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DESIGN OF A TRANSCRIPTIONAL REPRESSOR-BASED BIOSENSOR IN *E. COLI*

A Thesis in

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by

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Abstract

The development of *in* vivo high-throughput screening techniques for the detection of polyketides is essential to alleviate our dependence of expensive synthetic and time consuming methods for the production these industrial bioactive molecules. Nature provides a vast array of polyketides in a myriad of organisms, produced from highly malleable biosynthetic pathways. Protein transcriptional regulators offer a gateway to mitigate the current processes of polyketide extraction and/or chemical synthesis. By naturally having an affinity for molecules such as polyketides and with the genetic tools available today, transcriptional regulators can be evolved to detect other industrially significant ligands and novel bioactive compounds through directed evolution. This will allow for the design of inexpensive high yield processes when expressed in a genetically manipulative host such as *Escherchia coli*. In this study, we describe the design of transcriptional repressor-based biosensor for the *in vivo* detection of polyketides in *E. coli*. The biosensor was engineered with ActR, a TetR-like transcriptional repressor protein, controlling expression of the *gfp* reporter gene which produces a green fluorescent protein (GFP). The incorporation of a GFP reporter allows for high-throughput screening of ActR and polyketide biosynthetic pathway combinatorial libraries using fluorescence-activated cell sorting (FACS). The ActR mutants will be screened based on their affinity and selectivity for a specific product produced through an engineered polyketide biosynthetic pathway, which the pathway can then be screened for increased yields. The broad range of genetic techniques available for manipulation of *E. coli* combined with the ActR-based biosensor engineered in *E. coli*, a new window of opportunity has been presented for engineering more efficient microbial production of polyketide compounds.

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Chapter 1: Introduction

Plant and microbial natural products are among the most important pharmaceutical compounds due to their ability to treat many human ailments including neurological, infectious, cardiovascular, and oncological diseases (Butler 2008). Natural products have been priceless as front-line structures for the discovery of novel compounds and tools for deciphering the logical progression of biosynthetic pathways (Clardy and Walsh 2004). The complexity of most such compounds demands production through powerful whole-cell biosynthetic pathways instead of rigorous *in vitro* or chemical synthesis methods. Despite their complexity, these molecules stem from simple primary metabolites within the natural organism but in minute concentrations (Clardy and Walsh 2004). The unavailability of synthetic methods and low yields from host organisms has been the driving force for gene identification, cloning, and heterologous expression of biosynthetic pathways of these compounds in high-producing, amenable host organisms. In the late 1990's and early 2000's there were hundreds of predicted and identified secondary metabolite gene clusters discovered (Walsh 2004), adding tools to an ever growing, versatile bioengineering toolbox. With the gene clusters being defined, categorized, and engineered, natural products and novel unnatural products with therapeutic activity can be discovered as well as produced on large scale quantities from host organisms,

but there are a lack of high-throughput options for detecting these products from combinatorial libraries composed of various genetic elements involved in their biosynthesis.

1.1 Background

Combinatorial biosynthesis aims to alter or improve the activity of enzymes towards desired products using mutagenesis and iterative rounds of screening. Conversely, rational design uses logical computational iterations to determine the appropriate alterations to an enzyme to alter or improve activity based upon detailed knowledge of the enzyme. Biosynthetic pathways are made up of a cluster of enzymes, many of which have not yet been classified or well-characterized, thus making rational design arduous or unavailable. Combinatorial biosynthesis is a viable option when little about the pathway is known, which dissolves the complication of knowing specifics of a protein structure or gene regulatory mechanisms. Linking the final product to a measurable phenotype is limited to specific compounds or is non-existent. Combinatorial biosynthesis of enzymes in biosynthetic pathways is subject to the classic adage "you get what you screen for", thus leading to the bottleneck, reporting the production of the compound of interest. By engineering natural detectors such as previously-identified transcriptional regulators involved in biosynthetic pathways of similar compounds to recognize compounds of interest and produce a measurable phenotypic response, engineered heterologous pathways conferring microbial production of natural and novel therapeutically active compounds can readily be identified.

Some of the most studied and desired natural products are a class of compounds called polyketides. Due to their high occurrence of bioactivity they are highly desired compounds for

instance the antibiotic tetracycline and the potent anticancer doxorubicin. Polyketides are produced from a variety of host organisms, for example the production of erythromycin from actinomycete *Saccharopolyspora erythraea* and the anti inflammatory flavenoid Luteolin found in many vegetables such as celery and green peppers (Shimoi et al. 1998). They are derived from acyl-coenzyme A (CoA) monomers and are constructed through enzymatic pathways expressing polyketide synthases (PKS). PKSs are macroscopic multifunctional proteins responsible for the production of polyketides. They are comprised of several functional groups organized in a logic fashion. Each functional group catalyzes a reaction relative to the neighboring functional group's product, thus creating an assembly line of enzymatic reactions to tailor the resulting polyketide (Zhang and Tang 2008). The resulting product is determined by four definitive degrees of freedom: (1) the length of the polyketide chain, dependent upon length of poly-β-ketone backbones produced dependent upon the number of functional groups in the PKS; (2) the specific primer and extender units incorporated, each regulated by the acyl transferase domains; (3) Reduction of the polyketide backbone determined by the enzymatic site of each functional group in the PKS; and (4) alkyl and hydroxyl substituted centers' specific stereochemistry managed by the functional group responsible for creating the stereocenter in question (Cane et al. 1998). Due to the intricacy of the PKSs and the variables involved with producing polyketides, successful combinatorial manipulations aimed at producing novel compounds or at improving heterologous biosynthesis of a desired product would require screening of enormous libraries of PKS mutants. Screening or selection of the mutants is currently hindered by the lack of sensitive high-throughput screening (HTS) techniques for polyketides. Recently, the PKS4 of *Gibberella fujikuroi* was dissected and reassembled as a

functional macrosynthase expressed in *Escherichia coli*, producing complex bacterial aromatic polyketides (Zhang et al. 2008). This demonstrated the first successful expression of PKSs in *Escherichia coli*, which has the potential to be used as a powerful tool for engineering PKSs for understanding and production of novel aromatic polyketides. Like a hammer without a handle, heterologous expression of PKSs in *E. coli* would be an inefficient tool for future combinatorial manipulations without a robust HTS technique.

In the recent decade, there have been major advancements in combinatorial design of molecular reporters for the detection of small molecules *in vivo*. One such advancement has been in the implementation of transcriptional regulators to respond to the presence of the molecule of interest. A gene encoding a protein with a measurable phenotypic output (reporter) is placed downstream of an operator respective to the regulator. The regulator is subsequently mutated to bind the molecule of interest, where specific mutations are usually determined using structure models of the ligand binding domain (LBD). Mutants are screened or selected based upon the reporter expression. The Cirino Laboratory was able to demonstrate success in screening a library of AraC mutants employing iterative rounds of fluorescence-activated cell sorting (FACS) to obtain mutants with altered specificity from L-arabinose to D-arbinose (Tang, 2008). Now, they can readily design and screen libraries with up to 3×10^7 mutants using their AraC system as a platform for novel and exciting combinatorial design of transcriptional regulators in response to atypical ligands (Tang, unpublished).

Though AraC is an effective platform for the detection of small molecules similar in structure to L-arabinose, AraC would not make an effective platform for large multicyclic polyketides due to spatial constraints in the LBD. A more suitable platform would be a

transcriptional regulator of a polyketide pathway. A specific family of homodimeric transcriptional regulators known as TetR-like proteins comprised of thousands of polyketide-responsive transcriptional regulators most of which have yet to be characterized. TetR-like proteins range from regulators like QacR, with a broad range of effectors, to TetR, with a highly restricted range of effectors. The crystal structure and specific genetic markers have been recently developed for the TetR-like protein ActR (Tahlan et al. 2008; Willems et al. 2008), which shows induction by a semi-restricted range of polyketide effectors structurally similar to the aromatic polyketides produced in *E. coli* from the engineered PKS referred to in Fig 1.1 (Tahlan et al. 2008). ActR would then make an ideal platform for the engineering of a molecular reporter system for aromatic polyketides of interest.

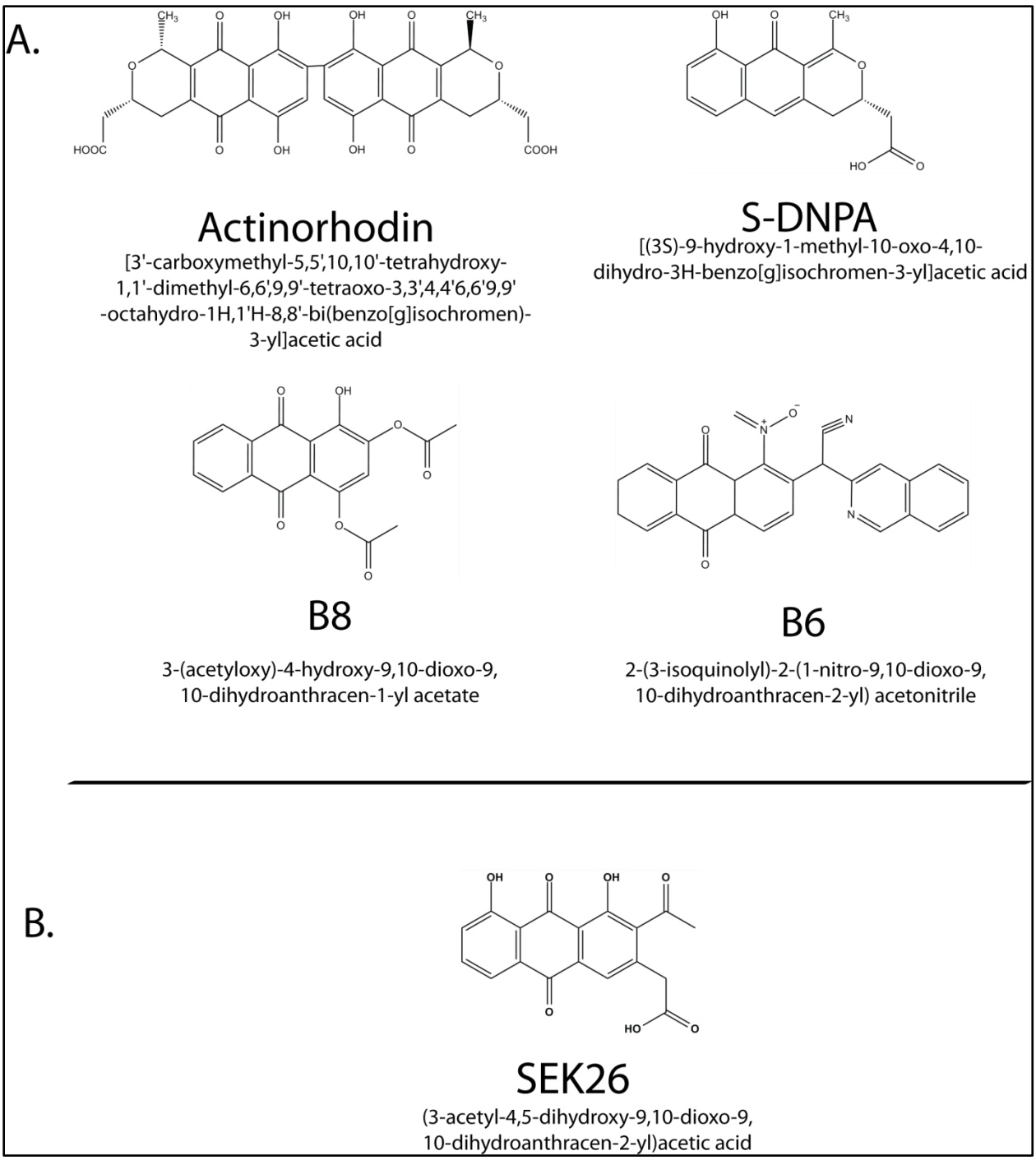


Fig 1. 1: Structures of ActR ligands and the polyketide engineered to be produced endogenously in *E. coli*. (A.) Structures of ActR ligands as reported by Tahlan and coworkers (Tahlan et al. 2007; Tahlan et al. 2008). (B.) Structure of polyketide produced through the incorporation of a polyketide megasynthase heterologously expressed in *E. coli*.

1.2 Goals and Specific Aims

The main goal of this thesis is to design and implement an ActR-based transcription reporter in *Escherchia coli* toward the ultimate goal of a tool in the genetic toolbox for combinatorial manipulations of PKSs using FACS as a HTS technique in detecting desired PKS mutants. The specific aims of the thesis are as follows:

i. Design an in vivo reporter system in *E. coli* regulated by the ActR repressor protein
ii. Engineer ActR regulated reporter for improved "on"/"off" function
iii. Show experimentally that ActR repression can be relieved by natural ligands
iv. Demonstrate the ability to alter ActR effector specificity

Chapter 2: Literature Review

2.1 Flow cytometry and Fluorescence-Activated Cell Sorting (FACS)

Flow cytometry is a powerful technique in combinatorial applications, using multiple optical properties to detect variations of individual cells or compartments (particles) such as fluorescence, size, and internal complexity. Fluorescence-activated cell sorting (FACS) utilizes flow cytometry to detect the relative fluorescence of a particle, and depending upon a predetermined desired relative fluorescence (gating) the particle will be charged negative or positive or will be left neutral. FACS is dependent upon a linkage between the phenotype of interest with the fluorescence of the particle. The implementation of FACS is vast and is discussed in many reviews (Becker et al. 2004; Farinas et al. 2001; Georgiou 2001) The particles are subsequently separated based upon their charge. FACS screens cells up to 30,000 particles sec^{-1} approaching the limits of library screening and providing a paramount HTS technique for combinatorial protein engineers.

Perhaps the simplest case is when the fluorescence producing interactions are maintained intracellular. The desired proteins are expressed *in vivo* and remain in the cell throughout the entire process, which the proteins subsequently interact with a fluorescent producing molecule or a genetic operator regulating a fluorescent protein such as GFP. The latter case was recently

implemented using the *E. coli* dual regulatory protein *araC* from the *ara* operon. Exploiting directed evolution, two libraries of *araC* mutants were engineered using the activation of the expression of *gfpuv* downstream of the AraC promoter, Pbad, in the presence of D-arabinose and not responds to the natural effector L-arabinose. FACS was used to screen each library, composed of mutants with site-saturations at the genomic level translating to four different sites around the binding pocket of AraC for each library. Both libraries produced mutants with altered specificities from L-arabinose to D-arabinose (Tang 2008).

Due to the nature of combinatorial metabolic pathway engineering for natural compounds screening methods based upon the binding of an effector using *in vitro* techniques such as mRNA display, ribosome display, and phage display would not be plausible where they are used to screen for protein equilibrium affinity, stability, and folding. These display techniques allow for unhindered linkage of the genotype to the phenotype, which would be advantageous for connecting molecular recognition to gene expression but not for increase production rate of products synthesized *in vivo*. Another *in vitro* screening technique, *in vitro* compartmentalization (IVC), employs purified genetic elements and proteins and/or crude cell lysates emulsified in either w/o or w/o/w droplets often used to screen libraries of enzymes for catalytic activity (Aharoni et al. 2005a; Taly et al. 2007). Most utilization of IVC has been used as a proof of concept or method development. Complex polyketide biosynthetic pathways would prove unnecessary to screen for increased production rate of endogenous polyketides using compartmentalization, but may be beneficial if the polyketide is excreted form the cell. Use of compartmentalization for the screening of small molecule production rate has yet to be reported. There have been many implications and techniques of flow cytometry and FACS for

enzyme engineering which are discussed in many excellent review articles (Aharoni et al. 2005b; Boersma et al. 2007; Lin and Cornish 2002; Link et al. 2007; Olsen et al. 2000a; Olsen et al. 2000b; Taylor et al. 2001), but more relevant applications for intracellular fluorescence are discussed in this section.

2.2 Aromatic Polyketide Biosynthetic Pathways

Polyketides are a diverse group of aromatic natural products, a group which is comprised largely of significant bioactive molecules. One of the most familiar and relevant aromatic polyketides is the antibiotic class of tetracyclines, prescribed in response to many bacterial infections. Noteworthy polyketides are derived from the soil dwelling *actinomyces* producing cancer, antibacterial, antifungal, and antiviral compounds. Polyketides are synthesized in organisms through polyketide synthases (PKSs) and tailoring proteins.

PKSs differ between prokaryotes and eukaryotes. Bacterial PKSs are made up of several disslocated enzymatic components, whereas fungal PKSs are megasynthases made up of a single polypetptide with several multifunctional domains capable of catalyzing the production of long chain poly-β-ketones (Cox 2007). The functional groups of the PKS megasynthases have been determined to have two specific essential domains that consist of a ketosynthase-chain length factor (KS-CLF) heterodimer and an acyl-carrier protein (ACP). The modularity of PKSs makes them receptive to manipulations so new and potentially more bioactive compounds can be discovered. Not only does the modulation of PKSs allow for accessible manipulations for discovery, but for high production of already known bioactive compounds as well.

Yin and coworkers designed a high-throughput phage display method for cloning of biosynthetic clusters of PKSs and nonribosomal polypeptide synthases, useful for extracting rich sources of natural products from unculturable organisms (Yin et al. 2007). Unfortunately, the success of combinatorial approaches to manipulating PKSs has been limited, but some groups have been successful.

Watanabe and coworkers were able to split up and recombine the PKSs modules to form an active enzymatic chain (Watanabe et al. 2003). Zhang and coworkers were successful in heterologously expressing the *Gibberella fujikuroi* PKS megasynthase in *E. coli*. The functionally expressed PKS was shown to produce an expected nonapeptide anthraquinone, SEK26. The production of SEK26 was scaled-up attaining titers up to 3 mg mL^{-1} (Zhang et al. 2008). Their method for determining the correct structure of products to make sure they were producing SEK26, was to use MS-LC, which requires time and the extinction of cells and is not high-throughput. The approach of engineering PKSs for the production of novel and/or increasing the production of bioactive polyketides has been discussed in several excellent reviews (Das and Khosla 2009; Khosla and Keasling 2003; Neumann and Neumann-Staubitz ; Walsh 2004; Zhou et al. 2008)

2.3 Regulatory Proteins Engineering

Transcriptional regulation is controlled by several genetic factors, as in regulatory proteins (i.e. repressors and activators) and transcription factors (i.e. sigma factors and promoters), but regulatory proteins are amenable tools providing a valuable link between the intracellular environment to a phenotype. Many prokaryote regulatory proteins have been synthetically

incorporated in biological circuits for executing specific expression systems (de las Heras et al. 2010). LacZ (β-galactosidase), being a well established example of an adaptable transcriptional regulatory proteins (TRPs), has been widely used as a genetic tool for example in plasmid cloning vectors. The cloning vector containing mutated *lacZ* gene containing a multiple cloning site is used to clone desired genetic element such as genes into a plasmid. Without the inserted gene, *lacZ* is expressed producing LacZ, seen as blue colonies on a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) containing agar plate, and with a gene inserted into the multiple cloning site in *lacZ*, LacZ is no longer expresses, seen as white colonies on a X-gal containing agar plate, know as blue and white screening (discussed below). The genetic toolbox for genetic manipulations contains a myriad of techniques and methods to finesse genetic structures making the manipulation of transcriptional regulatory proteins almost trivial. Thus, TRPs are ideal for designing new tools for engineering metabolic pathways and deciphering those pathways along with studying general gene expression in both prokaryotes and eukaryotes.

Tang and Cirino demonstrated a new approach to metabolic engineering using the transcriptional dual-regulatory protein AraC (unpublished data). Exercising directed evolution, AraC was engineered to respond to a novel inducer (mevalonate), and the mevalonate reporter system was then used to screen for improved mevalonate synthesis, increasing the yield from ~6 mM up to 17 mM mevalonate. Mevalonate is a precursor to a pathway of isoprenoids, a large class of industrial valuable secondary metabolites, and mevalonte synthesis is the rate-limiting step in isoprenoid production in *E. coli* (Pfleger, 2006). The almost exclusive eukaryote mevalonate pathway was reported to be functionally engineered in *Escherichia coli* (Martin et

al. 2003; Pfleger et al. 2006). The first step in engineering AraC was to find a mutant to respond in the presence of mevalonate, previously determined not to be an inducer of AraC (Tang 2008). A library of AraC mutants containing variants with five site-saturated mutations in the ligand binding domain was screened isolating clones for responsiveness to exogenously introduced mevalonate using FACS. After several rounds of positive and negative sorting, a single mutant was isolated showing greatly enhanced relative concentrations of GFPuv when introduced to mevalonate compared to without. The mutant was also confirmed with chromosomally integrated *lacZ* controlled by Pbad where they reported increased LacZ expression responding to both exogenous and endogenous mevalonate. The second step was to screen a library of ribosomal binding site mutants of the optimal expression of *tHMGR*, where cell growth and mevalonate production is sensitive to *tHMGR* expression levels. Since mevalonate can passively transport across the cellular envelope, endogenous production of mevalonate in a single mutant can be cross linked activating AraC in a neighboring cell if there was no barrier, such as in liquid cultures. Therefore, they revisited the use of LacZ, where mutants endogenously producing mevalonate were grown on solid media containing X-gal. Improved mutants were screened visually, picking single colonies with increased LacZ activity. After screening the six bluest colonies based on mevalonate production in liquid cultures, the top three mutants were tested for activity levels of the *tHMGR* reuslting protein, HMG-CoA reductase, which showed two of the mutants had significantly lower levels of HMG-CoA reductase compared to the wild type expression (Tang, unpublished).

Before engineering a TRP recognizing and responding to a novel inducer, basic knowledge of which residues in the protein structure show the most significant impact would be

convenient. Sholz and coworkers explored this concept with the tetracycline operon repressor (TetR). TetR was engineered to recognize and respond to the naturally non-inducible tetracycline (tc) analog 4-de(dimethylamino)-6-demethyl-6-deoxy-tc (cmt3) through directed evolution using LacZ activity assays. The first round of evolution was focused on isolating a library of cmt3-inducible mutants created using DNA shuffling of the TetR(BD) chimera. The library produced mutants with relaxed specificity, inducible 100% by tc and between 6.2% and 82.3% by cmt3. The second round of *in vitro* evolution pinpointed the most important mutations in the eight best mutants they screened for in the first round. They found the TetR H64L mutation conferred changed specificity. In the third round of evolution, they again implemented *in vitro* techniques to construct a library of mutants, but this round focused on structural configurations in the ligand binding domain. The previously described mutants were not inputs to mutants of this round of evolution. Site-saturated mutagenesis was employed to mutate spatially determined residues located in four different parts of the primary structure. This round yielded the TetR S135L mutation, which was then combined with the H64L mutation. The resulting combined mutations produced a TetR mutant 95% inducible with cmt3 and only 2.9% with tc. A final fourth round of evolution was used to increase the specificity of TetR towards cmt3. Construction of a new library by site-directed mutagenesis of *tetR H64K S135L* using the same sites they mutated in the third round was screened and mutants were found with up to 100% inducibility by cmt3 (Scholz et al. 2003). Their success demonstrated specific insight into the induction mechanism of TetR, which proved to be useful in future characterization of TetR (Klieber et al. 2009).

Another engineered TRP, LuxR was chosen to study the plasticity of a broad range inducer TRP, specifically inducer-dependent upon acyl-homoserine lactones (AHL). LuxR is an activator naturally found in the *lux* operon for quorum-sensing of *Vibrio fischeri* controlling expression of the 3-oxohexanoyl-homoserine lactone (3OC6HSL) production gene, *luxI* (Dunlap 1999). A previously determined mutant reported to respond to a diverse group of AHLs but not butanoyl-HSL (C4HSL) acted as a parent allele for 2 rounds of directed evolution screening for mutants with specificity toward C4HSL. Mutations were incorporated into the LuxR mutant first by error-prone PCR and mutants were screened for C4HSL inducibilty using a plate based fluorescence assay, from the production of GFPuv controlled by the Plux promoter. Third - generation mutants were again mutated, but by DNA shuffling producing several mutants with an average half maximal dose response of 150 nM of C4HSL (parent did not respond to C4HSL). The mutations in the engineered mutants also corresponded to increase induction by the intrinsic ligand, 3OC6HSL, from half maximal dose response in the wild-type of 26 nM to ≤1 nM averaged between the final mutants. The applications of TRPs for engineering metabolic pathways and deciphering puzzling progressions are available for future engineering of new and diverse TRPs.

2.4 TetR-like Proteins

The tetracycline transport operon transcriptional regulator, TetR, of the transposon Tn10 has been highly characterized. The crystal structure was solved by Hinrichs and coworkers(Hinrichs et al. 1994) and vast amount of thermodynamic properties of the TetR have been determined (Hillen and Berens 1994; Kleinschmidt et al. 1988). TetR naturally binds to the

two different operators with different levels of affinity found in the region between the *tetR* and *tetA* genes, functionally repressing the transcription of both in absence of tetracycline ($[tc{\cdot}Me]^+$). The binding constant for TetR binding to the *tet* operator is $2 \times 10^{11}\ M^{-1}$. TetR shows to have a greater affinity for the promoter controlling expression of the *tetA* gene most likely do to the toxicity of TetA, a tetracycline transporter with twelve cytoplasmic membrane spanning α-helices. The general structure of TetR is an omega (Ω)-shaped homodimer. A crystal structure of TetR in complex with $[tc{\cdot}Me]^+$ was determined with a 2.1 Å resolution by Hinrichs and coworkers (Hinrichs et al. 1994). A TetR monomer is composed of ten α-helices as shown in Fig 2.1. The C-terminal of the TetR protein contains the hydrogen bonding network of the dimer and the ligand binding domain (LBD), each monomer binding to a single $[tc{\cdot}Me]^+$ with an association constant of $3 \times 10^9\ M^{-1}$ (Hillen and Berens 1994) The N-terminal of the monomer contains the DNA binding domain (DBD) including a helix-turn-helix motif similar to many other repressors as reviewed by Harrison and Aggarwal (Harrison and Aggarwal 1990). Since the classification of TetR, many transcriptional regulators have been discovered and all convey similar structures to TetR. The family of transcriptional repressors with similar structures to that of TetR was aptly named TetR-like proteins. The family of TetR-like proteins includes but is not limited to ActR, QacR, EthR, and TtgR (Alguel et al. 2007; Engohang-Ndong et al. 2004; Grkovic et al. 1998; Willems et al. 2008). A detailed review of TetR-like proteins was written by Ramos and coworkers (Ramos et al. 2005).

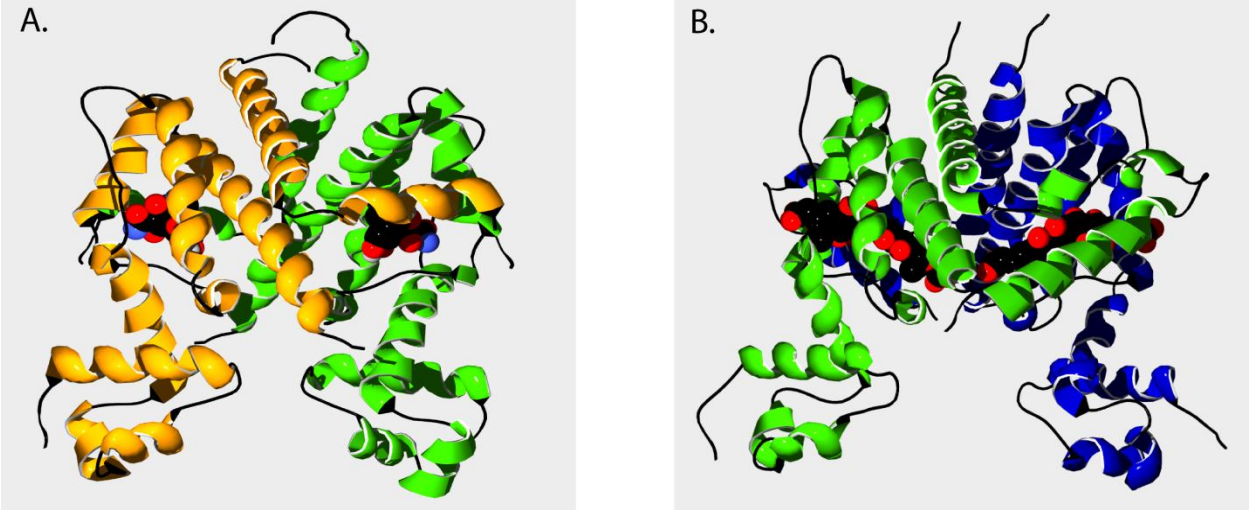


Fig 2. 1: Comparison of TetR and ActR Structures. (A.) TetR crystal structure bound to the natural ligand tetracycline. (B.) ActR crystal structure bound to the natural ligand actinorhodin.

The soil dwelling *Streptomyces coelicolor* synthesizes a large library of secondary metabolites and its genome encodes for more than 150 TetR-like proteins (Bentley et al. 2002). *S. coelicolor* produces a blue pigment under alkaline conditions from the production of the hexacyclic polyketide antibiotic actinorhodin by a type II PKS thus conferring tricyclic intermediates such as 4-dihydro-9-hydroxy-methyl-10-ox-3-H-naphtho-[2,3-c]-pyran-3-(S)-acetic acid ((S)-DNPA). Several putative transmembrane proteins are chromosomally encoded for the transport of γ-actinorhodin (lactone of actinorhodin) out of the cell acting as a self resistance to actinorhodin. The regulation of the transmembrane proteins, specifically ActA, is controlled by the TetR-like protein, ActR. Also like TetR, ActR self regulated its own transcription (Willems et al. 2008). The crystal structures for apo ActR, ActR bound to actinorhodin, and ActR bound to (S)-DNPA have all been solved with a 2.05 Å, 3.05 Å, and 2.30 Å resolution, respectively (Willems et al. 2008). The resulting crystal structure revealed each monomer as being made up of 3 distinct regions: (1) helices α1 through α3 made up the DBD located on the N-terminus; (2) helices α4 through α8 and α11 made up the core of the

monomer where the LBD was located at the C-terminus; (3) helices α9 and α10 were the connecting helices from the DBD to the LBD. The LBD was round to be a cavity roughly 800 $Å^3$, which could be divided into two separate polar regions by a hydrophobic region in the center of the ligand pocket. Each polar region was named for their location relative to the outside of the protein, where the region closer to the center of the protein was named the proximal region and the region closer to the outside of the protein was called the distal region. Only one molecule of the hexacyclic actinorhodin was bound in the ligand pocket for each monomer, where as two molecules of the tricyclic (S)-DNPA were bound, but the binding of either ligand had similar results. When either of the ligands was bound, the DBD arms were stabilized in the homodimer in the position relating to derepression of ActR, but if they were not bound the arm moves freely. The mechanism of binding of the ligand to ActR suggested when the ligand is not bound the ActR protein freely moved "on" and "off" the operator sequence. The crystal structure of ActR revealed a large amount of information for future adaptations of the protein.

2.5 Permeabilizers

For preliminary studies of reporter design, compounds of interest must enter the cell from an external source such as the growth medium or a wash buffer. Compounds, such as tetracycline and aminoglycosides, are passively transported into the cell, but many compounds have difficulty migrating into the cell. Many methods for whole-cell reporters require transport of a compound of interest through the cell membrane and into the cytoplasm of the cell where in prokaryotes the compound can easily interact with a vast number of proteins. Many variations of whole-cell permeability methods have been successfully employed to

transport small molecules and proteins into and out of the cell as reviewed by Chen (Chen 2007).

One such method is to use chemicals to weaken the cellular membrane. A toluene wash has been used to permeabilize the cytoplasmic phospholipid layer of gram-negative bacteria in the presence of Mg^{+}, making the cell more susceptible to low molecular weight molecules (De Smet et al. 1978). Dupont and Clarke used toluene to permeabilize *Proteus miribilis* cells for the uptake of specifically radiolabelled nucleotide precursors to study the biosynthesis of O-acetylated peptidoglycan. They found evidence confirming the hypothesis of O-acetylation of peptidoglycan is synthesized outside of the cytoplasm in the membrane. Though toluene has been proven as a permeabilizer, there are many other less toxic options available for example the anionic detergent Triton X-100, which was demonstrated by Miozzari and coworkers. They used a low concentration of Triton X-100 to freeze *Saccharomyces cerevisiae* cells and after thawing, the cells were immediately used in enzymatic assays. They reported proteins with molecular weights up to 70 kDa able to cross the cellular membrane (Miozzari et al. 1978).

Other methods of permeabilization include the use of polymyxin, cyclic polycationic peptide with a fatty acid chain attached to the peptide through an amide linkage. Polymyxin B is a natural antibiotic by disrupting the lipopolysaccharide layer of gram-negative bacteria. Polymyxin B is an effective bactericide and disrupting the stability of the cellular membrane at low concentrations (Storm et al. 1977). A less disruptive alternative to polymyxin B is a deacylated derivative of polymyxin B, polymyxin B nonapeptide (PMBN). PMBN mainly acts on the outer membrane of the cell wall producing ultrasonic damage, allowing passage of certain hydrophobic antibiotics into the cell and leakage of proteins out of the cell (Dixon and Chopra

1986). The low destabilization of the outer membrane and high susceptibility of hydrophobic antibiotics to passively transport into the cell make PMBN a viable solution for the transport of aromatic polyketides into *E. coli*.

Chapter 3: ActR Biosensor Design

Currently the lack of sensitive and/or rapid screening and selection methods of natural and unnatural metabolites has hindered the discovery and engineering of genetic elements involved in synthesis of desired metabolites, specifically polyketides (several significant polyketides are shown in Fig 3.1). The TetR-like family of proteins responds to small molecules ("effectors") such as polyketides, thus providing a premiere platform to engineer a reporter system in response to specific polyketides. The polyketides of interest, shown in Fig 3.2, for this thesis are variations of tri- and tetra- cyclic anthraquinones, similar to the *Streptomyces coelicolor* naturally produced hexacyclic antibiotic actinorhodin and its precursor 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-H-naphtho-[2,3-c]-pyran-3-(S)-acetic acid ((S)-DNPA). The actinorhodin transport operon in *S. coelicolor* is regulated by the TetR-like repressor, ActR, where actinorhodin, (S)-DNPA, and several other compounds containing an anthraquinone core have been found as effectors for relieving ActR repression (Tahlan et al. 2008). Also, establishment of a biosensor based ActR system was reported using a luciferase based assay. Luciferase based assays are presently limited in functioning as high-throughput screening (HTS) technique being plate based. A much better HTS technique can be achieved using the reporter system presented in this chapter using a Green Fluorescent Protein (GFP) based assay.

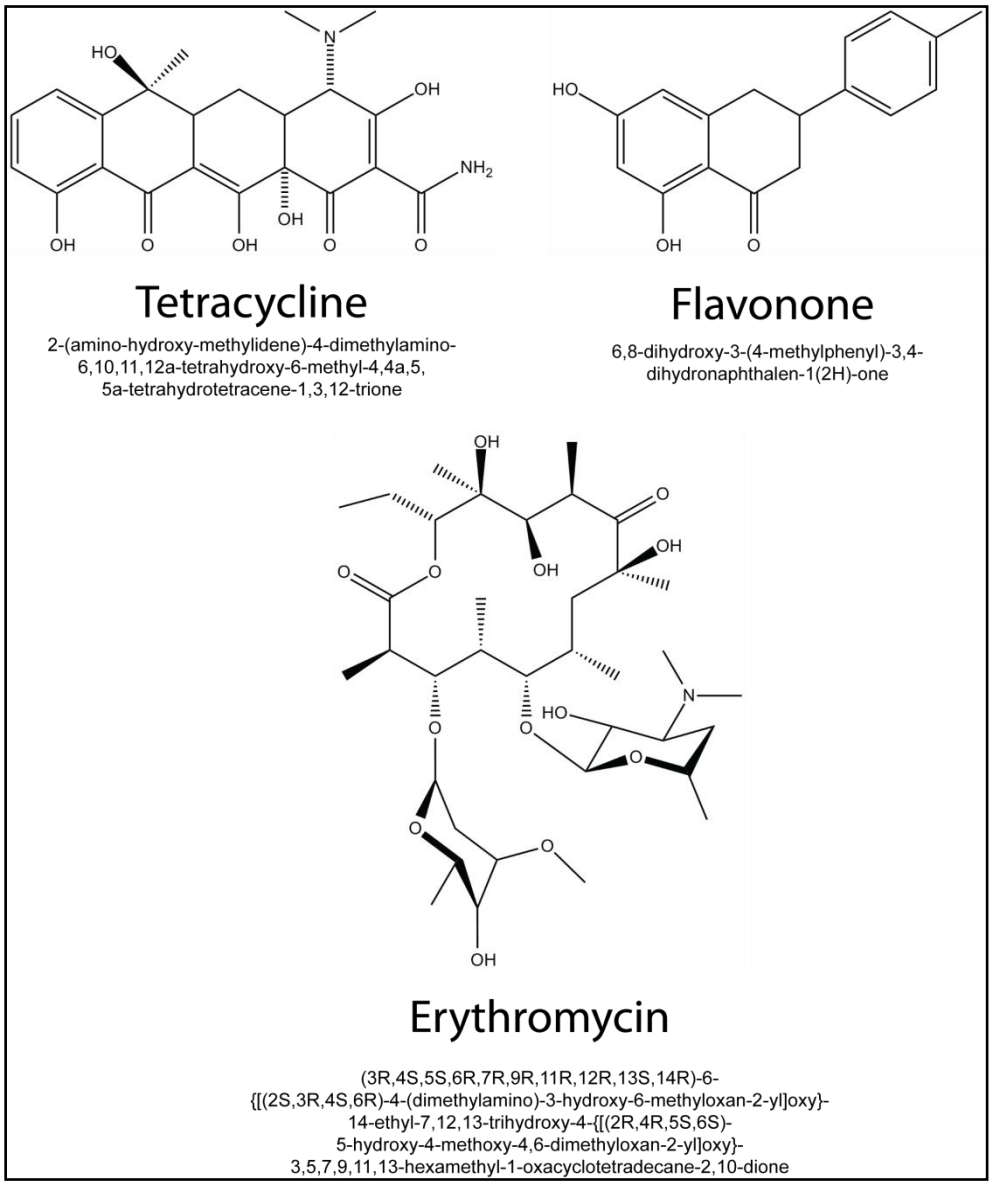


Fig 3. 1: Pharmaceutically important bioactive polyketides.

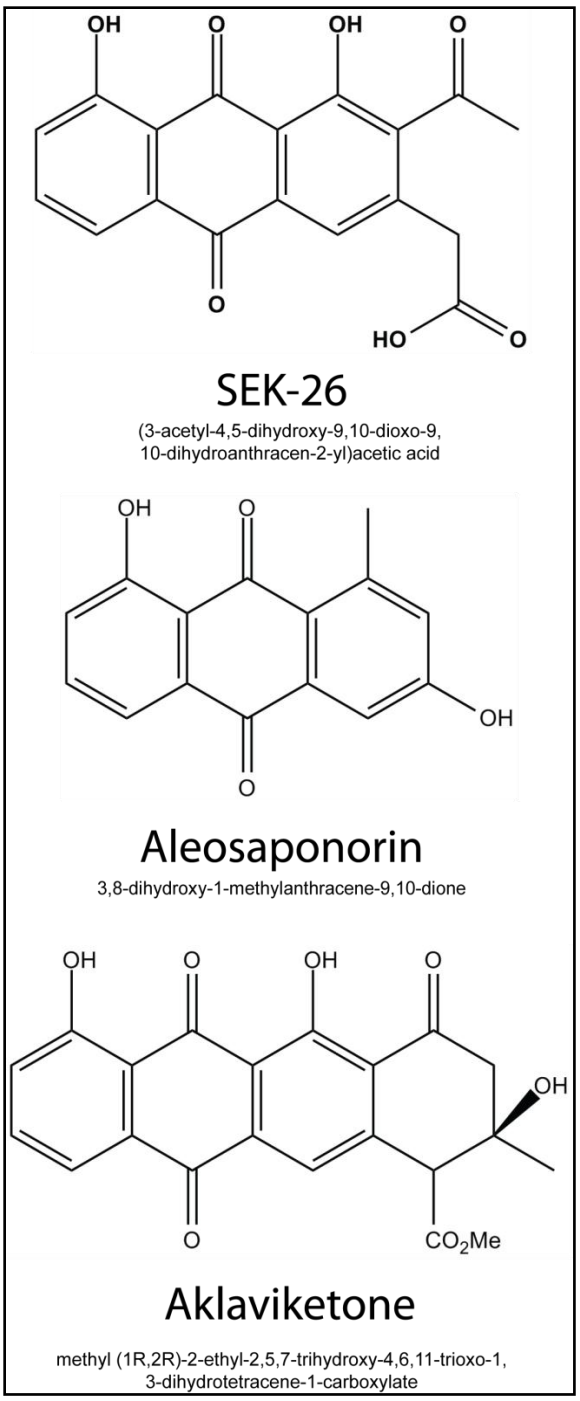


Fig 3. 2: Structures of the target polyketides of interest to engineering the ActR-based biosensor to respond to. SEK-26 has already been produced in *E. coli* at high titers (Zhang, 2008), but improved yields are desired using the ActR-based biosensor responding to SEK-26. Aleosaponorin and Aklaviketone are target polyketides for the engineering of a PKSs biosynthetic pathway in *E. coli* using the ActR-based biosensor.

3.1 Foundation of ActR-Based Biosensor

Initial success using GFP based AraC biosensor in *E. coli* from the Cirino lab (Tang 2008) provided a stable foundation to build a GFP assay based ActR reporter system from. Their dual plasmid system consisted of the repressor (AraC)-expressing construct pPCC423 (AprR , pBR322 origin, *araC*, Ptac1) with *araC* gene controlled by the Ptac promoter, which is inducible by the addition of isopropyl β-D-1-thiolgalactopyranoside (IPTG). The second plasmid was the reporter (GFPuv)-expressing construct pPCC442 (CmrR , RSF1031 origin, *gfpuv*, Pbad) where gene encoding for GFPuv was regulated by the Pbad promoter inducible by L-arabinose using wild-type Arac. pPCC423 and pPCC442 were used to clone the *actR* gene and the Pact promoter containing the reported operator of ActR (Oact)(Tahlan et al. 2007), respectively. The pPCC442 construct was initially designed form an engineered high copy plasmid compatible with commonly used cloning vectors, pDHC29 (Phillips et al. 2000). The Pact promoter was initially synthesized from both ActR based biosensors constructed by Tahlan and coworkers and Tang and coworkers (unpublished). The Oact sequence was from Tahlan and coworkers (Tahlan et al. 2007). The construction of the promoter region was completely produced *in vitro* using a modified overlap extension PCR (oe-PCR) method from Stemmer and coworkers (Stemmer et al. 1995), where they synthesized an entire 1.1-kb nucleotide fragment using oe-PCR with 40 nt primers. Here, two long primers with 20 nt overlap were annealed in a single PCR to form a synthesized promoter. The PCR created a primer containing the Oact operator which was then denatured and implemented as a forward primer for the 2-step amplification of the GFP genes used in this thesis (the first step being the creation of the long primer containing the promoter

region). All primers were designed to have a 20 nt overlap with their pairing sequence, whether that was from another primer or from a gene the primer was amplifying (Table A.1).

3.2 Construction of Dual plasmid ActR Repression System

The large forward primer containing a synthesized ActR promoter (Pact1) amplified the *gfpuv* gene from pPCC442 with another smaller reverse primer annealing to the region of the *gfpuv* gene's stop codon in a single PCR, replacing the Pbad promoter of pPCC442. Genetic elements of each plasmid used in this thesis are listed in Table 3.1. The resulting plasmid, pPCC601 (CmrR , RSF1031 origin, *gfpuv*, Pact1) now would express *gfpuv* constitutively with the lack of ActR repressor in the cell. Since the Pact1 promoter was modeled off of previous work of Tahlan and coworkers (Tahlan et al. 2007), a single nucleotide mutation was incorporated into the promoter, presented in Table 3.2 comparing the promoter components of each reporter plasmid constructed. Based upon a study to determine the effects of the nucleotides in the Oact operator on ActR binding, the mutation made should not affect the binding of ActR (Ahn et al. 2007).

Plasmids	*Gene of Interest*	*Promoter*	*Resistance Marker*	*Origin of Replication*
pDHC29*	n/a	n/a	Cmr	RSF1030
pFG10	*egfp*	Pact3	Cmr	RSF1030
pFG100	*actR*	Ptac2	Apr	pBR322
pPCC423*	*araC*	Ptac1	Apr	pBR322
pPCC442*	*gfpuv*	Pbad	Cmr	RSF1030
pPCC601	*gfpuv*	Pact1	Cmr	RSF1030
pPCC602	*actR*	Ptac1	Apr	pBR322
pPCC607	*gfpuv*	Pact2	Cmr	RSF1030
pPCC610	*egfp*	Pact2	Cmr	RSF1030
pPCC622	n/a	n/a	Apr	pBR322
pWJ290**	*egfp*	Pact*	Kan	pMB1
pYR20**	*actR*	Ptac*	Kan	pMB1

Table 3. 1: List of plasmids used in this thesis.

*-Plasmids obtained from Cirino Laboratory

**-Plasmids obtained from Tang Laboratory

Plasmid	*Promoter*	*Immediate upstream of -35 region*	*-35 region*	*Oact operator*	*-10 region*	*Spacer to the GFP Start codon*
S. coelicolor				CGCGACCACCGTTCCAT		
pOactLux*		TCGAG	TTGACA	CGCGACCACCGTTCCAC	TTATTT	TACCA
pWJ290	Pact	CATTAGGG	TTGACA	CGCGACCACCGTTCCAC	TTATTT	TACCATTAATTAAGAAGGAGATATACC
pPCC601	Pact1	CGGTACCG	TTGACA	CGCGACCACCGTTCCAC	TTATTT	TACCATTAATTAAGAAGGAGATATACC
pPCC607	Pact2	CGGTACCG	TTGACA	CGCGACCACCGTTCCAC	TATAAT	AGATCTGAGGAGGTATACAT
pPCC610	Pact2	CGGTACCG	TTGACA	CGCGACCACCGTTCCAC	TATAAT	AGATCTGAGGAGGTATACAT
pPCC625	Pact2	CATTAGGG	TTGACA	CGCGACCACCGTTCCAC	TTATTT	TACCATTAATTAAGAAGGAGATATACC
pFG10	Pact3	CCGTCGACCGCGACCACCGTTCCAC	TTGACA	CGCGACCACCGTTCCAC	GATACT	GGCACATCAGCAGGACGCACTGACCGA
						GCTCGCTAGCAAGCTTTCGTCGCGAAC
						*CGACGTAAATA AGGAGG TCT*CAT

* - Sequence was taken from Tahlan and coworkers (Tahlan et al. 2007)

-*Italicized* nucleotides indicates seqeunce synthesized from RBS calculator (Salis et al. 2009)

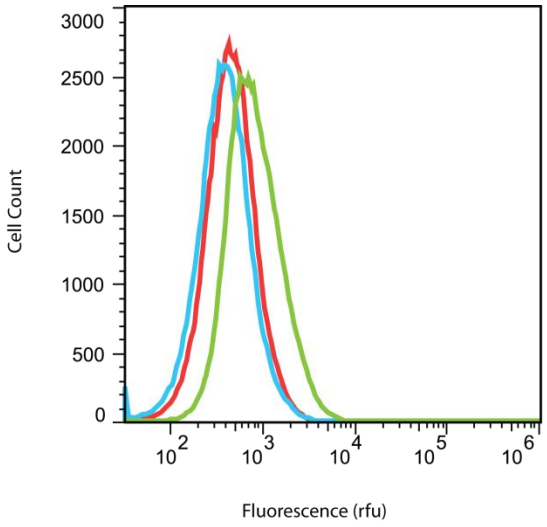
-Underlined nucleotides indicate a mutation from the natural Oact operator

-Pink letters indicate ribosomal binding site

-Red letters indicate the Oact operator

Table 3. 2: A comparison of Pact promoters used in the development of the ActR-based biosensor.

Meanwhile, the ActR producing plasmid was constructed from PCR amplifying the *actR* gene from pYR20 (Kan^R, pMB1 origin, *actR*), provided by Tang and coworkers (unpublished), and cloned into pPCC423 in place of the *araC* gene. The resulting plasmid pPCC602 (Apr^R, pBR322 origin, *actR*, Ptac1) expressed the *actR* gene controlled by the Ptac1 promoter. The Ptac1 promoter contains the operator for the transcriptional repressor LacI, which is constitutively expressed from the same plasmid, therefore the expression of *actR* is inhibited when the plasmid is present in the cell. *actR* expression can then be induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), a well established inducer of the LacI. The IPTG inducible expression of *actR* was incorporated for three reason: (1) there were no previous studies on the toxicity of the ActR protein at high levels of expression; (2) there may be an optimal concentration of ActR in the cell for repression and derepression; (3) the lack of expression results in a control for the repression ("OFF") and the derepression ("ON") of the reporter. Unfortunately as seen in histogram of Fig 3.3, the expression of GFPuv was too low resulting in near indistinguishable ON and OFF peaks after being read on a flow cytometer.

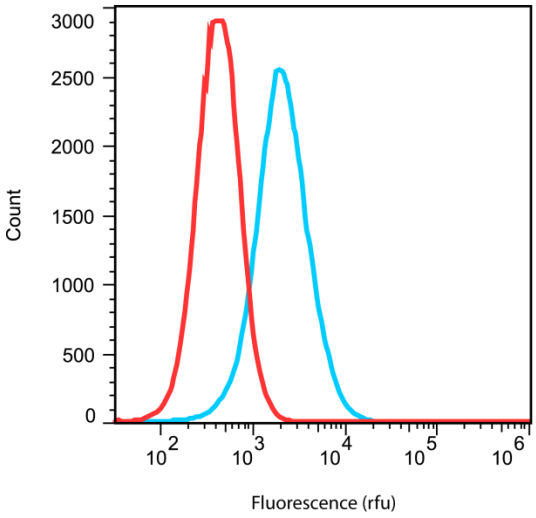


Sample Name	Mean FL (rfu)	CV (%)
GFPuv(Pact1)/Control	1000	81.7
GFPuv(Pact1)/ActR(Ptac1) OFF	474	112
GFPuv(Pact1)/ActR(Ptac1) ON	545	121

Fig 3. 3: Histogram of fluorescent cells comparing the "ON" and "OFF" states of GFPuv expression from the first attempt of the ActR-based biosensor construction. The histogram shows the highly overlapping peak of the "ON", "OFF", and control samples. From this data, the need for an improved system was realized.

Since the AraC project reported FL up to the 10^4 magnitude relative fluorescence units (rfu) expressed from the Pbad controlled *gfpuv* and expression of *gfpuv* from the Pact1 promoter was barely on the 10^3 order of magnitude, engineering of the Pact1 promoter was examined. Several elements in the Pact1 promoter were found to be inconsistent with other reports of a consensus or other strong promoter in *E. coli*. The main elements of a promoter effecting the expression of the gene it is controlling are: (1) the space between the -35 and -10 region; (2) the sequence of the -35 and -10 regions (Mijakovic et al. 2005). Jensen and hammer reported an optimal spacer length between the -35 and -10 regions to be 17 base pairs (bp) (Jensen and Hammer 1998). The Oact operator synthesized in that spacer region was 17 bp so this was not examined any further. The sequence of the -35 region was consistent with the

consensus region reported by Jensen and Hammer, but the -10 region did not resemble a consensus sequence (specifically, the Pribnow box (Pribnow 1975)). The -10 region was therefore altered to the sequence corresponding to a Pribnow box. Also, mutations in the -10 region of the RBS were made to represent a more conserved RBS sequence, so instead of 'TTATTT' the sequence was mutated to 'TATAAT'. The new Pact2 promoter was cloned upstream of the *gfpuv* gene in place of the Pbad promoter pPCC442, producing the plasmid pPCC607 (Cmr^R , RSF1031 origin, *gfpuv*, Pact2). After transforming pPCC607 into *E. coli*, the cells were about five times more fluorescent than cells expressing *gfpuv* from pPCC601 with the Pact1 promoter as seen in Fig 3.4. When the ActR plasmid was co-transformed into the cell, the levels of *gfpuv* expression were similar. Again, distinguishing between the repressed and derepressed peaks was difficult with less than a two-fold decrease in fluorescence when ActR was expressed.



	Sample Name	Mean FL (rfu)	CV (%)
	GFPuv (Pact1)	484	127
	GFPuv (Pact2)	2550	79.2

Fig 3. 4: Histogram of fluorescent cells comparing the expression of GFPuv from the Pact1 promoter and the Pact2 promoter. The shift in the peak representing GFPuv (Pact2) represents an increase of GFPuv expression showing the incorporation Pact2 was successful.

At this time, observations had been made of high levels of FL when pWJ290 was transformed into *E. coli* (each colony was bright green in natural room lighting). The enhanced GFP cloned into the pWJ290 (Kan^R , pMB1 origin, *egfp*, Pact1) plasmid was producing much higher levels of FL than any previous plasmid, but the plasmid conferred kanamycin resistance. The repressor construct, pPCC602, currently being used conferred apramycin resistance, which was determined able to grow in LB media under normal growth conditions containing kanamycin (data not shown). The 3-*N*-acetyltransferase (Apr resistance enzyme) has been shown to confer cross resistance to other aminoglycosides such as kanamycin and neomycin (Davies and Oconnor 1978). The incompatible antibiotic markers lead to two possibilities: (1) design a new plasmid to incorporate the Pact2 promoter in a Cmr^R vector; (2) clone the entire gene and promoter region (Pact1) out of pWJ290 and into a Cmr^R vector. Both options were

explored, but since the previous promoter, Pact2, was able to increase the FL of cells expressing *gfpuv*, the Pact2 promoter and the *egfp* gene were cloned into pDHC29 (vector which pPCC442 was derived) first. The resulting plasmid, pPCC610 (Cmr^R, RSF1031 origin, *egfp*, Pact2) showed almost a 100-fold improvement of FL over the initial GFPuv construct with Pact1. Also, when ActR expression was induced, a 10-fold decrease in fluorescence was observed. A 6-fold decrease in FL was more than both of the other constructs, but the FL was still high for the repressed state. At this time Pact1 controlling EGFP was revisited since the FL of pWJ290 was producing brighter FL colonies on plates than pPCC610, and by doing this the hope was that the gap between the ON and OFF peaks may increase. The region containing *egfp* and Pact1 were digested from pWJ290 and cloned into the pDHC29 vector, thus constructing pPCC625 (Cmr^R, RSF1031 origin, *gfpuv*, Pact1).

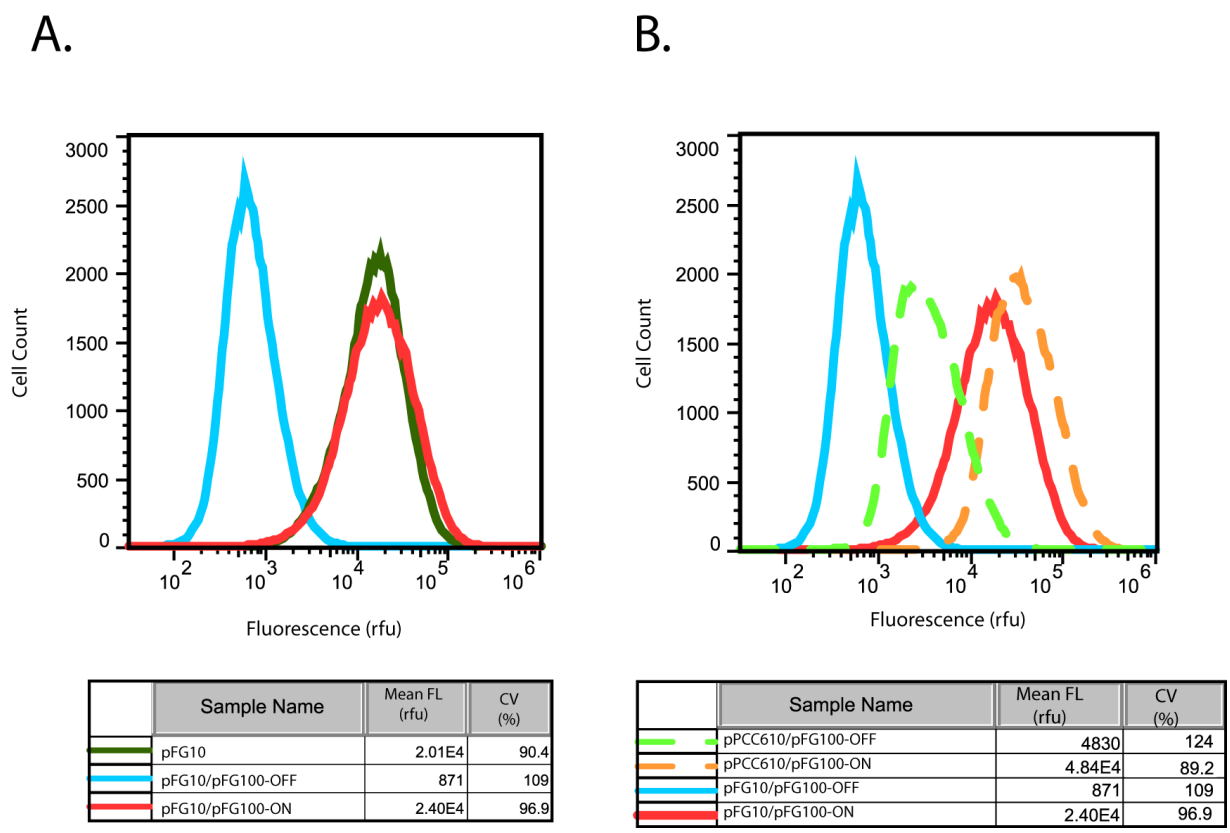
Another approach at promoter construction using a well defined operator sequence was to use multiple operators in the promoter region as reported by Lutz and Bujard where they enhanced the repression of Ptet promoter by TetR 5000-fold (Lutz and Bujard 1997). Based on their findings, a new promoter was synthesized, Pact3. The Oact operator was designed between the -35 and -10 region as before but also immediately upstream of the -35 region. Another alteration in constructing a new plasmid was to incorporate a calculated RBS from Salis and Voigt (Salis et al. 2009), where the translation initiation rate was chosen to be 10^6 on a relative scale. Also the DNA spacer region transcribing the 5'-unstranslated region was lengthened based on the same region reported by Lutz and Bujard. The new construct, pFG10 (Cmr^R, RSF1031 origin, *egfp*, Pact3) alone was 2-fold brighter than the pPCC610 alone. When the ActR plasmid pPCC602 was co-transformed with pFG10, the ON state FL showed about a 5-

fold decrease compared to pPCC610 with pPCC602 in the ON state. The new plasmid, pFG10 was more responsive to the leaky expression of ActR from pPCC602. Also, the repression of EGFP in the OFF state was still only around a 6-fold decrease in FL over the ON state FL.

Concurrently to the construction of pFG10, a new ActR expressing construct was cloned, pFG100 (Apr^R, pBR322 origin, *actR*, Ptac1). pFG100 was constructed from a new modular plasmid of pPCC423, pFG1 (Apr^R, pBR322 origin, *araC*, Ptac2). The pFG100 plasmid was constructed due to the lack of modularity of the pPCC423 plasmid for insertion of other genes to be mutated. The pPCC423 plasmid contained many restriction sites (RS) downstream of the *araC* gene, but was limited to only one NdeI RS, which for cloning genes such as *tetR* gene in place of the *araC* gene there is also a NdeI site in the *tetR* gene. More RS were sought to clone upstream of the start codon of the gene of interest placed into pPCC423. New England Biolabs recently started selling high fidelity versions of common restriction enzymes (RE), so four RSs of the new high fidelity REs conferring single cut digestions within the plasmid were cloned upstream of the -35 region. pFG1, with the new promoter Ptac2, was used to clone in the *actR* gene digested from pPCC602 in place of the *araC* gene found in pFG1. Though the reason is unclear, Pact2 helped reduce the leaky expression of ActR.

When the new pFG100 plasmid was co-transformed with pPCC610, containing the Pact2 promoter and the expression of ActR was induced, a 10-fold decrease in FL was seen from the ON state to the OFF state. The real improvement came from the pFG100 and pFG10 dual plasmid system. When the system was in its ON state, the FL increased 6.6-fold over the pPCC602 and pFG10 system, and in the OFF state the FL only increased 1.4-fold over OFF state of the pPCC602 and pFG10 system. This ended up improving the repression from a 6-fold

decrease in FL to approximately a 28-fold decrease in FL. Clean single peaks arose from the data collected from samples run on the flow cytometer as well. Incorporation of the dual ActR operator into pFG10 was attributed to the improved repression as seen in Fig 3.5. We assumed ActR is binding to both operators, thus making a more efficient repression. Though a modified RBS was also included in pFG10, the overall expression of EGFP was decreased compared to the pPCC610 system. We can assume the decreased amount of EGFP expression was not due to leaky expression of ActR based on the peak overlap of pFG10 and the ON state of pFG10 and pFG100. A final comparison showing the progression of improvement between all reporter constructs is represented in Fig 3.6 as fold repression after 12 hrs. The reporter system was ready for preliminary tests of optimal repression and determining the amount of derepression. The ActR biosensor would then be ready for library creation and screening, but first a reproducible culturing technique had to be developed.



	Sample Name	Mean FL (rfu)	CV (%)
	pFG10	2.01E4	90.4
	pFG10/pFG100-OFF	871	109
	pFG10/pFG100-ON	2.40E4	96.9

	Sample Name	Mean FL (rfu)	CV (%)
	pPCC610/pFG100-OFF	4830	124
	pPCC610/pFG100-ON	4.84E4	89.2
	pFG10/pFG100-OFF	871	109
	pFG10/pFG100-ON	2.40E4	96.9

Fig 3. 5: (A.) Histogram of pFG10 and pFG100 system "ON and "OFF" states compared to pFG10 alone. The peak overlaps of the "ON" state and pFG10 alone shows the leakiness of ActR is minimal. (B) .) Histogram comparing the "ON" and "OFF" states of the final ActR-based biosensor systems. The dashed lines represent the pPCC610 and pFG100 system. The solid lines represent the pFG10 and pFG100 system. Comparing the peak overlaps of "ON" and "OFF" states for the two systems, the pFG10 and pFG100 system show a much greater amount of repression over the pPCC610 and pFG100 system

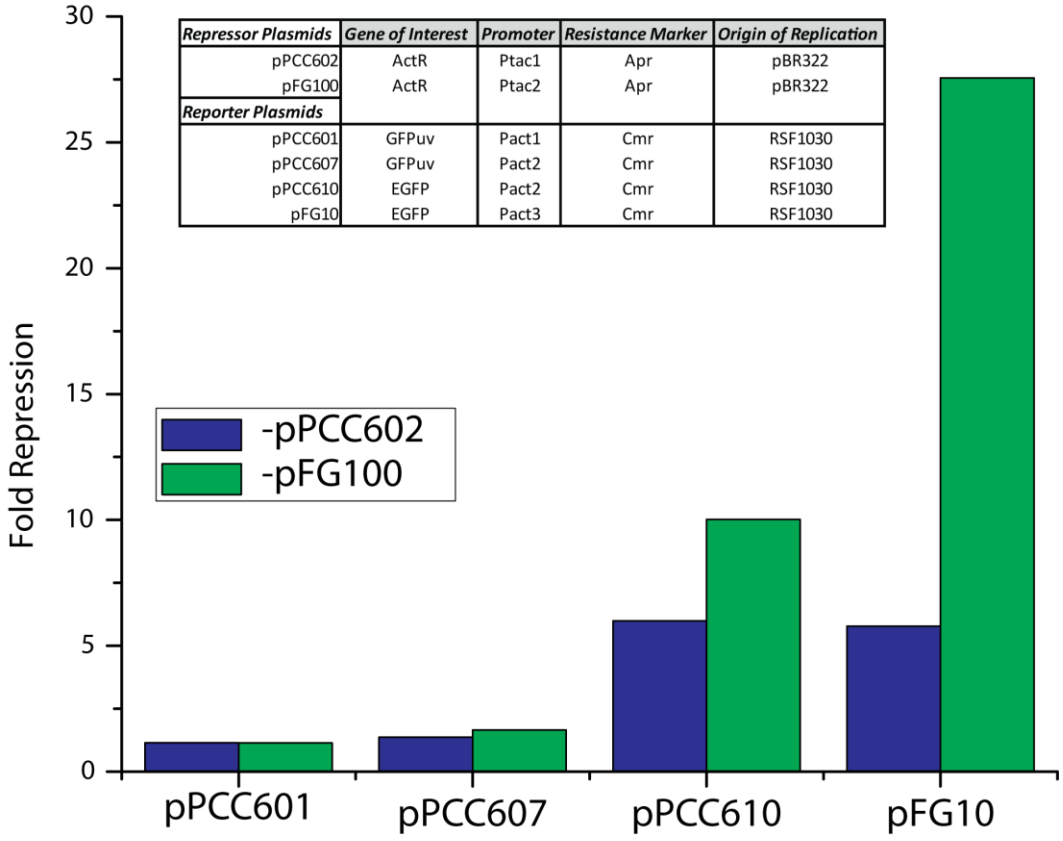


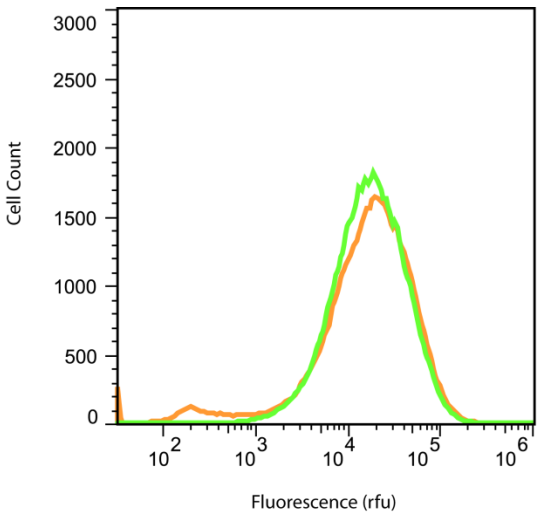
Fig 3. 6: Comparison of Fold repression between all ActR-based biosensor systems. The fold repression was determined by dividing the "ON" state mean fluorescence by the "OFF" state mean fluorescence. The chronological progression of reporter plasmids is represented from left to right. The blue and green bars represent the original and the new plasmid expressing ActR, respectively. The system composed of pFG10 and pFG100 shows about a 30 times better fold repression than the original system, pPCC601 and pPCC602. Each system showed improvement over the prior system.

3.3 Optimizing Fluorescence Measurements and Culturing Conditions

For preliminary experiments, fluorescence data was recorded using a fluorescence plate reader in 96-black well plates, but the fluorescence plate reader was not sensitive enough to detect the subtleties between samples. Reading the samples as a bulk could not show dual populations and other estranged data (data not shown), whereas using a flow cytometer the

fluorescence of individual cells is measured. By measuring individual cell fluorescence, dual populations can be resolved and the system can be better understood

All data of samples measured with a flow cytometer were interpreted from a histogram based on the intensity of relative fluorescence. Once the flow cytometer was incorporated into determining the FL of samples, most samples were showing dual populations, broader peaks, and inconsistent data from the output of the flow cytometer. This motivated work to optimize culture conditions (incubation time, inoculation technique), leading to increased histogram reproducibility. As seen in Fig 3.6, samples were compared during late log phase (12 hours) and late stationary phase (24 hours). Samples after 12 hours of incubation showed sharper single peaks compared to the 24 hour samples, where tailing was starting to occur most likely representing dying or dead cells. Baffled flask cultures would also lead to inconsistent results, thus switching to non-baffled flasks reduced the inconsistencies (data not shown). Also, Starting from an inoculation optical density (600 nm) around 0.1 resulted in inconsistent data from the flow cytometer, showing multiple peaks (up to 4 different peaks sometimes). This was alleviated by inoculating with a single colony from a LB agar plate containing only the respective antibiotics.



	Sample Name	Mean FL (rfu)	CV (%)
	12 hrs- EGFP(Pact3)/ActR(Ptac2)	2.40E4	96.9
	24 hrs- EGFP(Pact3)/ActR(Ptac2)	2.50E4	104

Fig 3. 7: Histogram of fluorescent cells comparing the different culturing times. The green curve represents a sample taken after 12 hours, whereas the orange curve represents a sample of the same culture after 24 hours. The tailing effect seen toward the left of the histogram indicated cell death after 24 hours.

Chapter 4: Controlling ActR Repression

4.1 Induction of ActR Repression

Once a working repressible system was in place, specifically using the dual plasmid system of pFG10 and pFG100, the optimal amount of IPTG induction was determined from a dose repose curve for the maximum amount of repression. The first dose response curve was generated over a small range of IPTG concentration (50 µM, 100 µM, 200 µM, and 400 µM) based upon previous experience, but all concentrations of IPTG tested in the first dose response were almost completely in the OFF state. The second dose response performed used a much broader range of IPTG from 5 µM up to 800 µM. From the second dose response curve shown in Fig 4.1, an optimum level of IPTG was chosen to be either 50 µM or 100 µM. Both concentrations use the least amount of the IPTG compound while still maintaining complete repression.

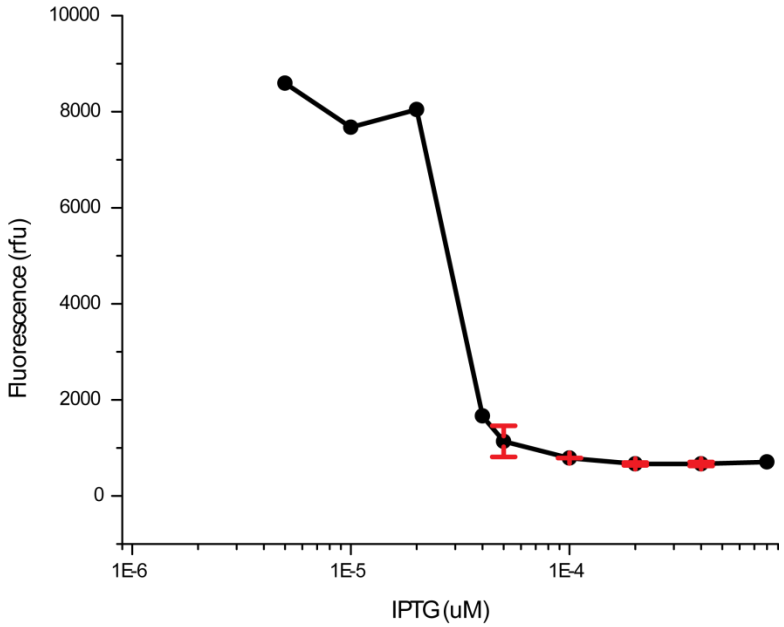


Fig 4. 1: The IPTG dose response of ActR repression based on the relative fluorescence of samples with varying concentrations of IPTG taken after 12 hours of culturing. The dose response showed that almost full ActR repression can occur with small titers of IPTG down to 40 µM.

4.2 Derepression of ActR

Now that an optimum amount of repression was established, the ActR reporter system was ready for testing derepression using reported ligands of ActR. The first and most reproducible ligand was not a purified compound, but the spent media of the actinorhodin producing *S. coelicolor* M145 strain. Several groups had been successful in purifying the compound from the spent media (Bystrykh et al. 1996; Taguchi et al. 2000), but Tahlan and coworkers were able to show derepression using just the spent media (SM) from *S. coelicolor* M145 strain grown in phosphate-limited RG-2 media. They also reported using SM of *S. coelicolor* M145 in an actinorhodin production medium from Taguchi and coworkers (Taguchi et al. 2000), but were unsuccessful in producing derepression with their luciferase reporter. Both SMs of *S. coelicolor* were tested for derepression in E. coli using 20% SM supplementation of

the LB growth media. Neither of the SMs showed any derepression of ActR in E. coli strain MC1061 harboring plasmids pFG10 and pFG100, meaning either the desired inducer small molecules in the *S. coelicolor* medium (γ-actinorhodin and (S)-DNPA) did not interact with ActR, were not crossing the *E. coli* envelope, or the SM medium was not correctly cultured considering the sensitivity of the antibiotic production to the media and culture conditions (Paradkar and Jensen 1995). Since Tahlan and coworkers had success with the phosphate-limited RG-2 medium and the molecules were proven *in vitro* to induce ActR (Tahlan et al. 2007), the SM was assumed to be the lacking the ligands. The derepression with *S. coelicolor* M145 SM has not been pursued any further to this point.

Select molecules from a library of anthraquinones purified at McMaster University High Throughput Screening Laboratory were reported as being recognized by ActR and derepressing it (Tahlan et al. 2008). Of the 20 anthraquinone compounds screened by Tahlan and coworkers, two were chosen based on their high *in vitro* and *in vivo* ActR response. The *in vivo* induction of ActR was shown using *S. coelicolor* M145 instead of *E. coli*, so there was a prior concern of the transport of the molecules across the *E. coli* cell envelope. The two compounds chosen were B8 (3-(acetyloxy)-4-hydroxy-9,10-dioxo-9,10dihydroanthracen-1-yl acetate (B8) and 2-(3-isoquinolyl)-2-(1-nitro-9,10-dioxo-9,10-dihydroanthracen-2-yl) acetonitrile (B6), structural representations of which are shown in Fig 4.2 along with all other compounds added to the cultures. The compounds were suspended in DMSO so that the concentration of DMSO in the culture would not exceed 5% as will be discussed in section 5.3. The first compound tested was B8, but when the compound was added to culture of pFG10/pFG100 containing cells with IPTG

present, fluorescence means and Gaussian distributions were similar to the repressed state shown in Fig 4.3.

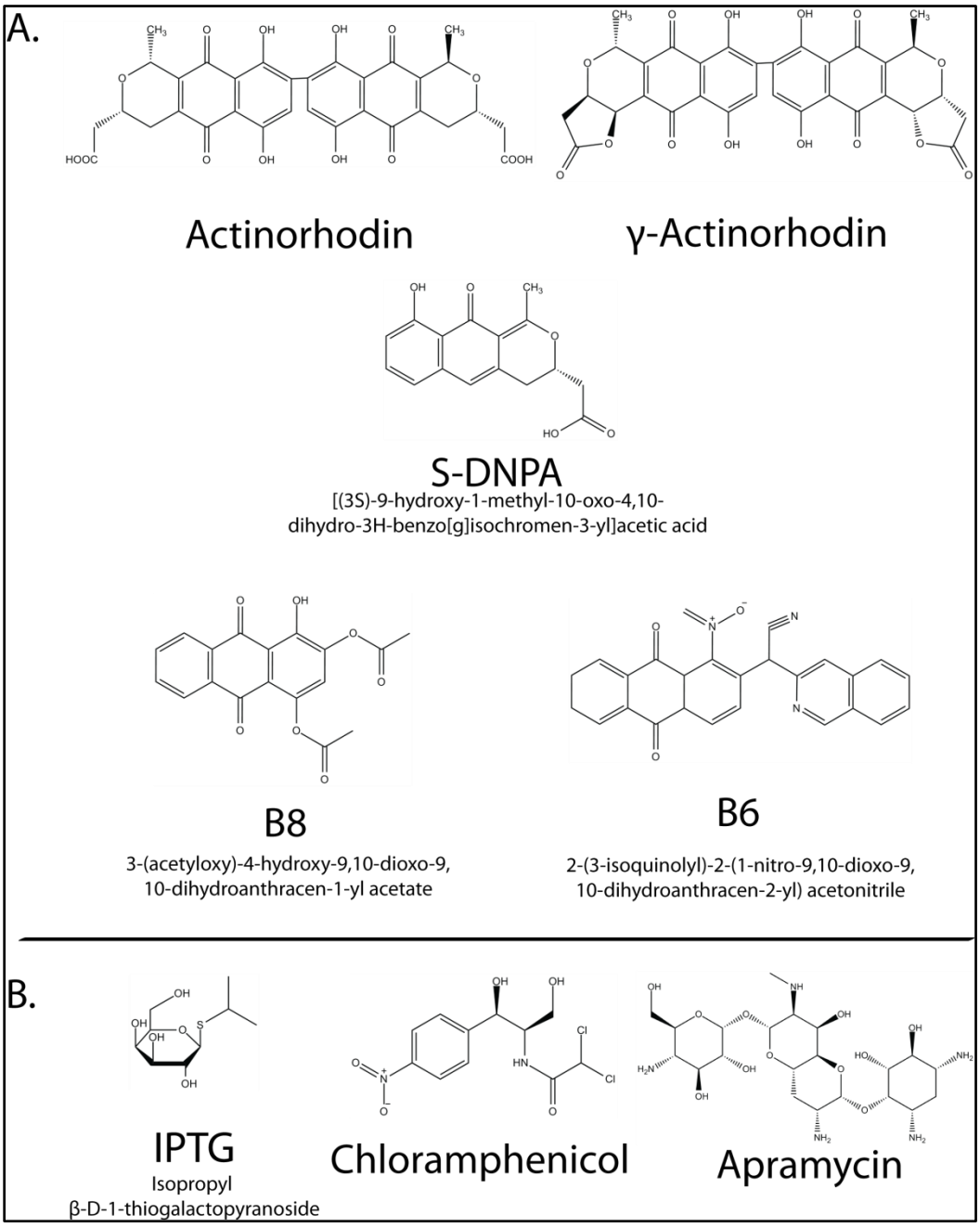


Fig 4. 2: Structures of natural ligands and other reported ligands of ActR along with the structures of other compounds added to the cultures.

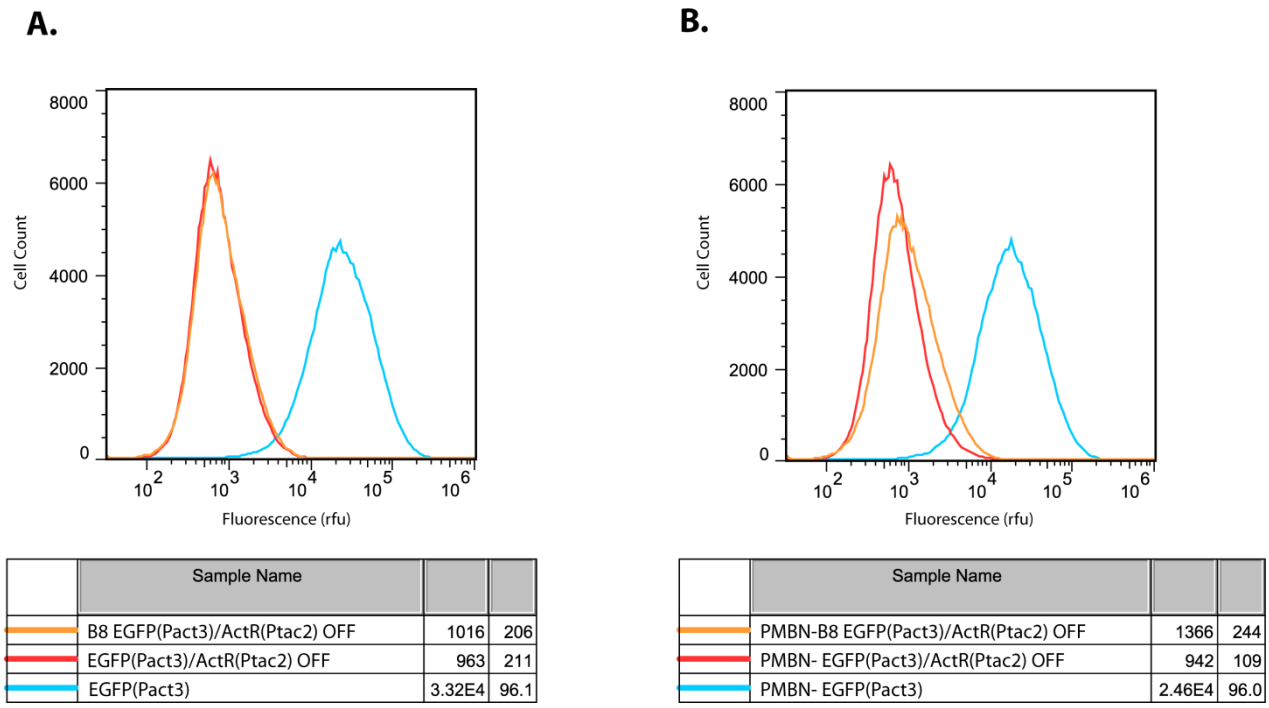
4.3 Delivery of the Ligands into the Cell

The two compounds, B8 and B6, were either not transported across the cell envelope or the compounds were inhibited, altered, or metabolized. As discussed earlier, the apramycin resistance protein, 3-*N*-acetyltransferase confers resistance to a wide range of antibiotics, and the system reported by Tahlan and coworkers does not produce apramycin resistance. Though the apramycin resistance cross talk may be an issue, the transport of the molecules into the cell was further studied. As reviewed by Rachel Chen (Chen 2007), there are many techniques that have been developed and are being developed for increasing the permeability of the *E. coli* without lysing the cell. There is no universal solution to this problem, and relevant parameters influencing success include the relative polarity/hydrophobicity of the compound of interest and concentration and toxicitiy of permeabilizer and ligand.

Several different permeabilizers were examined. Growth curves were initially determined for the effects on increasing concentration of the permeabilizers DMSO, a polar aprotic solvent, and Triton X-100 (Miozzari et al. 1978), a detergent. The growth curves pictured in Fig A.1 of the appendix showed DMSO should be used above a 5% v/v and Triton X-100 should not be used above 10% v/v due to a decrease in growth rates.

The compounds were already dissolved in DMSO, so cultures cells containing the pFG10/pFG100 system were inoculated into a culture with DMSO to determine the permeability of DMSO with the 50 µM B8 compound. The result was negative for derepression of ActR after running on the flow cytometer.

Instead of trying Triton X-100, Polymyxin, a cell membrane disrupting antibiotic, was explored as a permeabilizer. Polymyxin though is quite toxic to cells at low levels , so a less toxic polymyxin B nonapeptide (PMBN) with a reduced affinity for the lipopolysaccharide of the cell membrane of gram-negative bacteria was used (Morris et al. 1995). When the PMBN was added to cultures at a concentration of 5 µg mL^{-1} with 50 µM IPTG and 50 µM B8, the Gaussian peak (+) B8 shifted to the right of the Gaussian peak (-) B8 shown in Fig 4.3. The shift in the peak (+) B8 was reproducible, but the shift decreased with increasing IPTG concentrations (shown in the Appendix). The shift represented a derepression by ActR.



	Sample Name		
	B8 EGFP(Pact3)/ActR(Ptac2) OFF	1016	206
	EGFP(Pact3)/ActR(Ptac2) OFF	963	211
	EGFP(Pact3)	3.32E4	96.1

	Sample Name		
	PMBN-B8 EGFP(Pact3)/ActR(Ptac2) OFF	1366	244
	PMBN- EGFP(Pact3)/ActR(Ptac2) OFF	942	109
	PMBN- EGFP(Pact3)	2.46E4	96.0

Fig 4. 3: Histograms of fluorescent cells comparing the addition of polymyxin B nonapepide (PMBN) to cultures to increase the transport of ligands of ActR across the cell envelope. All cultures contained 50 µM IPTG and were cultured in 3 mL of LB for 12 hours with respective antibiotics. (A.) Histogram representing cultures without PMBN. As can be seen, there was no derepression of ActR when the ligand (50 µM B8) was added to the culture. (B.) Histogram representing cultures containing PMBN. A shift to the right in the presence of the ligand (50 µM B8) indicated a slight derepression of ActR.

To confirm the B8 compound was not itself showing fluorescence activity, samples of the B8 compound were measured for their FL in a 96-well black plate on a fluorescence plate reader and in cultures without ActR where the samples were then run on a flow cytometer. The B8 compound actually showed reduced FL over the background FL of PBS in the 96-black well plates. The histograms from the cytometer data showed similar results, where the peaks (+) B8 and (-) ActR shifted left compared to cultures (-) B8 and (-) ActR, representing a decrease in FL. The other compound selected from the work of Tahlan and coworkers, B6 was tested for inducibility of ActR. Again, adding 50 µM of the B6 compound in cultures with 5 µg mL^{-1} PMBN and 50 µM IPTG resulted in a slight shift to the right of the Gaussian peak of the culture without B6, though the shift was less significant than the B8 shift. Though minor, the derepression of ActR by anthraquinones B6 and B8 has been confirmed, A genetic reporter system for screening repression and depression by ActR or variants therefore has therefore been constructed, although in order to use this system for library screening a stronger induction response is desired and will be further engineered.

Chapter 5: Conclusions and Future Work

5.1 Conclusions

The transcriptional repressor, ActR, has been successfully heterologously expressed in the versatile organism *Escherichia coli* and shown to repress the expression of a GFP reporter gene under control of the Pact promoter containing the Oact operator. Also, the expression of ActR was shown to be tunable by varying the concentration of isopropyl β-D-1-thiogalactopyranoside in the liquid cultures, allowing for optimal ActR concentrations and defining an ON and OFF state of expression of the GFP gene. The Pact promoter was designed and implemented upstream the reporter gene for repression by the binding of ActR, and several genetic elements of the Pact promoter were manipulated bestowing a greater transition between the ON and OFF state. The ActR-based biosensor was able to detect the presence of specific pure reported ligands of ActR endogenously from an exogenous source using the permeabilizer polymyxin B nonapeptide. Without the presence of polymyxin B nonapeptide, no change was observed to indicate derepression of ActR. The ActR-based biosensor is now ready for further analysis of ligand recognition and library screening.

5.2 Future Work

The future work with the ActR-based biosensor has already been extensively studied and preliminary experiments have been done. A strategy was first derived for screening for the polyketide SEK26 molecule, produced endogenously by Zhang and coworkers in *E. coli* (Zhang et al. 2008). The ligand binding domain (LBD) of an ActR monomer can support the binding of up to two molecules of SEK26 based on spatial configurations.

The LBD is devised of two distinct but similar regions aptly named for their location, the proximal region being centralized in the ActR dimer and the distal region being close to the outer extremity of the dimer. The proximal region being linked to hydrogen bonding network of the dimer (Willems et al. 2008) could potentially incur more non-functional mutants in a site-saturated mutagenesis library due to a lose in stability in the hydrogen bonding network than a library of mutants with mutations made in the distal region.

Sites (4) were chosen in the distal region to mutate which would produce a library of approximately 10^6 mutants. From protocols already designed in the Cirino lab for library construction, 10^6 mutants would be trivial to construct through overlap extension PCR. The primers for oe-PCR have already been designed to incorporate the desired mutations of the distal region of the LBD at the DNA level by incorporating four NNS codons translating to amino acid positions V92, N134, G135, G138. Library construction, will be as follows: (1) using 3 sets of primers incorporating the four codon mutations, three DNA fragments (A, B, and C) of the *actR* gene will be amplified by standard PCR methods with a high fidelity polymerase; (2) oe-PCR will be used to anneal the overlap of fragments A and B and B and C, creating two larger

fragments (AB and BC); (3) Fragments AB and BC will be annealed through one more round of oe-PCR creating a large DNA fragment (ABC) encompassing the library of the *actR* gene; (4) Finally, the ABC fragment is cloned back into the original ActR vector. As for screening of the library, a unique technique will be implemented to screen for positive mutants with affinity and selectivity toward SEK26. As mentioned before, mutations were only made in the distal region of the LBD, but according to Willems and coworkers a molecule in both pockets must be present to relive the repression of ActR (Willems,). Screening is going to involve two separate ligands, one being the ligand of interest and the other being a ligand of similar structure but already relieves wild-type ActR repression. Directed evolution of the ActR protein will involve iterative rounds of positive and negative screening with and without ligands, respectively. For all of the rounds of screening, ligands will be delivered exogenously, so the use of polymyxin B nonapetide will be imperative based on the conclusions of this thesis. Once mutants have been screened for responsiveness to SEK26, other polyketides engineered to be produced endogenously by *E. coli* will be selected for screening using the ActR-based biosensor.

Chapter 6: Materials and Methods

6.1 General

Wild-type *E. coli* strains DH5α, *E. coli* B, MC1061 obtained from , and XL-1 obtained from the Yi Tang were used for culturing and/or cloning procedures. *E. coli* strains were maintained on Luria-Bertani (LB) agar plates (per liter: 10 g tryptone, 5 g Yeast extract, 5 g NaCl, and 15 g agar). LB liquid media was prepared with the same concentrations without agar. *Streptomyces coelicolor* strain M145 for actinorhodin (ACT) production was obtained through the John Innes Centre (Norwich NR4 7UH, UK). *Streptomyces coelicolor* strains were maintained on Mannitol-Soy agar plates (per liter tap water: 20 g Mannitol, 20 g Agar, and 20 g Soy Flour). Apramycin sulfate salt (50 μg mL^{-1}) (apr) and Chloramphenicol (25 μg mL^{-1}) (cmr) were added to cultures to maintain plasmids in the cell when necessary. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). Phusion High Fidelity Polymerase was distributed by New England Biolabs and manufactured by Finnzymes (Lafayette, CO) part of Thermo Fisher Scientific. Primers for PCR and sequencing were synthesized by Invitrogen (Carlsbad, CA). Purified ActR ligands were purified at McMaster University High Throughput Screening Laboratory and distributed by Ryan Scientific (Mt. Pleasant, SC). Electrocompetent cells were made based on a protocol in the Sambrook and Russel Molecular Cloning: A

Laboratory Manual (2001). Flow cytometry data was analyzed using FlowJo Software from ©Tree Star, Inc.

6.2 Plasmid Construction

Plasmid maps and primer sequences can be found in the appendix. The construction of each plasmid expressing ActR is as follows: for the construction of pPCC602 (AprR, pBR322 origin*, lacI, actR*) *actR* was amplified from pYR20 (Tang, unpublished) in 5x 100 µL polymerase chain reactions (PCR) using Phusion High Fidelity Polymerase following the manufacturer's guidelines for setup of the reaction and primers actR-for-NdeI and actR-rev-KpnI with the following thermal cycler program using BioRad Thermocycler: (1) 3 min 98 °C, (2) 10 sec 98 °C, (3) 30 sec 40 °C, (4) 20 sec 72 °C (repeat steps (2)-(4) 29 times), (5)1 min 72 °C, and (6) hold at 4 °C. The PCR products were run on a 0.8% agarose gel to verify the reaction, and then the products were combined and cleaned and concentrated using Zymo Clean and Concentrate kit. pPCC423 (AprR, pBR322 origin*, araC*) (Tang 2008) and the resulting nucleotide fragments containing *actR* were digested with NdeI and KpnI restriction enzymes (RE) for 3 hours at 37 °C. The fragments containing *actR* and the pPCC423 vector were gel purified using a 0.8% agarose gel, stained with ethidium bromide (EtBr), and extracted using UV light detection and Zymo Gel Purification Kit. The *actR* insert was ligated into the pPCC423 vector downstream of Ptac in place of *araC* at a 3:1 insert:vector molar ratio using NEB T4 DNA Ligase for 14 hours at 16°C. The ligation products were dialyzed using Millipore dialysis membranes for 30 min. The ligation product (15 µL) was transformed into 70 µL electrocompetent DH5a in a sterile 0.2 cm electroporation cuvette kept on ice until the mixtures were shocked using a BioRad

electroporator. Approximately 800 µL of SOC was added to the shocked cells. The cells were transferred to a 13x100 mm sterile glass culture tube and incubated for 45 min at 37 °C 250 rpm. The cells were then beaded onto agar LB plates containing apramycin (apr) and incubated at 37 °C overnight. The following day, 16 colonies were picked from the overnight plates and struck onto new agar LB plates with apr and incubated again overnight at 37 °C. Each colony was then used to inoculate a 3 mL LB with apr culture in 13x100 mm glass test tubes (overnight). Each culture was grown for 16 hours at 37 °C 250 rpm. Each culture was used to make a miniprep of the plasmid DNA using a Zymo Miniprep Kit. The minipreps were then digested with XhoI for 3 hrs at 37 °C to check that the insert was cloned into the vector, where XhoI would cut both the vector and the insert leaving two distinct bands when run on an agarose gel. After running the digestion products on a 0.8% agarose gel, one sample was correctly digested, so that sample was sent for sequencing at the Penn State DNA facilities using primers 602-for-seq-KpnI and 602-rev-seq-KpnI. The sequencing came back correct without mutations resulting in the verification of the cloning of pPCC602.

For better modulation of the pPCC423 plasmid for use in other libraries other than *araC*, pFG1 (AprR, pBR322 origin*, lacI, araC*) was created based on new high fidelity RE from NEB that show increased specificity and/or reaction rate . pFG1 was cloned (jointly with J.A. Gredell) from pPCC423. Ptac was amplified from pPCC423 in 9x50 µL reactions in order to perform gradient PCR using primers 423mcs4-for and 423mcs-rev and PHFP following the manufacturers guidelines and using the thermal cycler program: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 15 sec 40-60 °C, (4) 30 sec 72 °C (repeat steps (2)-(4) 29 times), (5) 7 min 72 °C, and (6) hold at 4 °C. The optimal annealing temperature was determined to be 58.6 °C, though all of the samples

resulted in proper amplification of the desired nucleotide fragment as determined by electrophoresis using an EtBr stained 0.8% agarose gel. The two samples with the highest concentrations from annealing temperatures of 58.2 °C and 56.2 °C were initially used for further cloning, but too much of the product was lost downstream before ligating. The next five samples from the gradient PCR with the highest concentration of PCR product were used corresponding to annealing temperatures of 60.0 °C, 52.6 °C, 47.6 °C, and 40.0 °C, where there were two samples at 52.6 °C. The samples were run on a 0.8% agarose gel and made visual by MB stain. The samples were combined and extracted using two columns from a Zymo Gel Purification Kit, eluting with 25 µL nanopure water. The two column elutions were combined equaling 46 µL. All 46 µL and a sample of a pPCC423 miniprep were digested using RE NdeI and NcoI for 2 hours at 37 °C. The digestion products of the Ptac were separated using a 1.5% agarose gel to obtain better separation of the smaller fragments. The digestion product of pPCC423 were run on a 0.8% gel for gel purification. The gels were stained with MB and the proper bands were extracted using a Zymo Gel Purification Kit. The Ptac and pPCC423 fragments were verified on 1.5% and 0.8% agarose gels, respectively, and the concentrations were determined based on a predetermined ladder run parallel to the samples on the gels. Ligation of the Ptac insert and pPCC423 vector was setup at a 3:1 insert:vector molar ratio using NEB T4 DNA ligase for 2 hours at 25 °C. The ligation was heat inactivated for 10 min at 60 °C and then dialyzed on Millipore dialysis membranes for 45 min. 2 µL of the remaining 10 µL of the ligation was mixed with 45 µL of electrocompetent MC1061 cells and transferred to a sterile 0.2 cm electroporation cuvette all kept on ice, where the mixtures were shocked using a BioRad electroporator. Approximately 800 µL of prewarmed SOC was added to the shocked cells. The

cells were transferred to a 13x100 mm sterile glass culture tube and incubated at 37 °C 250 rpm for 60 min. The culture was diluted and plated onto agar LB plates containing apr. The transformation efficiencey was determined to be 5.6 x 10^7 cfu ng^{-1} DNA. Four randomly chosen colonies were struck onto a fresh agar LB plate containing apr and simultaneously used to inoculate 3 mL of LB containing Apr in 13x100 mm sterile glass culture tubes. The 3 mL cultures were incubated overnight at 37 °C and then used to make minipreps using a Zymo Miniprep Kit. The samples were verified by digestion analysis using RE BamHI and NotI, digesting for 2 hours at 37 °C. The samples were run on a 0.8% agarose EtBr stained gel, showing all of the samples produced the proper banding in the gel. Two of the samples were sent for sequencing at the Penn State DNA facility to check for the proper sequence without any mutations. The samples were sequenced using primers 423lib-for-NcoI, AraC-rev-4, and 423-mcs-rev. The sequencing results were checked using Invitrogen's VectorNTI ABI software, showing all of the sequencing results were a match without any mutations. The sequence of pFG1 was verified and ready for further manipulation.

From pFG1 and pPCC602, pFG100 (AprR, pBR322 origin*, lacI, actR*) was created (jointly with Jeremy Sargent). Minipreps of pPCC602 and pFG1 made using a Zymo Miniprep Kit were digested with KpnI-HF and NdeI REs for 2 hours at 37 °C resulting in the extraction of an *actR* insert and pBR322 origin vector, respectively. The digested DNA fragments were then gel purified after being run on a 0.8% agarose gel for verification. All of the digestion products were added to the large wells of a 0.8% gel and run for 25 min at 90 V. The gel was stained with EtBr and bands were visible on a UV lamp, and the band containing the vector was cut out of the gel and extracted using the Zymo Gel Purification Kit. A Shimadzu Biospec was used to

measure the concentration of the eluted product. The vector was ligated using a 3:1 insert:vector molar ratio and NEB T4 DNA ligase at 25 °C for 4 hours and was subsequently heat inactivated at 65 °C for 10 min incubated in a BioRad thermocycler. The ligation products were dialyzed on Millipore dialysis membranes for 45 min and 2 µL of the ligation products were mixed with 45 µL of electrocompetent MC1061 cells and transferred to a sterile 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 55 min at 37 °C. Serial dilutions of the culture in fresh prewarmed SOC were made and 100 µL of the dilutions were beaded onto LB agar plates containing apr. The plates were incubated overnight at 37 °C and the colonies were counted the following day. Four of the colonies were randomly selected and struck out onto fresh LB agar plates containing apr. The first two colonies were used to inoculate 3 mL overnights containing apr, which were used to make minipreps using a Zymo Miniprep Kit. The two minipreps were sent for sequencing to verify the deletion of *araC* and the insertion of *actR* at the Penn State DNA Facility using the primers 423lib-for-NcoI and 423lib-rev-XbaI. The sequencing results verified the insertion of *actR* with no mutations, thus the cloning of pFG100 was successful.

The ActR control plasmid, pPCC622 (AprR, pBR322 origin*, lacI*), was constructed from pPCC423. A miniprep of pPCC423 was digested with NcoI and EcoRI for 2.5 hours at 37 °C, cutting out *araC* and the Ptac promoter. The digestion enzymes were heat inactivated at 80 °C for 20 min using a thermocycler. After the heat inactivation, the digestion products' resulting 5'-end overhangs were filled in using NEB Klenow. The reaction was setup according to the manufacturer's instructions and the reaction was incubated at 25 °C for 15 min using a

thermocycler. The DNA fragments were then gel purified after being run on a 0.8% agarose gel. The gel was stained with MB, and the band containing the vector was cut out of the gel and extracted using the Zymo Gel Purification Kit. A Nanodrop ND2000 was used to measure the concentration of the eluted product. The vector was self ligated using NEB T4 DNA ligase at 25 °C for 4 hours and was subsequently heat inactivated at 65 °C for 10 min incubated in a BioRad thermocycler. The ligation products were dialyzed on Millipore dialysis membranes for 20 min and 2 µL of the ligation products were mixed with 45 µL of electrocompetent MC1061 cells and transferred to a sterile 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. Serial dilutions of the culture in fresh prewarmed SOC were made and 100 µL of the dilutions were beaded onto LB agar plates containing apr. The plates were incubated overnight at 37 °C and the colonies were counted the following day resulting in a transformation efficiency of 4.3×10^8 cfu μg^{-1} vector DNA. Four of the colonies were randomly selected and struck out onto fresh LB agar plates containing apr. The first two colonies were used to inoculate 3 mL overnights containing apr, which were used to make minipreps using a Zymo Miniprep Kit. The two minipreps were sent for sequencing to verify the deletion of *araC* and Ptac at the Penn State DNA Facility using the primers 423lib-for-NcoI and AraC-rev-4. The sequencing results verified the deletion with no mutations, thus the cloning of pPCC622 was successful.

The cloning of the ActR reporter plasmids with GFP were constructed as follows: the first reporter plasmid containing the GFPuv gene, pPCC601 (CmrR, RSF1030 origin, GFPuv), was derived from pPCC442 (Tang 2008) and pDHC29. The reported ActR operator, Oact, was fully

designed and constructed *in vitro* using two overlapping primers GFPuv-for-1-KpnI and GFPuv-for-2 synthesized by Invitrogen. The two primers were annealed using PHFP in 10 x 20 µL reactions and the following program in a BioRad thermocylcer: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 30 sec 65 °C, (4) 15 sec 72 °C (repeat steps (2)-(4) 9 times), (5) 1 min 72 °C, and (6) hold at 4 °C. The resulting 84 bp long oligonucletide (50 µL), GFPuv-for-II, along with the manufacturer recommended concentration of GFPuv-rev-ClaI were used as the primers in amplifying the GFPuv gene from pPCC442 in 5 x 100 µL reactions using PHFP in a BioRad thermocylcer and the following program: (1) 3 min 98 °C, (2) 10 sec 98 °C, (3) 30 sec 44.2 °C, (4) 20 sec 72 °C (repeat steps (2)-(4) 29 times), (5) 1 min 72 °C, and (6) hold at 4 °C. PCR products (8 µL) were run on a 0.8% agarose gel, which verified a product was produced. PCR products were clean and concentrated using Zymo Clean and Concentrate Kit. PCR products and a miniprep of pDHC29 were digested using RE ClaI at 37 °C for 3.25 hours. The digestion products were cleaned and concentrated using Zymo Clean and Concentrate Kit, followed by a second digestion using RE KpnI at 37 °C for 3.25 hours. Digestion products were cleaned and concentrated using a Zymo Clean and Concentrate Kit. The GFPuv insert and pDHC29 vector were purified using a 0.8% agarose gel stained with EtBr and visualized using UV light. The insert and vector were extracted from the agarose gel using a Zymo Gel Purification Kit. GFPuv insert and pDHC29 vector were ligated using a 3:1 insert:vector molar ratio with an insert concentration of 380 fmol. NEB T4 DNA Ligase was used for the ligation and the reactions were incubated for 14 hours at 16 °C. Ligation products were dialyzed using Millipore dialysis membranes for 30 min before transforming the ligation (15 µL) into electrocompetent DH5a cells (70 µL). Ligation and cells were mixed together and transferred to a 0.2 cm electroporesis

cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. The cultures were plated onto LB agar plates containing cmr. The following day, 16 colonies were picked from the overnight plates and struck onto new agar LB plates with cmr and incubated again overnight at 37 °C. Each colony was then used to inoculate a 3 mL overnight containing cmr. Each culture was grown for 16 hours at 37 °C 250 rpm and was used to make a miniprep of the plasmid DNA using a Zymo Miniprep Kit. The minipreps were then digested with NcoI for 3 hrs at 37 °C to check that the insert was cloned into the vector, where NcoI would cut both the vector and the insert leaving two distinct bands when run on an agarose gel. After running the digestion products on a 0.8% agarose gel, two samples were correctly digested, so those two samples were sent for sequencing at the Penn State DNA facilities with primers 601-for-seq and 601-rev-seq. The sequencing came back correct for the one sample without mutations resulting in the verification of the cloning of pPCC601.

Since the expression of GFPuv was low from pPCC601 with the synthesized ActR operator, pPCC607 (CmrR, RSF1030 origin, GFPuv) was constructed from pPCC442 and pDHC29. The reported ActR operator, Oact, was fully designed and constructed *in vitro* using two overlapping primers GFPuv-for-KpnI-fwd-C and GFPuv-for-rev-C synthesized by Invitrogen. The two primers were annealed using PHFP in 10 x 20 µL reactions and the following program in a BioRad thermocylcer: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 30 sec 65 °C, (4) 15 sec 72 °C (repeat steps (2)-(4) 9 times), (5) 1 min 72 °C, and (6) hold at 4 °C. The resulting 84 bp long oligonucletide (50 µL), GFPuv-for-KpnI-C, along with the manufacturer recommended concentration of GFPuv-rev-XmaI-B were used as the primers in amplifying the GFPuv gene

from pPCC442 in 10 x 50 µL reactions using PHFP in a BioRad thermocylcer and the following program: (1) 3 min 98 °C, (2) 10 sec 98 °C, (3) 30 sec 44.2 °C, (4) 20 sec 72 °C (repeat steps (2)-(4) 29 times), (5) 1 min 72 °C, and (6) hold at 4 °C. PCR products were clean and concentrated using Zymo Clean and Concentrate Kit. PCR products and a miniprep of pDHC29 were digested using RE KpnI at 37 °C for 3.5 hours. The digestion products were cleaned and concentrated using Zymo Clean and Concentrate Kit, followed by a second digestion using RE XmaI at 37 °C for 4 hours to ensure complete digestion. The GFPuv insert and pDHC29 vector were purified using a 0.8% agarose gel stained with Methylene Blue and visualized in natural light. The insert and vector were extracted from the agarose gel using a Zymo Gel Purification Kit. GFPuv insert and pDHC29 vector were ligated using a 3:1 insert:vector molar ratio with an insert concentration of 300 fmol. NEB T4 DNA Ligase was used for the ligation and the reactions were incubated for 10 hours at 16 °C. Ligation products were dialyzed using Millipore dialysis membranes for 30 min before transforming the ligation (15 µL) into electrocompetent XL-1 cells (70 µL). Ligation and cells were mixed together and transferred to a 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. The cultures were plated onto LB agar plates containing cmr. The following day, 10 colonies were picked form the plate and plated onto two separate cmr containing LB plates using a toothpick. The plates were incubated overnight at 37°C. The next day one of the plates was used to screen for positive colonies showing fluorescence by introducing the plate to a UV lamp. Colonies (3) were picked based on their fluorescence and sent for sequencing using primers

607-for-seq-long and 601-rev-seq-long. The sequencing came back correct for the all sample without mutations resulting in the verification of the cloning of pPCC607.

Since the expression of GFPuv was low from pPCC607 was still low with the re-synthesized ActR promoter Pact2, pPCC610 (CmrR, RSF1030 origin, EGFP) was constructed from pWJ290 (Tang, unpublished) and pDHC29. The reported ActR operator, Oact, was fully designed and constructed *in vitro* using two overlapping primers GFPuv-for-KpnI-fwd-C and EGFP-for-rev synthesized by Invitrogen. The two primers were annealed using PHFP in 4 x 50 µL reactions and the following program in a BioRad thermocylcer: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 30 sec 65 °C, (4) 15 sec 72 °C (repeat steps (2)-(4) 9 times), (5) 1 min 72 °C, and (6) hold at 4 °C. The resulting 84 bp long oligonucletide (50 µL), EGFP-for-KpnI, along with the manufacturer recommended concentration of EGFP-rev-XmaI were used as the primers in amplifying *the egfp* gene from pWJ290 in 5 x 100 µL reactions using PHFP in a BioRad thermocylcer and the following program: (1) 45 sec 98 °C, (2) 15 sec 98 °C, (3) 40 sec 44 °C, (4) 30 sec 72 °C (repeat steps (2)-(4) 29 times), (5) 5 min 72 °C, and (6) hold at 4 °C. PCR products (8 µL) were run on a 0.8% agarose gel, which verified a product was produced. PCR products were clean and concentrated using Zymo Clean and Concentrate Kit. PCR products and a miniprep of pDHC29 were digested using RE KpnI at 37 °C for 3 hours. The digestion products were cleaned and concentrated using Zymo Clean and Concentrate Kit, followed by a second digestion using RE XmaI at 37 °C for 3 hours. Digestion products were cleaned and concentrated using a Zymo Clean and Concentrate Kit. The EGFP insert and pDHC29 vector were purified using a 0.8% agarose gel stained with Methylene Blue and visualized using natural light. The insert and vector were extracted from the agarose gel using a Zymo Gel Purification

Kit. EGFP insert and pDHC29 vector were ligated using a 3:1 insert:vector molar ratio with an insert concentration of 120 fmol. NEB T4 DNA Ligase was used for the ligation and the reactions were incubated for 14 hours at 16 °C. Ligation products were dialyzed using Millipore dialysis membrane for 30 min before transforming the ligation (10 µL) into electrocompetent XL-1 cells (90 µL). Ligation and cells were mixed together and transferred to a 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. The cultures were plated onto LB agar plates containing cmr. The following day, 34 colonies were picked form the plate and plated onto two separate cmr containing LB plates using a toothpick. The plates were incubated overnight at 37°C. The next day one of the plates was used to screen for positive colonies showing fluorescence by introducing the plate to a UV lamp. Colonies (2) were picked based on their fluorescence and sent for sequencing using primers 601-for-seq-long and 601-rev-seq-long. The sequencing came back correct for the all sample without mutations resulting in the verification of the cloning of pPCC6010.

Since the expression of EGFP was much greater from pWJ290 but construct was kanamycin resistant, pPCC625 (CmrR, RSF1030 origin, EGFP) was constructed from pWJ290 and pDHC29. The plasmids pWJ290 and pDHC29 were digested with NotI and BamHI at 37°C for 2 hours. The digestions were subsequently inactivated at 65°C for 20 min. The EGFP insert and pDHC29 vector were purified using a 0.8% agarose gel stained with Methylene Blue and visualized using natural light. The insert and vector were extracted from the agarose gel using a Zymo Gel Purification Kit. EGFP insert and pDHC29 vector were ligated using a 3:1 insert:vector molar ratio. NEB T4 DNA Ligase was used for the ligation and the reactions were incubated for

4 hours at 25 °C followed by inactivation by incubating for 10 min at 65°C. Ligation products were dialyzed using Millipore dialysis membranes for 20 min before transforming the ligation (2 µL) into electrocompetent MC1061 cells (45 µL). Ligation and cells were mixed together and transferred to a 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. The cultures were plated onto LB agar plates containing cmr. The following day, 4 colonies were picked based on their visual fluorescence (without UV lamp) from the overnight plates and struck onto new agar LB plates with cmr and incubated again overnight at 37 °C. The first two samples were sent for sequencing at the Penn State DNA facilities using preimers 601-for-seq-long and 601-rev-se-long. The sequencing came back correct for the one sample without mutations resulting in the verification of the cloning of pPCC625.

Since the expression of EGFP was much greater from pPCC610 but the repression was only 6-fold, pFG10 (CmrR, RSF1030 origin, EGFP) was constructed from pPCC625 and pDHC29. The reported ActR operator, Oact, was fully designed and constructed *in vitro* using two overlapping primers actO-for and actO-rev synthesized by Invitrogen. The two primers were annealed using PHFP in 5 x 100 µL reactions and the following program in a BioRad thermocylcer: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 10 sec 44 °C, (4) 10 sec 72 °C (repeat steps (2)-(4) 9 times), (5) 1 min 72 °C, and (6) hold at 4 °C. The PCR resulted in a 113 bp long oligonucletide, actO-KpnI-HindIII, and three of the samples were combined and cleaned and concentrated using a Zymo Clean and Concentrate kit. EGFP was amplified from pPCC625 using PHFP and primers EGFPrbs-for-HindIII and EGFP-rev-BamHI in 8 x 50 µL reactions with the

following protocol in a Biorad thermocycler: (1) 30 sec 98 °C, (2) 10 sec 98 °C, (3) 30 sec 40-60°C gradient, (4) 20 sec 72 °C (repeat steps (2)-(4) 29 times), (5) 7 min 72 °C, and (6) hold at 4 °C. All PCR products (2 µL) were run on a 0.8% agarose gel, which verified correct products were produced. PCR products were clean and concentrated using Zymo Clean and Concentrate Kit. Of the resulting PCR fragments, two were combined. PCR products and a miniprep of pDHC29 were digested as follows: (1) pDHC29 was digest with KpnI and HindIII; (2) actO-KpnI - HindIII was digested with KpnI-HF; (3) EGFP fragment was digest with HindIII and BamHI. All digestions were setup using manufacturer's recommendations for buffers. The samples were digested for 2 hours at 37°C. The digestion products were cleaned and concentrated using Zymo Clean and Concentrate Kit, followed by a second digestion of the acto-KpnI-HindIII fragment using RE HindIII at 37 °C for 2 hours. Digestion products were cleaned and concentrated using a Zymo Clean and Concentrate Kit. The EGFP digested insert, actO-KpnI-HindIII digested insert, and pDHC29 digested vector were purified using a 0.8% agarose gel stained with Methylene Blue and visualized using natural light. The inserts and vector were extracted from the agarose gel using a Zymo Gel Purification Kit. The inserts and vector were ligated using a 3:3:1 insert(EGFP):insert(actO-KpnI-HindIII):vector(pDHC29) molar ratio with insert concentrations of 117 fmol. NEB T4 DNA Ligase was used for the ligation and the reactions were incubated for 2 hours at 25 °C. The ligation products were heat inactivated at 65°C for 10 min. Ligation products were dialyzed using Millipore dialysis membranes for 30 min before transforming the ligation (2 µL) into glycerol stocked electrocompetent MC1061 cells (45 µL). Ligation and cells were mixed together and transferred to a 0.2 cm electroporesis cuvette all kept on ice. The mixtures were shocked, approximately 800 µL of prewarmed SOC was

added to the shocked mixtures, and the cultures were incubated for 45 min at 37 °C. The cultures were plated onto LB agar plates containing cmr. The following day, 15 colonies were picked from the overnight plates and struck onto new agar LB plates with cmr and incubated again overnight at 37 °C. Each colony was then used to inoculate a 3 mL overnight containing cmr. Each culture was grown for 16 hours at 37 °C 250 rpm and was used to make a miniprep of the plasmid DNA using a Zymo Miniprep Kit. The minipreps were then digested with NheI and NcoI for 2 hrs at 37 °C to check that the inserts were cloned into the vector, where NcoI would cut both the vector and NheI woul cut the insert leaving two distinct bands when run on an agarose gel. After running the digestion products on a 0.8% agarose gel, all samples were correctly digested, so two samples were selected and sent for sequencing at the Penn State DNA facilities using primers 601-for-seq-long and 601-rev-seq-long. The sequencing came back correct for the one sample without mutations resulting in the verification of the cloning of pFG10.

6.3 Crude Actinorhodin Production

Spent media production containing ACT was adapted from Bystrykh and coworkers (Bystrykh, 1996). A single colony of *Streptomyces coelicolor* M145 strain was struck from a MS plate onto a fresh MS plate and incubated for a week at 30°C. Glass beads (~15) were added the plate three separate times and gently rocked until all of the beads were covered with a thin film of spores. The beads were then transferred to a sterile 50 mL VWR centrifuge tube containing 10 mL of Resuspension buffer composed of 0.05 M Tris-HCl (pH 8), 0.001% Triton X-100, and 20% glycerol. Suspended beads were briefly vortexed and incubated at 60°C for 5

min. The suspended beads were then vortexed once more and subsequently, the optical density (450 nm) of the culture was determined. In a 2 L baffled Erlenmeyer flask RG-2 liquid media (200 mL) composed of: 50 mM glucose, 200 mM sucrose, 5 mM K2HPO4, 20 mM KNO3, 80 mM KCl, 4 mM MgSO4, 50 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-KOH, and 0.75 mL liter^{-1} trace elements solution (stock solution: 10 mM ZnCl2, 10 mM FeCl3 · 6H2O, 10 mM CuCl2 · 2H2O) was inoculated with the 9.5 mL of the suspended cell mixture. The culture was incubated for 4 days at 30°C 250 rpm. After 4 days, the culture was dense blue. The culture was then added to 250 mL Nalgene polycarbonate centrifuge tubes and centrifuged for 20 min 3750 rpm. The supernatant (spent media) was sterile filtered through a Nalgene 0.2 µm filter into a sterile Nalgene glass bottle. The SM was directly used in cultures from here but for a more concentrated solution of SM the following steps were taken. The pH of the sterile filtered spent media (SM) was then adjusted to a pH of 3 from 7.36, which produced a fine red precipitate. Chloroform (25% v/v) was added to the SM and vortexed in sterile 50 mL VWR centrifuge tubes. The SM was then centrifuged for 20 min at 3570 rpm. The organic layer was extracted and dried in a vacuum overnight producing approximately 54 mg of dried crude ACT extract. DMSO (5% of the initial culture volume) was added to the crude ACT extract producing a 20x SM solution.

6.4 Flow Cytometry

In general, samples of cultures were either diluted 1:4 or 1:10 in 100 mM phosphate buffered saline (Sambrook and Russel) in a final volume of 1 mL. The samples were then transferred to a sampling tube and measured using FC500 Benchtop Cytometer or Coulter XL-

MCL at the Huck Institute (Life Sciences, University Park, PA). The cytometer was programmed to read 100,000 events. The flow cytometer provided side scatter data (cell complexity), forward scatter data (cell size), and relative fluorescence data (relative GFP protein concentration) of individual cells.

Samples specifically for running on the flow cytometer were made up using freshly transformed cells on agar containing LB plates. Single colonies from a plate were picked with a sterile wood stick and used to inoculate 3 mL of liquid LB containing the appropriate antibiotic(s) and other compounds specific to the experiment. The cultures were incubated for 12 hours at 37°C 250 rpm. After 12 hours, the samples were prepared for the flow cytometer as described above.

6.5 Protein Detection

Proteins expression was determined by SDS-PAGE gel electroporesis. A 1 mL aliquot of the specific culture was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in Tris-EDTA (pH8) to a final optical density (600 nm) of 20. In a new microcentrifuge tube, a 50 µL aliquot of the resuspension and 50 µL of SDS buffer (v/v: 85% Tris-EDTA, 5% SDS, 10% β-mercaptoethanol (BME)) were mixed together. The samples were then submerged in boiling water for 5 min. The samples were then centrifuged for 5 min and the supernatant was transferred to a new microcentrifuge tube. In nanopure water, 1:10 dilutions of the supernatant were made. A 5 µL aliquot of the 1:10 lysate dilution was mixed with 10 µL SDS-loading buffer (v/v: 5% BME and 95% SDS protein dye). The samples were submerged in boiling water for 5 min and the entire

sample was loaded on a 10% stacking/20% resolving polyacrylamide gel. The gel was then run for 20 hrs at 50 V in a BioRad SDS-PAGE gel electroporation box. The box was kept in a ice bath overnight to prevent overheating. After 20 hrs, the gels were extracted from their casts and stained with BioRad Coomasie Stain for 1 hour. The stain was rinsed away with two washes with DI water. The gel was left in DI water until bands were visible and then imaged using a standard scanner. The image was enhanced using Adobe Photoshop to observe difficult to see bands (Note: images in no way were used for quantitative measurements).

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Appendix

Table A. 1: List of primers

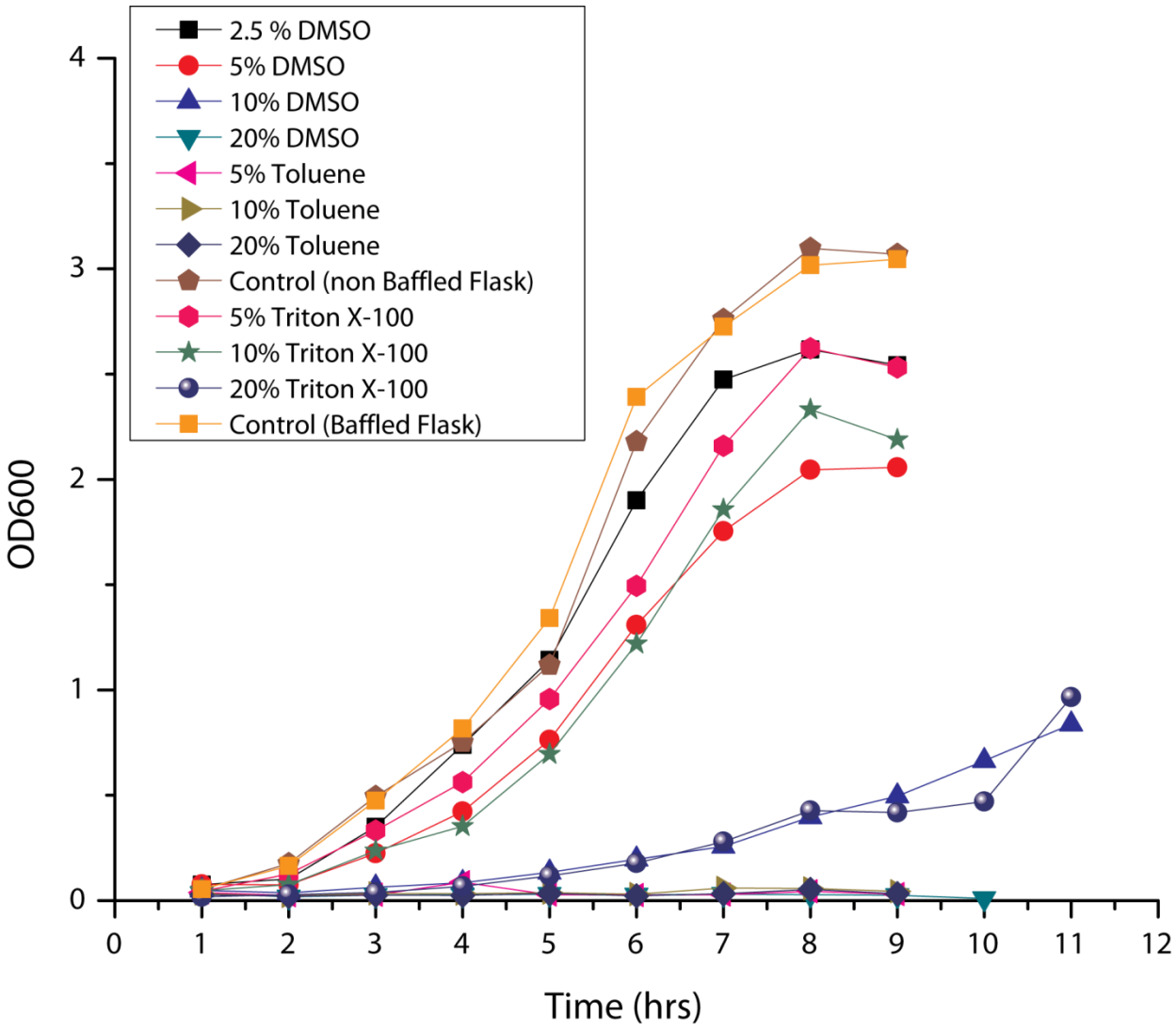
Primer name	*Sequence (5'-3')*
423lib-for-NcoI	GGC GCT ATC ATG CCA TAC CG
423mcs4-for	GCT AGG CCA TGG GAA TTC GCT AGC GCG GCC GCG AGC TGT TGA CAA TTA ATC A
423mcs-rev	GTG ATA CCA TTC GCG AGC CT
601-for-seq	GCG CGT AAT ACG ACT CAC TA
601-for-seq-long	GGA TGT GCT GCA AGG CGA TTA AG
601-rev-seq	TGT TGT ATG CTC GGC CTT CG
601-rev-seq-long	CGG CCG CTC TAG AAC TAG TGG AT
602-for-seq-KpnI	TGC GCC GAC ATC ATA ACG GT
602-rev-seq-KpnI	CGC GGT ACC TTG CCG CCA GTG TGC TGG AAT TCA
607-for-seq-long	AAC GTG GAC TCC AAC GTC AA
actO-for	GAT CGA GGT ACC GTC GAC CGC GAC CAC CGT TCC ACT TGA CAC GCG ACC ACC GTT CCA CGA TAC TGG CAC ATC
actO-KpnI-HindIII*	GAT CGA GGT ACC GTC GAC CGC GAC CAC CGT TCC ACT TGA CAC GCG ACC ACC GTT CCA CGA TAC TGG CAC ATC AGC AGG ACG CAC TGA CCG AGC TCG CTA GCA AGC TTA CTG GA

Primer name	*Sequence (5'-3')*
actO-rev	GTG AAC TGT GCG CTG GTG GCA AGG TGC TAT GAC CGA TCG TTC GAA TGA CCT
actR-for-NdeI	GTC AGT CTG AGG ATC GCA TAT GTC GCG AAG CGA GGA AGG
ActR-N134G135G138-comp-for	ATG GAG CGC ACC ATG AAC CT
ActR-N134G135G138-rev	AGG TTC ATG GTG CGC TCC ATS NNC ACC ATS NNS NNG GGT CCC AG
actR-rev-KpnIp	ATG CAT GAG CTG TCA TGA GTC CGC GGG AGG CG
AraC-rev-4	ATT GCT GTC TGC CAG GTG ATC
EGFP-for-KpnI*	GTG CGG TAC CGT TGA CAC GCG ACC ACC GTT CCA CTA TAA TAG ATC TGA GGA GGT ATA CAT ATG GGC AGC AGC CAT CAT CA
EGFP-for-rev	TGA TGA TGG CTG CTG CCC ATA TGT ATA CCT CCT CAG ATC TAT TAT AGT GGA ACG GT
EGFPrbs-for-HindIII	GTA CGT AAG CTT TCG TCG CGA ACC GAC GTA AAT AAG GAG GTC TCA TAT GGG CAG CAG CCA TCA TCA

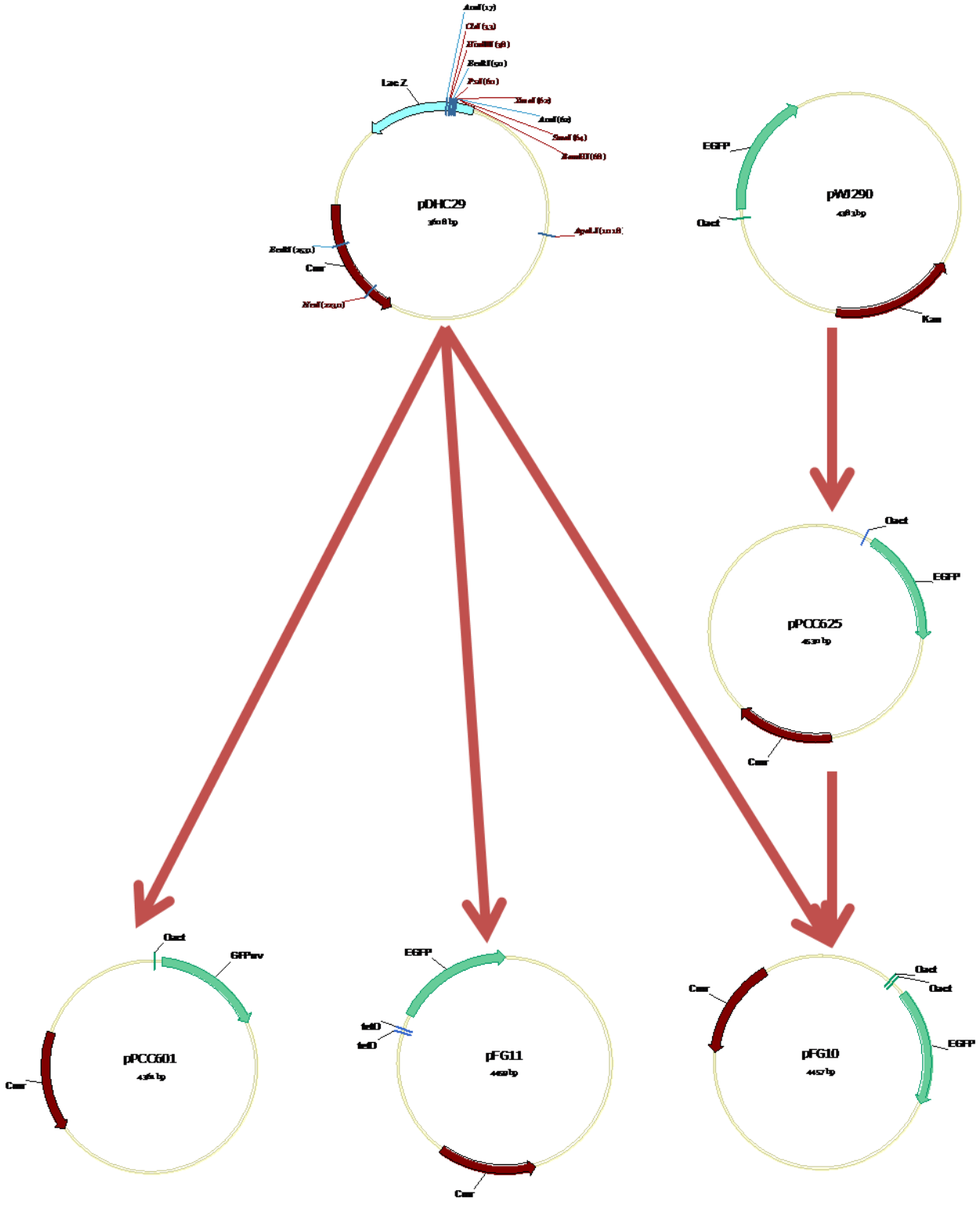
Primer name	*Sequence (5'-3')*
EGFP-rev-BamHI	GTA CGT GGA TCC TCA CTC GTA CAG CTC GTC CA
EGFP-rev-XmaI	AGC ACT CGC CCG GGC GCC GAA TCC TCA CTC GTA CAG CTC GTC CA
GFPuv-for-1-KpnI	GCG GGT ACC GTT GAC ACG CGA CCA CCG TTC CAC TTA TTT TAC CAT TAA TTA
GFPuv-for-2	TTC TTC TCC TTT GCT AGC CAT GGT ATA TCT CCT TCT TAA TTA ATG GTA AAA TAA G
GFPuv-for-II*	GTG CGG TAC CGT TGA CAC GCG ACC ACC GTT CCA CTA TAA TAG ATC TGA GGA GGT ATA CAT ATG GCT AGC
GFPuv-for-KpnI-C*	GCG GGT ACC GTT GAC ACG CGA CCA CCG TTC CAC TTA TTT TAC CAT TAA TTA AGA AGG AGA TAT ACC ATG GCT AGC AAA GGA GAA GAA
GFPuv-for-KpnI-fwd-C	GTG CGG TAC CGT TGA CAC GCG ACC ACC GTT CCA CTA TAA TAG A
GFPuv-for-rev-C	GCT AGC CAT ATG TAT ACC TCC TCA GAT CTT ATA ATG TGG AAC GGT

Primer name	***Sequence (5'-3')***
GFPuv-rev-ClaI	CGC ATC GAT TTA TTT GTA GAG CTC ATC CA
GFPuv-rev-XmaI-B	CTC GCC CGG GCG CCG AAT CCT TCA TTA TTT GTA GAG CTC A

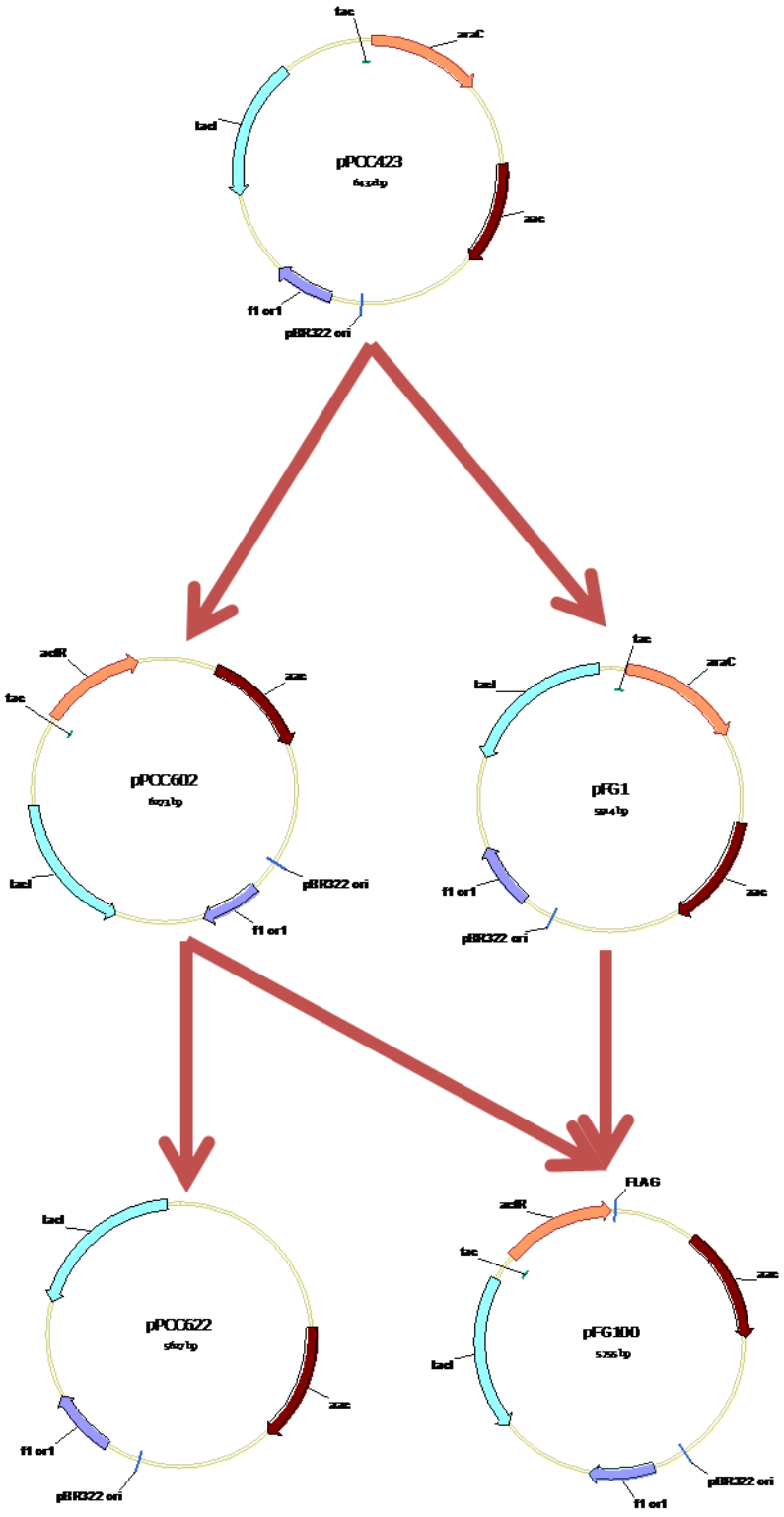
*-Synthesized from oe-PCR



A. 1: Growth curves of DH5a grown in 30 mL LB with permeabilizers. The permeabilizers tested were dimethyl sulfoxide (DMSO), toluene, and Tritin X-100. The DMSO was severely toxic to the cell above 10%, toluene severely inhibited growth for all concentrations, and Triton X-100 was severely toxic above 10%.



A. 2: Flow chart of reporter vector maps, starting with the parent vectors at the top and their derivatives below.



A. 3: Flow chart of repressor vector maps of the reporter constructs starting with the parent vector at the top its derivatives below.