horseradish peroxidase enzyme conjugated to the secondary antibody activity was monitored by purple color formation.

Expression analysis of the fusion protein attached to virions

Phage particles with the attached fusion protein were harvested and concentrated for SDS PAGE and Western blot analysis. The same protocols and antibodies used for the colony lift assays were also used for the Western analysis. STBL 4 cells containing any one of the two phagemid constructs containing the tandem array fusion of R67 DHFR described were grown in 500ml LB with 20µg/ml TMP for 2-5 hours at 30°C. JM107 cells containing any one of the two single R67 DHFR gene fusion constructs described were grown in 500ml LB with 20µg/ml TMP for 2 hours at 37°C. Then cultures were inoculated from a stock of M13K07 helper phage and incubated 24-40 hours. Cells were spun down twice at 6K rpm. The phage particles in the supernatant

were precipitated overnight at 4°C after making the solution 6% in PEG (8000 MW) and added NaCl to 0.3M. Phage particles were then spun down at 15K rpm. The pellets were resuspended in 2-5 ml Tris Hcl pH 8. The phage solution was dialyzed overnight in 0.03M Tris pH 8 using a Spectra/Por® cellulose ester membrane from Spectrum Labs with a 100,000 dalton nominal molecular weight cut off to remove most of the PEG (8000 MW), NaCl, and any other smaller extra cellular proteins. Samples were stored at –20°C and assayed. Additional harvests were done to concentrate the phage particles further by lyophilization. For the expression analysis and activity assays, the lyophilized phage particles were resuspended in 20-30µl of 0.3 M Tris Hcl pH 8.

Phage samples were also purified by CsCl density centrifugation after the phage were PEG/NaCl precipitated and spun into a pellet. The method was similar to the protocol used by Smith and Scott (39). The samples were resuspended in 3-5 ml 0.5 M Tris Hcl pH 7. CsCl was added to bring the density in the total volume of sample to 1.3 gm/ml CsCl. The amount added was calculated by the following formula:

$$wt_{CsCl} = {}^{0.3124 * \text{wt of solution with phage}}/_{0.6876}$$

The weight of the solution is measured in grams. This formula was extrapolated from the formula given in Current Protocols for CsCl density determination (40). After adding the CsCl, samples were divided and balanced in the polyallomer ultracentrifuge tubes (Beckman) that held around 3 ml volume each. Samples were placed in a Beckman SW Ti 50.1 rotor and centrifuged at 30,000 rpm for 30 hours. Bands were collected with a syringe and transferred to the same membrane tubing used as described above to be

dialyzed overnight in 0.3 M Tris pH 8. After removing the CsCl salt, the samples were stored in eppendorf tubes at –20°C and used for expression analysis and activity assays.

The preparations of the samples with the phage for the gel electrophoresis were modified from standard methods. The samples were either resuspended or already stored in 0.03 M Tris pH 8. 20ul of the phage sample was added to 5ul of 6% Brij and sat at room temperature for 20-30 minutes. Then an equivalent volume of reducing sample buffer was added and boiled for 2 minutes before being loaded onto the gels.

Activity Assays

Activity assays were performed to measure the relative velocity of the catalyzed hydride transfer reaction from NADPH to DHF. Assays were done on samples of the supernatant after the cells were spun down, samples of the dialyzed phage particles resuspended in 0.03 M Tris Hcl at pH 8 to remove the PEG and NaCl, the lyophilized phage samples, and the purified phage particles from CsCl density centrifugation. Samples were assayed using a Perkin-Elmer lambda3a spectrophotometer interfaced with an IBM PS2. The computer program UVS (41) analyzed the change in NADPH and DHF concentration over time at 340nm. A computed slope gave the relative velocity of the reaction over a three minute time period. The observed rate of μmol substrate consumed per minute was calculated from the measured velocity of the reaction using the following formula:

$$(\Delta A_{340}\ s^{-1})/12.3M^{-1}*(630/20)*60s$$

where ΔA_{340} s^{-1} is the slope of the reaction for the change in ligand concentrations over time and 12.3 $M^{-1} \cdot cm^{-1}$ is the molar extinction coefficient for the DHFR reduction of DHF (42).

Assays were performed at 30°C in a polybuffer containing 50mM MES, 100mM Tris, and 50mM acetic acid at either pH 6 or 7 (43). The polybuffer maintains a constant ionic strength from pH 4.5-9.5. Phage were suspended in 30 mM Tris pH 8 and 20 µl aliquots of the phage sample were added to the reaction cuvette after adding a predetermined amount of prepared stocks of DHF and NADPH. The DHF suspension was thawed on ice, pelleted, and resuspended in T.E. pH 8. NADPH was dissolved in T.E pH 8. Concentrations of DHF and NADPH in the reaction cuvette were determined spectrophotometrically. This was usually 10 µl of each stock that would give an absorbance at 340 nm of 0.93 – 0.96 corresponding to 150 mM for NADPH and 120 mM for DHF. Ligand concentrations were determined using the extinction coefficients for DHF (28,000 ε at 282 nm (44)) and NADPH (6220 ε at 340 nm (45)). These concentrations were well above saturation for the wild type R67 DHFR enzyme's NADPH and DHF K_m values. The total ligand concentration at the start of each reaction gave an Abs_{340} of 1.95-1.9. The cell culture supernatant samples were assayed by just adding the predetermined amounts of substrate and cofactor only.

The relative amount of R67 in the activity assays was determined by performing phage titres of the samples after CsCl centrifugation and dialysis. 5ul of the phage suspension was diluted in a series of tubes with 1X TBE. STBL 4 *E. coli* cells with no

phagemid were grown overnight in YT media. 10µl from each dilution of phage was added to 200µl of cells and incubated for 20 minutes at 37°C. 6ml YT top agar was melted and cooled to 55°C. Then each sample of cells infected with a dilution of the phage were mixed with the top agar which then was poured onto YT plates and incubated overnight at 37°C. Plaque forming units were counted to give the concentration of phage. This concentration was used to calculate the relative amount of R67 in the reaction cuvette by using the following formula:

$$\{[(^{Pfu}/_{5ul} * 20ul \text{ volume added to cuvette})*(\text{estimated copy number displayed})]/6.02x10^{23}\} * (1x10^{6}\ \mu mol/630ul)_{\text{assay dilution}} * (1x10^{6})_{\text{conversion}} \cong \mu M \text{ of R67 displayed on phage}$$

Minimal media plates for selection

Minimal media plates were used to apply a selection pressure on *E. coli* cells containing the phagemid and expressing the fusion protein to grow in the presence of TMP. The media consisted of 1X M9 salts, 2mM $MgSO_4$, and 0.4% glucose. In addition to the salts and glucose, thiamine was added to a concentration of 1 µM. A series of plates were poured with different TMP concentrations ranging from 1 µg/ml to 30µg/ml. *E. coli* cells with the phagemid were grown in YT media overnight with 200µg Amp/ml added. Then cells were streaked onto the plates to grow isolated colonies at 30°C. Plates were examined after 24–30 hours to observe any growth within this time frame as controls grew within 24-26 hours expressing the wt R67 DHFR enzyme. If no growth was observed, plates were examined every 12 –24 hours up to 60 hours from the initial inoculation to observe any colonies that may be a revertant or mutant.

Chapter III

Results

Construction of the four fusion genes

The phagemid vectors with the R67 DHFR gene linked to either the gene III or gene VIII of M13 bacteriophage were designed with the intent of maintaining an easy cloning strategy that would be interchangeable between both gene III and gene VIII systems. The cloning ultimately resulted with both systems utilizing Nsi I and Apa I restriction enzyme sites for inserting and properly orienting the R67 DHFR sequence into the correct reading frame of the M13 gene. This was advantageous for the construction of all four fusion genes desired – both the single copy and quadruple copy R67 DHFR sequence incorporated into the gene III and gene VIII systems. The way these systems are set up may also benefit the hypermutagenesis cloning strategy in future experiments.

The single copy synthetic gene sequence of R67 DHFR (31) was initially cloned into James Well's gene III phagemid - phGH-M13gIII (33). The gene III phagemid already had unique Nsi I and Apa I sites; therefore, PCR mutagenesis was done to engineer the complementary Nsi I and Apa I sites into the R67 DHFR sequence at its 5' and 3' ends respectively (figure 4). The primers described in the methods were used in the PCR reaction to amplify a 250 bp product that produced the R67 DHFR sequence with the genetically engineered sites (figure 5). However, both sites were located on the ends of the double stranded DNA fragment. Enzymatic digestion of this fragment was

very poor. To get around this problem, the PCR product was blunt end ligated into a pUC19 plasmid digested with Hinc II. Blue/white screening was done, and two colonies were found to contain the PCR product inserted into the plasmid. This was confirmed by digests with Nsi I and Apa I followed by gel analysis of the DNA fragments. Once identified, the R67 DHFR clone was effectively cut out by Nsi I and Apa I digests and ligated into the gene III containing phagemid. The R67 DHFR sequence was inserted in-frame between the stII signal or secretion sequence and the gene III sequence as shown schematically in figure 6. This construct expressed the 78 amino acid R67 DHFR monomer fused to the gene III minor coat protein and is named wlz1pIII.

Cloning of the single copy R67 DHFR sequence into the gene VIII phagemid used the wlz1pIII phagemid. David Baker's phagemid was used in constructing a fusion with the gene VIII protein; this construct had two different unique restriction enzyme sites that flanked the protein L sequence inserted in-frame with the gene VIII of bacteriophage. We decided to change the restriction sites on the gene VIII phagemid using the Stratagene® kit rather than introducing two new sites flanking the R67 DHFR sequence. The GCG program package from Wisconsin was used to examine the gene VIII phagemid sequence for any other restriction sites corresponding to Nsi I and Apa I sequences (46). Only one Apa I site was found to be within the protein L sequence – inconsequential to the overall cloning strategy. These sites were changed on the gene VIII phagemid to complement insertion of the R67 DHFR sequence already in the gene III phagemid of wlz1pIII. Two consecutive PCR reactions amplified the entire phagemid using two complementary sets of primers described in the methods. These primers

changed the Nco I site to an Nsi I site at the 5' end and the Mlu I site to an Apa I site at the 3' end of the protein L sequence inserted within the fusion gene. Transformed colonies were screened for the double mutant gene VIII phagemid by digesting the purified DNA with all four restriction enzymes. Several colonies were found to contain the double mutation (figure 7). Then the R67 DHFR sequence in the wlz1pIII phagemid was removed and cloned into the gene VIII phagemid containing the complementary sites. The R67 DHFR sequence was inserted in-frame between the OmpA signal or secretion sequence and the gene VIII sequence as shown schematically in figure 8. The construction of this phagemid expressed the 78 amino acid R67 DHFR monomer fused to the gene VIII major coat protein and is named blz1pVIII.

Construction of a four gene copy of R67 DHFR linked to either the gene VIII or gene III protein sequence was accomplished by a similar approach previously described by Bradrick et al in constructing the quadruplicated R67 DHFR gene sequence (32). The single copy R67 DHFR sequence was cloned into both phagemids and a quadruplicated gene sequence of R67 DHFR was already constructed (32). The method utilized one restriction site (Bgl II) within the R67 DHFR sequence. Additional copies of the R67 sequence were then inserted into the single gene copy linked to the gene VIII protein sequence. A partial digest was done on the quadruplicated gene sequence of R67 DHFR using Bgl II (0.5u/μl) in a reaction that ran for less than five minutes at 37°C in a buffer reportedly showing 50% enzymatic activity for the enzyme. The partial digestion reaction produced mixtures of 2 and 3 gene copy fragments of the quadruplicated R67 DHFR sequence. These fragments were separated on an agarose gel. DNA was extracted from

the gel and ligated into the blz1pVIII phagemid previously digested with the same enzyme (Bgl II) and treated with alkaline phosphatase. However, the cloning of the four gene copy of R67 DHFR into the gene VIII and gene III phagemids was complicated by the inefficient ligation of longer DNA fragments. The length of the DNA fragment cloned into the phagemid and the low concentrations of DNA extracted from the gel effectively lowered the ligation and transformation efficiencies. Eventually, a colony was obtained carrying a triple copy R67 DHFR sequence cloned in the gene VIII phagemid. The sequence was confirmed to be in the correct orientation by checking the fragment size of an Apa I and BstE II digest, where BstE II cuts at a different location within the R67 sequence and not any where else on the phagemid. This construct was then used as a template for cloning of the tandem array. A partial digestion of this phagemid cut once with Bgl II allowed subsequent ligation with a single copy R67 DHFR sequence. Since higher concentrations of the single copy R67 DHFR DNA could be extracted from an agarose gel, ligation proved more efficient. A colony was identified with the phagemid containing a quadruple copy R67 DHFR sequence linked in-frame to the gene VIII sequence. The orientation of the insert was verified and the sequence checked with automated fluorescent dideoxy-sequencing (provided by Neil Quigley). The four gene copy of R67 DHFR was then removed from the MRJpVIII vector and cloned into the MRJpIII vector by similar cloning methods described for constructing blz1pVIII. Figure 9 shows the overall construction of both phagemids. The MRJpVIII phagemid expressed the quadruple R67 DHFR protein fused to the gene VIII phage coat protein. The MRJpIII construct expressed the quadruple R67 DHFR protein fused to the gene III phage coat protein.

As shown in figure 10, all four constructs were characterized by gel electrophoresis of a double digest reaction with Nsi I and Apa I that cuts out the 239 bp R67 DHFR synthetic gene sequence or the 956 bp quadruplicated gene sequence of quadruple R67 DHFR. As a byproduct of the cloning, we also obtained a triple and double copy R67 DHFR sequence inserted into the gene VIII phagemid.

The phagemids expressing the fusion genes were cloned into bacterial strains that have an F' pili for phage attachment and infection. These cells can be infected with M13K07 bacteriophage, also called helper phage. Upon infection, these cells package the phagemid and the viral genome into a virion which is secreted out of the cells. M13 phage does not kill the cells. Portions of the phage coat should contain the R67 DHFR enzyme displayed on the surface of the virion. We initially transformed JM107 with both phagemid systems containing the single copy R67 DHFR gene insert. However, STBL 4 *E. coli* cells were transformed with both systems containing the quadruplicated R67 DHFR gene insert. This strain eliminated homologous recombination between identical gene copies of the R67 DHFR sequence.

Protein expression of the fusion proteins

All four constructs provided low levels of TMP resistance upon host *E. coli* cells, indicating the expression of the fusion product allowed the R67 DHFR portion of the fusion protein to properly fold and be active. Low TMP resistance could have been the

result of lower expression levels or altered function of the R67 moiety of the fusion protein. Several attempts to detect the fusion protein from cell extracts and membrane components were unsuccessful. However, colony lifts were able to provide information about the relative level of expression of all four fusion proteins.

Colony Lifts

For all four constructs, colony lifts were performed on M13K07 superinfected colonies of STBL 4 and JM107 *E coli* cells grown on 2xYT plates containing 20 μg TMP/ml. Both JM107 and STBL 4 *E. coli* cells transformed with any of the four phagemid constructs had growth rates that were much slower than those cells containing a control R67 DHFR gene carried in pUC18. This gene was under the control of a constitutive promoter and is named pLZ1. An anti-R67 DHFR polyclonal antibody was used to detect the expression of the fusion product for all four constructs (figure 11). The controls indicated the intensity of signal produced for R67 DHFR released into the extra cellular environment from the cytosol of bacterial cells due to cell death and necrosis. The intensity of signal from colony lifts of the strains containing the phagemids was much greater than the controls. This result was very interesting as the control stains grew to a much greater density on the plates. Therefore, it was likely that the resulting signal observed for the fusions was due to secreted fusion protein outside of the cell, possibly as part of virions. In support of this finding, colony lifts were also done using the anti-fd bacteriophage antibodies on the same set of plates, which detected the secretion of phage particles or virions from infected colonies (data not shown). This indicated that the cells

could package and secrete phage particles with the possibility of phages containing the fusion protein. In conclusion, these results supported the likelihood of the active R67 DHFR enzyme displayed on bacteriophage.

Expression of the fusion protein on the phage particle

Phage samples that were PEG and NaCl precipitated and then lyophilized were initially analyzed using Western blots to detect the fusion protein. We were uncertain if the activity of displayed R67 DHFR would be lost after CsCl density centrifugation. Therefore, virions were first harvested to determine if we could detect the fusion protein associated with phage particles using anti-R67 DHFR and anti-fd bacteriophage antibodies in Western blot analysis. The main objective of the PEG and NaCl precipitation (harvest) was to purify and concentrate the phage particles so that the Western blot protocol was at a level of sensitivity for the use of our antibodies to detect the fusion protein. Using the GCG software package (46), the fusion protein expressed from the MRJpVIII phagemid had an estimated MW of 39,041 daltons, and the fusion protein expressed from the MRJpIII phagemid had an estimated MW of 54,871 daltons. We used this information to look for bands on the Western blot that corresponded to the molecular weights of the fusion products. We were more interested in an active R67 DHFR enzyme displayed on phage; therefore, we concentrated our efforts on the protein analysis of the quadruple R67 DHFR-gene VIII/III fusion protein displayed on virions.

Initial gels and blots were run for the PEG/NaCl precipitation and lyophilization of phage (harvest) samples. Numerous additional bands were observed, so CsCl density centrifugation was used to further purify phage for these analyses. Data in figure 12 describe the protein analysis of phage samples purified using CsCl density centrifugation along with the Coomassie stained gels used for the transfer. The figure also shows a sample of phage that were PEG/NaCl precipitated followed by lyophilization (the harvest). We observed the same bands detected on the blots, but the data from the CsCl density centrifugation method gave a stronger signal and were cleaner and more conclusive. The quadruple R67 DHFR-gene VIII fusion protein was detected using both antibodies. Its molecular weight was estimated by MW markers and is within the range of 42,000 daltons. A doublet band was observed on both blots. The presence of two bands could have indicated some proteolysis of the R67 DHFR-gene VIII fusion protein. Alternatively, it could indicate cleavage of the signal sequence. The upper band of the doublet could correspond to the GCG calculated molecular weight of the gene VIII fusion with the signal peptide sequence (41,436 daltons). Purified R67 DHFR served as a positive control for the blot using the anti-R67 DHFR. Information provided with the anti-fd bacteriophage reported that it should bind to both the gene VIII and gene III proteins of fd bacteriophage. The phage antibody detected the major coat gene VIII protein as a smeared band near the bottom of the blot. This served as our control for the amount of phage protein transferred onto the blot.

The quadruple R67 DHFR-gene III fusion protein was not detected. We also did not observe the gene III protein with the use of the bacteriophage antibodies, but we did

detect the gene VIII protein indicating that phage was present. The level of sensitivity with the use of our antibodies and Western analysis methods was perhaps insufficient to detect the extremely low copy number of the fusion protein. A personal communication from David Baker indicated that their lab was also not able to directly observe their fusion proteins on Westerns due to low copy numbers. Other factors were taken into account such as the antibody dilution. The working dilutions of both antibodies were sufficient to detect the quadruple R67 DHFR-gene VIII fusion protein. However, the copy number is much higher for gene VIII protein (10-1000 per phage particle) as compared to the gene III protein (3-5 copies per phage particle).

Activity Assays

Activity assays indicated a low level of activity was associated with the R67 DHFR enzyme displayed on virions secreted from the bacterial cell. Activity assays were initially performed throughout the various stages of the phage precipitation and lyophilization. Then they were performed on samples using CsCl density centrifugation. Activity was not lost during purification and we obtained more conclusive data from those samples purified using CsCl.

We determined the relative activity of the fusion protein on virions. A control used was a phage prep sample that contained virions without fusion protein attached to their surface. Two other controls were also assayed in parallel to rule out the possibility of a background level of activity associated with the release cytosolic, chromosomal

DHFR or detached fusion protein. One control contained the wt R67 DHFR gene; this construct constitutively expresses the enzyme. Another control monitored fusion gene expression in hosts uninfected with helper phage. Table 1 shows the activity detected at different purification steps from these samples. For example, in Table 1 the activity that was observed in the supernatant was lost at the final stage of the preps for the cultures constitutively expressing the wt R67 DHFR. There was no activity observed at any stage for those preps only expressing the fusion product but not infected by virus. Apparently the concentration of unattached fusion protein was too low to contribute to the measurements, or if it was targeted to the membrane, it was not purified. In conclusion, phage purification removed any components in the preparation that could also reduce the substrate and cofactor in the assays, and the activity was not lost as a result of the conditions associated with both protocols – PEG/NaCl precipitation and CsCl purification.

Relative velocities of both quadruple R67 DHFR fusions are given in Table 2. Low activity levels were clearly observed for the display of the quadruple R67 DHFR enzyme fused to the gene VIII protein. A very marginal level of activity was observed for the R67 DHFR enzyme fused to the gene III protein*.

It was possible that the gene fusion subtly altered the R67 DHFR structure. Therefore, the relative activities were tested by doubling the concentrations of both

* The measured slope of consumed substrate and cofactor over time was only slightly steeper than the slope measuring the non-catalyzed control hydrolysis reaction. These measurements represent addition of similar concentrations of phage for both systems based on titres performed on the stocks that assessed the relative amount of DHFR added to the solution for activity assays.

ligands in the reaction cuvette. The initial DHF and NADPH concentrations were at saturating levels for wt R67 DHFR and doubling the ligand concentrations should have no effect if K_m values were not affected for the fusion proteins. However, the measured velocity doubled, indicating sub-saturating concentrations were present. The activity of the quadruple R67 DHFR-gene III fusion protein changed from undetectable to detectable under the same conditions. We can thus conclude that the K_m's have increased for both fusion protein types.

We could not easily address the effect of the low copy number (number of displayed fusion proteins per phage particle) that contributed to the overall activity levels. We believe the actual amount of displayed R67 is relatively high for the gene VIII system since we were able to detect the gene VIII fusion protein on Western blots. To try and quantify the protein concentration, phage titres were taken from the samples after CsCl centrifugation. We estimated the relative amount of displayed R67 DHFR enzyme by back calculating the phage concentration based on the titres. For simplicity, both the gene VIII and gene III fusion systems had titres of approximately 1 million pfu's per 5 µl of sample. Using 20 µl for the activity assays, an estimated 4 million phage particles were in the reaction cuvette. With the gene VIII system, there should be between 40 million to 400 million copies of the R67 DHFR enzyme displayed on the virions. However, for the gene III system, there should be only 4 – 5 million copies. This would correspond to estimated concentrations of 1.0×10^{-14} M of R67 DHFR for the gene III system and an average of 1.0×10^{-13} M of R67 DHFR for the gene VIII system. The activity observed for the gene VIII system was about 10 fold over that observed for the

gene III system corresponding to the relative copy number difference between each system. This calculation is surprising as it is difficult to monitor activity of wt R67 DHFR at pM levels, so there may, perhaps, be an inverse correlation between the level of R67 DHFR fusion protein and the infectivity of the phage.

Selection

A general selection strategy was developed for selection of R67 DHFR variants with potentially higher activity displayed on phage. Minimal media plates were poured containing only salts, glucose as a carbon source, and thiamine. A series of plates were poured with increasing amounts of TMP added (up to 30μg/ml). *E. coli* cells containing either construct did not grow on plates with TMP concentrations above 1-2 μg/ml. *E. coli* cells containing the pLZ1 that constitutively expresses the wt R67 DHFR enzyme grew at all concentrations of TMP tested. Therefore, a selection pressure can be applied to the R67 DHFR displayed on phage as its altered K_m values provide a potential means to induce possible revertants with higher activity. These revertants will be able to grow on minimal media plates containing higher concentrations of TMP. This can be applied to select for interesting variants deriving from a library of mutants generated from hypermutagenesis strategies.

Chapter IV

Discussion

Although the fusion of enzymes to phage coat proteins has been previously engineered, this thesis reports the first case where a tandem array of genes has been fused successfully with both the gene VIII and gene III proteins. The wild type R67 DHFR is a monomer that associates into a homotetramer forming a single active site pore with 222 symmetry. The display of the R67 DHFR monomer presents problems for study as it is uncertain whether it can associate as a homotetramer when displayed on the surface of virions. Therefore, a four gene copy encoding R67 DHFR was utilized to construct gene fusions for two phage display systems. The goal of phage display requires a functional enzyme in order to utilize affinity selection to screen libraries, ultimately leading to discovering more efficient enzymes.

Our work with two phage display systems provides the opportunity to test two different fusions for function, as well as two different expression levels. Bradrick had already shown that four copies of the 78 amino acid monomer could be fused to fold into an active enzyme with a k_{cat} slightly lower than the native enzyme (32). Therefore, it seemed likely that this quadruple R67 would properly fold into an active enzyme if the fusion with the phage coat protein did not perturb its structure. If the fusion product itself was not an issue, there were other factors to be considered concerning its physical display on the surface of phage. The use of two phage display systems provides two types of

environments for display of the fusion product. Each system has its advantages, and this gives us the opportunity of selecting which system works best.

An active quadruple R67 enzyme that has been altered as a result of the fusion product is displayed on phage using the gene VIII system and data infers the same for the gene III system

Our results indicated that both the gene VIII and gene III phage display systems constructed display an active quadruple R67 DHFR enzyme. Both constructs provided TMP resistance to host *E coli*. Furthermore, the quadruple R67 DHFR-gene VIII protein fusion was directly observed on Western blots for assaying phage, and DHFR activity assays showed a low activity associated with purified phage. Clearly, an active quadruple R67 enzyme was displayed on M13 phage using the phage display system that fuses it to the major gene VIII coat protein. However, the intrinsic properties of R67 DHFR have been altered due to the fusion. As a result, the K_m's of displayed quadruple R67 DHFR have changed.

What causes the changes in K_m values cannot be assessed exactly. Altered K_m values seem likely to be due to any minor stress or strain on the active site pore that accompanies fusion and display of the protein. Studies with the wt R67 DHFR have shown that altered K_m values can readily translate into decreased catalytic efficiencies (i.e. k_{cat}/K_m) (18). Whether any accompanying effects on k_{cat} occur is not clear as we are not able to measure k_{cat} directly.

The observation of low rates for purified phage displaying quadruple R67 DHFR-gene VIII protein may simply be due to the display of 10^{-13} and 10^{-14} M concentrations of the R67 enzyme. Activity observed at these very low estimated enzyme concentrations could mean that the enzyme's catalytic rate is unchanged, but this cannot be directly assessed.

A plausible explanation for altered K_m values and low activity measurements might be the length of the spacer. A short spacer might cause stress or strain on the structure due to the fusion. The folded structure of R67 could require more flexibility and a close proximity to the folded structure of gene III or gene VIII might become a problem.

Cleavage of the amino terminal 16 residues in R67 by chymotrypsin does not interfere with the activity of R67 DHFR. This sequence was, therefore, used as the linker for the construction of the quadruplicated enzyme (31,32). By computer modeling, these residues were found to be flexible enough to tether two monomers together and also to reach across the dimer-dimer interface to associate the dimers into a tetramer. In contrast, the sequence linking the C-terminus of quadruplicated R67 to the N-terminus of gene VIII or gene III protein is only Gly-Pro. A two amino acid sequence may not be long enough and may place some sort of strain or cause a perturbation near the C-terminal end of R67's sequence. This hypothesis can be tested by introducing a longer linker sequence.

In conclusion, the results from the colony lifts and activity assays show the display of active R67 DHFR on phage. It seems likely that an altered property of the fusion protein causes the change in K_m. The low activity associated with the fusion product on phage could be due to a decreased k_{cat}/K_m or to decreased k_{cat} rates. An additional possibility is display of a very low copy number (or amount) of the R67 enzyme.

Although we did not directly observe the quadruple R67 DHFR-gene III fusion protein on Western blots, it was inferred from the colony lift data and activity assays that this system also works for displaying the active quadruple R67 enzyme fused to the gene III protein. The colony lifts showed the expression and display of the quadruple R67 DHFR-gene III fusion protein. In addition, the host bacteria containing the gene III construct grew slowly and gave stronger signals than the controls. Activity was determined on purified phage samples with the fusion protein. The activity measured for the gene III system was marginal, probably because of the much lower copy number of the displayed quadruple R67 DHFR-gene III fusion protein. The lower copy number was also a factor with its protein analysis. Western blots performed using both antibodies to R67 and gene III protein were not sensitive enough to detect the fusion protein. The wt gene III protein was also not detected in any of the phage prep samples using the anti-fd phage antibodies. The inability to detect these two proteins was likely due to the very low amounts in samples, but it was also considered that the anti-fd antibody purchased may not have been able to bind to the gene III protein. Regardless, a similar pattern

obtained from the colony lifts and activity assays, as seen for the gene VIII system, inferred that the gene III system also displayed the active R67 enzyme at a 10 fold lower level (at least).

The benefits gained working with two types of phage display systems

The use of two phage display systems gives us more options. Since both phage display systems work, we can examine and utilize the advantages each system has to offer.

The main advantage for using the gene VIII system is the higher copy number display of the quadruple R67 DHFR. This allows for a higher yield of variants or a greater success in screening variants with altered binding and/or catalytic properties to be selected from a population using affinity matrices (1). Valency becomes an issue, though, by which the selection of a variant displayed on virions with higher affinities for the tethered molecule or analog are co-selected with those virions displaying a higher copy number of another variant with moderate affinities for the tethered molecule or analog. However, those candidates possessing higher valency over higher affinity can be reduced or eliminated using repeated rounds of selection. Until now, no other larger catalytic enzyme has been reported as a fusion to the gene VIII protein.

On the other hand, the gene III system has been reported successful with other enzymes displayed as fusions (28-30,33). The fusion protein is likely to properly fold

without altering the properties of R67 as a fusion. The gene III fusion system is very flexible fusion protein in accommodating very large enzymes. The problem, however, is the copy number. One to two copies of quadruple R67 DHFR are displayed at the head of the virion using the mosaic display approach. Desired variants could become difficult to cull from a population. The success of the selection for desired rare variants of R67 would depend on higher affinity for the analog chosen, thus the method of selection becomes an essential factor with the gene III phage display system. However, one advantage the gene III system has over the gene VIII system may be that steric factors (referring to the association of displayed enzyme to molecules tethered to affinity matrices) are not as likely to become a problem.

Using two phage display systems improves the chances of generating a variant with increased catalytic efficiency. The same type of mutation in the R67 gene that can result in increased k_{cat} values for the enzyme may not exhibit the same properties as a fusion in one of the phage display systems.

We can focus on improving the function of R67 by technically improving the display of the R67 enzyme. Since we postulate that the fusion product itself results in the altered function of R67, it can be reasonably assumed that this perturbation is the result of the short linker sequence that fuses the proteins. Longer spacers are reported with other phage display of active enzymes (29). Lengthening the linker sequence may undo this alteration for R67 displayed on phage. This may become a necessity if the selection

methods utilized do not produce a quality of desired variants using the constructs reported in this thesis.

Selecting for variants takes advantage of the altered properties of the fusion protein until an analog molecule can be used to find variants with increased catalytic efficiency

Until we design, synthesize, and test an affinity molecule that can be used for selection, we can use the altered properties of the displayed enzyme to select for mutations that partially or fully restore its activity. The technique uses the TMP resistance that is conferred upon host bacteria transformed with the phagemid. Cells are grown in media only containing glucose and salts and supplemented with thiamine. TMP added to the media places pressure on the cells expressing the fusion protein to select for any mutants present that increase R67's activity. Phage collected from colonies that grow on these plates are used to reinfect cells containing no phagemid grown under the same conditions. These steps are taken to eliminate other possible factors that can lead to increased TMP resistance.

Factors associated with the cell can be distinguished from those factors associated with the phagemid that lead to an increase in TMP resistance. For example, an increase in TMP resistance could relate to an increase in K_i of the inhibitor for chromosomal DHFR, or some strains with mutations in the thymidylate synthetase enzyme can confer TMP resistance in host bacteria (7). It is even possible for the amount of TMP reaching the cytosol of the cell that may be affected by the incorporation of the fusion product in

the inner membrane (4). These factors are considered but may be less likely under the restricted nutrient conditions, and it is not likely that they are passed onto virgin host bacteria. The phagemid transferred via the virus/host system is likely to have the mutation that confers the increased level of TMP resistance in virgin host cells. However, the mutation may not be specifically an altered property of the fusion protein increasing catalytic efficiency but, for example, an increased expression of the R67 DHFR or also an increased K_i of the inhibitor for the enzyme (4,6,7). Nevertheless, the chances of selecting a reinfected colony growing under the same TMP stress would be greater for that colony having the mutation pertaining to the fusion gene on the phagemid.

Selection of mutants that confer higher TMP resistance in cells is a general strategy that does not allow for specific selection of variants with increased catalytic efficiency. However, this method could be the first step in screening libraries of *E. coli* cells containing phagemids subjected to hypermutagenesis procedure in future experiments. A more specific selection would utilize an affinity step. Therefore, it is crucial to design an analog that can effectively select for those variants with altered binding and catalytic properties. The analog must bind with greater affinity for the transition state or other intermediate such as those reported with the selection for β–lactamase and GST phage display (28,30). A transition state molecule would benefit our selection for rare R67 DHFR variants, yet no such analog is available, so we propose to start investigating use of a bisubstrate analog as previously discussed. Some examples of bisubstrate compounds are presented in figure 13. These compounds may potentially

allow us to find those desired rare variants more effectively than using TMP resistance in hosts.

Conclusions

Quadruple R67 DHFR is displayed on both phage display systems and is active. The K_m values for the gene VIII fusion are altered and are likely to be a result of intrinsic changes to the fusion protein itself, possibly due to the length of the spacer. Two selection methods can be investigated by which mutants can be obtained, potentially generating one with improved catalytic abilities. The first selection utilizes the altered K_m values, allowing for revertants of the fusion protein under the selective pressure of TMP resistance in hosts. The second selection utilizes a constructed library of mutants which an affinity ligand selects for those desired variants. It would be more efficient to apply the second method, allowing us to find variants that would eventually provide information into R67 DHFR's binding and mechanism of catalysis.

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List of References

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Appendix

List of Figures

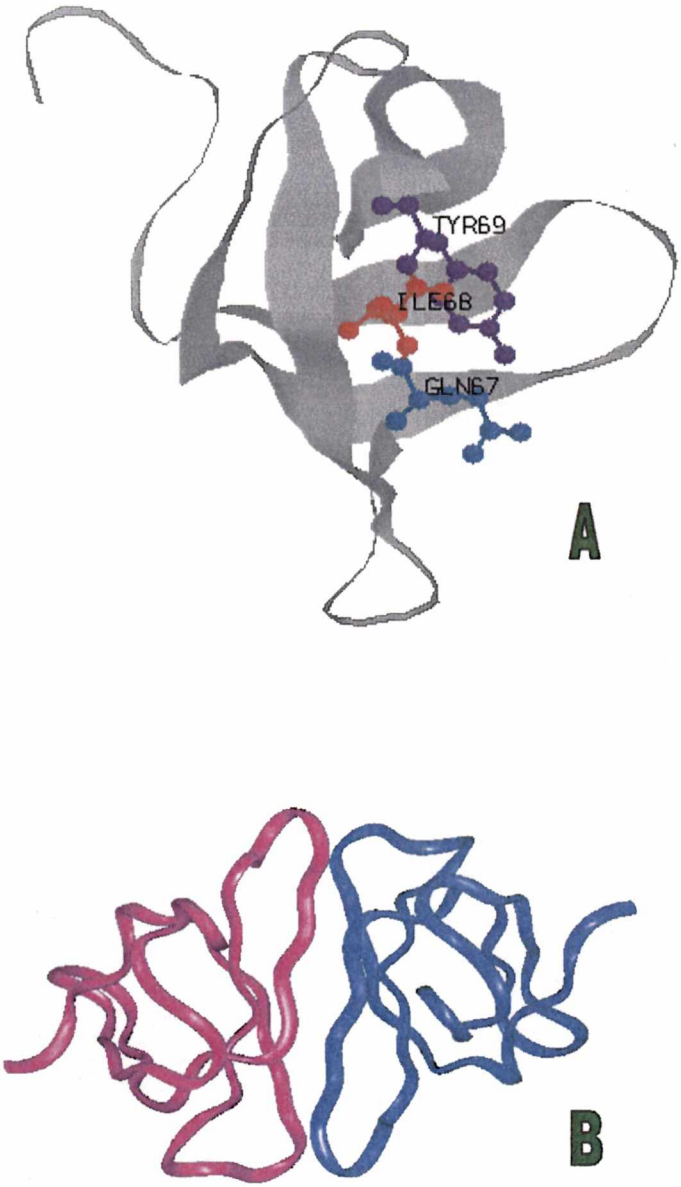


Figure 1. Images of the monomer structure of R67 DHFR depicting some residues in the active site (A) and the association of two monomers into a dimer (B). (A) Three residues in the active site (glutamine 67, isoleucine 68, tyrosine 69) are shown with respect to their position on the protein (ribbon). The image was generated using Rasmol (Sayle, R. and Milner-White, J. *TIBS* (1995). Vol. 20; No. 9: 374). (B) The carbon backbone structure of dimeric R67 DHFR shown as a ribbon. The intersubunit β-barrel appears in the center of the image.

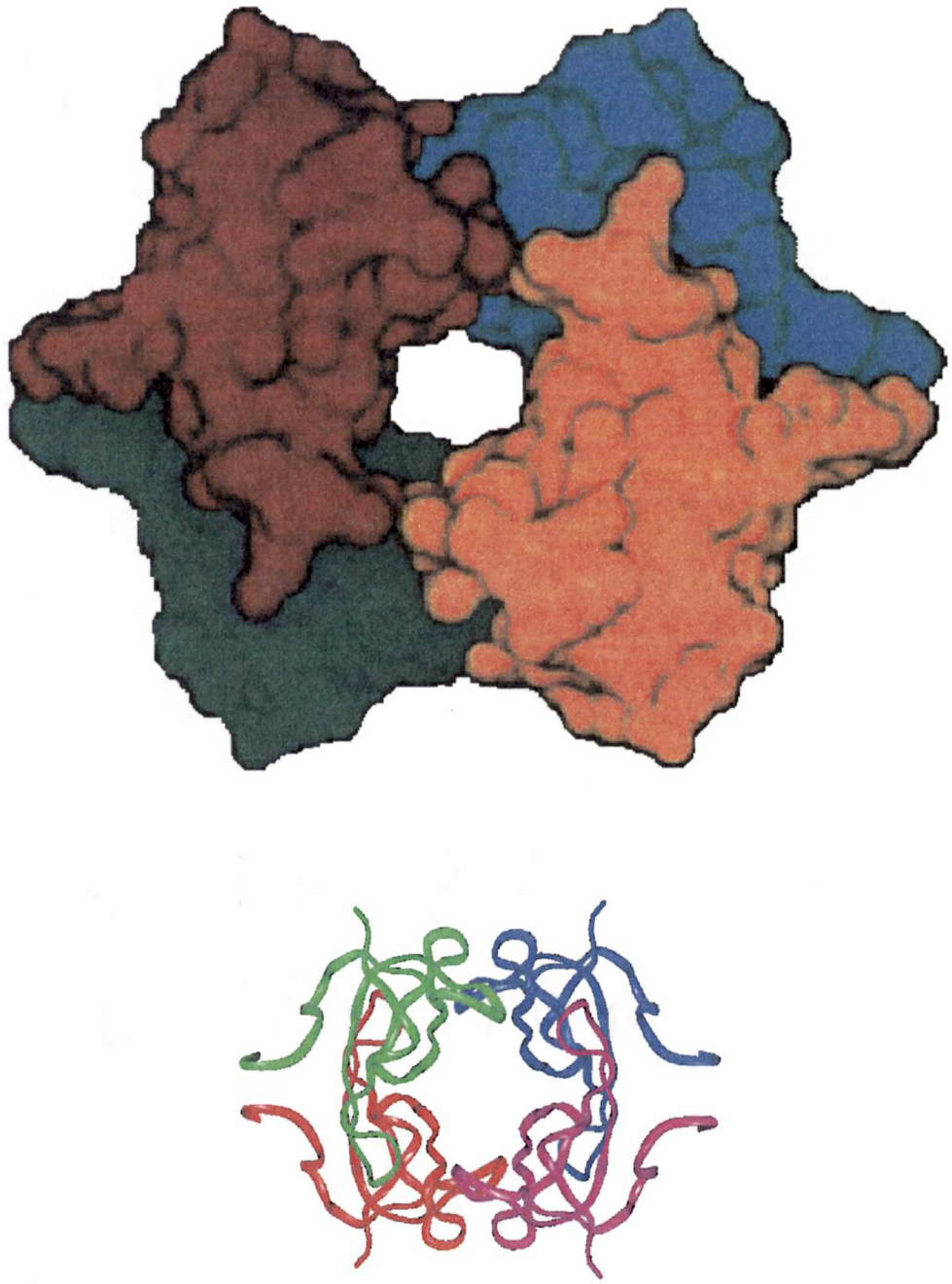


Figure 2. Two depictions of the homotetramer of R67 DHFR. The active site traverses through the center of the pore. The top image shows the CPK surface while the bottom image shows the α-carbon backbone as a ribbon.

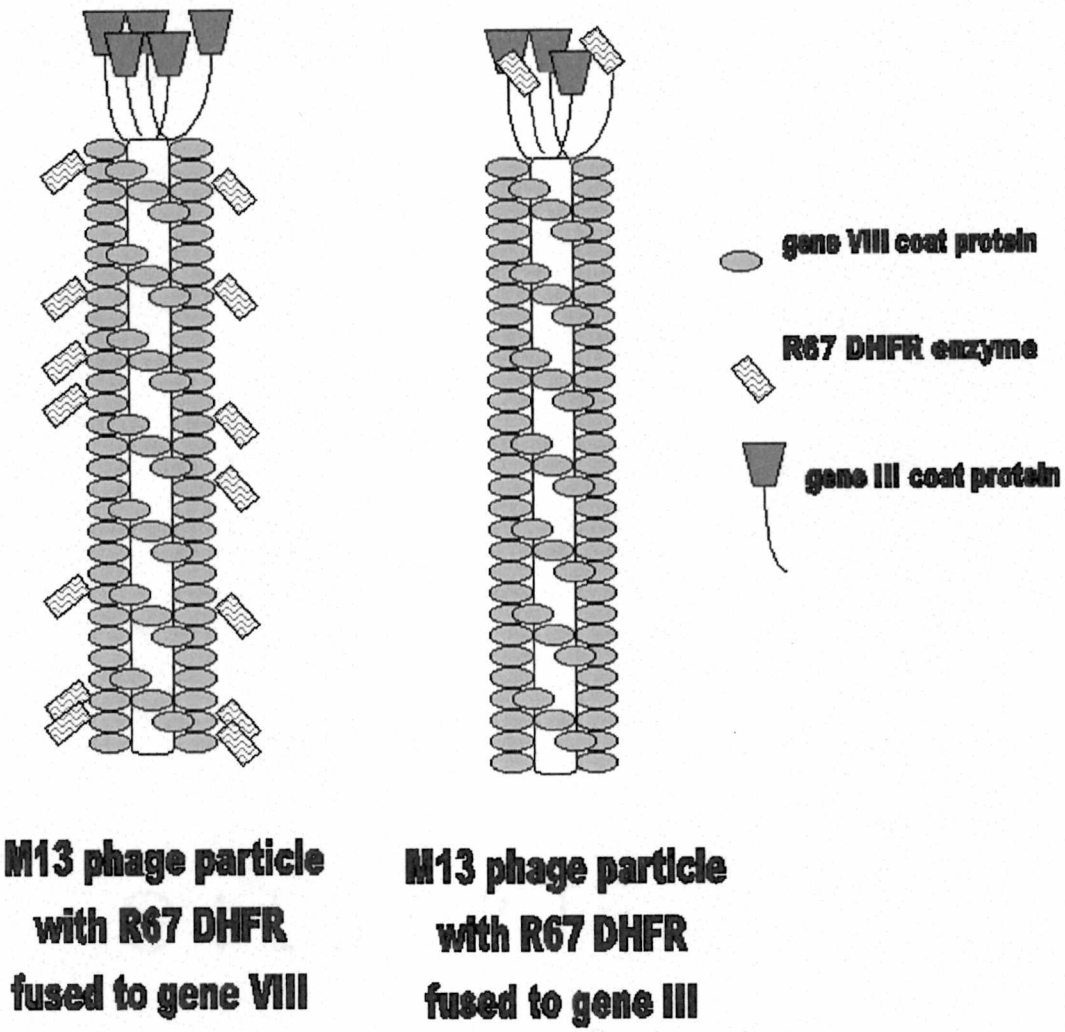


Figure 3. A drawing representing the mosaic display of R67 DHFR on the surface of the M13 phage using the gene VIII system (left) and gene III system (right). The DNA is carried inside the protein coat.

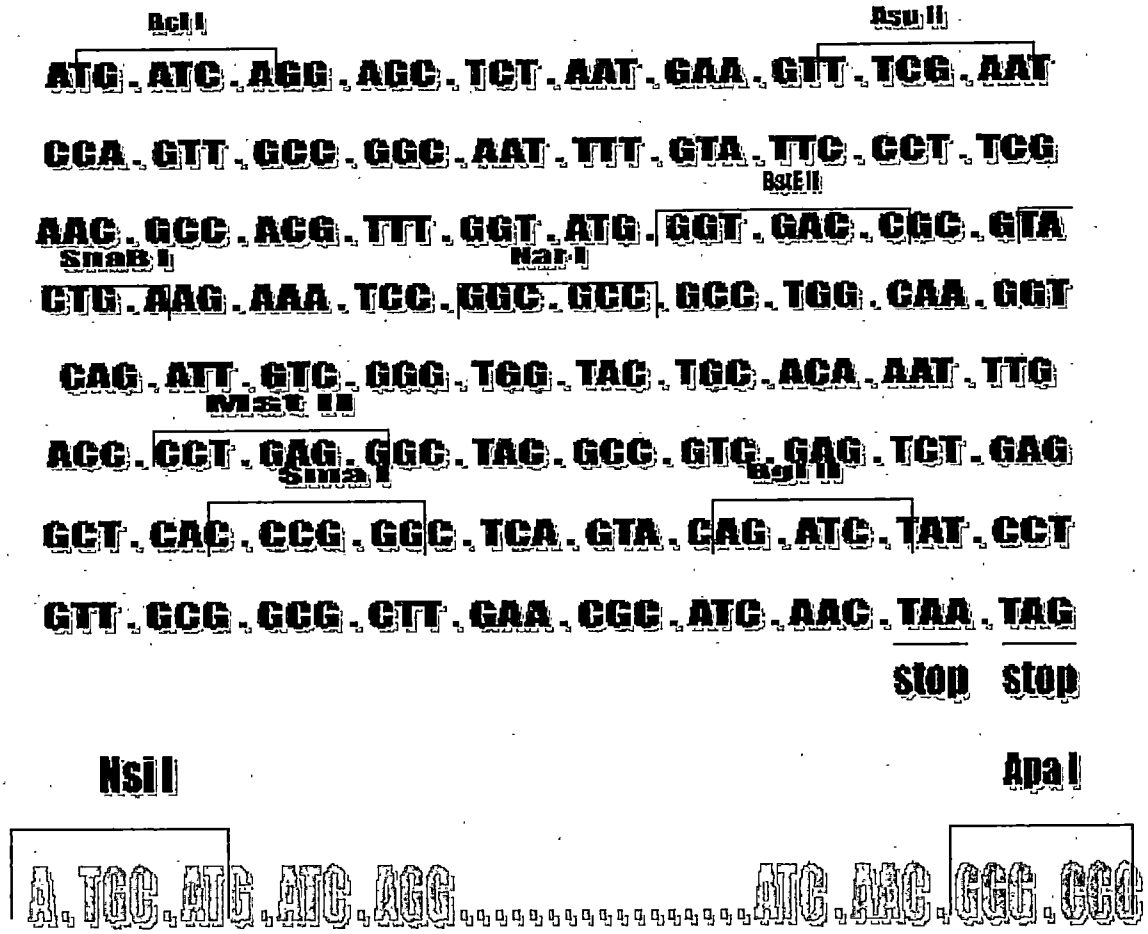


Figure 4. The sequence of synthetic R67 DHFR. PCR mutagenesis was used to introduce an Nsi I site at the 5' start of the sequence as well as replace the two stop codons at the 3' end with the Apa I site. The Apa I site is also the spacer that links the R67 sequence to the gene III and gene VIII protein sequence.

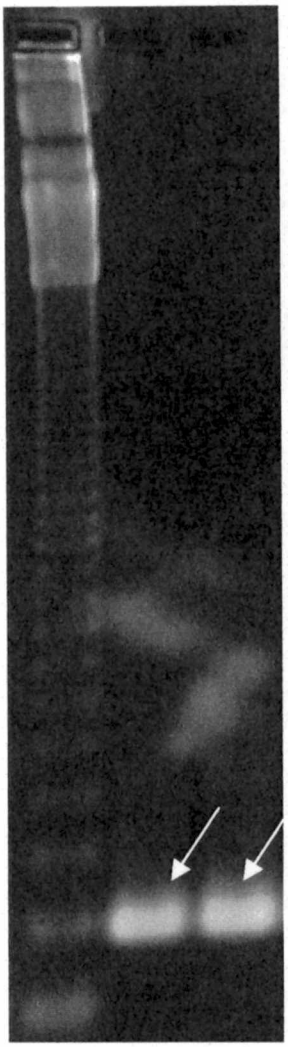


Figure 5. The 250 bp PCR product for the double mutant R67 sequence with an Nsi I site at the 5' end and an Apa I site at the 3' end. Samples from the PCR reaction were loaded and separated onto a 1.2 % agarose gel. Two samples are represented. Bands were visualized with ethidium bromide stain. The smeared anomaly above the bands represents some overloading of the sample.

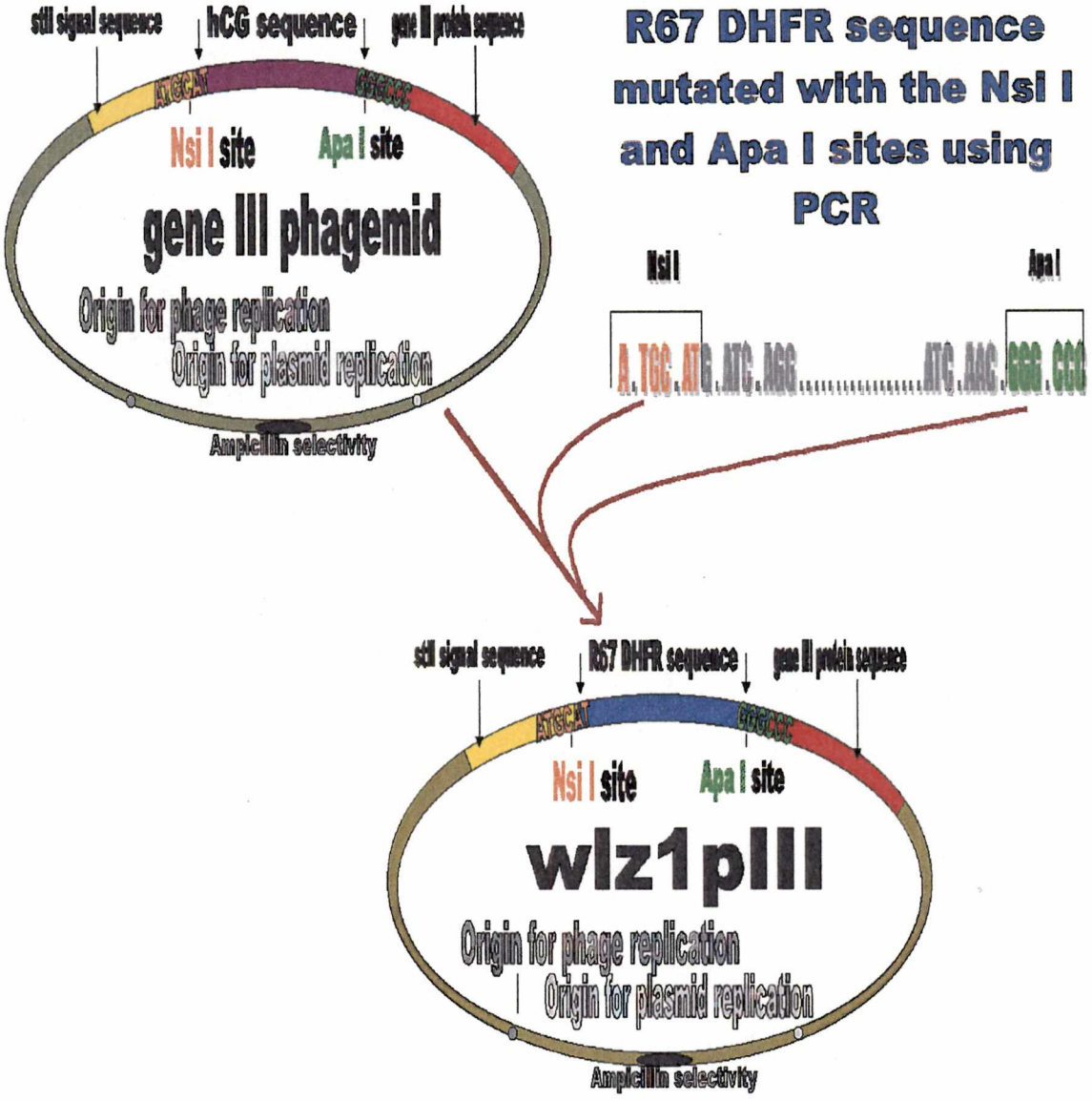


Figure 6. Construction of the wlz1pIII phagemid. The PCR mutated fragment of the R67 DHFR gene sequence with Nsi I and Apa I sites (excised from pUC19) was inserted into the gene III phagemid that possesses the complementary restriction sites. The R67 DHFR gene replaces the hCG sequence in the original construct obtained from James Wells.

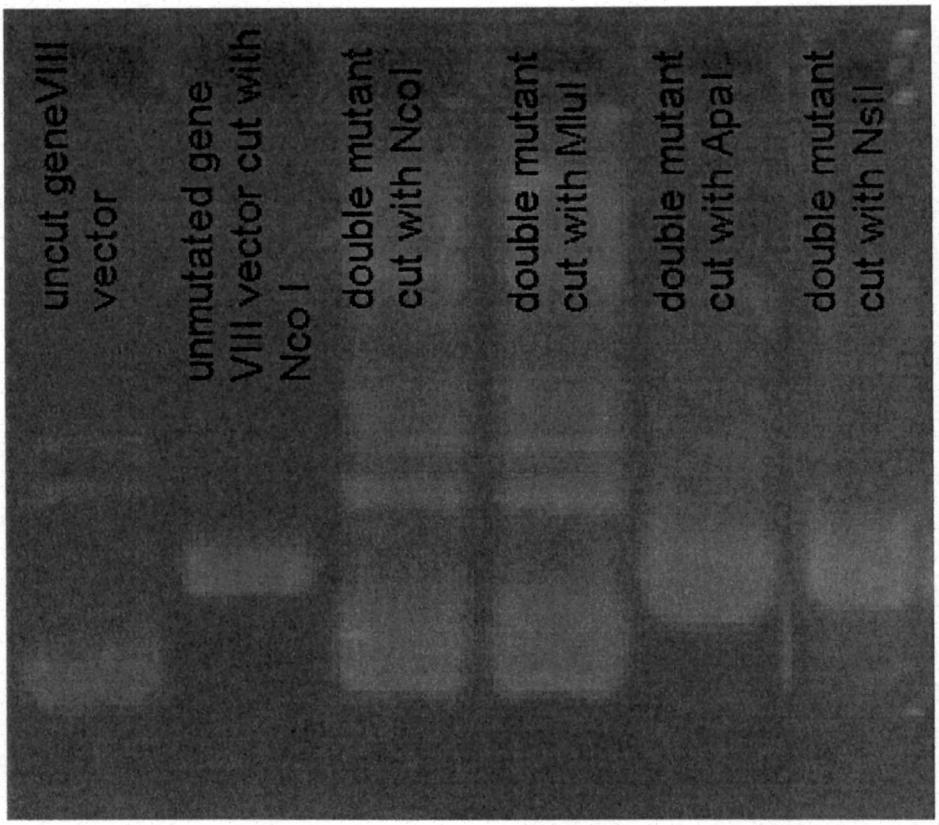


Figure 7. A 1.2% agarose gel used to screen for the double mutant gene VIII phagemid with an R67 gene insert. In this construct the Nco I restriction site is replaced by an Nsi I site and the Mlu I restriction site is replaced by an Apa I site. The double mutant is characterized by digesting the phagemid with each restriction enzyme. The Nco I and Mlu I enzymes no longer cut the DNA of the double mutant phagemid vector, thus the gel shows an uncut phagemid as observed with the control for uncut DNA. The Nsi I and Apa I enzymes do cut the double mutant phagemid vector generating a single cut within the sequence as seen with the non mutant vector cut with Nco I.

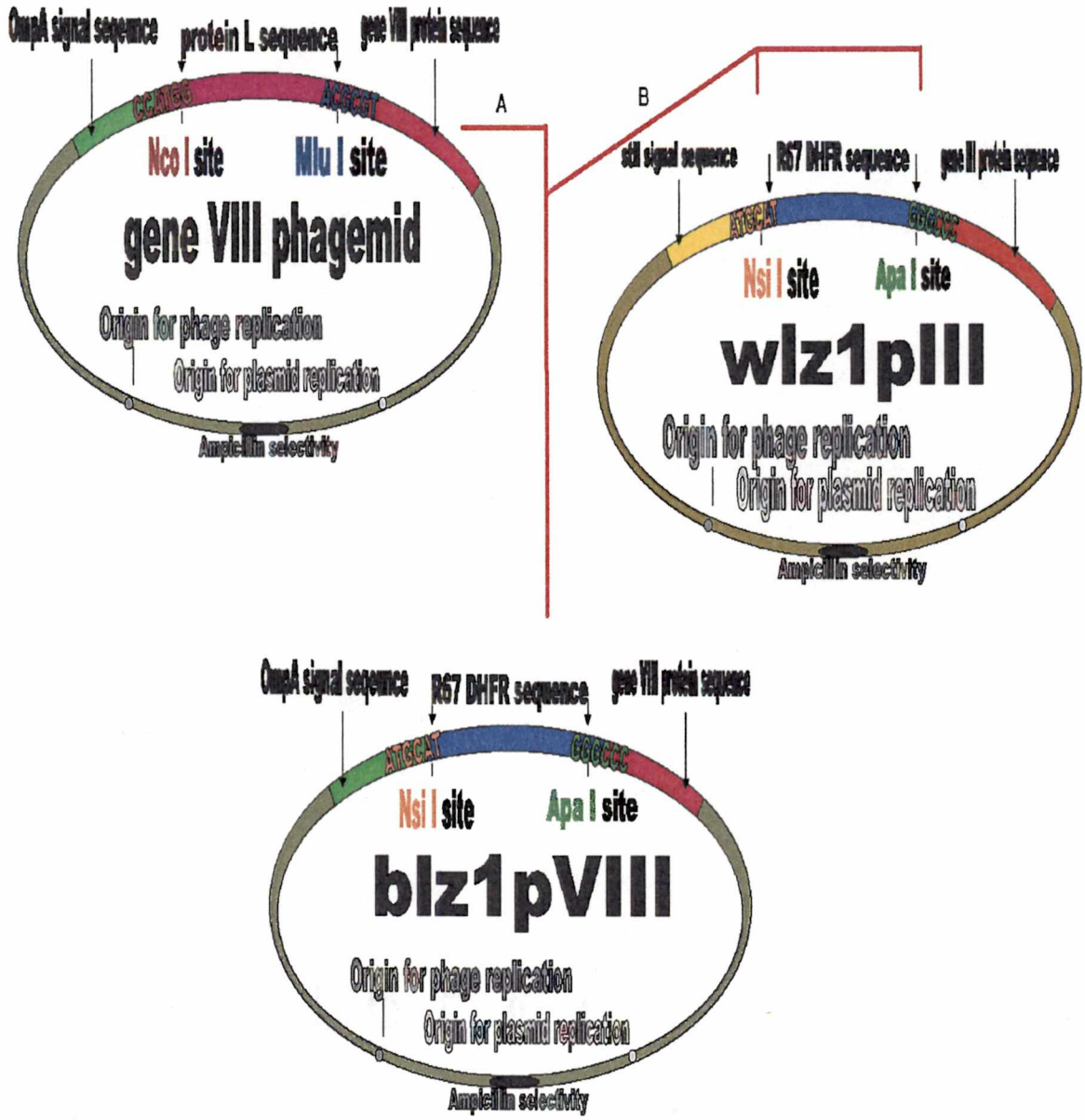


Figure 8. Construction of the blz1pVIII phagemid. Two consecutive PCR reactions result in replacement of an Nco I site by an Nsi I site as well as an Mlu I site by an Apa I site (A). This allows insertion of the R67 DHFR sequence excised with Nsi I and Apa I from the wlz1pIII phagemid (B).

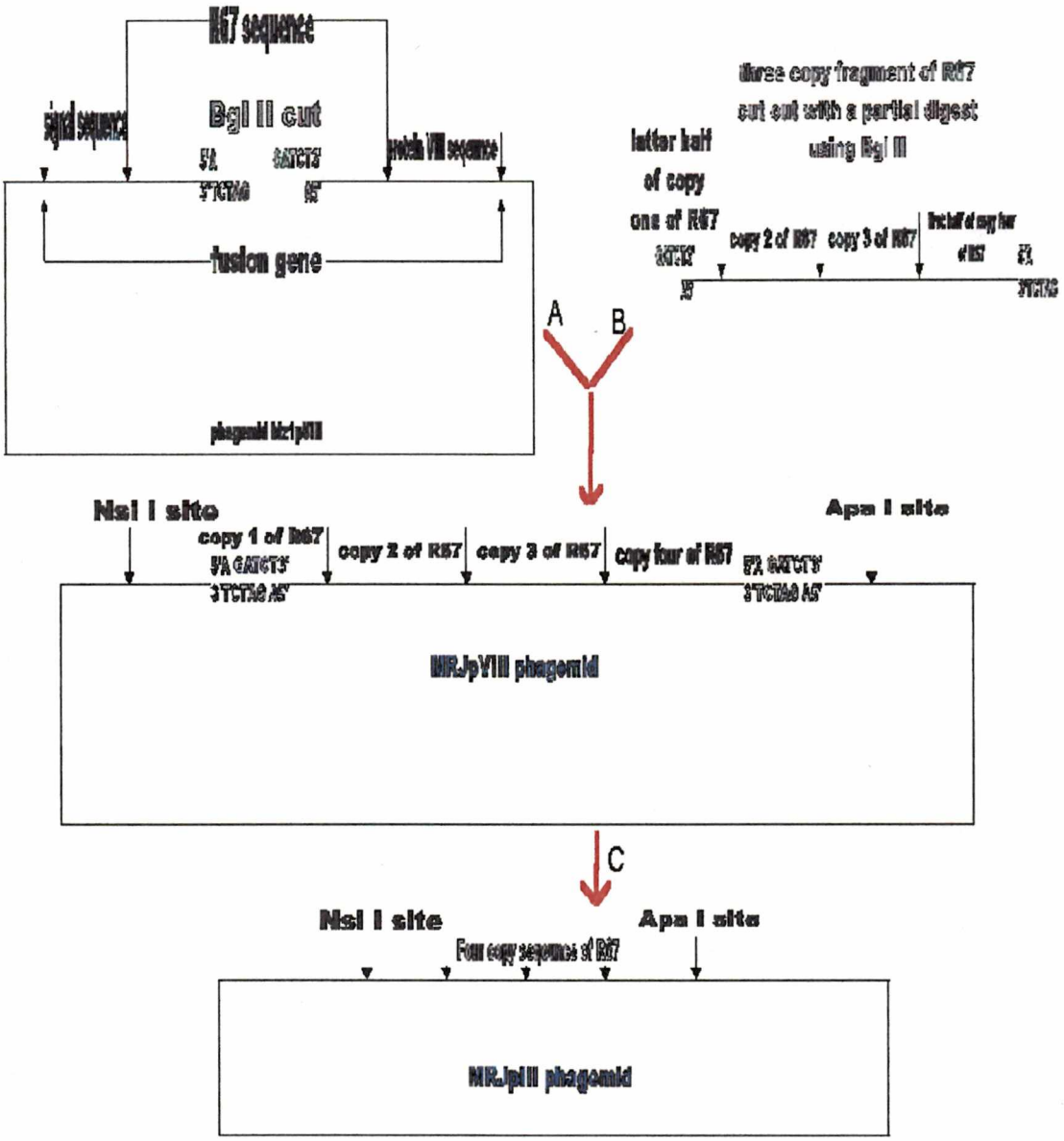


Figure 9. Construction of the MRJpVIII and MRJpIII phagemids (four gene copies of R67 DHFR sequence linked in frame to the phage gene coat protein sequences). Bgl II cuts at one location in the R67 DHFR sequence in phagemid bz1pVIII (A) to allow for insertion of a fragment that corresponds to three copies of the R67 sequence excised by partially digesting the four gene copy R67 DHFR construct in pUC8 using the same restriction enzyme (B). Nsi I and Apa I were used to excise the four gene copy of R67 in MRJpVIII for insertion into the gene III phagemid to produce MRJpIII (C).

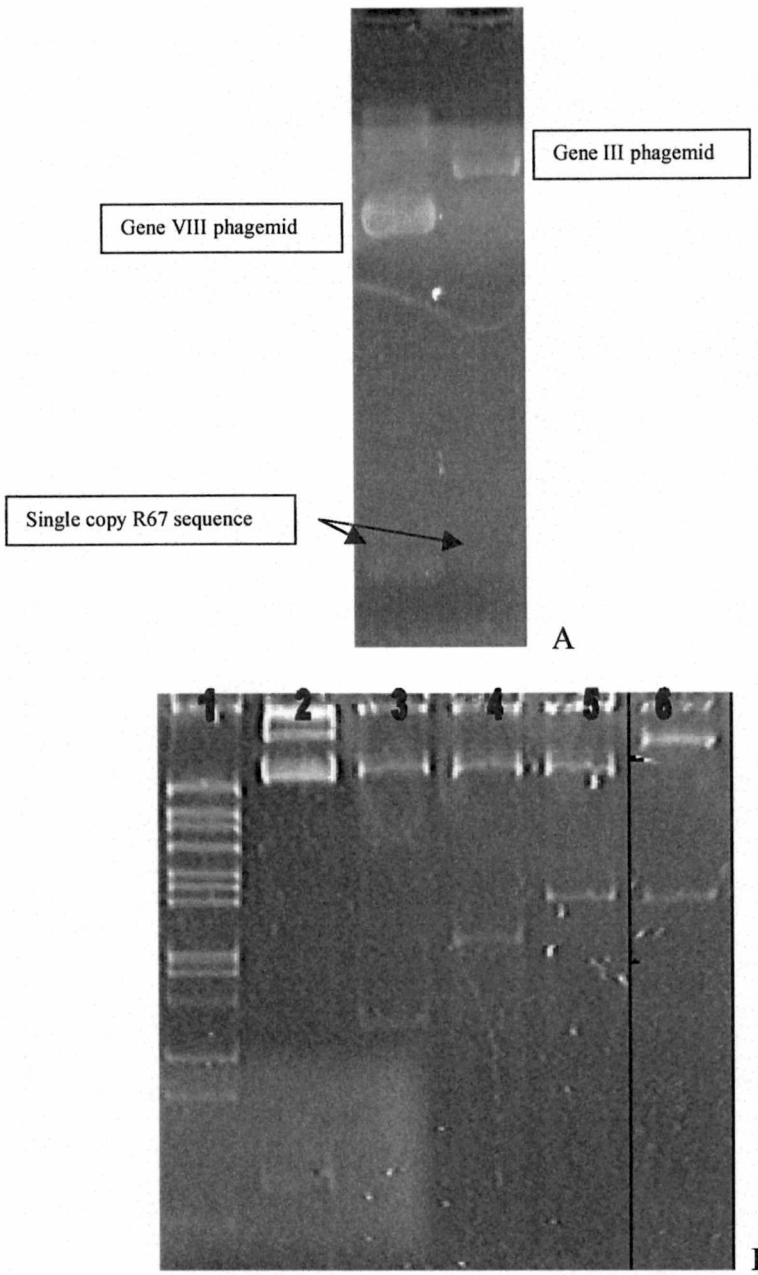
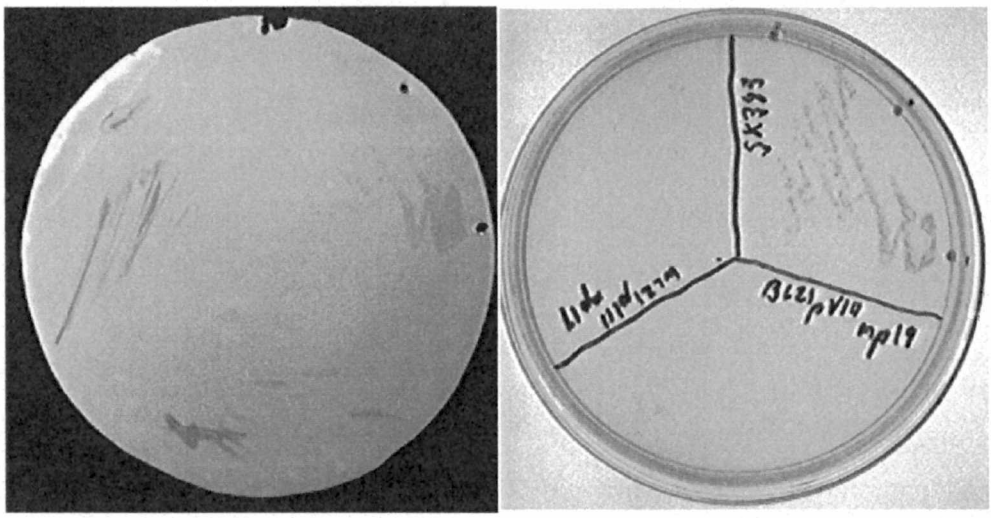
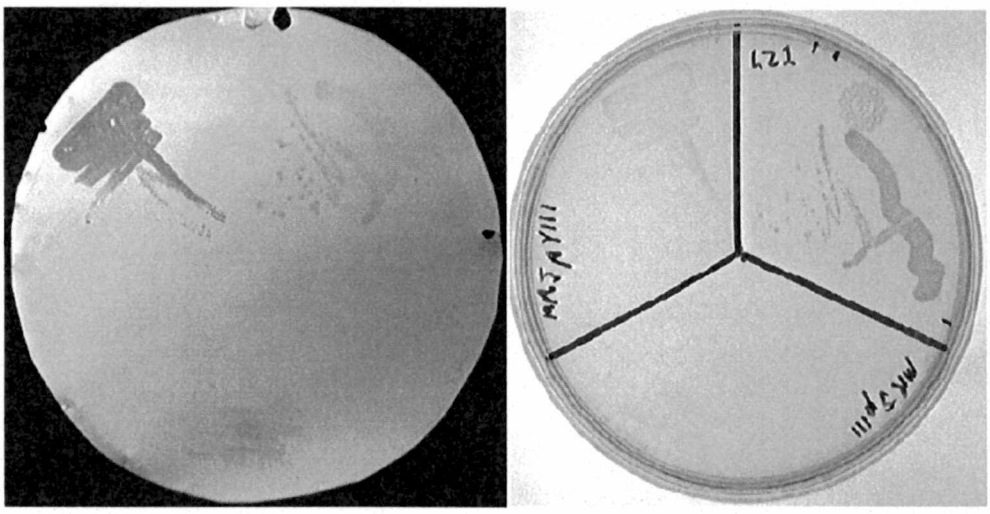


Figure 10. Double restriction enzyme digest of the four constructs with either single copy or four copy R67 DHFR sequence inserted into the gene VIII and gene III phagemids. The 1.2% agarose gel shows the single copy R67 insert cut out with Nsi I and Apa I from both phagemids (7A). In 7B, the 6% polyacrylamide gel shows the single, double, triple, and quadruple copy R67 inserts cut out from the gene VIII phagemid in lanes 2-5 respectively. Lane 6 shows the four copy R67 insert cut from the gene III phagemid.



A



B

Figure 11. Colony lifts detecting the four fusion proteins using the antibody for R67 DHFR. YT media with 200ug/ml Amp was first used to culture cells overnight. Cultures were super-infected and then were streaked onto the plates to grow individual colonies. Plates were incubated at 37°C for panel A and 30°C for panel B and for a time no longer than 24 hours for each. Controls grew to an optimal density on the plates. Cells with any of the four phagemids grew at much reduced rate forming very small colonies. Panel A shows the colony lifts for JM107 *E. coli* cells expressing the R67 DHFR monomer fused to the gene VIII protein (top left of lift) and gene III protein (bottom part of lift). Panel B shows the colony lifts for STBL 4 *E. coli* cells expressing the quadruple R67 DHFR fused to the gene VIII protein (top left of lift) and the gene III protein (bottom part of lift). The signal developed in 5-8 seconds for the *E. coli* strains transformed with the phagemids. The YT plates with 20 μg/ml TMP are shown on the right of each lift. SK383 *E. coli* cells transformed with the plasmid carrying the gene that constitutively expresses wt R67 DHFR are used as controls for the single gene R67 fusion constructs, and STBL 4 *E. coli* cells with the same plasmid are used as controls for the four gene copy R67 fusion constructs.

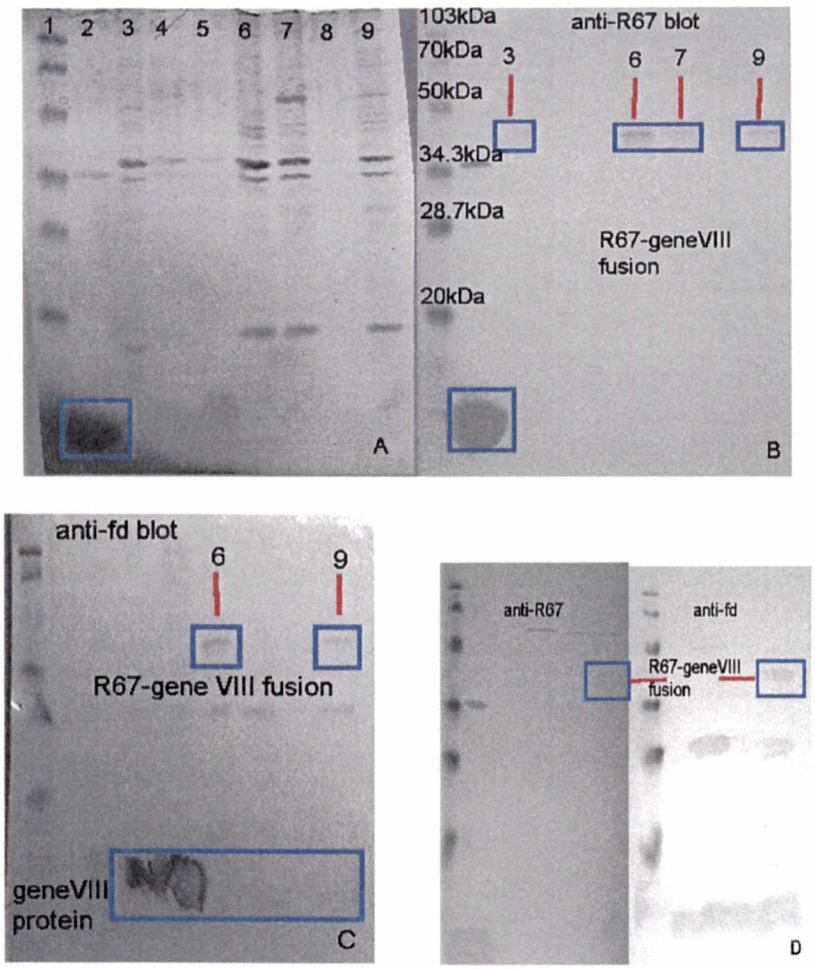


Figure 12. The protein analysis of phage particles with the attached fusion protein after CsCl density purification. The quadruple R67 DHFR-gene VIII fusion protein is detected (highlighted squares in upper parts of the blots) using the anti-R67 and anti-fd bacteriophage antibodies. The fusion protein falls within the range of the prestained standards, consistent with its predicted molecular weight (around 40kDa). Panel A shows the 13% polyacrylamide gel loaded with the samples: lane 1. prestained standards, 2. purified wt R67 DHFR, 3. a lyophilized R67 DHFR gene VIII fusion phage prep not purified using CsCl centrifugation, 4. a CsCl band of phage with no fusion product collected just above the PEG layer, 5. an upper level band of phage with no fusion product, 6,7, and 9. CsCl purified phage with the gene VIII fusion, 8. an upper level band collected from the same centrifuge tube as the band assayed in lane 7. The blot procedures are described in the methods. Panel B and C show the corresponding lanes that have the fusion protein. The anti-fd blot in C also detected the fusion protein for the samples loaded in lanes 3 and 7 as detected on the anti-R67 blot in B, but the signal was very faint. Panel D is the first blot done to detect the fusion protein in phage preps that were harvested and lyophilized. The faint signal appeared after 10 minutes for these blots. In contrast, the signal in panel B and C appeared in a matter of seconds for the phage preps after CsCl purification.

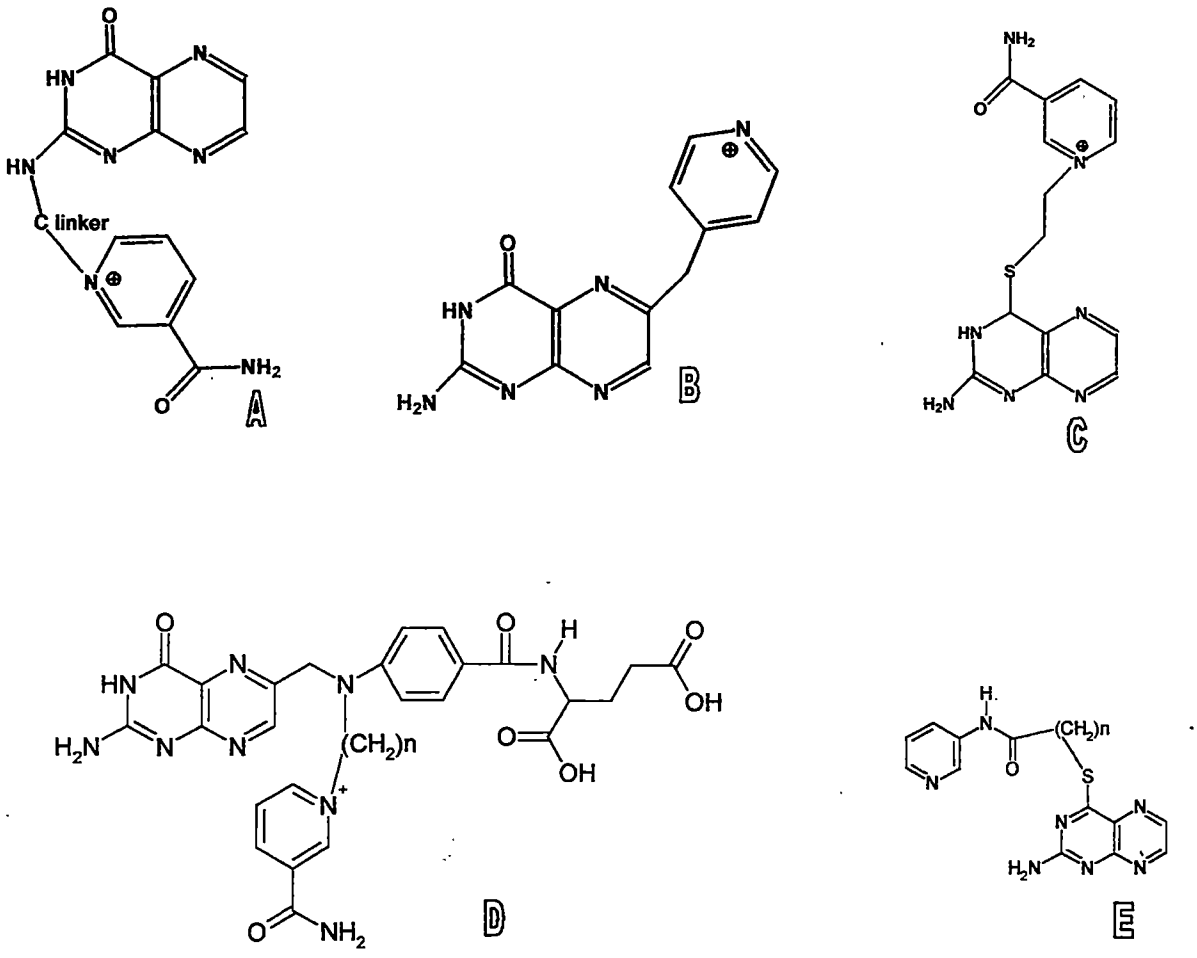


Figure 13. Some of the bisubstrate compounds that our collaborator Bruce Hathaway proposes to synthesize. Compound B is an ideal bisubstrate analog where the covalent association of the nicotinamide ring would be juxtapositioned to the C6 of the pteridine ring for hydride transfer. Compound D has more components of *p*-amino-benzoyl glutamic acid tail (PABA-Glu) for representing a folate analog. All bisubstrate compounds shown have the pteridine ring and nicotinamide rings parts in close proximity which would be the case in the active site of R67 DHFR.

List of Tables

Table 1. Presence of DHFR activity during phage purification.

Construct/culture	Cultures superinfected (Si) or uninfected (Ui) with M13K07	Detection of activity in the stages of the phage preps and those samples that were CsCl purified: (+) activity; (-) no activity; (x) not performed			
		Stage 1[†]	Stage 2[‡]	Stage 3[§]	CsCl density centrifugation
JM107 (or STBL 4) E. coli with no phagemid (control)	Si	-	-	-	-
	Ui	-	-	-	X
Gene expressing the wt R67 DHFR (control) in JM107 E. coli	Si	+	-	-	X
	Ui	+	-	-	X
Wlz1pIII in JM107	Si	-	-	-	X
	Ui	-	-	-	X
Blz1pVIII in JM107	Si	-	-	-	X
	Ui	-	-	-	X
MRJpIII in STBL 4	Si	-	-	+(low)	+(low)
	Ui	-	-	-	X
MRJpVIII in STBL 4	Si	-	+	+	+
	ui	-	-	-	X

[†] Activity assays on the supernatant of cultures.
[‡] 20ul of the phage suspension assayed in MTH polybuffer at pH 6 after PEG and NaCl precipitation and dialysis.
[§] 20ul of some of the lyophilized phage resuspended in 0.03 M Tris pH 8 assayed in MTH polybuffer at pH 6.

Table 2. Relative velocities of samples containing phage with the active quadruple R67 DHFR displayed on its surface. Samples assayed have been purified using CsCl density centrifugation.

Phage display system assayed	Observed rate measured as μmol substrates consumed per minute[†]
Gene VIII system displaying quadruple R67 DHFR	0.019[‡]
Gene III system displaying quadruple R67 DHFR	0.003[‡]

[†] Activity assay performed in MTH polybuffer at pH 7 and the reaction measured over a 3 minute time period.
[‡] Observed rate increased two fold when concentrations of substrate and cofactor were doubled.

Vita

Michael Robert Jackson was born in Memphis, TN on April 13, 1971. He lived there for three years and then moved to Crossville, TN where he grew up. He went to Cumberland County High School, playing football, marching in the band, and touring abroad in Europe in 1988 performing concerts. He also played in the jazz band in 1989 with his band winning first place in the amateur jazz competition at the Dogwood Arts Festival in Knoxville, TN of that year. He graduated from his high school in 1989 and enrolled into Memphis State University (now the University of Memphis) later that year. In 1994, he received a Bachelor of Science degree in biology with an emphasis in microbiology and chemistry. After graduating, he worked at the Cumberland Medical Center for about 14 months and then enrolled into the graduate program at the University of Tennessee, Knoxville. He entered as non-degree status in September of 1997 and then was accepted into the Biochemistry, Cellular, and Molecular Biology program for September 1998. He was awarded a Master of Science degree in August of 2001.