ABSTRACT

Title of Document: THE TRANSCRIPTIONAL REGULATOR MPHR(A): CHARACTERIZATION AND CIRCUIT ENGINEERING

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Directed By: Dr. T. Ashton Cropp, Assistant Professor, Department of Chemistry and Biochemistry

The MphR(A) transcriptional repressor protein was incorporated into high-efficiency expression vectors, purified and characterized. Initial screens for crystallography solvents were conducted along with preliminary gel-shift and intrinsic fluorescence quenching experiments to obtain the equilibrium dissociation constant for the protein-DNA interactions. The constant was found to be 0.5 – 1.9 μM, depending on the method used. The gene was also incorporated into positive feedback circuits to detect macrolide antibiotics using various reporter genes and plasmid constructs. Qualitatively, the circuits showed a change in output upon the addition of MphR to the system.

THE TRANSCRIPTIONAL REGULATOR MPHR(A): CHARACTERIZATION AND CIRCUIT ENGINEERING

By

Adam R. Smolinsky

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Advisory Committee:
Professor T. Ashton Cropp, Chair
Prof. Dorothy Beckett
Prof. Nicole LaRonde-LeBlanc
Prof. Herman O. Sintim

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List of Abbreviations

E. coli .....Escherichia coli

EDTA .....Ethylenediamine tetraacetic acid

FPLC .....Fast protein liquid chromatography

His-tag.....Polyhistidine tag

IPTG.....Isopropyl β-D-1-thiogalactopyranoside

MphR(A), MphR.....Macrolide 2'-phosphotransferase A Repressor

PCR .....Polymerase chain reaction

SAP .....Shrimp alkaline phosphatase

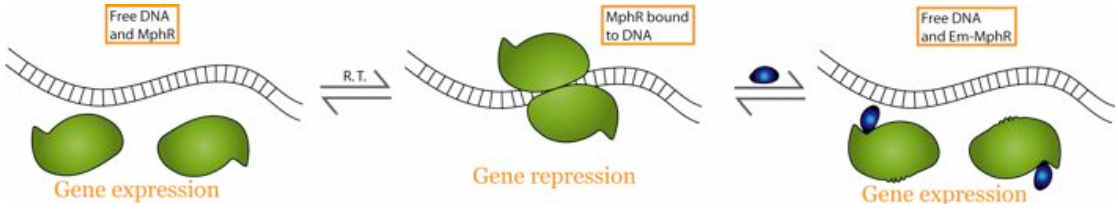
SDS-PAGE .....Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

Chapter 1: Introduction

1.1: Basic Protein Information

The Macrolide 2'-Phosphotransferase I [Mph(A)] protein isolated from *Escherichia coli* is a strong inactivator of macrolide antibiotics, such as erythromycin (Erm)[1]. This protein is induced by Erm in the natural strain (Figure 1-1) through the transcriptional repressor protein, MphR(A)[2]. Specifically, the repressor controls transcription of a gene sequence containing the *mph*(A)-*mrx*-*mphR*(A) operon, in which Mrx has an unidentified function[3]. The negative transcription factor, MphR(A), has only been crudely characterized, but presents a significant possibility for use in genetically encoded erythromycin sensing feedback loops.

Figure 1-1: Basic Illustration of MphR-Controlled Transcription



MphR can be included in a large family of transcriptional repressors responsive to molecules present in the bacterial system[4]. The most widely characterized of these repressors is TetR, which binds as a homodimer to a specific sequence of DNA that regulates the formation of tetracycline (Tc) in the cell. TetR is inducible by tetracycline, such that when the $[TcMg]^+$ complex binds the protein, a conformational change occurs and the protein dissociates from the DNA.

1.2: TetR Family of Transcriptional Regulators

1.2.1: Key Features

There are a number of features of MphR that appear to be consistent amongst a majority of similar transcriptional repressors[4]. The most important feature of the protein itself is the HTH motif. The sequence of α-helix-turn-α-helix is extremely highly conserved among members of this family, although it has been shown to appear mainly in the N-terminal region and occasionally in the C-terminal region of the primary sequence[4]. Additionally, the residues present in the HTH regions of the primary sequence are themselves highly conserved among these proteins.

Another feature of these protein-DNA interactions is the nature of the DNA sequence involved in protein recognition. Often, these sequences are palindromic or pseudo-palindromic—that is, they contain a complete or nearly complete inverse repeat at the protein binding site. This is further evidence to support the idea that these proteins tend to bind as some sort of oligomer.

An example of a protein with similar function is QacR regulator. This protein is responsive to a large library of drugs[4]. It regulates transcription of the quaternary ammonium compound resistance gene and thus must bind to a variety of drugs instead of just one, like TetR. The interactions between QacR and DNA are also different than TetR; a pair of dimers binds the palindromic sequence. However, the regions of the HTH motif still contain a highly conserved sequence of residues.

1.2.2: Sequence Homology

A BLAST search using ClustalW shows a high degree of sequence homology between MphR and other prominent members of the TetR family (Figure 1-2), particularly with the HTH motif in the N-terminal region.

Figure 1-2: ClustalX 2 Alignment of MphR with TetR Family Members

The boxed region represents the putative HTH motif.

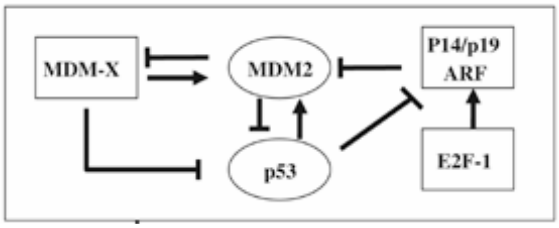
1.3: Regulatory Circuits and Biofeedback Loops

1.3.1: General Concept

In general, there are two types of biological response networks. Positive feedback involves an increase in some response variable, while negative feedback shows a decrease in the response variable. Feedback loops have evolved to cause a variety of responses. One of the most studied of these loops involves the p53 regulatory system involved in cell death[5]. Through an extensive list of possible activators and a somewhat complex response system involving many other intermediary proteins, a response from the p53 protein can be seen in cells of almost every possible function. Activation of the

p53 prevents the transcription of some genes and allows for the transcription of a number of other genes including the MDM-2 protein, which is, in turn, the major negative regulator of p53 (Figure 1-3). Any of a multitude of other proteins can act on some aspect of this circuit either increasing or decreasing the effect of p53.

Figure 1-3: p53 Regulatory Circuit[5]



According to Sayut, *et al.*[6] and others[7-9] many of these positive feedback loops exhibit "switch-like all-or-none bistability" which means that they have a binary response to the particular inducer of interest. Bistability requires some degree of ultrasensitivity to the inducer, although the property is not required for a working feedback loop. However, designing and engineering a loop to be bistable will give the most useful results.

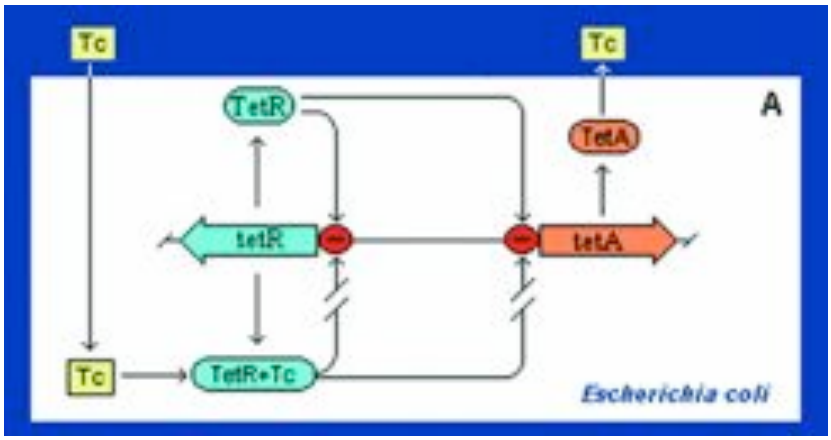
There are a number of advantages to using positive feedback loops to control gene expression. First, the binary activity means that the gene can be controlled simply and with tight regulation. Second, since many of the repressor proteins used to control the loops are derived from bacterial sources, they have the added advantage of being easily inducible by simple antibiotics and they are nontoxic to the host cells[6].

1.3.2: TetR Family Constructs

The TetR family of transcriptional repressors is also generally involved in regulatory circuits. As described for both TetR and QacR, above, the induction of the repressor protein is done by a molecule or set of molecules, the presence of which depends on the

binding equilibrium of that particular protein. Specifically, when TetR is bound to the DNA it prevents transcription of the protein that removes tetracycline from the cell, as well as its own transcription. When no Tc is present in the cell, there is no need for the protein to remove it from the cell, therefore TetR remains bound to the promoter sequence. By controlling its own transcription, TetR ensures that, after Tc has been added to the system, there will still be some protein that is not part of the TetR-TcMg complex and can therefore re-regulate the transcription of the TetA protein (Figure 1-4).

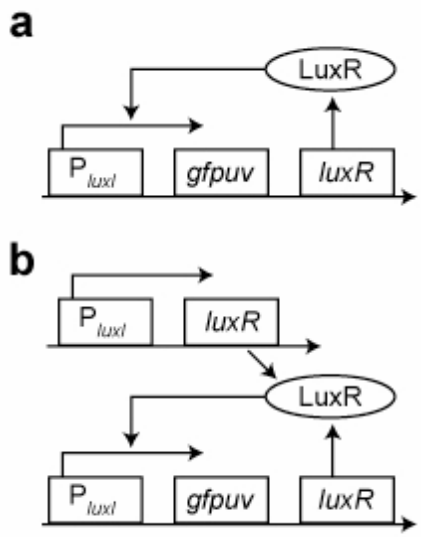
Figure 1-4: Regulatory Network Involving TetR[4]



TetR, among other members of this family have already been employed extensively in biotechnological applications that take advantage of their properties as self-regulatory[4]. Their use provides hope that, after characterization, MphR will be useful for analogous applications. In theory, any member of the TetR family of transcriptional repressors could be used in some feedback loop. In practice, however, this is not always possible. Many of these transcriptional effectors are involved in very complex regulatory systems[4] and would therefore be difficult to control externally. The complex systems would also present a problem when trying to determine which stage of the circuit is directly affected upon changes to the system. Some of these networks are comprised of

simple sequential controls and therefore have been studied and utilized to a great extent. In particular, the LuxR activator system has been investigated in many studies because of its natural, quorum-sensing properties[6]. This circuit (Figure 1-5) has also been shown to have significant potential in synthetic biology[10].

Figure 1-5: LuxR Positive Feedback Circuits[6]

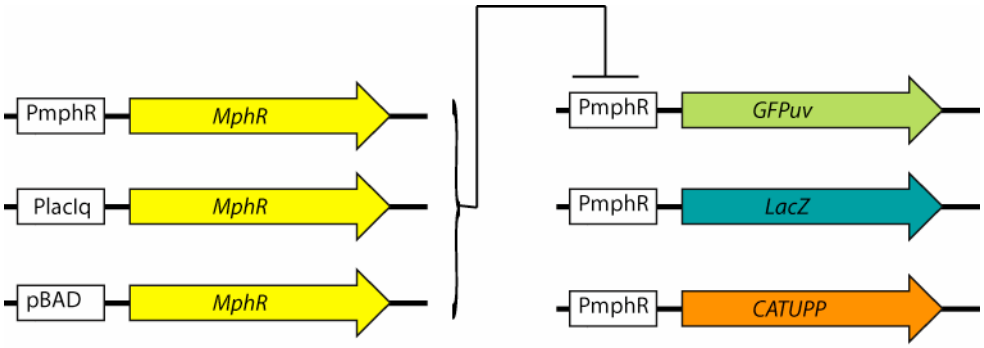


In nature, the LuxR activator is required for expression of the luciferase operon responsible for luminescence in *Vibrio harveyi* bacteria[4]. As a quorum-sensing protein, LuxR is responsive to a compound secreted from other bacteria, 3-oxo-hexanoyl-homoserine lactone. Sayut, *et al.*[6] used circuits shown to investigate the effect that mutant protein would have on the response of the system. They were interested in whether or not the sensitivity of the system could be improved by introducing mutations, which they did using error-prone PCR. They scanned the resultant library of mutants for response in the feedback loops showing that by enhancing the response to the intended inducer, the response of a feedback loop could be improved to require less inducer for activation.

1.3.3: MphR Constructs

In the case of MphR(A), like the majority of the TetR family of proteins, the circuit would involve positive feedback. Specifically, cells are engineered to make the MphR(A) protein independent of the presence of erythromycin. This protein can then bind to the native promoter sequence, as described above, which controls transcription of a specific reporter protein. In many cases, the GFP*uv* or β-Lactamase activity are used since their presence can be measured spectroscopically (Figure 1-6). The specific inducer—for MphR(A), erythromycin would be used—is then introduced to the system in some way resulting in increased reporter activity, which can be measured as a function of the amount of inducer in the system.

Figure 1-6: Basic Illustration of Potential MphR Circuits



PmphR is the DNA sequence recognized by MphR as found by Noguchi, *et al.*[3] and described below. PlacIq is the constitutive sequence of the lacI promoter. pBAD is the arabinose-inducible araBAD promoter. *GFPuv* is the gene encoding the Green Fluorescent Protein; *LacZ* is the gene encoding β-Galactosidase enzyme, and *CATUPP* is a gene that encodes the chloramphenicol acyltransferase and uracil phosphoribosyltransferase enzymes, which confer chloramphenicol resistance and cause 5-fluorouracil toxicity, respectively.

Circuits under the control of MphR with other reporters have been assembled[11]. The authors describe a luciferase-based sensor that can detect the presence of erythromy-

cin and other macrolides as directly delivered or as produced by bacteria that biosynthesize them. The work confirms the low specificity of wild-type MphR-based circuits, but presents promising results for quantifiable circuit assembly.

1.4: MphR Biochemistry

1.4.1: DNA Binding

Noguchi, *et al.*[3] performed crude analyses to determine the exact location of binding to the promoter region of the *mph*(A) gene. They found a partially overlapping region of 30 nucleotides on the coding strand and 29 nucleotides on the complementary strand to be protected by MphR(A) from DNase I footprinting. The net 35 base pairs protected encompass exactly the promoter sequence for transcription of the *mph*(A) gene.

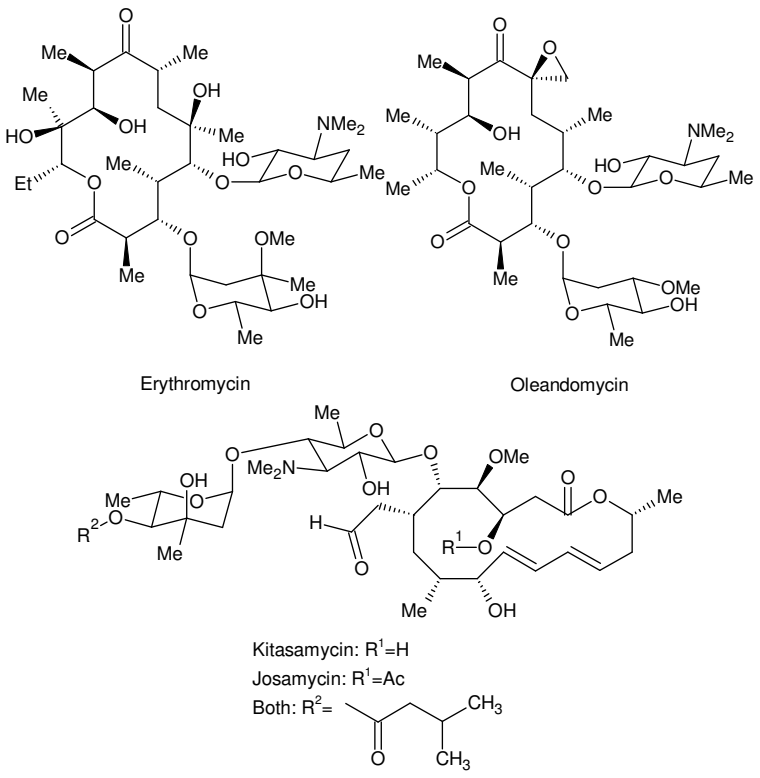
1.4.2: Inducer Sensitivity

MphR(A) is known to be inducible by erythromycin (Erm)[2]. In order to determine whether or not other macrolides could also induce transcription, Noguchi, *et al.*[3] also investigated binding of oleandomycin, josamycin, and kitasamycin to MphR(A). These compounds, which show a high degree of similarity to erythromycin (Figure 1-7), were all shown to induce the release of MphR(A) from its promoter. The relatively low specificity greatly limits the potential of this system for use in feedback loops. However, the group made no effort to quantify any binding interactions or ascertain the nature of inducer binding. That is, no information regarding which structural features—the macrocycle, sugars, etc.—of the macrolide contact the protein.

Response to a variety of inducers is not unusual. Many transcriptional repressors of the TetR family have been shown to respond to a variety of drugs. These include

MarR[12, 13], which is highly homologous to MphR(A). Some work by Vazquez-Laslop, *et al.*[14] has shown that when the structurally different ligands bind to BmrR, they make specific contacts with different residues in the binding pocket. Therefore, simple mutations to some of these residues will inherently preclude specific inducers from making contact with the protein increasing the specificity for the desired drug. Solving this problem is not so easy for MphR(A); the structural similarity between the macrolides that have already been shown to inactivate the protein would suggest that they contact similar residues in the binding pocket.

Figure 1-7: Macrolides Tested by Noguchi, *et al.*[3]



Unfortunately, since bistability is an important feature governing the usefulness of a particular feedback loop, the wild-type MphR(A) protein is not inherently useful. A variety of similar macrolides can bind the protein, inducing its release from the DNA

with approximately the same affinity[3] and relatively low selectivity for any one of the compounds. Conveniently, quite a bit of work has been done engineering specific mutants of similar proteins that have improved specificity for one inducer. In some cases, mutants have even been developed that recognize a completely different inducer[15].

1.5: Improving Specificity by Site-Directed Mutagenesis

The first step in engineering a mutant protein that can better recognize a specific chemical involves a different kind of characterization. The two general approaches to protein engineering have generally been used are random and site-directed mutagenesis. Random mutagenesis involves introducing mutations at random locations throughout the primary structure of the protein. While this method can give a lot of information, especially about the effect of certain residues on the kinetics and thermodynamics of protein folding, it is very difficult to obtain useful information with regard to binding interactions unless the specific mutation has little or no effect on the folding properties and the stability of the folded protein and also has some effect on the protein-ligand interface.

In contrast, site-directed mutagenesis is much more applicable for engineering new or better binding interactions because the method can give much more specific information related to protein-ligand interactions. This process involves keeping most of the protein sequence constant, as is done with random mutagenesis, and mutating only one residue. The regions of the sequence where mutations are introduced are specifically chosen because they have distinct interactions with the particular ligand. Often these residues are those found in or around the active site or binding pocket of the protein of interest.

Once a library of mutant proteins has been developed, each one can be compared to the wild-type for a multitude of important or useful properties. For example, when engineering the TetR protein to recognize a new inducer, Scholz, *et al*.[15] were interested in which mutations showed high affinity and selectivity for one particular inducer. In their experiments the initial mutations were screened for their affinity to the new inducer and tetracycline (Tc); those mutants that did not have relatively high affinity for the Tc analog of interest and relatively low affinity for Tc—as compared to the wild-type in both cases—could immediately be eliminated. The mutations that showed both of these properties were then held constant while a second round of mutagenesis was completed in the same regions as before. Further stages of mutations where then evaluated for their affinity to not only the two compounds of interest, but other Tc analogs. That way selectivity could be defined as a property of a protein such that the protein shows high affinity for one particular compound and low affinity for other possible relatives to the compound of interest.

The same process can be applied to a protein such as MphR(A) even when affinity is not a primary concern and the main purpose is to improve specificity for the primary inducer. However, site-directed mutagenesis does require some amount of *a priori* knowledge of how the protein and ligand interact natively. Since the process requires only changing amino acid residues that will have a direct impact on the ligand binding and not on the overall folded structure of the protein, it is important to know which residues should not be changed. In particular for proteins in the TetR family, it is important to hold the DNA-binding region constant so that improved specificity does not come at the cost of decreased usefulness in feedback loops.

The easiest way to determine which residues are viable options for mutation will be to obtain a crystal structure of the protein, especially if co-crystallization with the target ligand can be accomplished. However, if this information is unavailable, a number of other proteomic methods can be used in concert to discern the desired information. Specifically, by comparing the sequence to that of other proteins with similar function for which the crystal structure has been solved, the region of the MphR(A) involved in DNA binding can be approximated by the homology of the helix-turn-helix motif. By holding these residues constant, large-scale site direction can be completed to find a set of residues that are primarily responsible for ligand binding by simply investigating whether or not the ligand still binds to the protein after a particular mutation.

When engineering the binding pocket of an effector-responsive protein such as MphR(A) it is important to maintain most of the structural features of the protein. Since binding of the effector molecule is directly linked to binding or release of DNA, some allosteric change is involved[16-18]. Thus, it is important for a changed interaction with the drug to improve specificity but also maintain transmission of the allosteric information through to the DNA-binding domain. That is, even though a mutation may cause the protein to bind more specifically to one particular compound, if that mutation and subsequent binding does not also cause a change in the DNA-binding characteristics it is essentially useless for further feedback loop engineering.

1.6: Summary and Study Goals

Beginning with the basic characterization of the protein, there are clearly a number of steps in the process of designing a positive feedback loop using MphR(A) as the transcriptional repressor and erythromycin as its inducer. First, the thermodynamic rela-

tionships between the protein and itself (oligomeric form), the DNA sequence it recognizes, and the specific inducer must all be quantified. Then, the sequence of the protein must be analyzed in some way so as to determine which residues, when mutated, will have a direct impact on the affinity and specificity for the inducer alone. Finally, a library of mutations must be analyzed to determine which mutant gives the highest sensitivity to one particular inducer alone and thus will be the mostly likely to show bistability as part of a positive feedback loop. When combined, all of these steps will provide the most useful form of MphR for feedback loops.

Chapter 2: Materials and Equipment

2.1: Materials

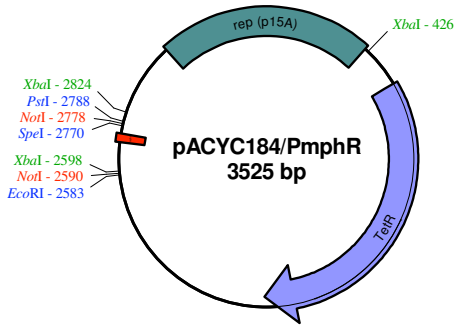
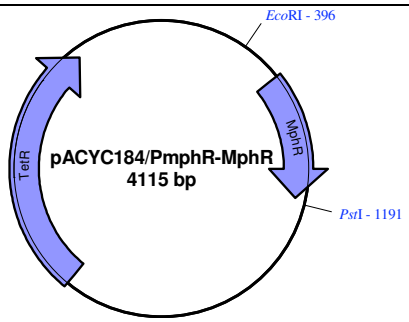
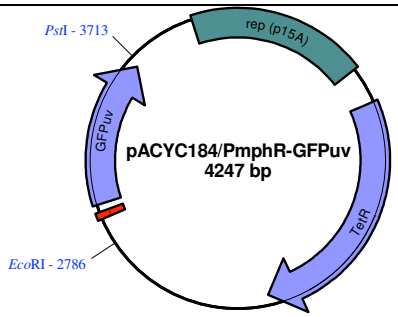
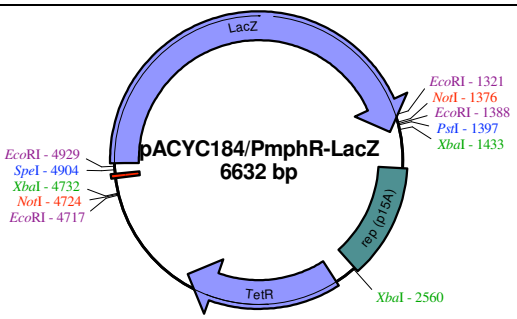
2.1.1: Chemicals and Plasmids

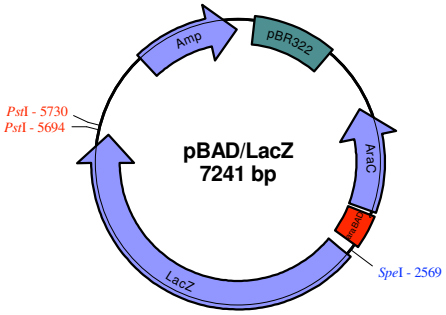
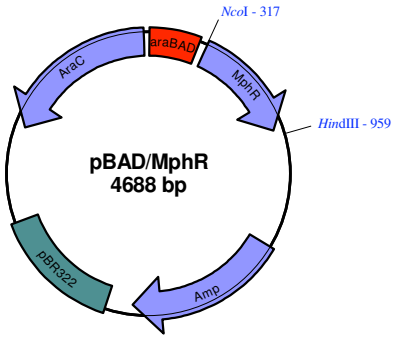
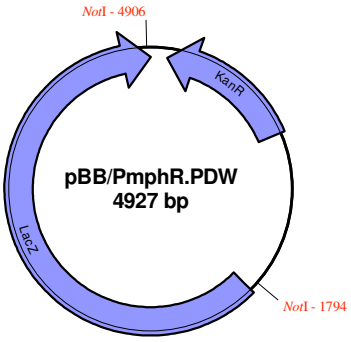
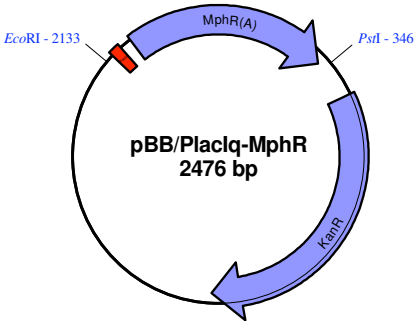
Table 2-1: List of Reagents

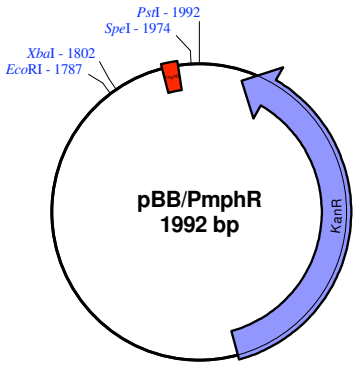
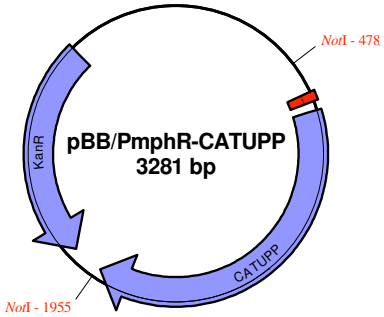
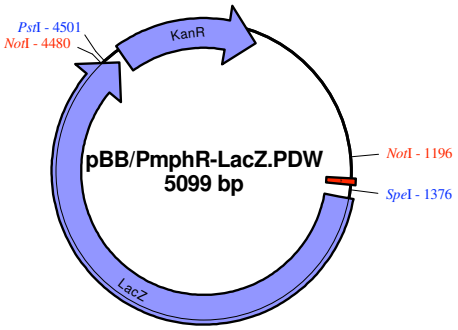
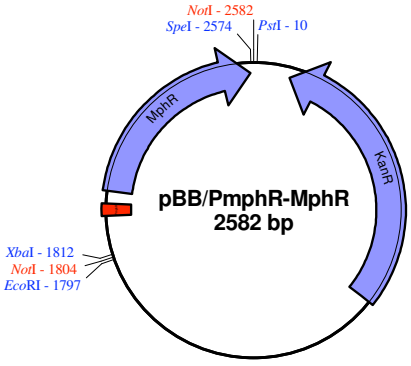
Chemical	Supplier
AG® MP-1 200-400 mesh chloride form analytical grade macroporous anion resin	BioRad
CloneJET™ PCR Cloning Kit	Fermentas Life Sciences
GeneJET™ Plasmid Miniprep Kit	Fermentas Life Sciences
Index Sparse Matrix Screens • Polyethylene glycol (PEG) 3350	Hampton Research
LB (10 g tryptone, 5 g yeast extract, 10 g NaCl / 1 L)	FisherBiotech
M9 Minimal Salts (6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl / 1 L)	US Biological
QIAquick Gel Extraction Kit	Qiagen
SOC (20 g tryptone, 5 g yeast extract / 1 L + 10 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$, 20 mM glucose	FisherBiotech
Superdex™ 200 prep grade gel filtration resin	GE Healthcare
Wizard™ I and II Sparse Matrix Screens	Emerald BioStructures
Wizard® *Plus* Midipreps DNA Purification System	Promega

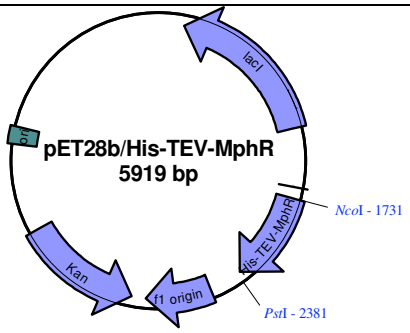
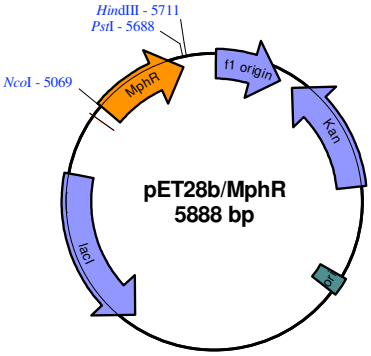
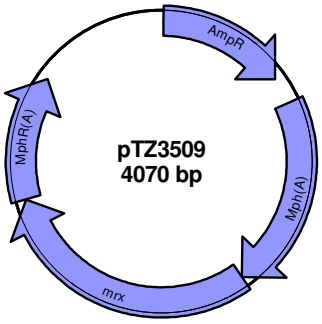
Table 2-2: List of Plasmids

Name	Assembled/Provided By	Vector/Restriction Map
p22-CATUPP	Prof. Ashton Cropp	
pACYC184/$P_{lac}I^q$-MphR	Adam Smolinsky	

Name	Assembled/Provided By	Vector/Restriction Map
pACYC184/P_{mphR}	Prof. Ashton Cropp	
pACYC184/P_{mphR}-CATUPP	Adam Smolinsky	
pACYC184/P_{mphR}-GFPuv	Prof. Ashton Cropp	
pACYC184/P_{mphR}-LacZ	Adam Smolinsky	
pACYC184/P_{mphR}-MphR	Adam Smolinsky	

Name	Assembled/Provided By	Vector/Restriction Map
pBAD/LacZ	Invitrogen	
pBAD/MphR	Prof. Ashton Cropp	
pBAD/*myc*HisA	Novagen	
pBB/LacZ	Adam Smolinsky	
pBB/$P_{lac}I^{q}$-MphR	Prof. Ashton Cropp	

Name	Assembled/Provided By	Vector/Restriction Map
pBB/P_{mphR}	Prof. Ashton Cropp	
pBB/P_{mphR}-CATUPP	Prof. Ashton Cropp	
pBB/P_{mphR}-GFPuv	Prof. Ashton Cropp	
pBB/P_{mphR}-LacZ	Adam Smolinsky	
pBB/P_{mphR}-MphR	Prof. Ashton Cropp	

Name	Assembled/Provided By	Vector/Restriction Map
pET28b/His-TEV-MphR	Adam Smolinsky	
pET28b/MphR	Adam Smolinsky	
pET28b/NadE	Prof. Barbara Gerratana	
pKQ/UPP	Prof. Ashton Cropp	
pRK793	Dr. David Waugh	
pTZ3509	Dr. Norihisa Noguchi	

2.1.2: Oligonuclotides

Oligonucleotides used for PCR amplification of genes and circuit components were purchased from Invitrogen. Oligonucleotides used for MphR-DNA binding ex-

periments were purchased from Invitrogen with end modifications as described (Table 2-3).

Table 2-3: List of Oligonucleotides

Name	Sequence	Use	Modifications
CL343	TTTCTGCAGC GGCCGCTACT AGTTTACGCA TGTGCCTGGA GG	Introduction of BioBrick™ end to *mphR* gene, Reverse	None[i]
CL350	AAAGAATTCG CGGCCGCTTC TAGATGATAG ATCCCGTCGT TTTACAACG	LacZ reporter with BioBrick™ ends, Forward	None[ii]
CL351	TTTCTGCAGC GGCCGCTACT AGTTTATTTT TTGACACCAG ACCAACTGG	LacZ reporter with BioBrick™ ends, Reverse	None[iii]
CL353	TTTCTGCAGC GGCCGCTACT AGTACTCCTG AGGGCTTGAC GGG	MphR operator with BioBrick™ ends, Reverse	None
CL476	CTGCCTCATC GCTAACTTTG C	MphR operator, Forward	5'-Fluorescein
CL640	CTGCCTCATC GCTAACTTTG C	MphR operator, Forward	5'-Biotin
CL652	TGCCGGATTG AATATAACCG ACGTGACTGT TACATTTAGG TGGC	Minimal MphR operator, Forward	None
CL653	GCCACCTAAA TGTAACAGTC ACGTCGGTTA TATTCAATCC GGCA	Minimal MphR operator, Reverse	None

[i] Red text denotes PstI restriction site.
[ii] Green text denotes XbaI restriction site.
[iii] Blue text denotes SpeI restriction site

Name	Sequence	Use	Modifications
CL699	AAACCATGGG GCATCATCAT CATCATCATT TGGAAAACCT ATACTTTCAA GGCCCCCGCC CCAAGCTCAA G	Introduction of TEV Protease recognition sequence to *mphR* gene, Forward	None[iv]

2.1.3: Competent Cells

Table 2-4: List of Competent Cells

Cell Line	Features	Competency
BL21(DE3)	Chromosome carries a gene for the expression of T7 RNA Polymerase that is IPTG-inducible	Chemical competent
Erm^r, ΔUPP	*UPP* gene is knocked out of the chromosome. Cells were made resistant to erythromycin by being grown in increasing concentrations of the antibiotic	Chemical competent
Mach1	High transformation efficiency	Chemical competent
NEB Turbo	Extremely high transformation efficiency	Chemical competent
Rosetta	Carries an additional plasmid that confers chloramphenicol resistance and expresses additional tRNA for rare *E. coli* codons.	
ΔUPP	*UPP* gene is knocked out of chromosome	Chemical competent
XL-1 Blue	Poor viability for use in blue/white screening	Electrocompetent

[iv] Dark Red text denotes NcoI restriction site.

2.2: Equipment

Table 2-5: List of Equipment

Unit	Function	Manufacturer
Avanti® J-E Centrifuge • JA-20 and JLA-9.1 Rotors	Large-scale (≥ 10 mL) centrifugation	Beckman Coulter
Axima-CFR	Mass Spectrometry	Shimadzu
BioLogic Duo Flow	FPLC Pump	BioRad
BioMax 5K Ultrafree	Small-scale (≤ 2 mL) protein concentration	Millipore Corporation
Centrifuge 5415 D	Small-scale (≤ 1.5 mL) centrifugation	Eppendorf
F-4500	Fluorescence Spectrophotometer	Hitachi
HiLoad™ 16/60 Column	FPLC column	GE Healthcare
iCON™ Concentrators, 7mL/9K	Large-scale (>2 mL) protein concentration	Thermo Scientific
IEC Clinical Centrifuge	Medium-scale (between 1.5 and 10 mL) centrifugation	International Equipment Co.
ImageJ 1.37v	DNA image analysis and quantification	http://rsb.info.nih.gov/ij/
MicroPulser™	Electroporation	BioRad
Phoenix Liquid Handling System	Crystallography solvent screening	Art Robbins Instruments
PowerPac Basic	Electrophoresis power supply	BioRad
Prism 4.03	Nonlinear regression modeling	GraphPad Software
PTC-200	Peltier Thermal Cycler	MJ Research
Select™ Series UV Transilluminator	Ethidium-stained DNA gel visualization	Spectroline
Sonifer 450 with microtip	Sonicator for cell lysis	Branson

Unit	Function	Manufacturer
STORM 850 Scanner	Fluorescent Imaging	Molecular Dynamics
Varian 50 Bio	UV/Vis Spectrophotometer	Cary
Vortex Genie 2	Vortex mixing	Scientific Instuments

Chapter 3: Characterization of MphR(A)

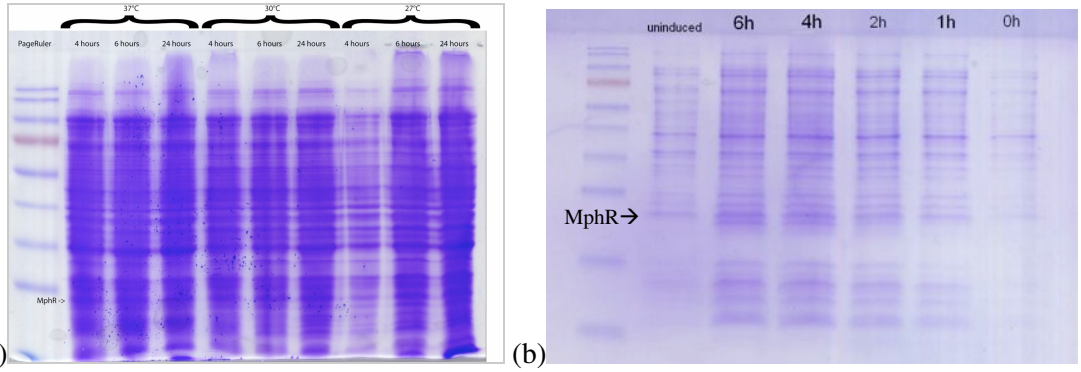
3.1: Expression

Initially, expression of MphR utilized the arabinose-inducible pBAD promoter. However, time course studies of MphR levels following induction showed less than ideal expression. It was determined that an even stronger promoter, particularly one for the T7 bacteriophage RNA polymerase, was necessary for efficient overexpression of the protein. The pET28b expression system contains a T7 promoter upstream of a multiple cloning site consisting of NcoI and HindIII sites, among others. The *mphR* gene was extracted from the pBAD/MphR plasmid with 5'-NcoI and 3'-HindIII overhangs. The sticky ends were then ligated into the expression plasmid.

The pET expression system also requires a source of T7 RNA polymerase for transcription. This was achieved by transforming the plasmid into BL21(DE3) chemically competent cells. The BL21 cell line contains the gene encoding T7 RNA polymerase transfected onto the chromosome under control of a lacUV5 promoter, which allows for induction upon the addition of IPTG. Once the cells with the correct plasmid were available, the conditions for overexpression were optimized. It has been shown that optimal induction takes place when the inducer is added while the growth is still in the log phase. The log phase was reached by growing 50-100 mL overnight and using it to inoculate a larger (1.5-2 L) culture which is allowed to grow until OD_{600} is between 0.5 and 1. When using the pBAD system, the optimal induction time is approximately 4 hours so induction times of 4-6 hours were used for initial trials.

It was found that expression in the initial stages after induction is relatively slow; up to 6 hours after addition of IPTG, the amount of MphR is not significantly greater than other soluble proteins (Figure 3-1). However, cultures induced overnight generally produced enough protein to be purified using the incorporated His-tag. The presence and molecular weight of MphR was confirmed by mass spectrometry (Figure 3-2), where the monomeric mass was shown to be 22.5 kDa.

Figure 3-1: Time-Course Studies of *mphR* Expression Using pBAD and pET28b Vectors



(a) (b)

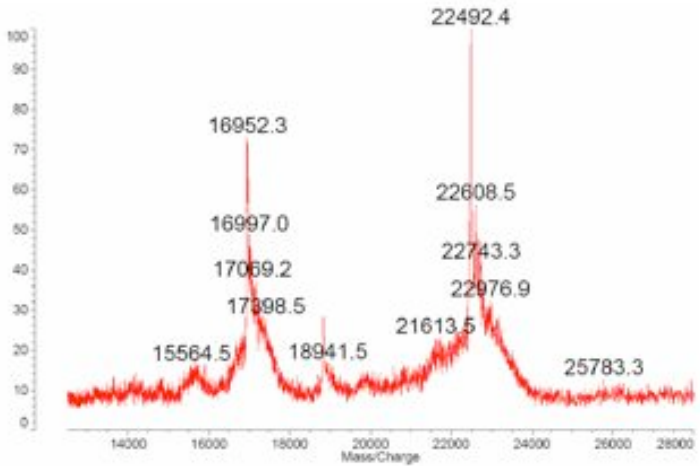
Panel (a): SDS-PAGE gel showing soluble proteins of pBAD/MphR cells induced at 37, 30 and 27 °C for 4, 6, and 24 hours each, from left to right.

Panel (b): SDS-PAGE gel showing soluble proteins of pET28b/MphR cells induced at 37 °C for 0 – 6 hours.

3.2: Purification

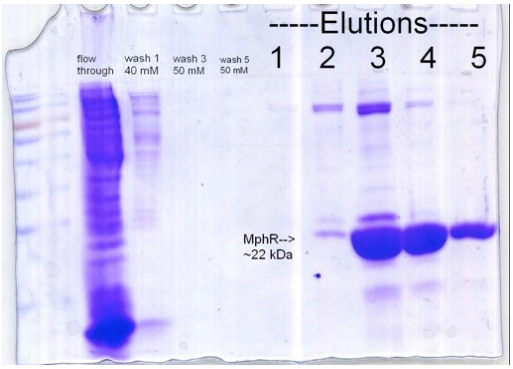
The *mphR* gene originally used in the pBAD and pET systems contains a C-terminal His-tag, primarily for purification purposes. The total volume of soluble proteins from an expression cultures was mixed with Ni^{2+} resin and increasing concentrations of imidazole were used to wash away non-tagged proteins and eventually elute the MphR. SDS-PAGE confirmed the overexpression and purification of MphR using this method (Figure 3-3).

Figure 3-2: MphR Mass Spectrum



MphR peak is at 22492.4 m/z. Apomyoglobin reference peak is at 16952.3 m/z

Figure 3-3: MphR His-tag Purification

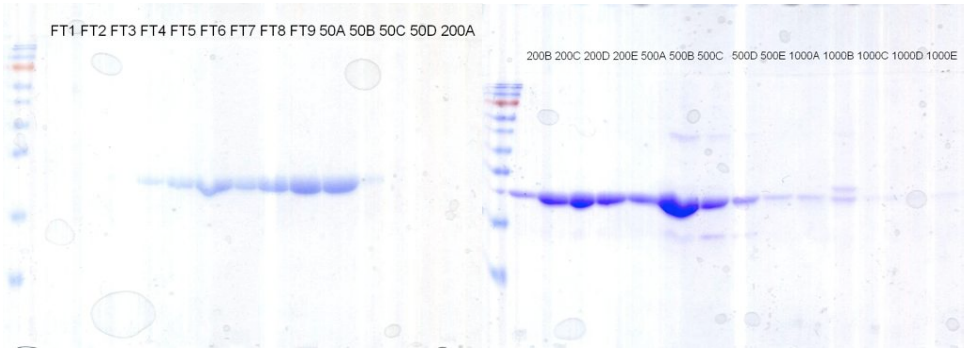


SDS-PAGE gel showing very large amounts of mostly pure MphR.

While the protein is pure enough for biochemical studies, it has been shown that one Ni^{2+} purification is insufficient for crystallography studies; at least one additional step must be incorporated prior to crystallization. The additional purification techniques might include ion exchange and size exclusion chromatography, both of which have been experimented with for this process. Early attempts at ion exchange chromatography yielded valuable information and promising results (Figure 3-4), but the optimization process was stopped short in favor of better alternatives.

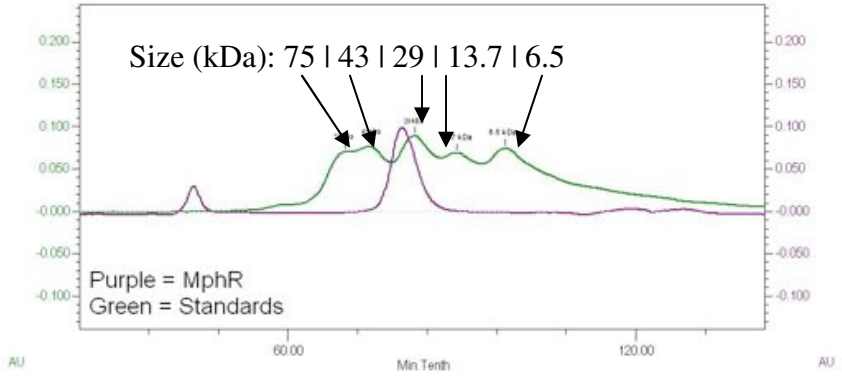
Most crystallography studies simply use FPLC size exclusion chromatography as a clean-up technique. It was determined that purification efforts would be focused on optimizing this method. A chromatogram overlaid with column standards (Figure 3-5) shows an aggregated protein peak that eluted off the column very quickly, and a purified protein peak between 23 and 49 kDa. This was reasonable based on the assumption that MphR exists in a state of monomer-dimer equilibrium and would run at the oligomeric weighted average between 22.5 and 45 kDa.

Figure 3-4: MphR Ion Exchange Purification



SDS-PAGE gels showing each 1-mL fraction collected. In both panels, FT1-9 are the fractions collected from the initial protein solution washed over the column. Numbered fractions correspond to washes with the appropriate salt concentration (50, 200, 500, and 1000 mM NaCl, respectively).

Figure 3-5: MphR Size Exclusion Purification



FPLC chromatogram overlaid with column standardization.

3.3: Binding Interactions

A variety of methods were tested to quantify the interactions between MphR and the operator sequence and between MphR and the inducer erythromycin. These methods included agarose and polyacrylamide gel electrophoresis and fluorescence spectroscopy described as follows.

3.3.1: MphR-DNA Binding Gel-Shift Experiments

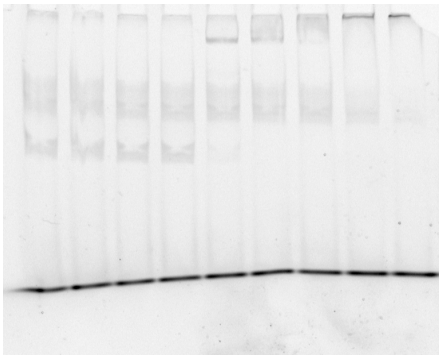
Two different gel-shift methods were used to determine the equilibrium dissociation constant, K_D, for the binding of MphR to the operator DNA sequence, as determined by Noguchi, *et al.* For both horizontal agarose and vertical polyacrylamide gel electrophoresis, different concentrations of pure protein were combined with a short DNA fragment and run on the gel. The DNA fragment used was approximately 100 base pairs—long enough to be seen on the gel, but short enough to show a significant shift when bound to the protein—and end modified with a fluorophore or biotin, depending on the visualization method to be used. By determining the amount of DNA in each band (bound and unbound), it was possible to determine the fraction of DNA bound for each concentration of MphR.

The data was then fit to a curve which provided a value for the dissociation constant and confirmation of the coupled equilibria between MphR oligomerization and DNA binding. The Hill equation was used based on the assumption that MphR exists as a monomer in solution, but the active form is a dimer. Thus, some amount of coupling must exist.

The first set of experiments used a fluorophore-modified DNA strand, which was generated by PCR using primers CL476 and CL 353 with pTZ3509 as the template. The

reaction mixtures were run on both 8% polyacrylamide and 3% agarose gels. The polyacrylamide gels were imaged using a Storm scanner, but were difficult to interpret (Figure 3-6). However, the same reactions visualized well on agarose gels, which provided initial values for the constants; the apparent K_D from this data was 0.2 μM. This data is shown in more detail in Appendix A.

Figure 3-6: MphR-DNA Binding Gel-Shift Experiment; Fluorescent Label Imaging



8% Polyacrylamide gel; imaging excitation wavelength was 450 nm and detected with high sensitivity.

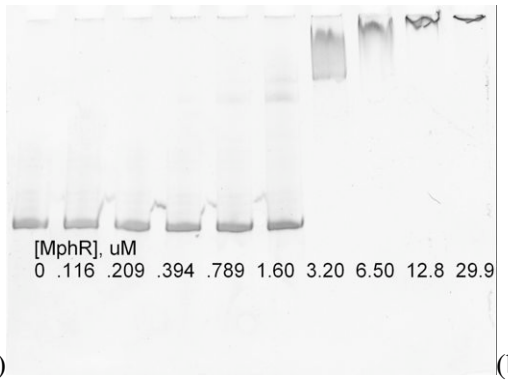
There were some drawbacks to using 3% agarose gels: (1) while easy to work with, the high percentage of agarose affected the light refraction during imaging, and (2) visualization required ethidium intercalation, which in turn required very concentrated DNA and protein solutions. Unfortunately, the short DNA fragments used required a high-percentage gel for efficient separation. Therefore, efforts were made to avoid using agarose gels for further experiments.

The second set of binding experiments used biotin-modified DNA, which was generated by PCR using primers CL640 and CL353 with pTZ3509 as the template. Because of the high affinity of streptavidin to biotin, visualization by streptavidin probe would require a much smaller amount of protein. The reaction mixtures were run on 8%

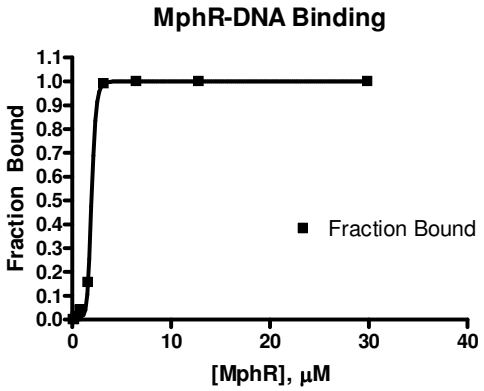
polyacrylamide gels and cross-linked to a membrane in a procedure similar to Southern blotting. However, the procedure proved to be difficult to work with for double-stranded DNA molecules and it was unclear whether any DNA was transferred to the membrane.

The same polyacrylamide preparation was stained with ethidium bromide solution and conveniently produced clear bands (Figure 3-7). The intensity of each band (bound and unbound) was found and converted to fraction bound for each protein concentration; lanes where the complex did not run at all were assumed to be in a bound state although some higher-order protein interactions are obviously at work. Based on this data, K_D was found to be 1.9 μM with the appearance of extremely cooperative binding. It was incorrect to assume that this data is accurate, unfortunately. What is clear, however, is that significantly more points that show intermediate fractions bound are necessary to deduce true values.

Figure 3-7: MphR-DNA Binding Gel-Shift Experiment; Ethidium Bromide Staining



(a)



(b)

Panel (a): 8% polyacrylamide gel visualized with UV transilluminator.

Panel (b): The data was plotted as fraction bound vs. [MphR], in μM. The DNA concentration was 24 ng/μL. The curve shown was from fitting the data to the equation

$$y = low + \frac{high - low}{1 + \left(K_D / x \right)^n}$$

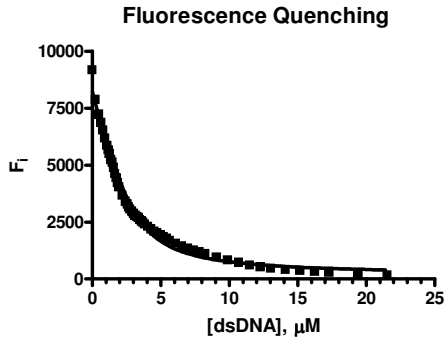
At the writing of this thesis, it was known that similar experiments would have given an apparent K_D for the binding of the MphR-DNA binding complex to erythromycin and the subsequent release of DNA from the complex. However, the experiments had not yet been completed.

3.3.2: MphR-DNA Binding Fluorescence Quenching Experiments

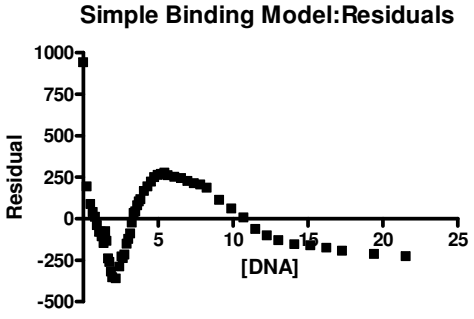
The intrinsic fluorescence of proteins is primarily due to the tryptophan residues and minimally due to tyrosine residues; there are three of each in the MphR primary sequence. Since these residues are aromatic, they are generally found in the interior of a folded protein, and often in the ligand-binding pocket, especially for interactions with DNA. Upon binding, the environment of the residues specifically involved—and therefore the fluorescence—changes and can be indicative of the amount of protein bound. For fluorescence quenching experiments, it was important to decrease the effect of DNA absorbance on the spectroscopy, so a minimal operator was prepared by mixing complementary oligonucleotides.

The ds-DNA was titrated into buffer containing 3 μM MphR and the fluorescence emission spectrum was measured at each concentration (Figure 3-8). The excitation wavelength was 285 nm and the emission maximum was 330 nm. The data was fit to a simple binding model, which suggested an apparent K_D on the order of 0.5 μM. It was also clear from the residuals of the fit that the model was altogether too simple and significantly more data would be necessary to completely understand the biophysical interactions involved in the complex binding system. At the writing of this thesis, the additional biophysical data had not been collected.

Figure 3-8: MphR-DNA Binding Intrinsic Fluorescence Experiment



(a)



(b)

Panel (a): Fluorescence Intensity vs. [dsDNA]. The [MphR] monomer concentration was 3 μM. The line represents the fit of a simple one-site model.

Panel (b): The residuals of the simple one-site model fit.

3.4: Crystallography

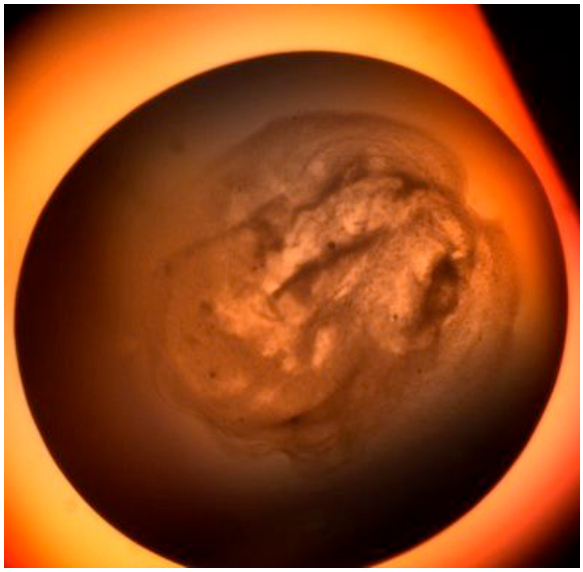
3.4.1: Initial Solvent Screening

Protein that was purified over Ni^{2+} resin and AG MP-1 anion exchange resin was concentrated to ~10 mg/mL in buffer containing 50 mM Tris and 300 mM salt. The concentration resulted in approximately 200 μL solution, which was sufficient to prepare crystallography screens with two complete sparse matrix systems: Wizard I and II and Index. The results from these screens (Figure 3-9) were inconclusive directly, but provided extremely promising results for future screens.

The precipitation patterns observed in about 75% of the solvents was indicative of two likely explanations: (1) reducing agent was not present in the protein solution and (2) higher order aggregates were present in the solution. Additionally, the high percentage of precipitated solutions suggested that the protein was not so soluble that it would remain in solution regardless of the solvent used. Further screens will be conducted following a

round of size exclusion and in the presence of a reducing agent—which will eliminate the aggregated protein and most of the precipitation. However, it was also found that after two months, one of the mixtures in the Index screen had grown very small crystals in solution containing 0.1 M sodium acetate trihydrate, and 25% PEG 3350.

Figure 3-9: Microscope Image of Precipitated Protein



3.4.2: Incorporation of the Tobacco Etch Virus Protease Recognition Sequence

In order to minimize the effect of the flexible His-tag, the *mphR* gene was redesigned such that the tag constituted the N-terminal residues. These residues were separated from the start of the protein primary structure by the recognition sequence of the tobacco etch virus (TEV) protease. The TEV protease was chosen because of its extremely high specificity for the given sequence and due to the fact that it leaves a single glycine residue scar, which should not affect the folding of the protein. The protease was also readily available because it could be obtained through overexpression procedures similar to those used for MphR using the pRK793 plasmid expressed in Rosetta cells.

The new *mphR* gene (Figure 3-10) was generated by PCR and cloned into the pET28b multiple cloning site.

Figure 3-10: His-TEV-MphR Primary Structure

MGHHHHHHLENLYFQGPRPKLKSDDEVLEAATVVLKRCGPIEFTLSGVAK
EVGLSRAALIQRFTNRDTLLVRMMERGVEQVRHYLNAIPIGAGPQGLWEF
LQVLVRSMNTRNDFSVNYLISWYELQVPELRTLAIQRNRAVVEGIRKRLP
PGAPAAAELLLHSVIAGATMQWAVDPDGELADHVLAQIAAILCLMFPEHD
DFQLLQAHA

The residues shaded light blue are those cut out by the TEV Protease. The underlined residues are the protease recognition sequence.

Chapter 4: Circuit Engineering

4.1: General Design

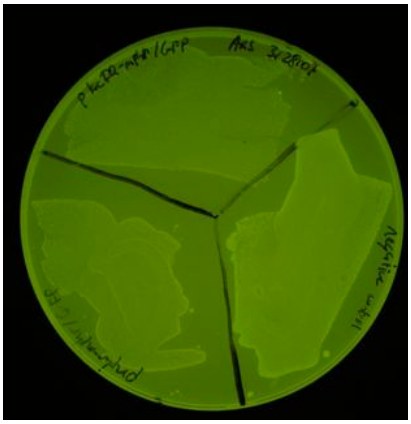
Some basic feedback loops that take advantage of the high specificity of the MphR-binding DNA sequence have been prepared. In the work described here, all of these circuits involved the expression of MphR on one plasmid and the expression of a reporter gene on another. The design of these plasmids made use of the classic 1.0 BioBrick™ format that incorporates EcoRI, NotI, and XbaI restriction sites to the 5'-end of each component and SpeI, NotI, and PstI restriction sites to the 3'-end. A vector based on pKQ—called pBB in this thesis—was used for high-copy pieces and a vector based on pACYC184 was used for low-copy pieces.

For the feedback loops, a variety of reporter proteins were available. These proteins include GFPuv, CATUPP, and LacZ, among others. Expression of the reporter proteins were controlled by the MphR promoter, P_{mphR}, as determined by Noguchi, *et al.*[3] Expression of the MphR protein was controlled by a variety of promoters—$P_{lac}I^{q}$ and P_{mphR}—in the BioBricks construct. Alternatively, in some cases, the arabinose-inducible pBAD/MphR plasmid was used for overexpression of the gene.

Some of the components had been previously cloned into one of the two vectors. In particular, the plasmids pBB/P_{mphR}, pBB/$P_{lac}I^{q}$-MphR, pBB/P_{mphR}-MphR, pBB/P_{mphR}-CATUPP, and pACYC184/P_{mphR}-GFPuv were already assembled. The first circuits assembled combined pACYC184/P_{mphR}-GFPuv with each of the two pBB/P-MphR plasmids and the pKQ/UPP plasmid as a negative control. The expression of GFPuv ap-

peared to be affected by the presence of MphR (Figure 4-1); however, quantification of the fluorescence would require the use of flow cytometry and has not yet been pursued.

Figure 4-1: LB-Agar Plate Illuminated with UV Light Showing *GFPuv* Expression Level



The copy number of each plasmid in the circuit could potentially have a very large impact on the output of the reporter alone and the whole circuit. The high-copy GFPuv reporter plasmid (pBB/P_{mphR}-GFPuv) had been previously assembled, but it was necessary to assemble the low-copy MphR expression plasmids pACYC184/$P_{lac}I^{q}$-MphR and pACYC184/P_{mphR}-MphR. Once built, the high-copy reporter was combined with each low-copy MphR plasmid and the p22/CATUPP plasmid as a negative control. However, the same problem with quantification requiring flow cytometry was still present, so these circuits have not yet been studied in great detail.

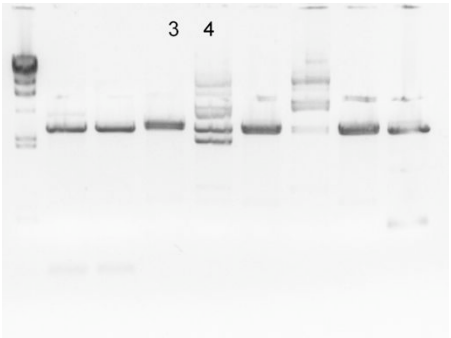
Expression of the *CATUPP* gene confers resistance to chloramphenicol and sensitivity to 5-fluorouracil, making the latter compound toxic. The reporter could be quantified by measuring the growth rate of cell cultures in the presence of each compound. Cells that expressed the gene would grow more quickly in the presence of chloramphenicol than cells that do not express the gene; conversely, expression of the gene would also prevent the cells from growing in the presence of 5-fluorouracil, while cells not express-

ing the gene would grow. The *upp* gene is generally found on the chromosome of standard *E. coli*, so the use of CATUPP reporter required UPP-knockout (*ΔUPP*) cells. Expression of the *lacZ* gene produces an enzyme, β-galactosidase, for which there is a well-established assay.

Circuits were assembled with both pBB/P_{mphR}-CATUPP and pACYC184/P_{mphR}-CATUPP. Each reporter was paired with both MphR expression plasmids on the opposite vector and the appropriate negative control. A series of different methods were tried in building the LacZ circuits. First, the alpha fragment was generated by PCR and cloned downstream of the MphR promoter in the pBB plasmid. However, the output of this particular reporter was lower than expected, which may have been related to the relatively weak promoter rather than poor enzyme activity. Regardless, the full-length gene was prepared by PCR and cloned into the pJET vector using the CloneJET™ kit. The resulting plasmid was used for months in the attempts to assemble the reporter plasmids, but on later evaluation turned out to be incorrect (Figure 4-2). Then, the *lacZ* gene was cut out of the pBAD/LacZ plasmid and cloned into the low-copy reporter plasmid downstream of the MphR promoter sequence.

It was important to use the low-copy reporter plasmids because expression of MphR on this plasmid was not significant enough to trigger a significant change in the expression of the reporter gene. High levels of MphR could be achieved by using one of the overexpression plasmids, but both of those systems could only be paired with pACYC184 reporters. These systems showed a distinct change in expression levels upon addition of MphR; the downside of using them was that initial reporter levels were also low, so the difference between gene expression and gene repression was not significant.

Figure 4-2: Agarose Gel Showing pJET1.2/LacZ Digested with BglII



Miniprepped plasmid DNA from 8 colonies on the plate of transformed ligation reactions was digested with BglII. The two correct fragments were 2.9 and 3.1 kb. Originally, Colony 4 was selected, but further analysis suggested that Colony 3 was, in fact, the correct choice.

Based on the difficulty measuring *CATUPP* expression in circuits where the *mphR* gene was in a pBB vector, the LacZ circuits were assembled using pBAD/MphR for *mphR* expression. As a control, the pBAD/*myc*HisA plasmid—from which pBAD/MphR was originally assembled—was included. That way, all circuits would be resistant to ampicillin and tetracycline.

4.2: Reporter Quantification

4.2.1: GFPuv Expression Circuits

As noted above, the quantification of *GFPuv* expression generally requires flow cytometry. As the equipment necessary for that procedure was not readily available, the *GFPuv* circuits were assembled and stored for potential future quantification while more easily quantifiable circuits were developed and explored.

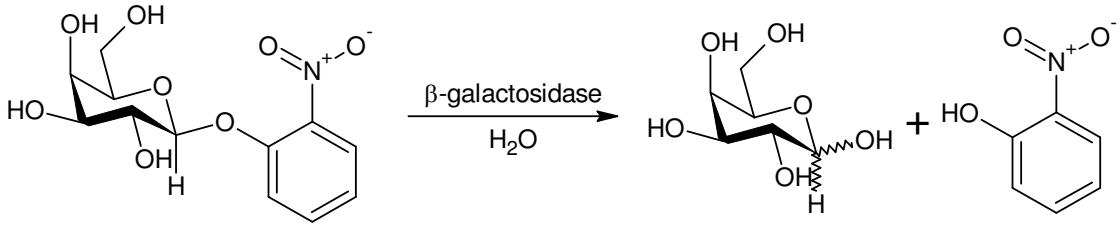
4.2.2: CATUPP Expression Circuits

Once the pBB/P_{mphR}-CATUPP reporter plasmid and the pBB/P_{mphR} control plasmid had been transformed into *ΔUPP* cells, they were grown on LB-Agar plates containing increasing amounts of chloramphenicol to approximately determine the level of resistance. It was shown that the reporter plasmid was resistant to chloramphenicol up to 700 μg/mL, depending on cellular concentration at the time of plating, while the control plasmid did not grow in any amount of antibiotic. The cells were also grown on M9-Agar (see Table 6-1) plates containing increasing amounts of 5-fluoruracil to approximately determine the level of toxicity. It was shown that 5-fluorouracil was not toxic to cells with the control plasmid—that did not express *CATUPP*—up to 5 μg/mL, depending on cellular concentration at the time of plating, while the nucleotide was immediately toxic to cells expressing the gene. Appendix A, below, contains pictures of these plates.

4.2.3: LacZ Expression Circuits

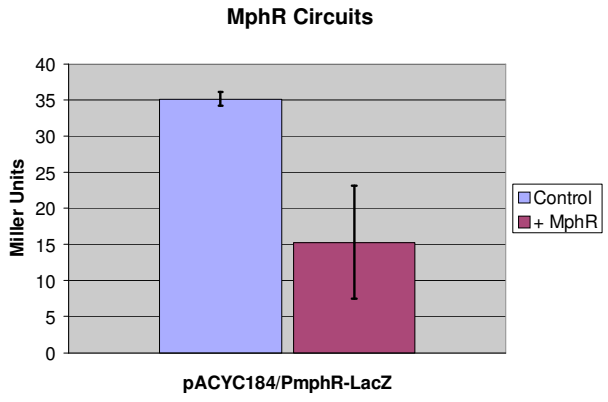
β-Galactosidase activity—and therefore LacZ expression—was measured using the Miller assay. The β-galactosidase enzyme hydrolyzes β-galactosides into a monosaccharide and an alcohol. When *o*-nitrophenyl-β-galactoside is used (Figure 4-3), the alcohol released is *o*-nitrophenol, which is yellow in color and absorbs light readily at 420 nm. By measuring the absorbance at that wavelength, the total activity of the culture was determined. The assay accounted for cell density and turnover rate to assign a value on the Miller unit scale. Control cultures that express low levels of LacZ (pUC18) gave values approximately 300 Miller units. Cultures that overexpress LacZ (pBAD/LacZ) gave values approximately 3000 Miller units.

Figure 4-3: β-Galactosidase-Catalyzed Hydrolysis Reaction



The pACYC184/P_{mphR}-LacZ culture measured 10.1 Miller units; the pBB/LacZ culture measured 0.94 Miller units. When incorporated into circuits, the value for pACYC184/P_{mphR}-LacZ increased to 15.3 ± 7.8 Miller units when paired with pBAD/MphR and 35.2 ± 1.0 Miller units when paired with pBAD/control (Figure 4-4). These values represent a significant difference between expression with and without MphR present. Unfortunately, difficulty was encountered when attempting to measure the effect of erythromycin. Initially, the *ΔUPP* cells used did not grow in the presence of the antibiotic. However, there was some non-quantitative evidence that introduction of erythromycin did increase LacZ expression (Appendix B). The circuits were transformed into *Erm^r, ΔUPP* cells but appropriate measurements of activity had not been measured as of the writing of this thesis.

Figure 4-4: LacZ Expression Levels in pACYC184/P_{mphR}-LacZ + pBAD Circuits



Chapter 5: Summary, Future Work, and Conclusions

5.1: Summary

5.1.1: Protein Biochemistry

Efficient overexpression and purification of MphR was demonstrated. Running the protein over a Ni^{2+} resin column produced protein that was over 95% pure and only one simple clean-up step (either size exclusion or ion exchange chromatography) was necessary to prepare the protein for crystallization studies. Initial solvent screening produced extremely promising results, including small crystals in one of the Index solvents. The incorporation of a TEV protease recognition site to remove the flexible His-tag was also completed successfully. At present, Jianting Zheng—another member of the research group—has been able to use this construct to grow and purify vast amounts of protein. The protein can grow into crystals that diffract x-rays at 2.8 Å resolution.

A variety of methods to determine the apparent K_D for binding of MphR to DNA were investigated, including gel-shift and intrinsic fluorescence quenching experiments. For gel-shift experiments, a variety of visualization methods were also tested. The results were generally difficult to interpret, but appeared to give reasonably consistent values for the K_D around 1 μM.

5.1.2: Circuit Assembly and Quantification

Circuits were assembled using a wide variety of the myriad of possible combinations. The circuits utilized the two-plasmid approach with *mphR* on one plasmid and the P_{mphR}-*[reporter]* construct on the other. *GFPuv* was used as a reporter on both plasmids, but quantification was not undertaken. *CATUPP* was initially expressed on the high-

copy plasmid, but circuits with *mphR* on the low-copy plasmid did not show a demonstrable difference in reporter expression. *LacZ* was expressed on the low-copy plasmid and saw expression decrease by approximately 50% in circuits containing MphR, as compared to control circuits.

5.2: Future Work

5.3.1: Protein Biochemistry

The incorporation of the TEV protease recognition site into the *mphR* gene should allow for improved protein purification without the use of size exclusion chromatography. Since the protease itself carries a His-tag, that enzyme and all other proteins that bind non-specifically should stick to the Ni^{2+} resin while cleaved MphR flows through. Once large amounts of pure protein are obtained, further crystallization screens can be completed. A "hit" will provide a solvent for larger-scale crystal growth and subsequent x-ray scattering experiments. Obtaining crystal structures for the protein alone and bound to erythromycin is crucial for site-directed mutagenesis to improve inducer specificity and should be completed soon.

In addition to a crystal structure, more detailed biophysical information is necessary to improve inducer specificity. It will be important to understand the basal affinities for various inducers of the wild-type protein before comparing the affinities of mutants. Thus, it may be necessary to ascertain binding constants for more complex interactions than what was explored in the work presented here. In particular, the state of the monomer-dimer equilibrium should be understood. That information will assist in the generation of an appropriate model for protein-DNA binding interactions, as well as the potential cooperative nature of inducer binding.

5.3.2: Circuit Assembly and Quantification

The effect of erythromycin on reporter expression needs to be quantified. It is unclear whether *Erm^r, ΔUPP* cells will allow for any significant uptake of erythromycin in addition to resistance. Expression of the circuits in erythromycin-sensitive cells could potentially provide the desired information. Additionally, it may be of interest to quantify more than one reporter, even though *lacZ* expression is clearly the simplest and most direct.

5.3.3: Circuit Optimization Using Protein Engineering

Since the ultimate goal of the entire project is to design a biofeedback system that is extremely sensitive to erythromycin only, a certain amount of protein engineering must be used. As stated above, obtaining a crystal structure of the inducer binding pocket and understanding the affinity to the inducer are two key intermediates in this process. It is also important to understand the nature of the wild-type protein in the feedback loops. Once structural and biochemical information is obtained, however, it will be a relatively simple exercise to engineer optimized MphR mutants.

Site-directed mutagenesis can be focused on the region surrounding the inducer binding pocket. Rather than having to express the mutants and analyze their biochemical interactions, the mutated plasmid (since mutations take place at the genomic level) can simply be incorporated into the established feedback loops for screening. Those mutants that show an increased sensitivity to erythromycin—that is, a smaller amount of erythromycin is required for full reporter expression—will be easily identifiable. Similar comparisons can be made using other macrolides to quantify the specificity of the mutant proteins.

5.3: Conclusions

The work for this thesis produced sufficient demonstrable results: significant progress was made towards developing quantifiable circuits. It was found that the various biophysical constants associated with MphR are more complex than originally anticipated; understanding the oligomeric state of the protein is crucial to modeling all other interactions. A great amount of headway was also made with regards to finding a crystal structure of the important allosteric states. A wide variety of biofeedback loops were assembled with different reporters and quantification of the effects of MphR on reporter expression was begun. The circuits expressing the *lacZ* reporter gene are very promising for use as exactly measurable circuits.

Chapter 6: Methods

6.1: Protein Expression

For all inducible protein expressions, an overnight culture of 50 mL LB broth containing the appropriate antibiotics (100 μg/mL ampicillin for pBAD and pRK vectors, 50 μg/mL kanamycin for pET vectors, and 35 μg/mL chloramphenicol for vectors expressed in Rosetta cells) was used to inoculate a 2-L LB culture also containing the appropriate antibiotics. The large culture was grown until the log phase was reached and the appropriate inducer (0.2% arabinose for pBAD vectors and 1 mM IPTG for pET and pRK vectors) was added when the OD_{600} reached 0.8.

When using the pBAD expression system, the culture was incubated with shaking at 37 °C for 6 hours. The pET28b expression system required incubation at 37 °C overnight, while the pRK (Rosetta) vector was incubated at 30 °C for 6 hours in order to maximize the amount of soluble protein. Once the incubation was complete, the cells were pelleted by centrifugation at 5,000 rpm for 20 minutes. At that stage, the pellet could either be stored at -80 °C or immediately lysed for protein purification.

Table 6-1: Buffer Compositions

Buffer	Composition
Binding Buffer	50 mM Tris (pH = 7.9) 300 mM NaCl 10 mM imidazole 10% glycerol

Buffer	Composition
Wash 40 Buffer	50 mM Tris (pH = 7.9) 300 mM NaCl 40 mM imidazole 10% glycerol
Wash 50 Buffer	50 mM Tris (pH = 7.9) 300 mM NaCl 50 mM imidazole 10% glycerol
Wash 60 Buffer	50 mM Tris (pH = 7.9) 300 mM NaCl 60 mM imidazole 10 % glycerol
Elution Buffer	50 mM Tris (pH = 7.9) 300 mM NaCl 300 mM imidazole 10% glycerol
Permeabilization Solution	100 mM Na_2HPO_4 20 mM KCl 2 mM $MgSO_4$ 0.8 μg/mL hexadecyltrimethylammonium bromide (CTAB) 0.4 μg/mL sodium deoxycholate 5.4 μL/mL β-mercaptoethanol (BME)
Substrate Solution	60 mM Na_2HPO_4 40 mM NaH_2PO_4 1 mg/mL o-nitrophenyl-β-D-Galactoside (ONPG) 2.7 μL/mL BME
Stop Solution	1 M Na_2CO_3

Buffer	Composition
10X T4 DNA Ligase Reaction Buffer	500 mM Tris (pH = 7.5) 100 mM $MgCl_2$ 10 mM adenosine triphosphate (ATP) 100 mM dithiothreitol (DTT)
M9 Minimal Media	1X M9 Minimal salts 0.4% glucose 0.2% casamino acids
AP 7.5	100 mM Tris (pH = 7.5) 100 mM NaCl 2 mM $MgCl_2$
AP 9.5	100 mM Tris (pH = 9.5) 100 mM NaCl 50 mM $MgCl_2$

6.2: Protein Purification

6.2.1: Ni^{2+} Resin His-tag Purification

The post-induction cell pellet was resuspended in 15 mL Binding Buffer (Table 6.1) per liter of original culture. The suspension was frozen completely at -80 °C and thawed completely in warm water in two cycles. This solution was then sonicated at 50% output power for 2 minutes in 20-second on/off intervals. The cell debris was pelleted by centrifugation at 15,000 rpm for 45 minutes. The lysate was then mixed with Ni^{2+} resin (1 mL pelleted resin per 10 mL cell lysate) pre-equilibrated in Binding Buffer and incubated at 4 °C for 2 hours with occasional mixing to bind the protein.

Resin bound to the protein was resuspended in the buffer and poured into a glass column (1 cm diameter × 20 cm length); the column was allowed to pack using gravity

while the buffer flowed through the column. The binding vessel was rinsed with ~15 mL Wash 40 buffer as the initial wash step. This was followed by five 15-mL washes—2 × Wash 40, 2 × Wash 50, 1 × Wash 60. The protein was eluted using 5 mL Elution Buffer for each liter of original culture.

6.2.2: AG MP-1 Anion Exchange Chromatography

The total binding capacity of the resin was 4.2 meq/g dry resin or 1.2 meq/mL resin bed. Based on the theoretical pI of MphR and the pH at which the column would be run, it was assumed that each mole of protein would carry four equivalents of charge. Thus, 1 gram of dry resin was used for each mmol of protein. All buffers used for the purification contained 250 mM Tris (pH = 7.9). The resin was equilibrated in buffer containing 50 mM NaCl and the protein was dialyzed into the same buffer. The protein solution was poured over the resin and 1-mL fractions (FT) were collected.

The various proteins were eluted using washes of 5 mL / mL resin. The optimal purification of MphR was found to take place when the three washes contained 100 mM NaCl, 300 mM NaCl, and 1000 mM NaCl, respectively. With those conditions, the MphR primarily eluted in the 300 mM salt fractions, although some protein was lost in 100 mM fractions and some remained through 1000 mM fractions. The lost protein was not a problem, as long as enough protein was loaded onto the column.

6.2.3: Superdex™ 200 prep grade Size Exclusion Chromatography

Protein previously purified using Ni^{2+} was concentrated to a maximum of 2 mL. The previously packed column was then equilibrated into running buffer that contained 25 mM Tris (pH = 7.9), 50 mM NaCl, and 1 mM EDTA. The column was run on a Bio-

Rad instrument at 1 mL/min and 2-mL fractions were collected beginning after 37 minutes until 143 minutes.

6.3: Plasmid Generation

6.3.1: MphR Expression Plasmids

The pBAD/MphR plasmid was digested with NcoI and HindIII and the 642-bp fragment was extracted from the 1% agarose gel. The pET28b/NadE plasmid was digested with NcoI and HindIII and the 5246-bp fragment was extracted from the 1% agarose gel. The two fragments were ligated together and transformed into Mach1 chemical competent cells. The resulting plasmid was named pET28b/MphR.

The *HisTEVmphR* gene was PCR amplified using oligonucleotide primers CL343 and CL699 and the template pET28b/MphR plasmid. The product was purified and digested with NcoI and PstI and the 650-bp fragment was extracted from the 1% agarose gel. The pET28b/MphR plasmid was digested with NcoI and PstI and the 5269-bp fragment was extracted from the 1% agarose gel. The two fragments were ligated together and transformed into XL-1 Blue electrocompetent cells. The resulting plasmid was named pET28b/His-TEV-MphR.

The pBB/$P_{lac}I^{q}$-MphR and pBB/P_{mphR}-MphR plasmids were digested with EcoRI and PstI and the 689-bp and 792-bp fragments respectively were extracted from the 1% agarose gel. The pACYC184/P_{mphR}-GFPuv plasmid was digested with EcoRI and PstI and the 3320-bp fragment was extracted from the 1% agarose gel. Each insert was ligated with the vector and transformed into Mach1 chemical competent cells. The new plasmids were named pACYC184/$P_{lac}I^{q}$-MphR and pACYC184/P_{mphR}-MphR, respectively.

The pBB/P_{mphR} plasmid was digested with EcoRI and PstI and the 205-bp fragment was extracted from the 1% agarose gel. This fragment was ligated 3320-bp pACYC184 vector fragment and transformed into NEBTurbo chemical competent cells. The new plasmid was named pACYC184/P_{mphR}.

6.3.2: Reporter Plasmids

The pBB/P_{mphR}-CATUPP plasmid was digested with NotI and the 1477-bp fragment was extracted from the 1% agarose gel. The pACYC184/P_{mphR}-GFPuv plasmid was digested with NotI and SAP and the dephosphorylated 3337-bp fragment was extracted from the 1% agarose gel. The two fragments were ligated together and transformed into XL-1 Blue electrocompetent cells. The new plasmid was named pBB/P_{mphR}-CATUPP.

The pBAD/LacZ plasmid was digested with SpeI and PstI and the 3125-bp fragment was extracted from the 1% agarose gel. The pBB/P_{mphR} plasmid was digested with SpeI and PstI and the 3507-bp fragment was extracted from the 1% agarose gel. The two fragments were ligated together and transformed into XL-1 Blue electrocompetent cells. The new plasmid was named pBB/P_{mphR}-LacZ.

The pBB/P_{mphR}-LacZ plasmid was digested with NotI and the 3284-bp fragment was extracted from the 1% agarose gel. This fragment was ligated with the dephosphorylated 3337-bp pACYC184 vector fragment and transformed into XL-1 Blue eletrocompetent cells. The new plasmid was named pACYC184/P_{mphR}-LacZ.

6.4: LacZ Activity Miller Assay

A 2-mL culture of the cells of interest was grown in LB broth with the appropriate antibiotic(s) overnight. A new culture was inoculated using 100 µL of the overnight cul-

ture and any additional components (erythromycin, arabinose, etc.) were added. The new culture was incubated at 37 °C until $OD_{600} \approx 0.5$. The exact OD_{600} was measured and 20 µL of the culture was added to 80 µL permeabilization solution. The mixture and substrate solution were incubated at 30 °C for 30 minutes before 600 µL of the substrate solution was added to the lysed cells.

The reaction was allowed to proceed until a yellow color was observed. 700 µL of the stop solution was added and the total reaction time, in minutes, was recorded. The reaction mixtures were centrifuged at 13,200 rpm for 5 minutes and the absorbance at 420 nm was measured of a sample taken from the top of the reaction tube. The Miller units of a particular culture were calculated using Equation 6-1.

Equation 6-1: Miller Units Calculation

$MU = 1000 * \frac{A_{420}}{(OD_{600})(V)(t)}$, where V is the volume of cell culture sampled (0.02 mL) and t is the reaction time in minutes.

6.5: Other General Methods

6.5.1: Gel-shift Binding Experiments

Fixed amounts of DNA were mixed with varying amounts of MphR. Each reaction was diluted to 20 µL total volume and incubated at room temperature for 30 minutes. 4 µL of 6X DNA Loading dye containing bromophenol blue and xylene cyanol. 3% agarose gels were run in TAE buffer at 120 V and 8% polyacrylamide gels were run in TBE buffer at 100 V. All gels were run until the bromophenol blue dye ran off the edge of the

gel. For staining, the gel was incubated for 1 hour in 200 μL of the appropriate buffer containing 10 μL of ethidium bromide (stock solution of 10mg/mL).

Polyacrylamide gels with fluorescent-tagged DNA were scanned directly using the Storm scanner. Biotinylated DNA was transferred to a positively charged nylon membrane in a cassette run at 60 V for 20 minutes. The DNA was cross-linked to the membrane under short-wave UV light for 10 minutes. The membrane was blocked by rinsing with 10 mL AP 7.5 buffer + 2% dry milk and incubating at 37 °C for 15 minutes. The blocking solution was removed and 10 mL AP 7.5 buffer with 2% dry milk and 5 μL concentrated streptavidin alkaline phosphatase was added. That solution was incubated room temperature for 90 minutes to bind to the biotinylated DNA. The binding solution was removed the membrane was washed twice with 50 mL AP 7.5 buffer and once with 50 mL AP 9.5 buffer at room temperature for 10 minutes each. The membrane was then stained with a solution of 20 μL nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate in 1 mL AP 9.5 buffer for 15 minutes in the dark at room temperature.

6.5.2: Fluorescence Binding Experiments

MphR was diluted to 3 μM monomer concentration (as determined by A_{280}) in buffer containing 50 mM Tris (pH = 7.9) and 100 mM NaCl. The fluorescence excitation spectrum was obtained and the maximum was found at 285 nm. At 285, the fluorescence emission spectrum was obtained and the maximum was found at 330 nm. Small volumes of concentrated DNA were titrated into the protein solution and the emission spectrum at each point was obtained in triplicate after allowing the mixture to equilibrate for 2 minutes. The fluorescence intensity at 330 nm was averaged for each DNA concentration.

6.5.3: PCR Eperiments

Most PCR reactions were completed as described here. Minor modifications were made as necessary to obtain maximum results. The reaction mixture was prepared using 40 μL H_2O, 5 μL 10X ThermoPol buffer, 1 μL template, 1 μL forward primer, 1 μL reverse primer, and 1 μL 10 mM dNTP's. Using a thermo cycler, the following program was run:

1. 95 °C for 3 minutes;
2. 95 °C for 30 seconds;
3. 5 °C below the lowest primer T_M for 30 seconds;
4. 72 °C for 15 seconds plus 1 minute per 1000 bases in the product;
5. Return to step 2 for 29 repetitions;
6. 72 °C for 3 minutes;
7. 4 °C for ever.

1 μL Taq Polymerase was added during the initial heating step at 95 °C. The completeness of the reaction was verified by agarose gel electrophoresis.

6.5.4: Circuit Assembly and Transformations

All chemical transformations were completed using 100 μL of flash-frozen chemical competent cells mixed with 10 μL of each plasmid. The mixture was incubated on ice for 30 minutes followed by 90 seconds at 42 °C and 2 minutes on ice again. 500 μL of SOC media was then added and the culture was incubated at 37 °C for 45 minutes. 150 μL of the culture was then smeared on an LB-Agar plate containing the appropriate antibiotic(s) which was then incubated at 37 °C overnight.

All electroporations were completed using 50 μL of flash-frozen electrocompetent cells mixed with 2 μL of the ligation reaction solution. The mixture was added to a cold

2-mm gap electroporation cuvette and shocked using the Ec2 setting of the electroporator. 500 μL of SOC media was immediately added and the culture was incubated at 37 °C for 45 minutes. 150 μL of the culture was then smeared on an LB-Agar plate containing the appropriate antibiotic(s) which was then incubated at 37 °C overnight.

The various circuits were assembled by transforming equal amounts of the two plasmids simultaneously. For appropriate reporter expression, all circuits expressing CATUPP and LacZ were transformed into *ΔUPP* cells. Ultimately, pACYC184/P_{mphR}-LacZ circuits were transformed into *Erm^r, ΔUPP* cells for measurements of the effect of erythromycin on the reporter expression.

6.5.5: DNA Purification—Phenol/Chloroform Extraction and Isopropanol Precipitation

When the denaturation of an enzyme—typically a polymerase used for PCR—was necessary, phenol/chloroform extraction was used. The extraction was always followed by isopropanol precipitation. Water was added to approximately double the volume of the DNA solution. A volume of 1:1 phenol/chloroform mixture pH equilibrated with TE buffer equal to the volume of the original DNA solution was added and the mixture was vortexed thoroughly for 1 minute. The sample was then centrifuged at 13,200 rpm for 5 minutes and the aqueous layer was removed. A volume of 100% chloroform equal to the removed aqueous layer was added and vortexed. The mixture was centrifuged at 13,200 rpm for 5 minutes, the aqueous layer was removed and the chloroform steps were repeated again. After finally removing the aqueous layer, the DNA was ready for precipitation.

Alternatively, when extraction was not required, the DNA was simply precipitated. NaOAc was added to a final concentration of 0.3 M. Then an equal volume of

isopropanol was added and the mixture was cooled at -20 °C for 1 hour. The mixture was centrifuged at 13,200 rpm for 5 minutes and the supernatant was removed. The precipitate was washed with 70% EtOH (H_2O), cooled at -20 °C for 10 minutes and centrifuged at 13,200 rpm for 5 minutes. The supernatant was removed and the wash process was repeated with 100% EtOH. The DNA was finally resuspended in a volume of water or TE buffer (pH = 8.0) depending on the desired concentration and eventual use.

6.5.6: DNA Manipulation Methods

Plasmid DNA was isolated from cell cultures using either the GeneJET™ Plasmid Miniprep kit or the Wizard® *Plus* Midiprep kit, depending on the culture size and the amount of DNA needed. DNA fragments were extracted from agarose gels using the QIAquick Gel Extraction kit. Ligation reactions were completed by mixing the insert and vector in ratios that ranged from 3:1 to 5:1, depending on the fragment sizes. The DNA was mixed together with the necessary volume of water and heated to 85 °C for 10 minutes to melt the strands. The solution was cooled to 4 °C for 30 minutes to anneal the strands and sticky ends. Since all ligations were completed in 20 µL reactions, 2 µL 10X Ligase Buffer and 1 µL T4 DNA Ligase were added. The solution was then incubated at 16 °C overnight for best results. A negative control reaction containing more water instead of insert DNA was always completed simultaneously.

6.5.7: Mass Spectrometry

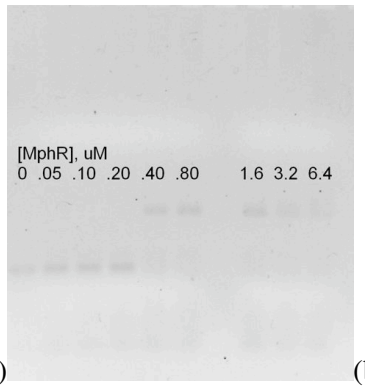
Mass spectra were obtained in linear mode at 90% maximum intensity. The spectrometer was optimized for 23,000 m/z and each spectrum was obtained using 500 laser shots.

Appendices

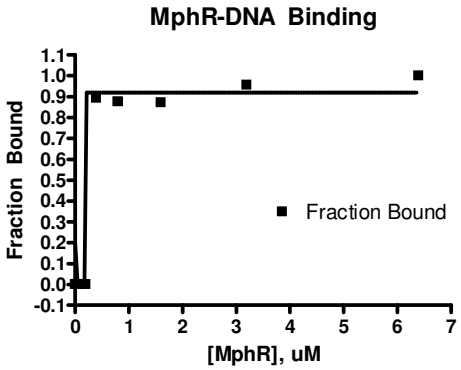
Appendix A: MphR-DNA Binding Experiment

The fluorescent-labelled DNA-binding gel-shift experiment was difficult to visualize. Ethidium bromide staining requires a large amount of DNA—more than was present in the reaction mixtures—for adequate illumination. Additionally, the high percentage gel increased the diffraction due to the gel. The two effects combined to result in DNA bands that were not significantly darker than the background (Figure A-1) with a low signal to noise ratio.

Figure A-1: MphR-DNA Binding Gel-Shift Experiment; Agarose Gel



(a)



(b)

Panel (a): 3% agarose gel visualized with UV transilluminator.

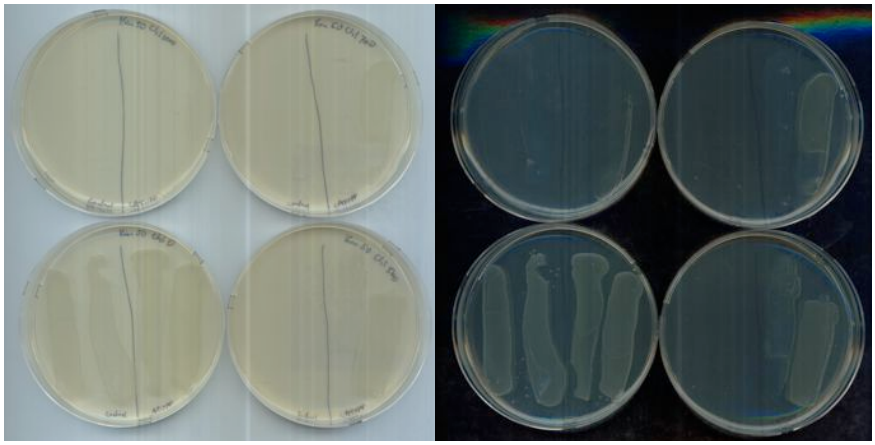
Panel (b): Data plotted as fraction bound vs. [MphR] in μM. The DNA concentration was 225 ng/μL.

Appendix B: Crude Quantification of CATUPP Expression

For chloramphenicol resistance test, LB-Agar plates containing 50 μg/mL kanamycin and a concentration of chloramphenicol between 0 and 1000 μg/mL were prepared. Cultures of pBB/P_{mphR}-CATUPP (CATUPP) and pBB/P_{mphR} (control) were grown

in liquid LB with 50 μg/mL kanamycin overnight. A portion of the stationary phase culture was diluted 10X in liquid LB. 50 μL of each culture and each dilution were smeared on the chloramphenicol plates and incubated at 37 °C for 48 hours (Figure A-2).

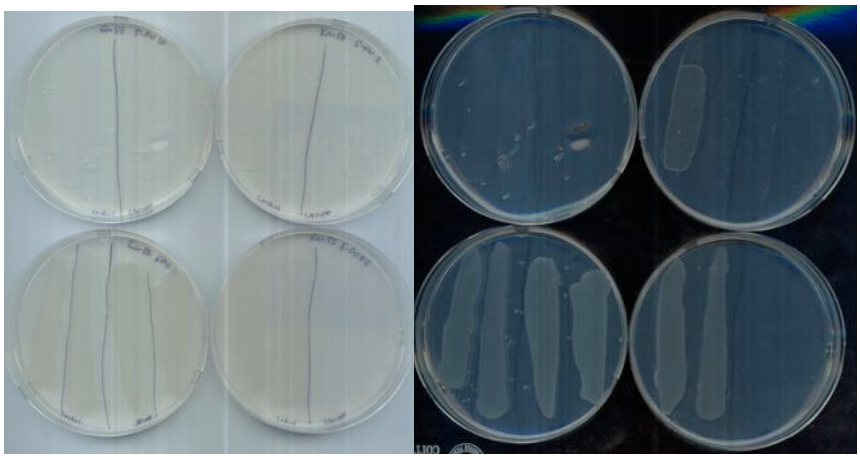
Figure A-2: Chloramphenicol Resistance Test



There is no difference between the two panels except for background color. Clockwise from top left: 1000, 700, 500, and 0 μg/mL chloramphenicol, respectively. On all plates, the outer smears are from stationary phase cultures and the inner smears are from the dilutions. On all plates, the left side is the control plasmid and the right side is the CATUPP plasmid.

For 5-fluorouracil toxicity test, M9-Agar plates containing 50 μg/mL kanamycin and a concentration of 5-fluorouracil between 0 and 10 μg/mL were prepared. Cultures of pBB/P_{mphR}-CATUPP (CATUPP) and pBB/P_{mphR} (control) were grown in liquid LB with 50 μg/mL kanamycin overnight. 1 mL of the stationary phase culture was spun at 8,000 rpm for 2 minutes and the pelleted cells were resuspended in 1 mL M9 media. A portion of the suspension was diluted 10X in M9 media. 50 μL of each culture and each dilution were smeared on the 5-fluorouracil plates and incubated at 37 °C for 48 hours (Figure A-3).

Figure A-3: 5-Fluorouracil Toxicity Test



There is no difference between the two panels except for background color. Clockwise from top left: 10, 2, 0.5, and 0 μg/mL 5-fluorouracil, respectively. On all plates, the outer smears are from stationary phase cultures and the inner smears are from the dilutions. On all plates, the left side is the control plasmid and the right side is the CATUPP plasmid.

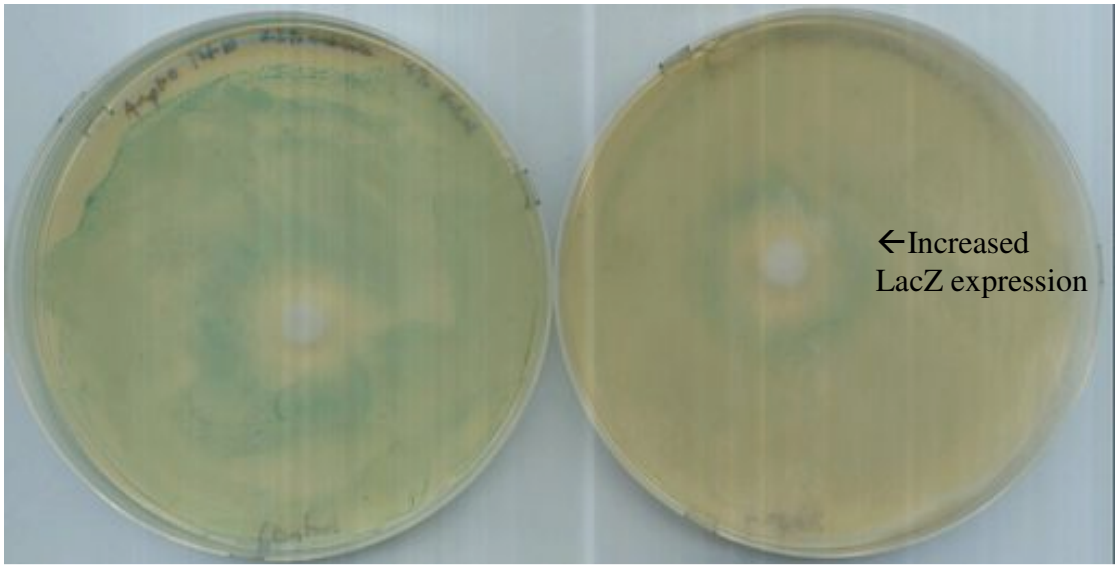
Appendix C: Crude Qualification of the Effect of Erythromycin on LacZ Expression

To check the potential effect of erythromycin on LacZ expression, LB-Agar plates containing 100 μg/mL ampicillin, 10 μg/mL tetracycline, 0.2% arabinose, and 4% (v/v) X-Gal were prepared. Additionally, small discs of filter paper were created using a standard desk hole-punch. One drop of 100 μg/mL erythromycin in 100% ethanol was placed on each disc and the ethanol was evaporated, drying the discs.

Cultures of pACYC184/P_{mphR}-LacZ + pBAD/MphR (+MphR) and pACYC184/P_{mphR}-LacZ + pBAD/*myc*HisA (control) were grown in liquid LB containing 100 μg/mL ampicillin and 10 μg/mL tetracycline overnight. 100 μL of each culture was smeared on a plate and the dry erythromycin disc was placed on top in the center of each plate, which was then incubated at 37 °C for 48 hours (Figure A-4). The lawn of blue colonies on the control plate demonstrated that LacZ expression was not affected by the

introduction of erythromycin in this culture. Conversely, the ring of more pronounced blue colonies near the disc suggested that the presence of small amounts of erythromycin increased the density of blue colonies, which signaled an increase in LacZ expression.

Figure A-4: Effect of Erythromycin on LacZ Expression



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