ENGINEERING OF *STREPTOMYCES* TO ENHANCE ANTIBIOTIC PRODUCTION

ENHANCING ANTIBIOTIC PRODUCTION THROUGH THE GENETIC ENGINEERING OF *STREPTOMYCES* SPECIES

By

PATRICIA PAK, BSc

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AUTHOR: Patricia Pak, B.Sc. (McMaster University)

SUPERVISOR: Professor Marie A. Elliot

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ABSTRACT

Antibiotics have a prominent role in the human healthcare system. With natural sources, such as bacteria, acting as the main source of antibiotics, it is no wonder that the actinomycete bacterium *Streptomyces coelicolor A3(2)* is so prominent in this field of research. Although none of the four known antibiotics it produces have any clinical use, there is a wealth of genetic information and tools available for this model *Streptomyces* (producers of over two-thirds of the world's antibiotics).

In recent years, there has been a gradual decline in the discovery of new antimicrobial drugs despite the rising need to combat increasingly resistant strains of bacteria. As such, my work focused on investigating the various methods of antibiotic overproduction available through genetic manipulations. Phenotypic analysis, antibiotic assays, and RT-PCR demonstrated the effectiveness of the *ermE** promoter from *Saccharopolyspora erythraea*, in conjunction with the *tuf1* ribosome-binding site, in the overexpression of the *atrA* gene in *S. lividans*. This provided support for the incorporation of both these regulatory elements in an effective heterologous overexpression vector for *Streptomyces*.

Overexpression of regulator genes as a method of stimulating increased and/or novel antimicrobial compounds is a common endeavor. Here, we investigated the effectiveness of expressing *S. coelicolor* genes in the Streptomyces wild isolate, Cu#39. Additionally, as a tool for antibiotic research, we created a *S. coelicolor* strain constructed to direct its metabolic resources towards a designated metabolite. By eliminating select endogenous secondary metabolites, this strain holds the potential of serving as a host for increased yields of heterologous molecule production. In these three projects, I explored the use of *Streptomyces* as a reservoir for the identification of new antibiotics.

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ABBREVIATIONS

Act: actinorhodin

BSL: Biosafety level

DNA: deoxyribonucleic acid

DNA (medium): Difco nutrient agar

dNTPS: deoxynucleotide triphosphates

DTT: dithiothreitol

LC/MS: liquid-chromatography/mass spectrometry

RBS: ribosome-binding site

Red: undecylprodigiosin

RNA: ribonucleic acid

RT-PCR: reverse-transcription polymerase chain reaction

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INTRODUCTION TO THESIS

Antibiotics and origins in soil bacteria

The dynamic soil environment is possibly one of the most complex environments in which an organism must survive. From fluctuating physical surroundings, to neighbouring bacterial competitors for a limited nutrient pool, this may account for the fact that some of these microorganism inhabitants are among the bacteria with the largest genomes (Bentley *et al.,* 2002; Challis and Hopwood, 2003; Ohnishi *et al.,* 2008). In the evolutionary struggle for survival against other bacterial species, the mightiest weapon can be the ability to produce secondary metabolites (in the form of antimicrobial substances) as a defence system. In addition to this prevailing view of antibiotic use, other functions have also been examined, including that of signaling molecules (Yim *et al.,* 2006).

The production of secondary metabolites, nonessential compounds for life derived from the products of primary metabolism, is a characteristic shared by taxa as diverse as animals, fungi, and bacteria (Reichenbach, 2001). Of the many bioactive compounds that can be harvested from these sources, antibiotics are among the most significant to humans. Soil bacteria belonging to the *Streptomyces* genus have provided humans with the majority of antibiotics in current use, along with other compounds used in such wide-ranging applications as medicine and agriculture (Baltz, 1998). Approximately half of reported bacterial secondary metabolites have come from the actinomycetes, of which 80% are derived from *Streptomyces* (Berdy, 2005). With over 8000 compounds identified to date, actinomycetes produces over five fold more than that of the next most productive bacterial genus, *Bacillus* (Talà *et al.,* 2009; Weissman *et al.,* 2009).

Streptomyces coelicolor as a model organism

As the model of the genus known for its complex multi-cellular life cycle, *Streptomyces coelicolor* A3(2) has been studied extensively for its potential to reveal insights into antibiotic production. Along with the fact that it has an arsenal of genetic tools and a fully sequenced genome (Bentley *et al.,* 2002), it also produces at least four different antibiotics: actinorhodin (Wright and Hopwood, 1976), undecylprodigiosin (Rudd and Hopwood, 1976), methylenomycin (Wright and Hopwood, 1976), and the calcium-dependent antibiotic (CDA) (Lakey *et al.,* 1976). Of these known antibiotics, two are conveniently pigmented for easy visualization and phenotypic analyses, as actinorhodin appears blue and undecylprodigiosin appears red (Bibb, 1996). Even though *S. coelicolor* is known to produce these four antibiotics, its genome in 2002 was revealed to code for at least twenty different secondary metabolite clusters (Bentley, 2002), suggesting a

discrepancy between its productivity and its genetic capability. As the presence of these "cryptic" clusters are ubiquitous in all other *Streptomyces* genomes currently available, these organisms represent a lucrative reservoir for antibiotics that have yet to be discovered.

Atypical to most bacteria, *S. coelicolor* progresses through several cellular forms in its complex, multicellular life cycle. Starting life as a single spore, this spore germinates and differentiates into a branched, substrate mycelium upon reaching a solid medium conducive to growth. Differentiation into aerial mycelia structures is eventually triggered, and it is on these spore-bearing structures that one round of the life cycle is complete (Chater, 2006). Secondary metabolism is developmentally regulated, and dependent on the growth environment. On solid agar, antibiotic production is initiated around the time of morphological differentiation (the transition from substrate mycelia into aerial mycelia), while in liquid culture, production is coordinated to approximately the entry into stationary phase (Gramajo *et al.,* 1993; Takano *et al.,* 1992). Whether or not this is determined even in part to nutritional deprivation and/or competition – a popular theory – has yet to be conclusively proven (Bibb, 1996; Chater, 2006).

Antibiotic discovery in the past

Every year, over 60 000 Americans die from resistant strains of bacteria acquired during hospital stays – this reality is further underscored by the fact that novel antibiotics are now over 50% less common than even a few decades ago (von Bubnoff, 2006). With the gradual decrease in the appearance of novel antimicrobial compounds in direct opposition to the rising emergence of antibiotic-resistant strains of pathogens, it has become that much more important to reinvest in the stalled search for new antibiotic compounds (Clardy *et al.,* 2006). Antibiotics are obtained from two main sources, those that are synthetic (man-made), and those obtained from natural sources. An example of the latter would be the first naturally-derived antibiotic penicillin, from the fungus *Penicillium notatum*. However, it was only after the discovery of the first bacterial-derived antibiotic in the 1940's that bacterial mining exploded, and the so-called golden era of antibiotic discovery, from approximately the 1940s-1970s, was founded (Knight *et al.,* 2003; Watve *et al.,* 2001).

What no one accounted for was the ease at which target pathogens would be able to accumulate resistance to antibiotics, resistance that was either innate (intrinsic) or gained (acquired) (Nguyen and Thompson, 2006). Due to the variable, and frustratingly inevitable, methods in which bacteria lose susceptibility to an antibiotic, there is a sense that humans are falling behind in the evolutionary arms race of antibiotic treatment versus bacterial resistance (Davies, 1994). Scientists are only now starting to unravel the extent of how easily one mutation or acquisition of a transferable element can render an antibiotic useless (Davies, 1994; Nguyen and Thompson, 2006). In a landmark

study, researchers at McMaster University demonstrated that bacteria isolated from diverse soil types were resistant on average to at least seven or eight of the twenty-one tested antibiotics (D'Costa *et al,* 2006). As this included drugs not yet in clinical rotations and thus prior exposure was not possible, this served to emphasize the ease at which a bacterium may be able to access or acquire drug resistance.

Contributing to this worry is that in the search for new biologically active compounds, laboratories were finding the same chemicals over and over again, suggesting that the easiest compounds to discover were being exhausted (von Bubnoff, 2006). In one approach to antibiotic production, scientists have recently focused their efforts on modifying the chemical structures of existing antibiotics, creating generations of so-called "me-too" drugs which remain similar enough to the original drug that resistant bacteria are able to easily adapt to the new incarnations (von Bubnoff, 2006; Walsh, 2000). As this is not a true solution to the problem of a lack of new drugs, there is an increasing realization that science must return to the original source of antibiotics, producer organisms with millions of years of evolutionary time to perfect their molecular weapons. As pointed out succinctly by scientist Richard H. Baltz, actinomycetes have been evolving in nature for close to a billion years, while we have only been capable of antibiotic production for approximately twenty five years (Baltz, 2008).

It has been estimated that less than 1% of soil bacteria have been able to be cultivated in laboratory environments; in consideration with the fact that over one thousand species of bacteria are expected in a gram of soil (von Bubnoff, 2006), and the extremely narrow range of the earth's soil that has been accessed (Baltz, 2005), it is no wonder that the idea of a wealth of secondary metabolites just waiting to be discovered is widely acknowledged. With the advent of technology in recent years, such as genome sequencing and metagenomics, scientists are beginning to view natural sources of antibiotics as a viable option once more. As the financial risks for the development of an antimicrobial product are much higher than that of a long-term drug for a chronically-ill population, pharmaceutical companies must be convinced of this necessary shift in research direction in order for this market to be viable.

The genetics of antibiotic production

Antibiotics are produced through complex, multi-step pathways, the genes for which are encoded in large biosynthetic gene clusters which may include the regulators, producers, resistance genes, and transporters of a particular product (Bibb, 1996; Fernandez-Moreno *et al.,* 1992). In general, these complex molecules are under the control of three recognized levels of regulation: (1) overarching regulators that have roles in both antibiotic production and morphological differentiation, (2) global regulators involved in the production of more than one antibiotic, and (3) pathway-specific regulators that affect only a

single antibiotic (Arias *et al.*, 1999). As one of the most rigorously studied antibiotic-producers, many *S. coelicolor* genes have been implicated in the production of its various antibiotics, as listed elsewhere (McKenzie and Nodwell, 2007) .

In recent years, a variety of innovative techniques have been developed by various groups for the upregulation of antibiotic production, such as introducing multiple drug resistant mutations as a form of ribosome engineering (Wang *et al.,* 2008), using mutant polymerases known to trigger unregulated activation of antibiotics (Talà *et al.,* 2009*)*, and using decoy oligonucleotides to disrupt normal antibiotic regulation (MacArthur and Bibb, 2008). There is no question fresh approaches to optimizing metabolite production are necessary (as reviewed in Olano *et al.,* 2008), and this has defined the direction of my projects, as described in the following sections. Metabolic engineering – defined loosely as the use of genetic manipulations to increase desired metabolic production (Olano *et al.,* 2008) – in various forms is the approach I have taken.

As has been suggested (Baltz, 2008), the complexity and scope of resources required to successfully overcome the current barriers in antibiotic research requires the input and collaboration of many, thus my projects were completed in conjunction with the Nodwell Lab in the Department of Biochemistry and Biomedical Sciences, as well as a biotechnology company based in Hamilton, JNE Biotech Inc.

CHAPTER 1: MAXIMIZING EXPRESSION IN A STREPTOMYCIN OVEREXPRESSION CONSTRUCT

1.1 Introduction

A fascinating idea is that of self-resistance in an antibiotic-producing organism. There are many ways in which a bacterium can protect itself against its own antibiotic, such as the modification of a target site, antibiotic inactivation, and use of efflux pumps to prevent dangerous intracellular levels of a toxic chemical (for a review, see Cundliffe, 1989). Dissemination of these traits form the basis of acquired antibiotic resistance in other species (Davies, 1994; D'Costa *et al.,* 2006).

It has been the last few years in which the idea of a link between antibiotic production and resistance has really taken off (Nodwell, 2007). In *S. coelicolor,* production of the blue-pigmented actinorhodin (Act) has been aided by the presence of an efficient exportation system that triggers the release of actinorhodin from the cell (Tahlan, 2007). As described in the paper by Tahlan and coworkers (2007), this proposed mechanism is similar to that of the well-characterized TetR and TetA tetracycline system, in which the expression of *tetA* is only possible in the presence of tetracycline, as this antibiotic ligand binds to and releases the repression caused by TetR binding. As *tetA* initiates production of a tetracycline exporter, this results in antibiotic resistance, as it is pumped from the cell before it has had a chance to act.

Similarly, an ActR gene has been identified in the biosynthetic cluster of actinorhodin that is responsible for the repression of antibiotic exporter production – a move that ensures the exporter is only created when antibiotic presence in the cell is imminent (Tahlan *et al.,* 2007). In this case, the ligands responsible for relieving repression are actinorhodin and several biosynthetic intermediates; because the intermediates appear to be more efficient, this suggests that this system has been designed to ensure the availability of exporters before the completed antibiotic appears, and becomes potentially dangerous. As this is not the only example of the use of intermediates or a product of an antibiotic pathway having a role in the regulation of the biosynthetic cluster, a link is drawn between the biosynthesis and resistance of an antibiotic within the same organism (Tung *et al.,* 2009; Wang *et al.,* 2009).

This led to the intriging hypothesis that antibiotic regulation may be increased by a forced upregulation in antibiotic efflux. If there is a cellular signal that microbes receive alerting them to stop antibiotic production due to approximity to a threshold, continuously ridding the antibiotic may cause the microbe to continuously produce the desired antibiotic. Here, the optimization of streptomycin production by natural producer *Streptomyces griseus* was

investigated; this study was completed in the context of determining elements of an overexpression construct that would maximize expression of a desired product.

Antibiotic self-resistance, transcription, and translation efficiency were all considerations in the creation of this vector. Given the growing regulatory link between all aspects of the antibiotic production pathway, we chose to overexpress streptomycin resistance genes (*strV* and *strW)* simultaneously with the biosynthetic activator (*strR).* As far as we know, overexpression of all three genes has not been attempted previously.

Elements of the streptomycin transport system and the activator were cloned behind the *melC* promoter in pSET152. Rather than using their native ribosome-binding sites, translational efficiency was addressed by incorporating the RBS of the highly expressed *tufl* gene. An idealized overexpression construct containing these elements, along with three vectors that isolated the effects of overexpressing these elements separately, was created and introduced into *S. griseus.* If they were able to function in a synergistic fashion to prevent antibiotic accumulation in the cell through upregulated resistance, overproduction of streptomycin was the expected outcome. Biological assays to follow the production of streptomycin were completed as a measure of these manipulations. Results indicated no significant increase in streptomycin production over the empty vector control.

1.2 Methodology

1.2.1 Media, culture conditions, and antibiotics

A list of all plasmids and bacterial strains used in all chapters can be found in Table I. Unless otherwise specified, growth conditions for all biological assay indicator strains (Table II) in liquid cultures involved incubation at 37°C, and shaking at 200 revolutions per minute (rpm) for approximately 16-20 hours. *Streptomyces* strains in this work (Table III) were grown in 25 mL glass bottles with polypropylene caps ("universals"), or 250 mL flasks, with a sterile spring to create disperse growth, and incubated at 30°C, shaking at 200 rpm. Growth of these bacteria on solid media were at the same temperatures. Appropriate antibiotics (Table IV) were added to liquid and agar media (Table V) to achieve a 1/1000 dilution.

1.2.2 Construction of streptomycin recombinant plasmids

Streptomycin genes (*strR, strV,* and *strW*) were amplified with the appropriate primers (see Table VI) from *S. griseus* genomic DNA (according to the manufacturer's instructions using the iProof High Fidelity PCR Kit). Each primer set was engineered with specific restriction enzyme sites for use in directional cloning. The strategy is diagrammed in Figure 1 (and completed vectors shown in Figure 2), but described below briefly. The backbone for all

constructs was the integrative pSET152 vector (Bierman *et al.,* 1992), containing the constitutive *melC* promoter from *Streptomyces antibioticus* (Schmitt-John and Engles, 1992), created in the Elliot Lab at McMaster University.

Construction of pSET152$_{melC}$-*strR* began with PCR amplification of the endogenous gene from *S. griseus* DNA (annealing temperature of 52°C, 30 second extension time) (see Table VII and Table VIII for standard PCR setup). The PCR product was run out on a 1% agarose gel using gel electrophoresis, before the fragment was gel purifed (QIAGEN MinElute™ Gel Extraction Kit) and prepared for digestion. In a standard digestion reaction (Table IX) at 37°C for 30-60°C minutes, the insert was prepared for ligation by digestion with the enzymes *Bgl*II and *Eco*RI. Ligation into the pSET152$_{melC}$ vector (digested with *Bam*HI and *Eco*RI) was completed with, and according to, the specifications of the Roche Rapid DNA Ligation Kit. Use of a dephosphorylated vector (1 µL of alkaline phosphatase [Roche] mixed into the digestion reaction and incubated for 30 minutes in a 37°C water bath for 2-3 rounds) helped prevent self-ligation of the vector.

Similarly, creation of the pSET152$_{melC}$-*strVW* recombinant plasmid was carried out as described above, except that the PCR-amplified product (64°C, 1 min 45 seconds) was digested with *Bam*HI and *Eco*RI before being moved into pSET152$_{melC}$. To create the pSET152$_{melC}$-*strRVW* construct, PCR amplification and restriction digest steps were carried out as described above. Both the purified *strVW* PCR product and the pSET152$_{melC}$-*strR* vector were digested with *Bam*HI and *Eco*RI, before ligation of the insert into the overexpression plasmid.

All completed vectors were cloned into Invitrogen™ Subcloning Efficiency™ (SE) DH5α™ cells as per the manufacturer's instructions. Three microlitres of the ligation mixture was mixed with the chemically competent cells, and incubated on ice for half an hour. A heat shock at 37°C was applied for twenty seconds, before being replaced on ice for a further two minutes. Approximately 800 µL of LB was added to the reaction, followed by incubation for an hour at 37°C. Cells were then pelleted and resuspended before being spread onto two plates of LB agar containing apramycin for selection. These ligations allowed for directional cloning, and were verified with PCR analysis and/or digestion, as well as DNA sequencing at the McMaster Institute for Molecular Biology and Biotechnology (MOBIX), before being moved into *S. griseus.* Due to limitations on the length of sequencing reads, multiple oligonucletides were required to completely sequence the larger constructs (see Table VI).

1.2.3 Streptomycin overexpression vectors were moved into *S. grisues* by conjugal transfer

Plasmid-containing strains of *E. coli* were cultured in Luria Bertani (LB) broth for growth until an OD_{600} of 0.4-0.6 was reached. Then, the culture was washed with LB to obtain a pellet that was resuspended in a final volume of 1 mL

of LB. Simultaneously with the above preparation of cells, approximately 10^8 *S. griseus* spores were added to 500 µL of 2X YT broth, and subjected to heat shock in a 50°C heating block for 13 minutes. The tube was cooled at room temperature for approximately 5-10 minutes before 500 µL of the *E. coli* suspension was added. The solutions were gently mixed before centrifugation and subsequent resuspension of the cells in approximately 50 µL of the supernatant. This suspension was divided onto three plates of mannitol soy flour (MS) medium containing 1M $MgCl_2$, and incubated overnight at 30°C.

Approximately 14-18 hours later, once resistance genes had enough time to be expressed, selection was carried out by flooding the agar media with 1 mL of sterile water containing 0.5 µg of naladixic acid and 1.25 µg of apramycin, before continuing the incubation at 30°C. After colonial growth was observed (approximately 4-7 days), a portion of the biomass was selected and streaked for single colonies onto MS agar containing the appropriate antibiotics.
Spore stocks were subsequently created for long-term storage at -80°C.

1.2.4 Biological assays

Biological assays were completed using an indicator strain to follow the induction (or absence) of antimicrobial activity following the introduction of recombinant plasmids. Experiments with *Streptomyces* strains as producers of antimicrobial molecules, were conducted using a variety of laboratory and pathogenic indicator strains (see Table II). On Difco nutrient agar (DNA; BD Biosciences) in petri dishes, approximately 100 000 spores of each producer strain were dispensed in equal-sized 5 µL spots, and left to air-dry. These *Streptomyces*-containing plates were then incubated at 27°C for a pre-determined amount of time (usually 24-28 hours). Antimicrobial activity, as seen in a zone of inhibited growth, was assayed by a 4 mL soft nutrient agar (agar:broth in a 1:1 ratio) overlay containing a 1/100 dilution of an overnight culture of an indicator strain (grown in LB broth). Zones of inhibition were measured and documented after overnight incubation at 37°C for 16 hours. Measurements were taken from the edge of the *Streptomyces* growth, to the outer edge of the zone of clearing. In all the data presented here, bioassay graphs (while including measurements and standard deviations) are meant as a guide for a semi-quantitative view of antimicrobial activity. Overall trends hold more weight than actual values.

Table I: Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype, description, or use	Reference or source
E. coli		
DH5α	Plasmid construction and subcloning	Invitrogen
ET12567/pUZ8002	Generation of methylation-free plasmid DNA	MacNeil *et al.*, 1992
BW25113	Construction of cosmid-based knockouts	Gust *et al.*, 2003
BT340	Vector carrying FLP recombinase (for use in *E. coli*)	Datsenko and Wanner, 2000
pUWLFLP	Vector carrying synthetic FLP recombinase (for use in *Streptomyces*)	Fedoryshyn *et al.*, 2008
S. lividans		
1326	Wildtype	Kieser *et al.*, 1982
SL1	*S. lividans* + pSET152$_{melC}$	This work
SL2	*S. lividans* + pSET152$_{melC}$-*atrA*$_{tuf1}$ RBS	This work
SL3	*S. lividans* + pSET152$_{melC}$-*atrA*$_{native}$ RBS	This work
SL4	*S. lividans* + pSET152$_{tuf1}$	This work
SL5	*S. lividans* + pSET152$_{tuf1}$-*atrA*$_{tuf1}$ RBS	This work
SL6	*S. lividans* + pSET152$_{tuf1}$-*atrA*$_{native}$ RBS	This work
SL7	*S. lividans* + pSET152$_{ermE*}$	This work
SL8	*S. lividans* + pSET152$_{ermE*}$-*atrA*$_{tuf1}$ RBS	This work
SL9	*S. lividans* + pSET152$_{ermE*}$-*atrA*$_{native}$ RBS	This work
S. griseus	Wildtype host	
	S. griseus + pSET152$_{ermE*}$	This work
	S. griseus + pSET152$_{ermE*}$-*atrA*$_{tuf1}$ RBS	This work
	S. griseus + pSET152$_{melC}$	This work
	S. griseus + pSET152$_{melC}$-*strR*	This work

***S. griseus* (continued)**	*S. griseus* + pSET152$_{melC}$-*strVW*	This work
	S. griseus + pSET152$_{melC}$-*strRVW*	This work
***Streptomyces coelicolor* A3(2)**		
M145	Wildtype host	Chater *et al.*, 1982
E310	M145 Δ*SCO5085-5089* (actinorhodin)	This work
E311	M145 Δ*SCO5085-5089* (actinorhodin)FLP	This work
E312	M145 Δ*SCO5877-5881* (undecylprodigiosin)	This work
E313	M145 Δ*SCO5877-5881* (undecylprodigiosin)FLP	This work
E314	M145 Δ*SCO5085-5089*Δ*5877-5881* (actinorhodin, undecylprodigiosin)	This work
E315	M145 Δ *SCO5085-5089*Δ*5877-5881* (actinorhodin, undecylprodigiosin)FLP	This work
E316	M145 Δ*SCO6266* (*scbA*)	This work
Ja#2b	Wildtype host	G. Wright strain collection
SJ1	Ja#2b + pSET152$_{ermE*}$	This work
SJ2	Ja#2b+ pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$	This work
Cu#39	Wildtype host	G. Wright strain collection
SU1	Cu#39 + pSET152$_{melC}$	This work
SU2	Cu#39 + pSET152$_{melC}$-*absA1*	This work
SU3	Cu#39 + pSET152$_{melC}$-*absB*	This work
SU4	Cu#39 + pSET152$_{melC}$-*abaB*	This work
SU5	Cu#39 + pSET152$_{melC}$-*afsQ1*	This work
SU6	Cu#39 + pSET152$_{melC}$-*afsR*	This work
SU7	Cu#39 + pSET152$_{melC}$-*afsS*	This work

Cu#39 (continued) SU8	Cu#39 + pSET152$_{melC}$-*eshA*	This work
SU9	Cu#39 + pSET152$_{melC}$-*scbR*	This work
SU10	Cu#39 + pSET152$_{melC}$-*metK*	This work
SU11	Cu#39 + pSET152$_{melC}$-*ppk*	This work
SU12	Cu#39 + pSET152$_{melC}$-*scbA*	This work
SU13	Cu#39 + pSET152$_{melC}$-*atrA*$_{tuf1\ RBS}$	This work
SU14	Cu#39 + pSET152$_{melC}$-*atrA*$_{native\ RBS}$	This work
SU15	Cu#39 + pSET152$_{tuf1}$	This work
SU16	Cu#39 + pSET152$_{tuf1}$-*atrA*$_{tuf1\ RBS}$	This work
SU17	Cu#39 + pSET152$_{tuf1}$-*atrA*$_{native\ RBS}$	This work
SU18	Cu#39 + pSET152$_{ermE*}$	This work
SU19	Cu#39 + pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$	This work
Plasmids		
pIJ790	Plasmid carrying λ-RED genes	Gust *et al.*, 2003
pIJ773	Template plasmid containing apramycin knockout cassette	Gust *et al.*, 2003
pIJ2925	Plasmid construction	Janssen and Bibb, 1993
pSET152	Plasmid construction (integrative vector)	Bierman, 1992
pMC500	pUC57 derivative containing *ermE** promoter	Elliot Lab (unpublished)
pMC145	pSET152$_{melC}$	Elliot Lab (unpublished)
pMC146	pSET152$_{melC}$-*strR*	This work
pMC147	pSET152$_{melC}$-*strVW*	This work
pMC148	pSET152$_{melC}$-*strRVW*	This work
pMC149	pSET152$_{melC}$-*absA1*	This work
pMC150	pSET152$_{melC}$-*absB*	This work
pMC151	pSET152$_{melC}$-*abaB*	This work
pMC152	pSET152$_{melC}$-*afsQ1*	This work

Plasmids (continued)	pSET152$_{melC}$-*afsR*	This work
pMC153		
pMC154	pSET152$_{melC}$-*afsS*	This work
pMC155	pSET152$_{melC}$-*eshA*	This work
pMC156	pSET152$_{melC}$-*scbR*	This work
pMC157	pSET152$_{melC}$-*metK*	This work
pMC158	pSET152$_{melC}$-*ppk*	This work
pMC159	pSET152$_{melC}$-*scbA*	This work
pMC160	pSET152$_{melC}$-*atrA*$_{tuf1}$ RBS	This work
pMC161	pSET152$_{melC}$-*atrA*$_{native}$ RBS	This work
pMC162	pSET152$_{tuf1}$	This work
pMC163	pSET152$_{tuf1}$-*atrA*$_{tuf1}$ RBS	This work
pMC164	pSET152$_{tuf1}$-*atrA*$_{native}$ RBS	This work
pMC165	pSET152$_{ermE*}$	This work
pMC166	pSET152$_{ermE*}$-*atrA*$_{tuf1}$ RBS	This work
pMC167	pSET152$_{ermE*}$-*atrA*$_{native}$ RBS	This work

Table II: Indicator strains that were used in biological assays as indicators of the production of antimicrobial activity. (+) indicates a Gram-positive strain, while (-) indicates a Gram-negative strain.

Indicator strains
Biosafety Level I:
Bacillus subtilis 168 (+)
Staphylococcus aureus 29213 (+)
Escherichia coli DH5α (-)
Micrococcus luteus (+)
Biosafety Level II:
Vancomycin-resistant Enterococcus ATCC #51299 (+)
Acinetobacter baumannii B0098426R (-)
Burkholderia cepacia CEP509 (-)
Methicillin-resistant *Staphylococcus aureus* (CMRSA1) (+)
Pseudomonas aeruginosa PA01 (-)

Table III: List of *Streptomyces* strains, both characterized and wild, used as heterologous hosts in this work. The * symbol denotes the six strains that were used in section 3.3.5.

***Streptomyces* strains**
Characterized strains:
S. coelicolor A3(2) M145
S. flavopersicus
S. griseochromogenes
S. venezuelae
S. lividans 1326*
*S. griseus**
S. sp. Mg1*
S. sp. SPB74*
Wild isolate strains:
Cu#39*
Ja#2b*

Table IV: List of antibiotics and their working concentrations as used in this work.

Antibiotic	Concentration
Ampicillin	100 μg/mL
Apramycin	50 μg/mL
Chloramphenicol	25 μg/mL
Kanamycin	50 μg/mL
Naladixic Acid	25 μg/mL
Hygromycin	50 μg/mL

Table V: Media and solutions used in this work.

Media	Reference
Agar Media (25 mL per plate)	
Minimal medium (MM)	Hopwood, 1967
$R2YE_{(s)}$	Thompson *et al.*, 1980
Mannitol, soy flour (MS) medium	Hobbs *et al.*, 1989
Difco nutrient agar (DNA)	Kieser *et al.*, 2000
Luria-Bertani (LB)	Bertani, 1951
MYM (maltose-yeast extract-malt extract)	Stuttard, 1982
Liquid Media	
Yeast extract-malt extract medium (YEME)	Kieser *et al.*, 2000
Tryptone soya broth (TSB)	Kieser *et al.*, 2000
2 X YT broth	Kieser *et al.*, 2000
$R2YE_{(l)}$	Kieser *et al.*, 2000
Solutions	
Protoplast (P) Buffer	Hopwood and Wright, 1978; Okanishi *et al.*, 1974
Modified Kirby Mixture	Kieser *et al.*, 2000

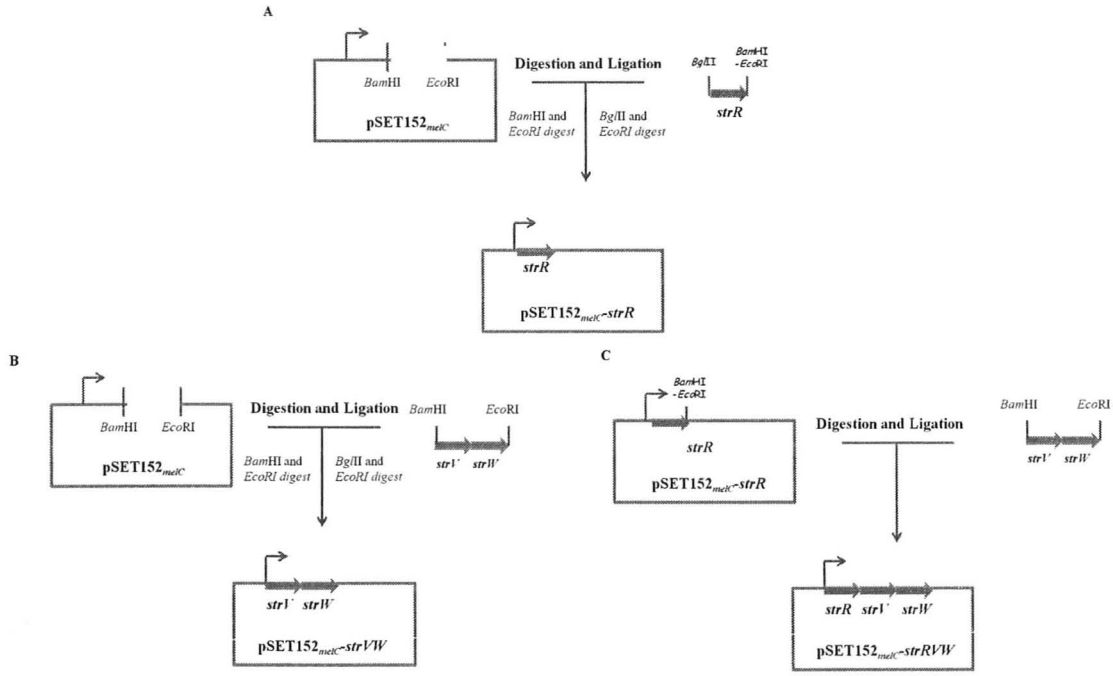


Figure 1: Schematic of cloning strategy used to create the three experimental streptomycin overexpression constructs (see text for additional details). A) Creation of the *strR* overexpression vector involved directional cloning of a PCR product digested with *Bgl*II and *Eco*RI into the pSET152$_{melC}$ vector that had been complimentarily digested with *Bam*HI and *Eco*RI. B) Creation of pSET152$_{melC}$ – s*trVW* involved directional cloning of the amplified *strVW* product digested with *BamH*I and *Eco*RI into the pSET152$_{melC}$ vector digested in the same manner. C) The experimental construct, pSET152$_{melC}$-*strRVW,* was created by cloning the *strVW* PCR product (with *BamH*I and *Eco*RI ends) behind the *strR* gene in the pSET152$_{melC}$ –s*trR* vector. Note: the control pSET152$_{melC}$ plasmid consisted of the pSET152 vector with the *melC* promoter from *S. antibioticus* cloned into the *Xba*I site.

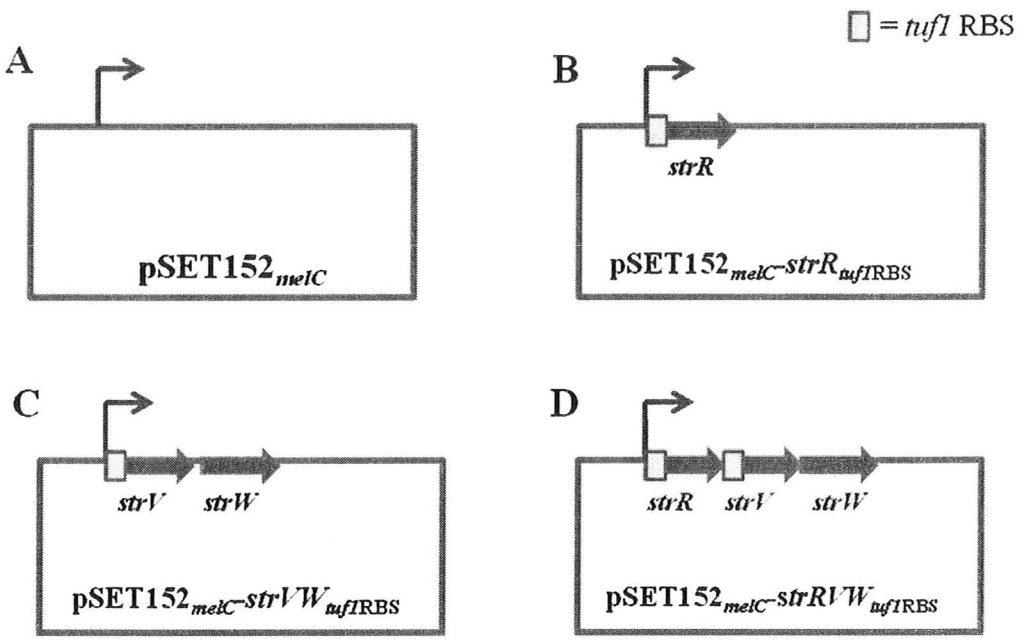


Figure 2: Streptomycin overexpression constructs created for this work, incorporating *strR* (activator gene) and *strV* and *strW* (resistance genes): A) empty vector control, B) *strR* overexpression construct, C) *strVW* overexpression construct, and D) *strRVW* overexpression construct.

Table VI: List of oligonucleotides used in this chapter.

Description of PCR oligonucleotide	**Oligonucleotide sequence (5'-3')**
Amplification of *strR*	Upstream: AAAAAAAGATCTAGGAGGACCCCAGTGGAGCA TATTTCAGGGAA Downstream: AAAAAAGAATTCAAAAAGGATCCTCATCCGAC ATCGCTCAAG
Amplification of *strV* and *strW*	Upstream: AAAAAAGGATCCAGGAGGACCCCAGTGTGCGC CCGCTCCCCGTCGCAGAT Downstream: AAAAAAGAATTCGGGTACGCCTTATTTCATT
strRVW sequencing primer 1	CCAGGCTTTACACTTTATGC
strRVW sequencing primer 2	CCGCGCTGTCGGCTT
strRVW sequencing primer 3	GGAGCGGCGCAGGTACC
strRVW sequencing primer 4	GCGGTACGGCGCGCTGG
strRVW sequencing primer 5	TGCGCTCGTCGATCA
strRVW sequencing primer 6	CGTGACCGACCACCAGTTGC

M13 universal primers flanking multiple cloning site of pIJ2925 and pSET152	Upstream: CGCCAGGGTTTTCCCAGTCACG Downstream: GCGGATAACAATTTCACACAGG

Table VII: Contents of a typical PCR reaction.

Standard PCR Reagent Concentrations	
Reagent	**Final Concentration**
Sterile water	Add as required to reach reaction volume
10x reaction buffer	1X
Mg^{2+} (if required)	2 mM
DMSO	7.5%
Deoxyribonucleotide triphosphates (all four bases)	200 µM
Primers (forward and reverse combined)	100 pmoles
Template DNA	1 – 15 ng
Enzyme	10 units for 50 µL reaction 5 units for 25 µL reaction
Total volume of reaction:	50 µL for large-scale amplification 25 µL for PCR checks

Table VIII: Standard PCR conditions for this study; the annealing temperature and extension time (both bolded) are variable for each reaction (depending upon melting temperature of the primers and length of the desired product, respectively). Steps 2-4 are repeated for a total of 30 cycles.

Standard PCR Program		
Action	**Temperature (°C)**	**Duration**
1) Initial denature	94	5 minutes
2) Denature	94	30 seconds
3) Anneal	**55**	30 seconds
4) Extension	72	**30 seconds**
5) Final extension	72	5 minutes

Table IX: The standard reagent conditions for a restriction enzyme digest.

Standard Restriction Enzyme Digest	
Reagent	**Final Concentration**
Sterile water	Add as required to reach total reaction volume
10X restriction enzyme buffer	1X
Bovine Serum Albumin (BSA; if required)	1%
DNA to be digested	10 – 100 ng for large-scale digestion; 5 – 50 ng for digestion check
Enzyme (each)	10 units
Total volume of reaction:	50 µL for large-scale digestions 15 µL for restriction analysis checks

1.3 Results

1.3.1 Creation and verification of an overexpression construct containing streptomycin activator and resistance genes

Constructs containing the streptomycin regulators in the integrative plasmid pSET152 $_{melC}$ were completed as described in section 1.2.2. In addition to the experimental construct containing the streptomycin activator and resistance genes, three other vectors were also created: an empty vector control, a vector carrying solely the *strR* activator, and a vector carrying solely the *strV* and *strW* resistance genes (Figure 2).

1.3.2 Bioassays did not reveal efficient overproduction of streptomycin by the strains carrying the overexpression constructs

Streptomycin bioassays with the four strains did not reveal any significant differences in streptomycin expression. A streptomycin-specific bioassay using *Bacillus subtilis* as the indicator strain revealed similarly sized zones of inhibition from *S. griseus* over a five-day period (Figure 3). This was confirmed by other streptomycin bioassays, including one completed over three days, which yielded comparable results. By themselves, these results suggest that streptomycin production was not engineered for overproduction. However, these unexpected results also supported a growing body of data generated by the Elliot Lab that the *melC* promoter was not effective in expressing cloned downstream genes.

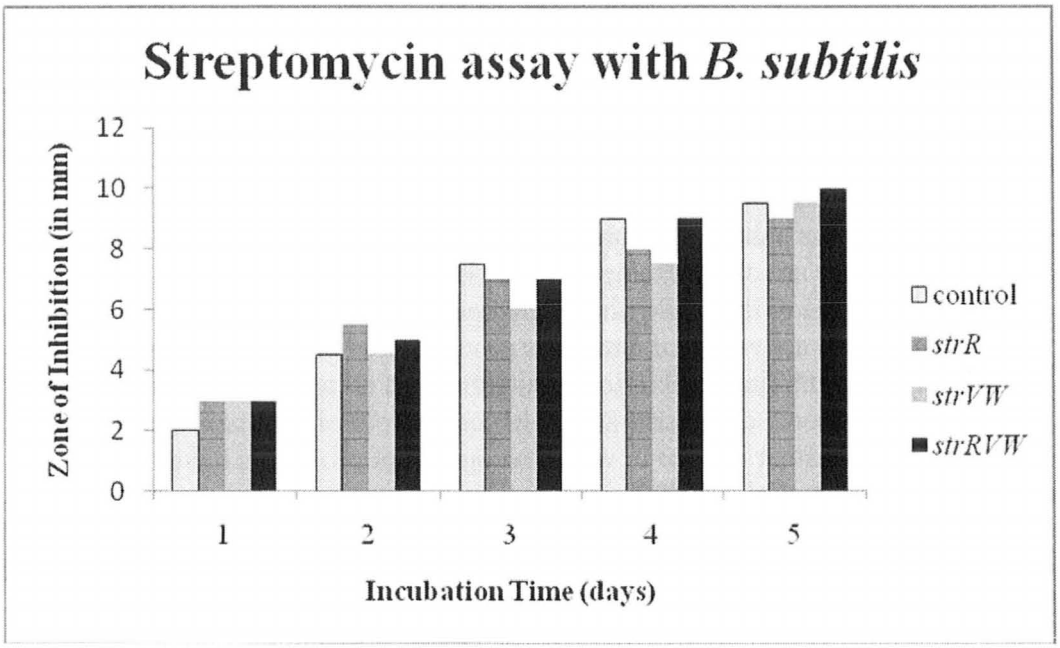


Figure 3: Streptomycin bioassay. Four different *S. griseus* strains were assessed for their streptomycin production levels, using *B. subtilis* as the indicator strain. *S. griseus* was spotted onto agar plates, and cultures were incubated for 1, 2, 3, 4, or 5 days at 27°C. Values were derived from the measurement of one spot. Reproducibility was approximated by two similar bioassays that also did not result in a significant difference between strains.

1.4 Discussion

Scientific papers have consistently supported the power of overexpressing a specific gene or group of genes in an organism for the purpose of function elucidation and generation of overt phenotypes to study. For practical reasons, microbes do not produce more metabolites than they require, and this level of expression must be enhanced in order to meet sufficient purification levels for human use. There are many examples in the literature of how this can be achieved, with one of the predominant methods being that of increasing the copy number of known activators of antibiotic production (Scheu *et al.,* 1997; Malla *et al.,* 2010a). If examining antibiotic production from all angles, the issue of self-resistance in producer organisms arises, hence the role of increased resistivity in these strains is another avenue in which recombinant producers have been engineered (Malla *et al.,* 2010b).

In this chapter, we set out to investigate which components in an overexpression construct should be optimized for effective gene upregulation. We chose to create an overexpression vector with the constitutive *melC* promoter from *Streptomyces antibioticus,* previously demonstrated to be a potent promoter of heterologous genes (Schmitt-John and Engels, 1992). This overexpression construct was constructed in the Elliot Lab, in which a modified version of the ubiquitous integrative pSET152 plasmid was engineered with the *melC* promoter (Bierman *et al.,* 1992). A practical reason behind the use of this vector was its capability for stable integration into the *Streptomyces* chromosome, thus rendering the need for antibiotic selection of the overexpression construct obsolete (Combes *et al.,* 2002). Along with stable expression, this was also vital as the presence of antibiotics for selection purposes in assays of novel antibacterial activity would confound results.

Investigations focused on the simultaneous overexpression of a streptomycin antibiotic activator, *strR* with its putative resistance genes, *strV* and *strW* (Beyer, *et al.,* 1996; Ohnishi *et al.,* 2008; Retzlaff and Distler, 1995). As the pathway-specific activator of streptomycin biosynthesis in producers *S. griseus* and *S. glaucecens, strR* functions through DNA-binding activation of target genes such as the the putative resistance-conferring ABC transporters, *strV* and *strW* (Beyer, *et al.,* 1996; Retzlaff and Distler, 1995). As seen in Figure 3, this combination did not result in a significant increase in streptomycin production. While the zones of inhibition seen against the indicator *B. subtilis* strain did increase in size over the duration of the five day trial, the zones did not vary significantly within a particular incubation period between the different strains. As the results of bioassays conducted in this work should be interpreted more on a qualitative level than quantitative, only a very large increase in antibiotic production (approximately 30% increase in production or more) would be considered significant, and this was not seen.

Interestingly, we didn't observe upregulation of streptomycin production, even when using the more "traditional" type of overexpression contruct containing solely *strR* (the pathway-specific activator). In many cases, overexpression of a pathway-specific regulator has successfully resulted in upregulation of target antibiotics (Malla *et al.,* 2010a; Vögtli *et al.,* 1994). Additionally, work completed with *S. peucetius* (producer of the anti-cancer drug, doxorubicin), has also found that overexpression of more global-acting regulators also caused increased production in the recombinant strains over the parent (Malla *et al.,* 2010a). In that case, upregulation of both direct regulators from the doxorubicin biosynthesis cluster, and general regulators involved in secondary metabolism were examined.

However, our findings do not necessarily negate the promise behind the theory guiding this work. For example, Malla *et al* (2010b) recently showed that recombinant strains of *S. peucetius* carrying overexpression constructs of resistance genes of doxorubicin resulted in higher titres of this drug. These resistance genes encoded both an efflux pump, and a DNA repair system, and overexpression of these elements (both separately and together) allowed for higher doxorubicin production. Malla and colleagues followed a similar experimental design as our work in their overexpression constructs containing resistance genes, though they did not include an activator protein in their experimental strains. Instead, they published two articles in 2010, one concerning the overproduction of positive regulators (Malla *et al.,* 2010a), and the other on the overexpression of resistance genes (Malla *et al.,* 2010b).

If the streptomycin resistance genes can confer similar increases in antibiotic production, then the addition of an overexpressed activator could enhance titres further. The success of the work with doxorubicin may support our working hypothesis, that ineffective production of streptomycin in the above work was due to inefficient overexpression constructs. Without sufficient overexpression of the desired genes in *S. griseus,* any increase in the level of streptomycin produced went potentially undetectable through the bioassays conducted. This result falls in line with northern blots in the Elliot lab that suggested the *melC* promoter was not effective for overexpression experiments (unpublished data). However, the use of this promoter in the literature suggests it may have some utility, therefore it may be premature to completely exclude it from future use (for example, it was used to initiate the work in chapter 3). As addressed in the next chapter, the *ermE** promoter from *Saccharopolyspora erythraea* may prove a more reliable promoter for future work (Doumith *et al.,* 2000). In the future, it would be interesting to see whether differences in streptomycin production can be observed, if the *ermE** promoter is used in place of the *melC* promoter to drive expression.

CHAPTER 2: MAXIMIZING EXPRESSION IN AN *atrA* OVEREXPRESSION CONSTRUCT

2.1 Introduction

AtrA (actinorhodin-associated transcriptional regulator) is involved in the production of antibiotics such as actinorhodin (*S. coelicolor*) and streptomycin (*S. griseus*) (Hirano *et al.,* 2008; Uguru *et al.,* 2005). As a transcription factor, it is rare as it performs its transcriptional activation of pathway-specific antibiotic regulators despite the fact that it is not associated with the target metabolic cluster (Uguru *et al.,* 2005). Specific in its target, it recognizes sites upstream of its regulated genes, such as the streptomycin activator, *strR,* in *S. griseus,* and the actinorhodin activator of *S. coelicolor, actII-ORF4* (Hirano *et al.,* 2008; Uguru *et al.,* 2005). Its association with the production of antibiotics in disparate *Streptomyces* species has been known for some time, but it was only recently that a potential link to nutrient metabolism was reported (Nothaft *et al.,* 2010).

With the overexpression of this gene holding the potential of activating a litany of metabolites in heterologous *Streptomyces* species, it was a candidate that was added to our regulator list in chapter three. Continuing our work from the last chapter, we decided to test the expression effectiveness of several vector features in this work; this was completed via a variety of overexpression constructs derived once again from pSET152 (Figure 7). In total, three different promoters were tested. The *melC* promoter, as described in the previous chapter; the promoter of the highly expressed *tuf1* gene, in a vector provided by JNE Biotech Inc (van Wezel *et al.*, 1994); and, the *ermE** promoter from *Saccharopolyspora erythraea* (Doumith *et al.,* 2000). The *atrA* gene was also amplified for cloning containing either its native promoter region, or with the ribosome-binding site (RBS) of the *tuf1* gene of *S. coelicolor.*

In this chapter, I manipulated overexpression of the *atrA* gene at both the transcriptional level and translational level in order to investigate the most efficient combination of regulatory elements. Results of *atrA* expression in the heterologous host *S. lividans* indicated that the *ermE** promoter and the *tuf1* ribosome binding site resulted in highly efficient expression of the desired gene.

2.2 Methodology

2.2.1 Construction of *atrA* recombinant plasmids

To begin elucidating transcriptional and translational regulatory elements for heterologous overexpression, nine different constructs, consisting of three promoters (*melC, tuf1,* and *ermE**) and two ribosome binding sites (that of the native *atrA* gene and the highly expressed *tuf1* gene) were created (see Figures 4 and 5). Phosphorylation of primers for PCR amplification of the *atrA* gene was

completed as per the manufacturer's instructions (Table X). PCR amplification (58°C, 30 seconds) was completed with different primers such that two different *atrA* inserts were obtained. One insert contained the RBS of the endogenous *atrA* gene, while the second insert utilized primers that would replace the native RBS with that of the *tuf1* gene. In both cases, the *atrA* gene fragment was subsequently purified to be cloned into the blunt-ended *Eco*RV site of pSET152 (containing either the *melC* or *tuf1* promoter).

To create the pSET152$_{ermE*}$-*atrA*$_{tuf1}$ and its control, *atrA* was first cloned into the Elliot Lab's pMC500 vector (unpublished data). Designed to overexpress small RNAs, pMC500 utilizes the pUC57 vector backbone, and contains the *ermE** promoter of *Saccharopolyspora erythraea* that is of interest to this study. As described above, successfully obtained transformants were verified each time. Here, orientation of the promoter and gene was vital, and needed to be confirmed upon blunt-ended ligation of the *atrA*$_{tuf1RBS}$ fragment into the *Eco*RV site of pMC500.

To transfer the *atrA* gene with the *ermE** promoter into the final pSET152 construct (for integration into the *Streptomyces* chromosome), pMC500$_{ermE*}$-*atrA*$_{tuf1RBS}$ was digested with *Bgl*II, which flanked the multiple cloning site, to obtain the synthetic insert of interest. Gel purification resulted in a blunt-ended fragment that was cloned into the *Bam*HI site of pSET152. pSET152$_{ermE*}$-*atrA*$_{native\ RBS}$ was similarly made, but utilized a different upstream primer to include the native RBS of *atrA*. Digestion with *Bgl*II as above was also completed to move the *ermE** promoter into pSET152 to obtain the empty vector control.

2.2.2 Pregermination of *Streptomyces* spores to be used as the inoculum for liquid cultures

Approximately 10^8 of *Streptomyces* spores were spun down in an Eppendorf centrifuge to obtain a pellet, and resuspended in 3 mL of TES buffer (0.05M, pH8) before being transferred to a universal bottle for heat shock at 50°C for 10 minutes. Spores were cooled under running water for 20 seconds, before an equal volume of double strength germination medium (1% Difco yeast extract, 1% Casaminoacids, 0.01M $CaCl_2$ at 2 µL per mL of medium) was added.

Appropriate antibiotics were added as specified above. This culture was incubated at 37°C shaking for 2-4 hours, before being spun down in a bench-top centrifuge at 3000 rpm for 5 minutes. The supernatant was carefully removed, and spores resuspended in the remaining liquid. The spores were dispersed by vigorously mixing on a VWR Analog Vortex Mixer, before being used as the inoculum in liquid cultures.

2.2.3 Phenotypic analysis of recombinant *Streptomyces* strains: Preliminary phenotypic analysis on rich agar

Phenotypic analysis was conducted visually by following the production of the blue actinorhodin antibiotic on rich R2YE agar (Table V). Equivalent amounts of spores of each strain were streaked out, and incubated for 5-7 days, with phenotypes documented daily.

2.2.4 Phenotypic analysis of recombinant *Streptomyces* strains: Quantification of actinorhodin production in liquid medium

In order to gain a better understanding of the degree of actinorhodin production in various strains, an antibiotic assay in liquid R2YE medium was conducted, as described previously (Kang *et al.,* 1998). Pregerminated *Streptomyces* spores were inoculated in R2YE and incubated at 30°C shaking. At each timepoint, 0.5 mL of the culture was combined with an equal volume of 2 M KOH, and thoroughly mixed before centrifugation at 10 000 rpm for 2 minutes. The supernatant was then subjected to a spectrophotometer reading at 640 nm in order to calculate the concentration of blue antibiotic produced. At each measurement, 0.5 mL of culture was also spun down (and supernatant discarded with a pipette) to be used as a measurement of dried cell weight, in order to gain insight into growth patterns.

2.2.5 RNA extraction (modified from Kieser *et al.,* 2000; Kirby *et al.,* 1967)

RNA extraction from liquid-grown *Streptomyces* cultures was utilized for the purpose of quantification of transcript levels through reverse-transcription PCR (RT-PCR). For RNA extraction, cultures were grown as outlined in section 2.2.2, using rich R2YE liquid. At each timepoint, a portion of the culture was aseptically removed and spun down for 10 minutes at 4000 rpm in a Sorvall® Legend RT table-top centrifuge that was maintained at 4°C. The following steps were carried out in 15 mL polypropylene tubes. The supernatant was removed from the pellet/tube before 5 mL of Kirby mixture [1% w/v N-lauroylsarcosine sodium salt, 6% w/v sodium 4-aminosalicylate, 6% v/v phenol mixture (pH 7.9), 50mM Tris (pH 8.3)] and 3-4 glass beads were added to the tube. This suspension was vigourously agitated on a vortex mixture for two minutes, before 5 mL of phenol/choloroform/isoamyl alcohol (ratio of 50:50:1) was added. Subsequently, this material was subjected to rounds of 30 seconds of vortex mixing, followed by 30 seconds on ice until the mixture remained homogeneous.

The homogenous solution was separated from the glass beads into a new tube, and spun at 7000 rpm for 5 minutes. The top phase of the now bi-phasal substance was removed to another tube already containing 5 mL of phenol/choloroform/isoamyl alcohol, before being subjected to vortex mixture

and spinning as above. These extraction steps were repeated until the interface between phases was clear (usually a total of three times). At this point, the upper phase was once again removed to a fresh tube, and all nucleic acids were precipitated through the addition of 1/10 of the total volume's worth of 3M sodium acetate (pH 6.0), and an equal volume of this new volume's worth of 100% isopropanol. This was throughly mixed by gentle inversion a few times, before storage at -80°C until all timepoints were collected.

Samples at all timepoints were thawed from frozen on ice, before being spun down at 8000 rpm for 10 minutes. The supernatant was separated from the pellet, and rinsed with 500 µL of 95% ethanol. The pellet was then air-dried for approximately 10-20 minutes, before being redissolved in 450 µL of sterile distilled water. DNase I treatment to remove DNA from this nucleic acid preparate was as follows: the redissolved solution was transferred to a 1.5 mL eppendorf tube, and incubated for 30 minutes in a 37°C water bath with 50 µL of 10X DNase buffer (0.5M Tris-HCl, pH 7.8, 0.05M $MgCl_2$) and 10 units of DNase I recombinant, RNase-free enzyme (Roche). Addition of another 10 units of DNase I after this initial treatment, was followed by another 30 minutes of incubation.This reaction was terminated by phenol/chloroform/IAA treatment, then on-ice precipitation for 30 minutes, as described above. RNA was pelleted by spinning at 13 000 rpm for 10 minutes, rinsed with 80% ethanol, and then resuspended in sterile water. Storage of RNA was at -80°C.

Assessment of RNA quality and quantity was subsequently completed (samples were thawed on ice first if frozen). For spectrophotometric analysis, a 1/500 dilution for each sample was made with the resuspended RNA above, and sterile water. Absorbance of the RNA solutions was read at A_{260} through UV spectroscopy, and each sample was freshly blanked with sterile water. The RNA concentration of each sample was then calculated, and 5 µg of RNA was run on a 1% agarose gel for visualization.

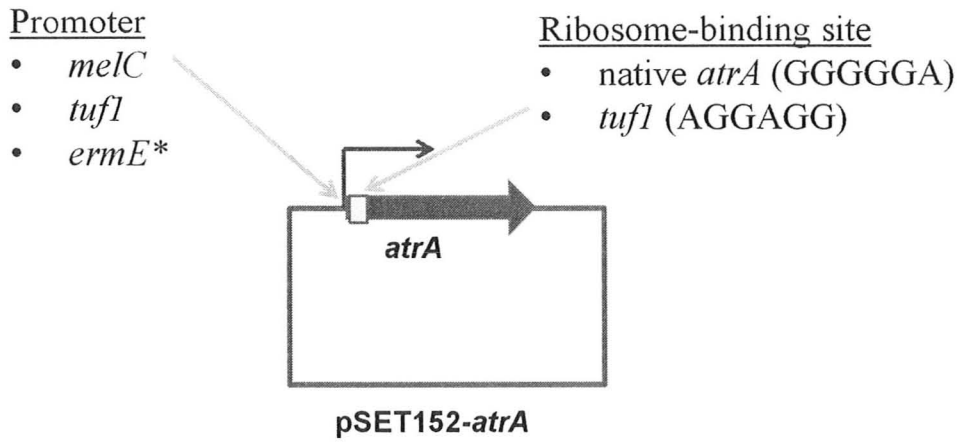
2.2.6 Reverse-transcription PCR (RT-PCR)

To generate cDNA from the RNA samples obtained above, 5 µg of RNA was incubated with 2 pmol of a specific reverse primer (see Table VI) for each desired PCR reaction, along with 10 nmol of dNTPs, and sterile water to top off the RNA-water mixture to 12 µL. Tubes were incubated at 65°C for 10 minutes, before being chilled on ice for a few minutes, followed by the addition of 2 µL 0.1M DTT (Invitrogen), 4 µL of 5X First Strand Buffer (Invitrogen), and 1 µL (40 units) of RNaseOut™ Recombinant Ribonuclease Inhibitor (Invitrogen). Further incubation at 42°C for two minutes was completed, then the addition of 200 units of SuperScript III reverse transcriptase (Invitrogen) enzyme. Subsequent incubations at 42°C for 50 minutes, and 70°C for 10 minutes completed the generation of complimentary DNA. This DNA was subsequently used as the template (2 µL per tube) for standard PCR reactions, as described above.

PCR setup to visualize the *atrA* (61°C, 30 seconds, 30 cycles) and *actII-ORF4* (61°C, 30 seconds, 25 cycles) transcripts was completed similar to Table VII, except half the usual concentration of primers was utilized. Primers specific to the 16S rRNA transcript were used for quality control in checking that similar amounts of cDNA template were used for each PCR reaction. The negative control reaction consisted of RNA (the same amount as was reverse-transcribed into DNA) as the template for the reaction. Amplification of bands in this case would indicate DNA contamination of the RNA samples. With each PCR reaction, a positive control (chromosomal DNA) was also conducted to ensure that amplified products were of the correct size.

In the case of the *actII-ORF4* transcripts, ImageJ software (for image processing and analysis) was used to quantify the intensity of the bands between the *atrA* overexpression and control strains (see Appendix A). The measurement of each band was normalized to the corresponding 16S rRNA transcript, as a way of controlling for RNA quality. Displayed values are the average of two independent RT-PCR reactions.

A



B

Native upstream *atrA* sequence:
ATTACC**GGGGGA**TTGTCTATGCATGTT

Engineered upstream *atrA* sequence with *tufl* RBS:
ATTACC**AGGAGG**TTGTCTATGCATGTT

Figure 4: Schematic of modifications made to the *atrA* overexpression construct. A) The efficiencies of the promoters *melC, tufl,* and *ermE** were used for exploring transcriptional efficiency. To gain insight into the translational process, the ribosome-binding site of the highly expressed *tufl* gene was engineered in front of the *atrA* gene. In comparison, the native RBS of the *atrA* was also used. B) Comparison of the genetic sequence upstream of the *atrA* gene shows that only the ribosome-binding site was modified in the *tufl* RBS containing insert. The start codon has been underlined.

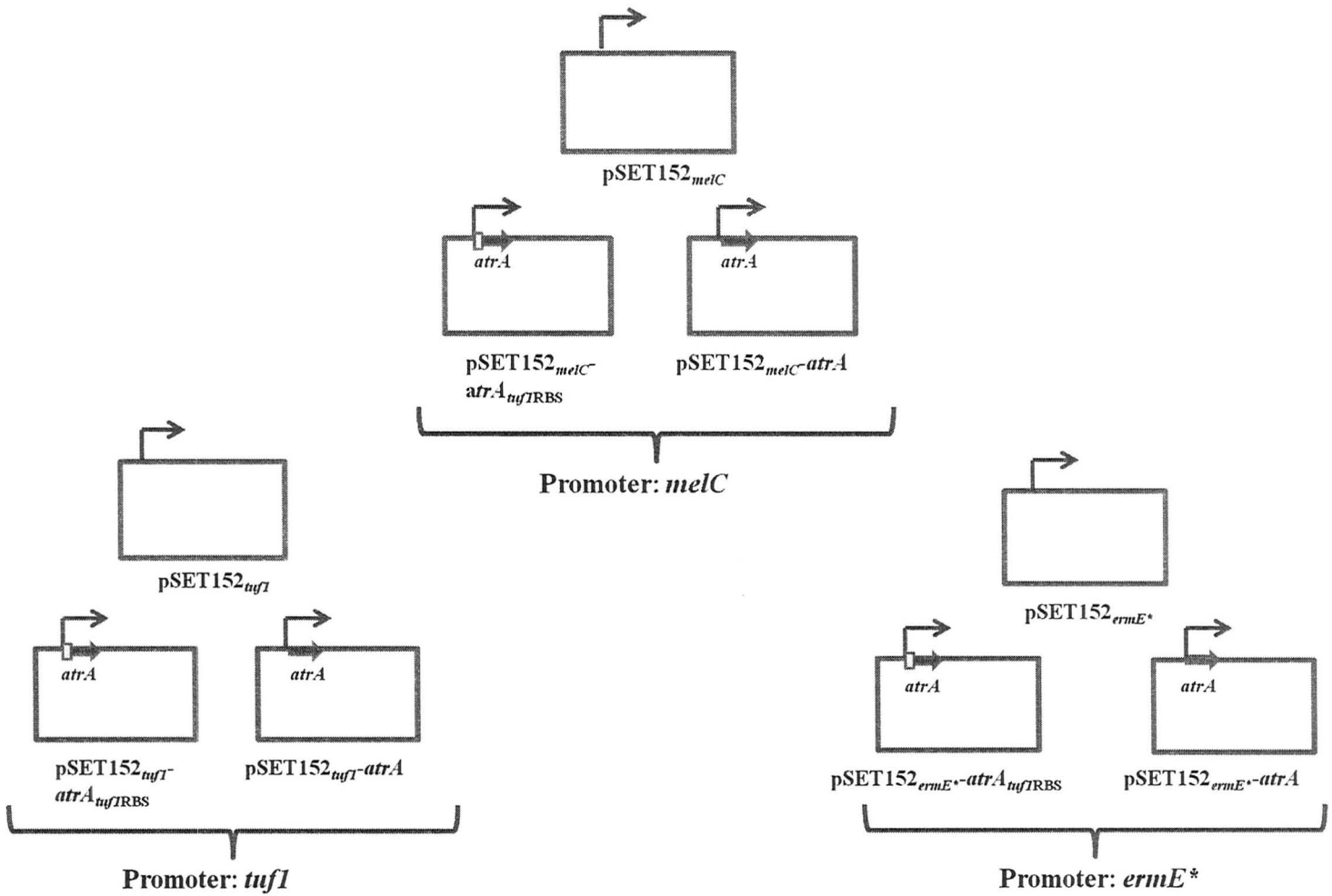


Figure 5: Diagrammatic overview of *atrA* overexpression constructs created for this work. Three different promoters were used, with each promoter incorporated into three different constructs (empty vector control, *atrA* cloned with its native RBS, and *atrA* cloned with the *tuf1* RBS.

Table X: List of oligonucleotides used in this chapter.

Description of PCR oligonucleotide	**Oligonucleotide sequence (5'-3')**
Amplification of *atrA*	Upstream (native RBS): AGGTCAGCGTGGGTGAGTGG Upstream (engineered *tufl* RBS): TTACCAGGAGGTTGT CTATGCATGTTCAGGA Downstream: GGTCAGCGTGCCATATTGGC
RT-PCR – 16S rRNA	Upstream: AGAGTTTGATCCTGGCTCAG Downstream: CGAACCTCGCAGATGCCTG
RT-PCR – *atrA*	Upstream: TCATTGGTCGTCCGCGTCG Downstream: TACACCGTGCCGACACCG
RT-PCR – *actII-ORF4*	Upstream: CAACTTATTGGGACGTGTCC Downstream: ACCAATTCCCGGTCGTCG C

2.3 Results

2.3.1 A variety of *atrA* overexpression constructs were created to investigate transcriptional and translational efficiencies

Following the results of the previous chapter, we decided to put the streptomycin overexpression project on hold until the uncertainty surrounding the *melC* promoter was cleared up. In searching for an effective promoter, the regulatory gene *atrA* was used to test different regulatory elements in overexpression constructs. The regulatory factors tested were two-fold: transcriptional regulation was investigated using three different promoters (*melC, tuf1,* and *ermE**), while translational regulation was explored using the native *atrA* ribosome-binding site GGGGGA, and the RBS of the highly expressed *tuf1* gene, AGGAGG (See Figures 4 and 5). The resulting nine constructs were then moved into *S. lividans* 1326.

2.3.2 The pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ construct activated actinorhodin production in *S. lividans* on rich media

Streptomyces lividans has the genetic capability to produce the blue-pigmented antibiotic actinorhodin that is also produced by *S. coelicolor,* but does not produce this under normal growth conditions (Kim *et al.,* 2001). As a direct, positive regulator of the actinorhodin pathway-specific activator gene *actII-ORF4, atrA* positively affects actinorhodin production in *S. coelicolor* (Uguru *et al.,* 2005). We proposed that sufficient heterologous overexpression of *atrA* in *S. lividans* would subsequently activate the production of actinorhodin in this species.

On rich R2YE agar, when equivalent number of spores of the various *atrA* overexpression constructs were streaked out, only one strain was able to significantly activate actinorhodin production in *S. lividans* after a week of growth. As seen in Figure 6, the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ construct containing the optimized *tuf1* ribosome binding site and *ermE** promoter, produced copious amounts of blue pigment. When compared to the pSET152$_{ermE*}$-*atrA*$_{native\ RBS}$ strain, the combination of the *ermE** promoter and the *tuf1* RBS proved more effective at actinorhodin activation than the *atrA* gene cloned with its native upstream regulatory region.

To quantify this observation more closely, a liquid actinorhodin spectrophotometric assay was completed, comparing production of the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$-containing strain and the corresponding plasmid-alone control (Figure 7). After approximately 48 hours, actinorhodin production in the *atrA* overexpression strain started to increase significantly, while the control strain had almost negligible production over the entire time course (the culture also never achieved a blue colour, even after allowing it to grow past the 162 hour

timepoint). This was reproducible over two independent trials; while the absolute values were different, the same overall trend was observed. Simultaneous analysis of the dried cell weight suggested this was not due to significant differences in growth rate or biomass.

2.3.3 Reverse-transcription PCR analysis of select *atrA* constructs in *S. lividans* confirmed increased *atrA* transcript abundance in the pSET152$_{ermE*}$-*atrA*$_{tufl\ RBS}$-containing strain

The data presented in previous sections was consistent with efficient expression of *atrA* using the *ermE** promoter. To investigate this further, semi-quantitative RT-PCR was utilized to follow the *atrA* transcript levels in the pSET152$_{ermE*}$-*atrA*$_{tufl\ RBS}$ and pSET152$_{ermE*}$ (its control) strains. At each of three timepoints, the *atrA* transcript was seen in the *atrA* overexpression strain, but undetectable in the control (Figure 8). To determine if the enhanced blue phenotype was indeed due to an increase in actinorhodin, expression of the actinorhodin pathway-specific regulator *actII-ORF4* was also followed by RT-PCR. We found that in the *atrA* overexpression strain, transcripts levels of *actII-ORF4* were not significantly different from those of the control strain (quantified using ImageJ processing software; Appendix A). These results have been corroborated by at least two RT-PCR trials, using two independent biological replicates.

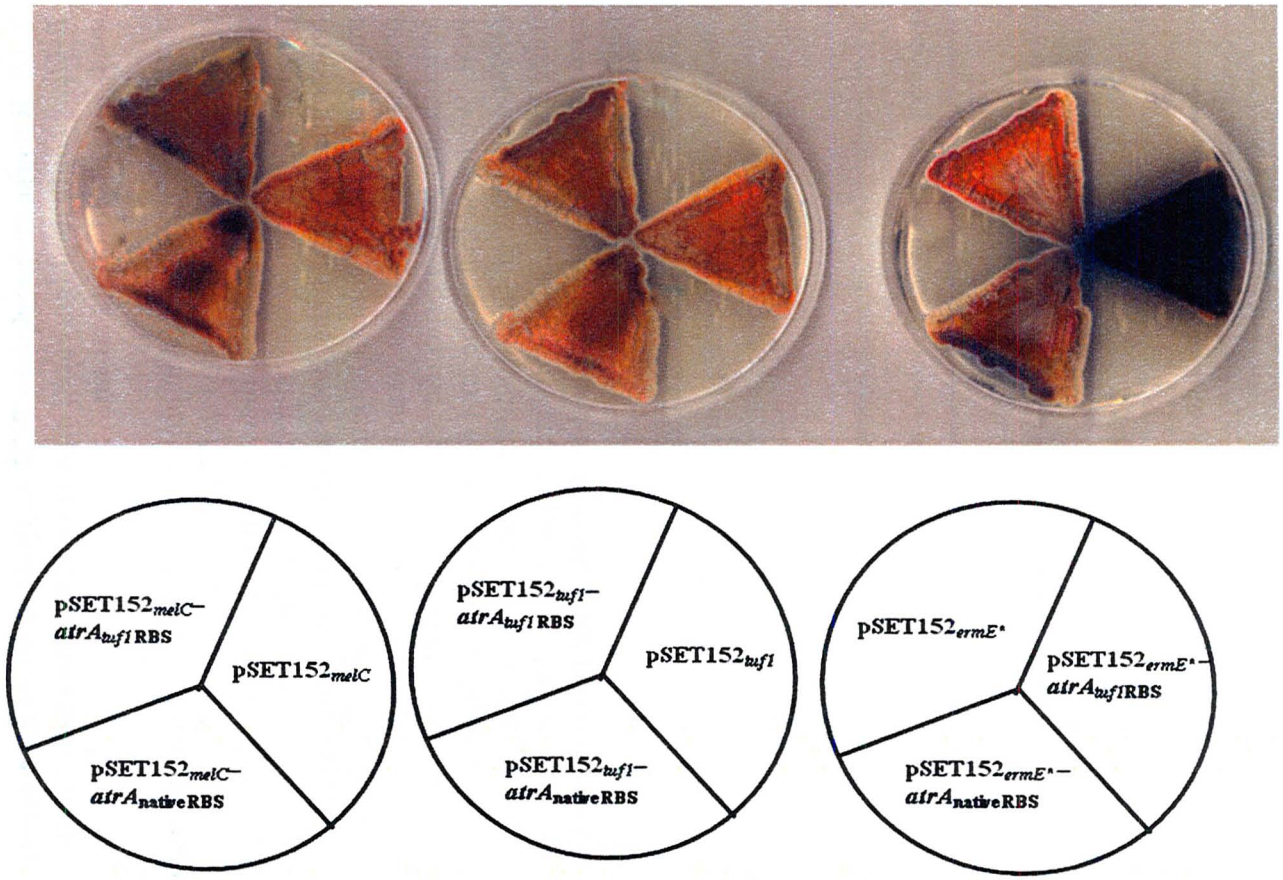


Figure 6: Following the blue actinorhodin production in *S. lividans* strains grown on rich R2YE agar for 7 days. The pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ construct was the only overexpression plasmid that significantly activated the production of actinorhodin, presumably through the upregulation of *atrA,* and correspondingly, *actII-ORF4.*

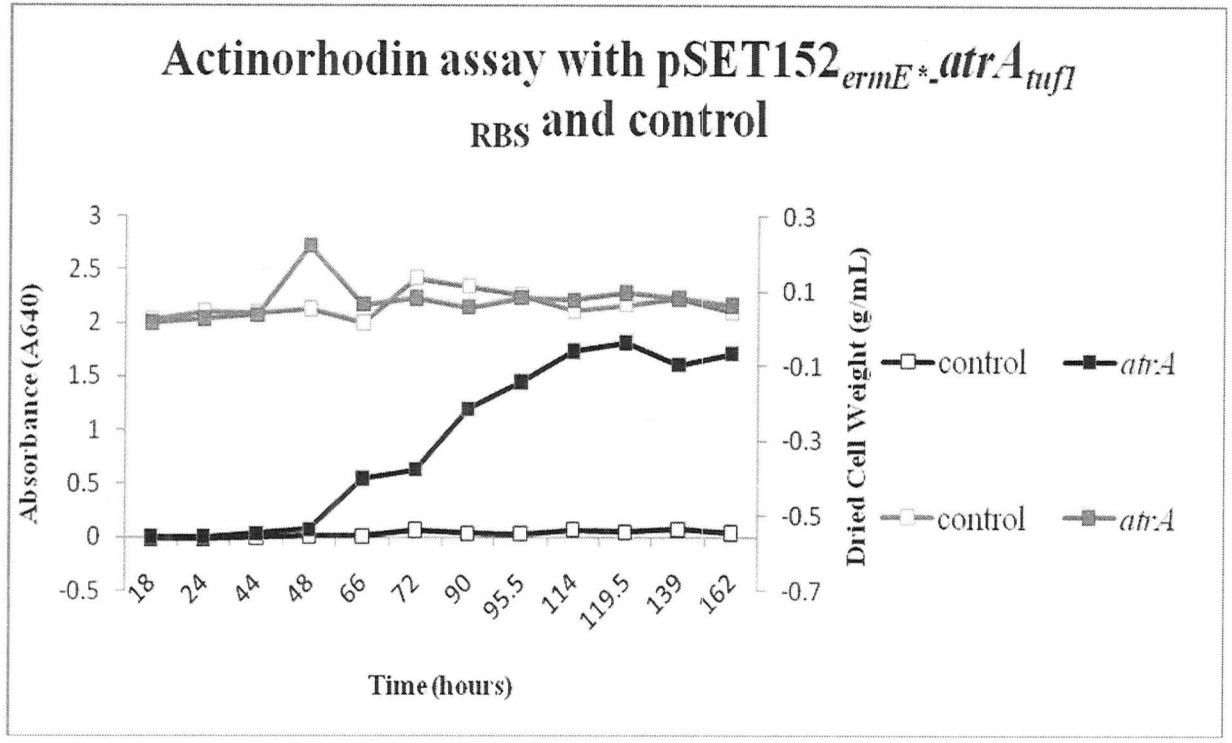


Figure 7: Liquid spectrophotometric assay quantifying the production of actinorhodin by the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS-}$ containing strain, compared with an empty-plasmid negative control (black lines). An analysis of growth is also shown on the secondary axis, using the dry weight of 1 mL of culture (grey lines). Two independent trials were completed, and the same trend was observed in both.

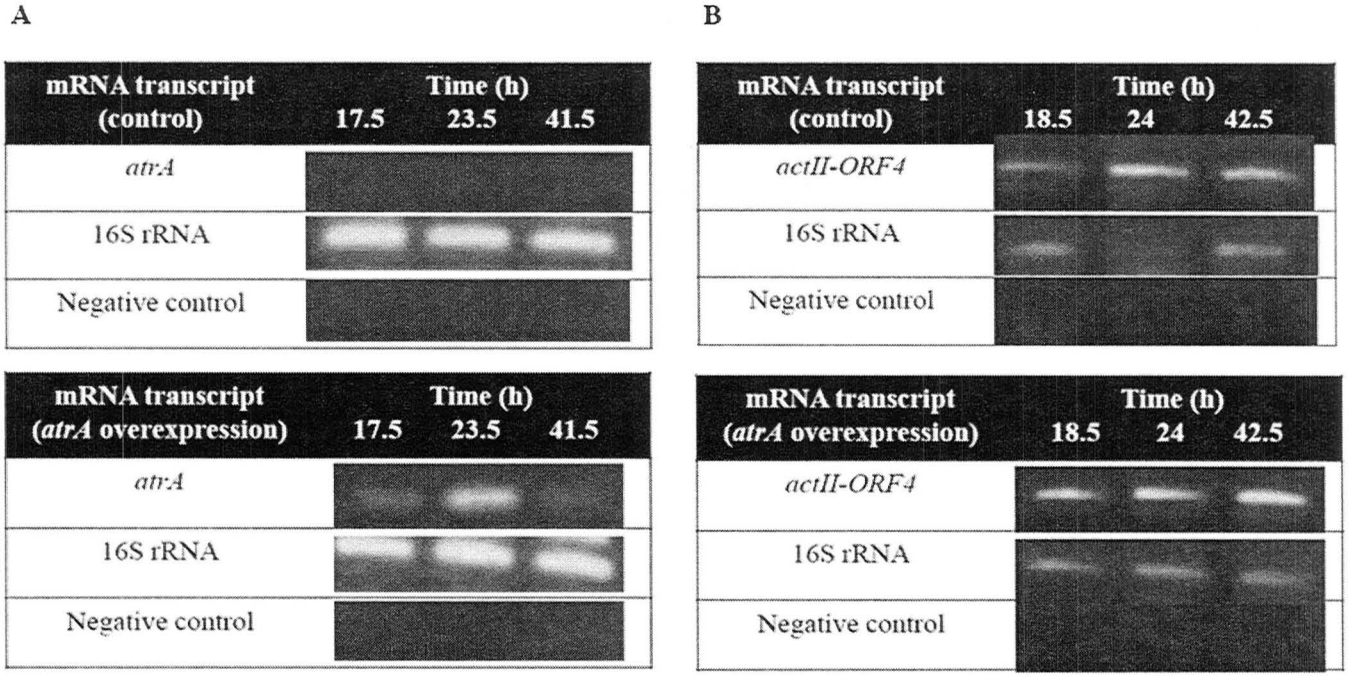


Figure 8: RT-PCR analysis of the (A) *atrA* gene, and its downstream target in the actinorhodin cluster, (B) *actII-ORF4*. Transcripts were compared between the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ and pSET152$_{ermE*}$ strains. The *atrA* transcript was amplified in a PCR program consisting of an annealing temperature of 61°C, extension time of 30 seconds, and was run through a 30 cycle reaction. The *actII-ORF4* transcript was amplified with the same parameters, except for a total of 25 cycles. For each of the four experiments, the top panel represents the transcript of interest (*atrA* or *actII-ORF4)*, the middle panel a control of RNA integrity and abundance, and the bottom a negative control showing a lack of contaminating DNA in the experiment. See Appendix A for quantification of *actII-ORF4* bands between the two strains.

2.4 Discussion

Over the years, a variety of different tools have become available for use in cloning, and as such, one must think carefully about the features to be included in a synthetic construct. It is not often that the effectiveness of experimental manipulation (like overexpression) is rigourously tested, leaving some question as to whether an observed phenomenon is actually due to upregulation of a desired product.

Here, we tested overexpression constructs carrying the *atrA* transcriptional regulator from *S. coelicolor*, making modifications at both the transcriptional and translational levels. As one of the regulatory genes used in the next chapter, *atrA* was chosen for this work as it had yet to be cloned, and this provided us the opportunity to experiment with its expression. The *melC, tufl*, and *ermE** promoters were all utilized, in conjunction with either the native *atrA* RBS, or the RBS of the *tufl* gene from *S. coelicolor*. As the *tufl* gene encodes for elongation factor EF-Tu (the most abundant protein in *E. coli),* responsible for bringing amino-acyl tRNAs to the ribosome during translation (Weijland *et al.,* 1992), its regulation was presumably optimized for high expression.

The first analysis involved looking for the appearance of the blue antitbiotic (actinorhodin) in *S. lividans,* a streptomycete that is genetically capable of producing this, but not under usual growth conditions (Kim *et al.,* 2001). As *atrA* has been proven to bind directly upstream of the actinorhodin activator *(actII-ORF4)* and activate its expression (Nothaft *et al.,* 2010), we decided to use actinorhodin as a quick visual indicator of *atrA* expression and activity.

Strikingly, we found that when equal numbers of spores for each strain were grown on rich media, only one strain effectively activated actinorhodin production: the strain that expressed *atrA* using the *ermE** promoter in conjunction with the *tufl* ribosome-binding site. Through comparisons with the other strains, solely the *ermE** promoter, or use of this RBS with other promoters, it was noted that either element alone did not have the same dramatic effect. Other than phenotypic analysis, additional lines of evidence supported this conclusion (RT-PCR results are discussed below). This suggested that effective overexpression required both, possibly enhancing efficiency at both the transcriptional and translational levels. The results in chapter 1 are supported by this finding, as the streptomycin regulator genes in those constructs only contained the *tufl* RBS, and not the *ermE** promoter (Figure 2). Presumably, we would expect to see an increase of streptomycin production in at least one of the streptomycin recombinant strains (in the overexpression of the *strR* regulator) with the addition of the *ermE** promoter.

To gain a better understanding of the extent of each modification on the efficient overexpression of *atrA*, *atrA* expression was explored more rigourously using semi-quantitative RT-PCR. This effectively focused on the activity of the *ermE** promoter. For this experiment, transcripts from the *S. lividans*

pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ strain and its vector control were examined using RNA extracted from culture-grown strains. The *atrA* transcript was only seen in the strain harbouring the overexpression construct, suggesting that the *ermE** promoter was able to produce *atrA* at a higher level than that of a strain with just the endogenous copy of *atrA*. This is in line with the notion that wildtype levels of *atrA* in *S. lividans* may not be sufficient to induce actinorhodin production. Although the *melC* and *tuf1* promoters were not assayed using RT-PCR, the phenotypic evidence in Figure 6 supported the idea that the *ermE** promoter produced a higher level of *atrA*.

However, when the expression of *actII-ORF4* was examined, there was not a corresponding increase in transcript level seen in the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ strain. For this gene, it appeared that transcriptional activity was not statistically different from the level of endogenous transcript produced (as seen in the control strain), which was unexpected. However, given the large standard deviation (see Appendix A), the testing of additional samples (and subsequent decline in standard deviation) would provide increased insight into this observation.

Oftentimes, the silent actinorhodin biosynthesis genes in *S. lividans* can be stimulated by the heterologous introduction of activator proteins from *S. coelicolor* (Horinochi *et al.,* 1989; Ishizuka *et al.,* 1992; Scheu *et al.,* 1997). Antibiotic stimulation can also occur with higher copies of a native *S. lividans* gene, such as *afsR2,* which acts through raising *actII-ORF4* transcript levels (Kim *et al.,* 2001; Vögtli *et al.,* 1994). Low basal transcription of specific regulator genes, resulting in insufficient expression of activators in the synthetic pathway, may explain why actinorhodin is not produced in usual wildtype situations. Due to the fact that sufficient ActII-ORF4 is normally the limiting factor in actinorhodin production (Gramajo *et al.,* 1993), it was odd that *actII-ORF4* transcript levels between the control and *atrA* overexpression strains were so statistically similar, yet the blue antibiotic was only seen with the latter strain. As it was unclear from the transcriptional level what triggered Act production in the *atrA* overexpression strain, perhaps an explanation lies within the post-transcriptional stage of this activator protein. Further experiments to follow actinorhodin production in these strains could look at specific Act biosynthetic genes that are triggered by ActII-ORF4 to determine if differences downstream in the Act pathway exist between the two strains tested in section 2.3.3.

On a genetic level, AtrA binds upstream of *actII-ORF4* in order to stimulate its production – this raises the possibility that perhaps the *S. coelicolor atrA* gene is unable to efficiently activate *actII-ORF4* in *S. lividans,* despite the high degree of homology between the strains (Jayapal *et al.,* 2004; Zhou *et al.,* 2004). Sequence analysis revealed identical *actII-ORF4* genes in both streptomycetes, but a stretch of approximately 200 nucleotides directly before the translational start site was not conserved. Although the *atrA* binding site upstream of *actII-ORF4* was conserved, this could conceivably explain a lack of

actinorhodin production (Nothaft *et al.,* 2010). It should be noted that *atrA* from *S. coelicolor* has the ability to bind upstream of *strR* in *S. griseus,* which suggests that it does not have a history of functional problems in heterologous species (Uguru *et al.,* 2005; Hong *et al.,* 2007). Another possibility is that *atrA* may have additional uncharacterized targets that may affect actinorhodin production, such as a target that may increase the production of Act precursors. At this time, our results cannot be explained by the literature as yet.

As far as we know, no other group has tested the components of their overexpression constructs as thoroughly as we have. While transcript levels have been examined in the use of overexpression constructs in the past, the multi-level analysis completed here was missing (for example, see Malla *et al.,* 2010a). Interestingly, the doxorubicin work described in Malla *et al* (2010b) also took advantage of the *ermE** promoter (also see Rodríguez *et al.,* 2002; Wilkinson *et al.,* 2002; Thuy *et al.,* 2005). As well, in a paper detailing the precise FLP-mediated excision of an antibiotic cassette (see Chapter 4), the authors also utilized the combination of an idealized RBS and the *ermE** promoter (Zelyas *et al,* 2009). Our work contributes to this growing awareness that rational engineering of genetic constructs can enhance the functional outcome of the cloning process.

In the future, it would be useful to study the extent of the effect of this *tufl* AGGAGG RBS on the overall expression of a particular gene product. Indirectly, this could be accomplished through the methods described above. For example, the actinorhodin assay can be utilized to gain a general idea of *tufl* effects between the pSET152$_{ermE*}$-*atrA*$_{tufl\ \mathrm{RBS}}$ and pSET152$_{ermE*}$-*atrA*$_{native\ \mathrm{RBS}}$-containing strains. Translational efficiency can also be studied directly by following AtrA using western blotting. Moving forward, this chapter provides strong evidence that the pSET152$_{ermE*}$-*atrA*$_{tufl\ \mathrm{RBS}}$ construct provides an effective regulatory model for the overexpression of genes *in vivo* in *Streptomyces,* and supports the use of this promoter and RBS combination in future overexpression and cloning constructs. This was subsequently put into practice in the latter part of chapter 3.

CHAPTER 3: USE OF ANTIBIOTIC REGULATORS TO ACTIVATE ANTIBIOTIC PRODUCTION IN HETEROLOGOUS HOSTS

3.1 Introduction

Antibiotic production is a complex, multi-step process that involves input and signals from many genes, not necessarily only those genes that comprise the antibiotic biosynthetic cluster. Due to the genetic importance of secondary metabolite research in *S. coelicolor,* in particular antibiotics, research groups around the world have already discovered many regulators that play a role in antibiotic production. For example, the appearance of pathway- specific regulators for actinorhodin and undecylprodigiosin (ActII-ORF4 and RedD, respectively) have been tightly linked with the activation of the biosynthetic genes of their respective antibiotics, as described in (Bibb, 1996). As well, overexpression of these genes have resulted in a corresponding increase in antibiotic production, representing a viable manipulation for antibiotic optimization.

Whether rooted in the ubiquitous use of antibiotics, or in the natural resistance of many bacteria, antibiotic resistance is an emerging threat to the human health care system. Microorganims have long been a vital source of novel antimicrobial agents, but a variety of reasons have contributed to the decline of research resources in this area (Clardy *et al.,* 2006; von Bubnoff, 2006). With modern technology unravelling entire bacterial genomes, it is becoming clear that bacteria are genetically capable of producing more secondary metabolites than is seen in present laboratory conditions. The activation of these cryptic (silent) gene clusters, with the potential of discovering novel antibiotics, is the focus of this project.

My project builds upon the results of a global regulator from *S. coelicolor* which was found to trigger the expression of cryptic antibiotic clusters upon expression in various *Streptomyces* strains. This work, conducted in the Nodwell lab, began with the isolation of a mutant AbsA1 sensor kinase capable of acting as both a kinase and phosphatase of its substrate transcription factor, AbsA2. In its phosphorylated form, AbsA2 represses production of several antibiotics; as such, with the mutated *absA1* gene unable to maintain the phosphorylated form of AbsA2, antibiotic production in *S. coelicolor* was increased (McKenzie and Nodwell, 2007).

The introduction of a plasmid-borne copy of this mutant gene, simultaneously with the introduction of a wildtype *absA1,* into seventeen different *Streptomyces* strains (both characterized with known antibiotic production capabilities, and uncharacterized soil isolates) resulted in new or increased antimicrobial activity in ten strains (McKenzie *et al.,* 2010). These bioactive molecules were assayed through two main complimentary techniques, (1)

extraction of secondary metabolites subjected to liquid chromatography/mass spectrometry (LC/MS) for chemical footprinting analyses, and (2) spotted growth of *Streptomyces* in a biological assay against targeted bacterial indicator strains (from harmless models such as *Micrococcus luteus,* to clinical pathogens such as *Burkholderia cenocepacia).*

The most promising result was the growth inhibition of *B. cenocepacia* (Speert, 2003) – an opportunistic pathogen of compromised patients (such as those afflicted with cystic fibrosis) – by the mutated *absA1* introduced into *Streptomyces flavopersicus. S. flavopersicus* produces only one known antibiotic, spectinomycin, which is not one of the two drugs known to inhibit *B. cenocepacia.* In fact, *B. cenocepacia* is a difficult infection to treat, as it demonstrates high resistance to most antibiotics (Speert, 2003) – making this discovery that much more significant. Isolation and analysis of the chemical mixture from *S. flavopersicus* responsible for this activity (using ^{13}C NMR and MS) revealed it to be a previously characterized compound named pulvomycin. With the novel function of this molecule discovered, along with its previously unknown production in a strain of *Streptomyces,* this work provided insight into the utility of such a strategy in the further exploration of activating cryptic gene clusters in *Streptomyces* using known antibiotic regulators from *S. coelicolor.*

Using the pulvomycin discovery as a model, it is reasonable to assume that the introduction of proven regulators associated with antibiotic production in the model *S. coelicolor,* into heterologous *Streptomyces* strains may prove fruitful in the search for new antimicrobial compounds. There is reason to believe that evolutionary relationships between strains will allow this ectopic gene expression to activate cryptic biosynthetic clusters in host species.

My project followed the successful model of antibiotic discovery set out by the Nodwell lab, our collaborators in this undertaking. As mentioned above, many antibiotic regulators of *S. coelicolor* have been characterized in the literature, where modulation of expression has resulted in increased or decreased levels of antibiotics. From this list, a selection of regulators was chosen for this work (Table XI). Overexpression constructs (similar to those constructed in previous chapters) carrying these regulators were introduced into the wild isolate Cu#39 strain, and these recombinant strains demonstrated inhibitory action against the highly resistant Biosafety Level II (BSLII) pathogens, *Acinetobacter baumannii* and *Burkholderia cenocepacia.* The process of characterizing the antimicrobial molecule(s) responsible are described, as well as the future direction of this project.

3.2 Methodology

3.2.1 Creation of overexpression constructs for introduction into heterologous *Streptomyces* species

As noted in Table XI, the creation of the expression vectors was divided between the Elliot and Nodwell labs. My role in the construction of the overexpression vectors in pSET152$_{melC}$ began with many of the constructs already in intermediate vectors, courtesy of previous cloning by two members of the Elliot Lab (Christina DiBerardo and Hindra). The primer combinations used in this chapter are listed in Table XII. With the regulators already cloned into an intermediate vector, pIJ2925 (Janssen and Bibb, 1993), I was able to clone the regulators into the pSET152$_{melC}$ vector in a straight-forward manner. The desired fragment containing the gene of interest was cloned either by directional digestion out of pIJ2925 and into the *Bam*HI-*Eco*RI site of pSET152$_{melC}$, or was PCR-amplified and cloned into the blunt *Eco*RV site. Construction of the *atrA* constructs was described in chapter 2. Completed vectors were confirmed through polymerase chain reaction (PCR), diagnostic digestions, and DNA sequencing.

3.2.2 Investigation into antimicrobial activity: Biological assays

Bioassays were completed as described above in section 1.2.4. *Streptomyces* incubation times were found to be optimal for antimicrobial production at 24 or 48 hours (depending on the indicator strain).

3.2.3 Investigation into antimicrobial activity: Chemical extraction and thin-layer chromatography

Further characterization of antimicrobial activity involved growing *Streptomyces* as confluent lawns on DNA agar. Agar and growth were chopped into fine pieces with a scalpel, and shaken for approximately 16 hours at 4°C in ethyl acetate, allowing for sufficient aeration and space for agar pieces to move freely. The ethyl acetate solution was then spun down to separate the agar pieces from the solution, and transferred to round bottom flasks to be evaporated using a Heidolph™ LABOROTA rotary evaporator.

Extracts resuspended in ethyl acetate after rotary evaporation were used for thin-layer chromatography analyses. Using a borosilicate glass capillary tube, ~ 2 µL of each sample was spotted on a silica gel (ALUGRAM SILG/UV254 0.2 mm, Camlab Ltd), where the mobile phase of chloroform:methanol (9:1) was used to separate fractions. Spots of antimicrobial activity were visualized by overlaying dried TLC plates with 20 mL of soft agar containing a 1/100 dilution of an overnight culture of an indicator strain. A colourimetric assay of viable/nonviable indicator cells was applied by overlaying 1.5 mL of 5 mg/mL of

thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) over the entire plate surface. After incubation at 37°C for one hour, a yellow clearing on the blue-dyed plate was indicative of death of the indicator strain, thus identifying zone(s) of inhibition by crude molecular fractions.

3.2.4 Investigation into antimicrobial activity: LC/MS

Alternatively, the extracts were resuspended in 200-500 μL of dimethyl sulfoxide (DMSO), before being subjected to liquid-chromatography/mass spectrometry (LC/MS) for chemical footprinting (searching for the presence of new molecules).

Table XI: Antibiotic regulators used in this work. The * denotes the overexpression constructs created in the Elliot Lab

Antibiotic regulator	Gene annotation	Antibiotic regulator	Gene annotation
absA1	*SCO3225*	*eshA**	*SCO7699*
*absB**	*SCO5572*	*scbR**	*SCO6265*
abaB	*SCO3919*	*metK**	*SCO1476*
*afsQ1**	*SCO4907*	*ppk*	*SCO4145*
*afsR**	*SCO4426*	*scbA*	*SCO6266*
afsS	*SCO4425*	*atrA**	*SCO4118*

Table XII: List of oligonucleotides sequences used in this chapter.

Description of PCR oligonucleotide	Oligonucleotide sequence (5'-3')
Amplification of *absA1*	Upstream: AAAAAAAGATCTAGGAGGACCCCAGTATGCACCGAT GGCAGGCCG Downstream: TTTTTTGAATTCCCTGGTGGTCAGAGCGTGTCCTT
Amplification of *absB*	Upstream: GGTCTGAGCGGCTGGTGAGAGG Downstream: CGTCGGCGACCGTTCGGTGG
Amplification of *abaB*	Upstream: GGACGAAACGACGGAATGACG Downstream: GAGAAATGTGGCCCGGCTGG
Amplification of *afsQ1*	Upstream: CCTCATGGGCCAGAATGAGC Downstream: GGTACCCCCCTGGTGTTCC
Amplification of *afsR*	Upstream: AAAAAAGGATCCAGGAGGACCCCAGTATGGACGGT GGACCGCGGG Downstream: TTTTTTGAATTCCGGGTTCACCGCGCCACACT
Amplification of *afsS*	Upstream: AAAAAAGGATCCAGGAGGACCCCAGTATGAGCGAC

	AAGATGAAGGG Downstream: TTTTTTGAATTCAGGTCTACTTGCCGTCGCCGT
Amplification of *eshA*	Upstream: GGTCTCCCCGTCGTGCAAGG Downstream: CGGAGTCGGGCATCGGTGG
Amplification of *metK*	Upstream: CGACCGATAAACTGTTCTCG Downstream: CGGATGAGCGCGAGCTGC
Amplification of *ppk*	Upstream: AAAAAAGGATCCAGGAGGACCCCAGTATGAAGCCG ACCGAGCCACA Downstream: TTTTTTGAATTCGGGTCAAGGTGTCGCTGTGCC
Amplification of *scbA*	Upstream: AAAAAAGGATCCAGGAGGACCCCAGTATGCCCGAA GCAGTAGTTTTGAT Downstream: TTTTTTGAATTCGGTGCTCAGCCGGAGAACGC
Amplification of *scbR*	Upstream: GGGAGACATGAACAAGGAGG Downstream: GACGTGGTGGCCTTTCTGG

3.3 Results

3.3.1 Overexpression constructs were created using the integrative pSET152 plasmid containing either the *melC* or *ermE* promoter

As a collaborative project involving the Nodwell lab in the Department of Biochemistry and Biomedical Science, the scope of this project necessitated a division of labour. Initially, work was split according to the regulator genes, with each lab introducing their overexpression strains into the each of the heterologous *Streptomyces* strains.

Of the initial group of genes assigned to me (*absB, afsQ1, metK, scbR, afsR,* and *eshA),* several were cloned into both pSET152$_{melC}$ and pSET152$_{ermE}$. Both promoters were used because the work in chapter 2 had not yet been initiated, thus there remained uncertainty regarding promoter effectiveness, despite their use in the literature (Bibb *et al,* 1985; Schmitt-John and Engels, 1992). In time, our collaborators at JNE Biotech Inc had positive results involving pSET152$_{melC}$ in expressing the streptomycin regulator gene, *strR,* thus my work in this chapter continued on using mostly the *melC* promoter.

3.3.2 Constructs were moved into heterologous *Streptomyces* strains to assay for antimicrobial activity

As a starting point to this research, the three strains that were among the top producers of increased antimicrobial production in the screen with *absA1* were selected to begin this work: *S. venezuelae, S. griseochromogenes,* and *S. flavopersicus* (McKenzie *et al.,* 2010). Over time, preliminary results and a division of work with our collaborating lab resulted in a shift of my focus. My efforts subsequently concentrated on introducing all regulator constructs into the wild isolate Cu#39, a strain that had also been effective in antimicrobial production in past work.

3.3.3 *Streptomyces* induced growth inhibition was seen against *Acinetobacter baumannii* and *Burkholderia cenocepacia*

Bioassays with Cu#39 were conducted against each of the BSLII organisms (Table II) using the full compliment of regulators (see Table XI; the various *atrA* constructs in Figure 5 were also included). The most significant growth inhibition was seen against *Acinetobacter baumannii* B0098426R and *Burkholderia cenocepacia* CEP509. With *A. baumannii,* growth inhibition by Cu#39 was greatest after 24 hours of *Streptomyces* incubation time (16h, 24h, 36h, 48h, 60h, 72h, 96h, 120h were all tested) (Figure 9). Of two timepoints tested with *B. cenocepacia,* the greatest inhibition was seen after 48 hours (Figure 10). Against both indicator strains, the empty vector controls had approximately the

same level of activity as the strains carrying overexpression vectors with regulators. While there were recombinant Cu#39 strains that showed statistically significant increases in antimicrobial activity (indicated by the asterisks), we applied caution in our interpretation of this. It was prudent to not assume this was necessarily an indication of major overexpression, as these bioassays were only semi-quantitative, and most differences were not large.

3.3.4 Thin layer chromatography gave insight into effects of *atrA* overexpression in Cu#39

Based on the bioassays completed in section 3.3.3, several regulators were moved forward to the next stage of analysis, as a preliminary exploration of the nature of the molecule(s) producing zones of inhibition against *A. baumannii.* The Cu#39 strains that were chosen were those overexpressing *atrA, afsR, metK,* their respective controls, and the wildtype Cu#39 strain. Chosen for their ability to inhibit the growth of *A. baumannii* after 24 hours of *Streptomyces* growth, these strains also exhibited activity against *S. aureus,* allowing this BSLI organism to subsequently be used as the representative indicator strain for antimicrobial activity following thin-layer chromatography (TLC).

When TLC was conducted (Figure 11), it was not clear whether there were different molecules produced by the various strains. The *S. aureus* zones of inhibition were found at approximately the same level on the chromatography gel, crudely suggesting a molecule, or molecules with similar chemical properties were responsible for inhibition (as mobility is dependent upon interactions between the solid and mobile phase of the chromatography reaction). Looking at lanes 1 and 4, there was a marked difference seen between the activity of the pSET152$_{ermE*}$-*atrA*$_{tufI\ RBS}$ construct and its control, making these candidates strains to move to the next stage of characterization. This difference would ease the identification of bioactive molecules, as the TLC suggested that they would have a significant presence compared to the control strain. This work is ongoing.

3.3.5 Using the *ermE** promoter to overexpress *afsR, metK,* and *atrA* in select *Streptomyces* species

Following the expression level results in 2.3.2 and 2.3.3, several of the more promising regulators (like above, we chose *afsR, metK* and *atrA*) were selected for incorporation into overexpression constructs containing the *ermE** promoter, and the *tufI* RBS. These overexpression constructs will subsequently be introduced into six select streptomycetes (four of which have publically available genome sequences), as this will facilitate the identification and characterization of specific biosynthetic clusters. The same methodology as described above for the search for new bioactive molecules will be followed.

At present, bioassays have been completed with pSET152$_{ermE*}$-$atrA_{tufI}$ RBS and its control in *S. griseus, S. lividans,* Ja#2b and Cu#39. Preliminary bioassays against all BSLI indicators (Table II) indicated that there was not significant novel antimicrobial activity induced upon *atrA* overexpression, except for the activity previously noted with Cu#39.

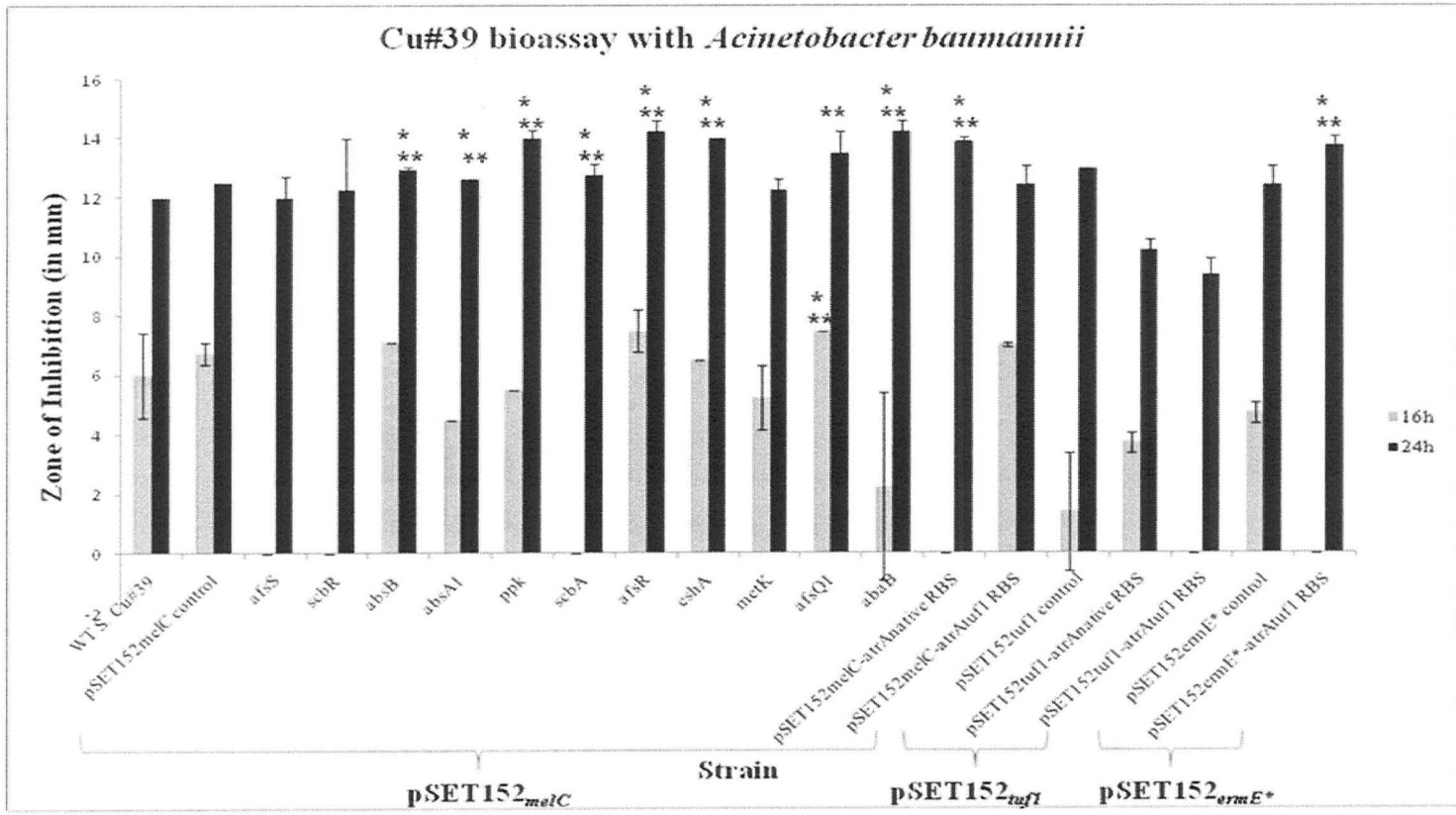


Figure 9: Bioassay of Cu#39 strains against *Acinetobacter baumannii* on Difco nutrient agar. Overexpression strains were created using one of three promoters, as delineated above, and were incubated for either 16h or 24h intervals. The average of two spots was taken to produce the data above. Strains that displayed a significant production of antimicrobial research over the WT (*) and/or empty vector control (**) have been indicated.

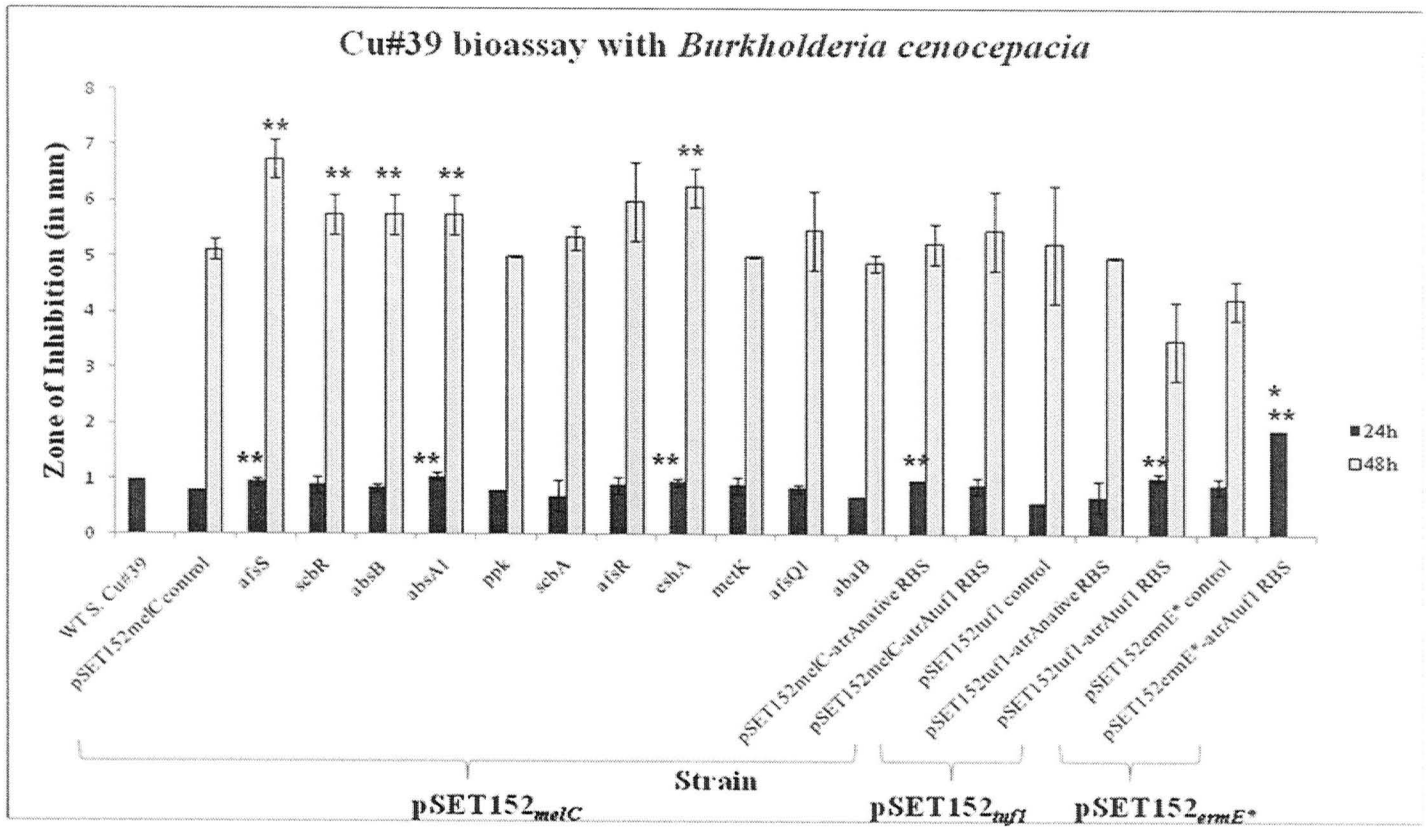


Figure 10: Bioassay of Cu#39 strains against *Burkholderia cenocepacia* on DNA agar. Overexpression strains were created using one of three promoters, as delineated above, and were incubated for either 16h or 24h intervals. The average of two spots was taken to produce the data above (one trial was completed). Strains that displayed a significant production of antimicrobial research over the WT (*) and/or empty vector control (**) have been indicated. Note: The WT strain was not included in the bioassay at 48h in error – all other strains displated antimicrobial activity as presented.

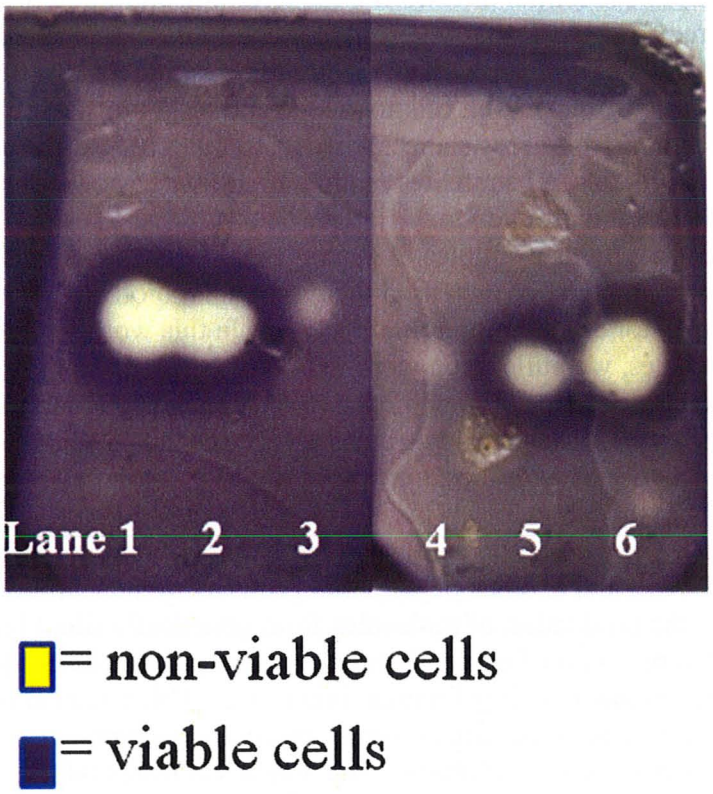


Figure 11: Thin layer chromatography (with *S. aureus* overlay and MTT visualization) showed the presence of spots of antimicrobial activity against *S. aureus,* derived from Cu#39 ethyl acetate extracts. Each extract was spotted using a closed-ended capillary tube, in the following order (left-right):
(1) pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS,}$ (2) pSET152$_{melC}$-*afsR*, (3) pSET152$_{melC}$-*metK*,
(4) pSET152$_{ermE*}$ control, (5) pSET152$_{melC}$ control, and (6) WT Cu#39.

3.4 Discussion

The rise of nosocomial infections and increasing antibiotic resistant pathogens has required increasing investment in antibiotic discovery. As the discovery of novel antibiotics has fallen off precipitously in the past few decades, there is a push towards the pursuit of knowledge and technology to regain the upper hand against microbial resistance; whether through new antibiotics, or modifying old antibiotics, all strategies require an intimate knowledge of antibiotic regulation and production.

The literature holds examples of strategies that have successfully activated novel antibiotic production, or increased the titres of antibiotics – both of which are important milestones in antimicrobial research. In this work with the *Streptomyces* species, we build upon work in investigating the activation of silent antibiotic clusters as a source of new bioactive molecules (McKenzie *et al.,* 2010; Schroeckh *et al.,* 2009). These 'cryptic clusters' are secondary metabolite clusters present in the genomes of microbes, but whose products are not expressed at detectable levels in the laboratory. This is a recurring theme in the sequenced streptomycete genomes currently available (for example, Bentley *et al.,* 2002 and Ohnishi *et al.,* 2008), and extends beyond this genus as well (Schroeckh *et al.,* 2009). Inducing the production of molecules from genetically silent loci could prove to be a rich reservoir of clinically important antimicrobials products, as well as other compounds that may be of human importance. Other than antibiotics, the *Streptomyces* genus is also recognized for its ability to generate immunosuppressants, enzyme inhibitors, and anticancer drugs (Horinouchi, 2007; Malla *et al.,* 2010a).

In this work, antibiotic regulatory genes from *Streptomyces coelicolor* were cloned into integrative overexpression constructs under the control of the *melC* promoter, before being moved into various *Streptomyces* heterologous hosts. Induction of antimicrobial activity was assayed against the basal level of the empty vector control. Biological assays conducted using the wild isolate Cu#39 strain revealed it to have antimicrobial activity against two highly resistant Gram-negative Biosafety Level II pathogens, *Acinetobacter baumannii* and *Burkholderia cenocepacia.* Inhibition of these Gram-negative bacteria was significant (regardless of whether it was induced by a heterologous regulator or not) because both these strains represent highly resistant strains that are prevalent in the clinical environment. Whether due to structural features that confer high intrinsic resistance, the ability to gain timely resistance, or an alternative, health care institutions are struggling at present with containing members of these strains (Gordon and Wareham, 2010; Speert, 2002).

Examining the bioassay results in section 3.3.3 revealed that for both indicator strains, the antimicrobial activity (when at its peak effectiveness, as evidenced by the largest zone of inhibition) was similar across all regulators tested. For those regulators that were calculated to have a significant raise in

antimicrobial activity over the wildtype or control strains, in almost all cases, the semi-quantitative nature of the bioassays and variable growth of *Streptomyces* took away from the significance of these findings. Despite care taken in obtaining the measurements of the zones of inhibition, human error also had to be accounted for.

An anomaly was the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ carrying strain of Cu#39 against *B. cenocepacia* at 48 hours. In this case, there was surprisingly no activity seen. As there were zones seen in the rest of the strains, as well as at the 24 hour timepoint for this strain, it is possible that the lack of bioactivity seen may have been due to error (spores not mixed well before being spotted on the agar, perhaps), rather than inactivity. It was possible to see antimicrobial activity disappear from one spot to another on a bioassay, or variations in inhibition zones, thus confirming that these bioassay results were subject to a range of variability. However, as this particular bioassay was only completed once, it must be completed again in order for definitive conclusions to be made.

Also of note was that the appearance of the antimicrobial activity against both indicator strains was maximally detected at different times (Figure 9 and Figure 10). This phenomenon was consistently observed: in two separate trials against *A. baumannii,* the Cu#39 strains incubated anywhere from 16-120 hours had the highest antimicrobial activity after 24 hours. This was seen once again in the bioassays completed against BSLI organisms in section 3.3.5.

The nature of the bioactive molecule(s) present remains unclear – indeed, there is no definitive answer as to the number of molecules that may be produced by Cu#39 active against *A. baumannii*. Due to the similar level of antimicrobial activity shown by the strains expressing various regulators, it is not hard to believe that each produced the same molecule, especially when similar activity was shown by the wildtype and control strains. The difference in the size of the inhibition zones may be attributable to varying sizes of the *Streptomyces* spots. There is the possibility that another molecule appeared after an 120 hour incubation, as consistently low levels of Cu#39 activity against *A. baumannii* shot up at that time point. If this was the same molecule(s) as the earlier timepoint, this may rule out molecule stability as the reason for the lost of activity after the 24 hour timepoint. Regardless, the number of molecules and its production levels are still speculative at this point, and require further experiments to elucidate.

From the bioassay results, investigations begun with the molecule(s) seen after 24 hours of Cu#39 growth. Select regulators we were particularly interested in were chosen for this further analysis: *afsR,* a pleiotropic regulator of actinorhodin and undecylprodigiosin (Santos-Beneit *et al.,* 2009); *metK,* producer of *S*-adenosylmethionine that has been implicated in the heterologous activation of secondary metabolites (Shin *et al.,* 2006); and the previously described *atrA* gene (carried in the optimized overexpression vector from chapter 2). Analyzing extracts from these strains of interest through TLC did not immediately answer the question of whether a single molecule was responsible for the zones of

inhibition. I have begun work to elucidate the identity of the molecule(s) with antimicrobial properties against *A. baumannii.* Experiments aimed at the chemical footprinting of molecular extracts from Cu#39 containing the pSET152$_{ermE*}$-*atrA*$_{tuf1\ RBS}$ plasmid, as compared to its vector control, did not yield definitive results due to too dilute concentrations of the extract needed for LC/MS. However, there were indications that several novel peaks existed in the *atrA* strain that were not present in the control. Future work will include determining the identity of the molecule(s) responsible for the inhibitory activity, and determining whether it represents a novel antimicrobial molecule.

While we have successfully identified at least one antimicrobial molecule of interest, we acknowledge that the possibility of Cu#39 producing additional molecules cannot be discounted. Among other factors, bioassays serve only as an early indication of antimicrobial activity. Variations in the growth media, incubation time, indicator strains, and solvent used in the extraction and subsequent resuspension are all factors that may exclude various molecules from being identified. These are all considerations in studying metabolite production, and are factors to be aware of in order to cover the widest possible field for molecule mining.

As mentioned above, a timed bioassay showed 24 hour incubation of Cu#39 provided the largest inhibition by a large margin, with the 120 hour timepoint second following that (at about half the zone of inhibition size). Extractions of the antimicrobial molecules were always after one day of growth. Whether or not completing extractions after 120 hours of growth would yield different molecules than a 24 hour extraction, is yet to be determined. Examples in the literature completed characterization analyses on molecules extracted from *Streptomyces* growth for at least 3-5 days (Elleuch *et al.,* 2010; McKenzie *et al.,* 2010), and performed extraction from cultures, so this is another avenue to explore.

The antimicrobial activity seen across all strains (Figure 9 and Figure 10), including the wildtype, did not support the initial hypothesis that overexpression of antibiotic activators would lead to the expression of cryptic metabolic clusters. Instead, at least for Cu#39, it presented the possibility of an innate molecule (produced irrespective of the presence of heterologous regulators) bearing responsibility for the antimicrobial activity observed. When further characterization was undertaken using TLC, two of the strains seemed to exhibit differential production of antimicrobial activity that was not clearly captured by these bioassays. This may represent either the increased sensitivity of TLC as a technique, or the extraction-TLC process representing a bottleneck step in eliminating possible antimicrobial molecules. These were the strains whose chemical extracts were subsequently used for LC/MS analysis. Another possible explanation for the lack of differential effect of the various regulators lies with the effectiveness of the *melC* promoter that was used for the majority of the overexpression constructs. It is possible that regulators were not expressed at

levels sufficient to trigger any cellular changes that would result in a significant increase in antimicrobial activity.

The *atrA* expression studies from chapter 2 indicated that moving particular regulators of interest into constructs containing the *ermE** promoter and *tufI* RBS may result in more effective overexpression – work that was initiated in section 3.3.5. By narrowing down the scope of this project by working with a subset of the antibiotic regulators, and introducing them into characterized *Streptomyces* (*S. griseus, S. lividans,* Mg1 and SPB74), or streptomycetes with proven antimicrobial activity (Cu#39 and Ja#2b), this will facilitate the screening and discovery of new molecules. Although preliminary results have indicated that the *atrA* gene appears to induce some antimicrobial activity, it remains to be seen whether an idealized overexpression construct could be used for other regulators as well.

In sum, it is not possible to definitively determine the success or failure of our proposed activation of silent metabolic clusters in heterologous *Streptomyces* strains through characterized antibiotic regulators. Due to the fact that the majority of the analysis of this work was completed in one strain (Cu#39), and with possibly ineffective expression constructs, it is not possible to conclude if these results are representative of what would be found on a larger, more complete screen of multiple strains. If indeed the presence of the antimicrobial molecule(s) seen in Cu#39 was native to the wild isolate, then this does provide support for the need to invest attention towards delving into exploiting uncharacterized (and possibly difficult to work with) microbes (Lewis *et al.,* 2010). As well, if the new direction undertaken in section 3.3.5 proves fruitful, it could serve as a platform for expanding this project through the use of constructs with a more efficient base of overexpression.

As discussed previously, there has been significant progress in understanding antibiotic regulation and production – along with the various creative methods for initiating antibiotic production, there is also significant literature on exogenous methods of improving titres (such as the work completed by Tanaka *et al.,* 2010). With the plethora of natural antibiotics not accessible by current approaches – there is a need to develop the strategies and technology needed to tap into this large pool of molecules (Lewis *et al.,* 2010; Watve *et al.,* 2001). In the next chapter, we discuss the progress made in the creation of a universal expression host strain of *S. coelicolor*, for the directed purification and production of metabolites of interest.

CHAPTER 4: DEVELOPMENT OF AN "ANTIBIOTIC-OVEREXPRESSING" STRAIN OF *STREPTOMYCES COELICOLOR*

4.1 Introduction

Boosting metabolite production in microorganisms, such as antibiotic production, can be achieved in many different ways. From antibiotic titre improvements through the modification of primary metabolism, to changing the presence of positive and negative regulators appropriately, strain improvement has been a desired goal in both academia and industry (Borodina *et al.,* 2008; Chen *et al.,* 2010). In recent years, technology has greatly improved the precision and scope of genetic manipulations to various cellular machinery and pathways (Baltz, 2006). This has resulted in increased metabolic yields, decreased expenses, and increased efficiency in both molecular engineering and industrial production. In general, almost every part of a metabolic pathway can be selected and optimized further.

As secondary metabolite clusters share the intermediates of primary metabolism for the building blocks of their products, it follows that the elimination of the production of certain secondary metabolites could increase the production of remaining molecules (Drew and Demain, 1978). This is the principle behind my last project, the creation of an antibiotic overproduction host strain of *Streptomyces coelicolor*. This *S. coelicolor* strain will serve as a host in which a specific metabolite can be driven to be expressed at a higher and more pure level than its native expression, thus facilitating investigations into that compound.

Strain improvement of wildtype *S. coelicolor* will be conducted through multiple deletions of known secondary metabolites, as identified in the publication of its genome (Bentley *et al.*, 2002). This will result in the creation of a *S. coelicolor* strain lacking select metabolites, freeing commonly shared precursor molecules, cellular energy and resources for the increased yield of a desired product. Introducing antibiotic production gene clusters, along with global regulators of antibiotic production, into this strain of *S. coelicolor* will enable the rapid characterization of new antibiotics. This follows from my previously described antibiotic work well, serving as a useful tool for the purification of small, naturally-produced molecules.

PCR targeting methodology, as described elsewhere (Gust *et al.,* 2002; Gust *et al.,* 2003), was used to knockout the key regulatory genes (or all genes in smaller sized clusters) of a particular secondary metabolite cluster. In an overview of the process, an antibiotic cassette (in this case, apramycin) was PCR-amplified with sequences homologous to the nucleotides flanking the targeted knockout area in the chromosome, such that homologous recombination would result in the replacement of the endogenous sequence with the selectable antibiotic cassette. This cassette was then removed from the chromosome, such that the same

antibiotic cassette was used to generate multiple knockouts in the same organism without losing its ability to select for chromosomal insertions (Fedoryshyn *et al.,* 2008).

Here, strains of *S. coelicolor* were generated that each contained the loss of a secondary metabolite, and as I accumulated deletion strains, I collected them into a singular strain that contained all the knockouts (the universal host strain).

4.2 Methodology

4.2.1 Isolation of the disruption (apramycin resistance) cassette

Elimination of secondary metabolites was completed through gene targeting of the relevant clusters in *S. coelicolor* using PCR-targeting methodology (Gust *et al,* 2002). In this procedure, a targeted chromosomal location was replaced with a selectable antibiotic resistance cassette (apramycin, in this work). Isolation of the plasmid pIJ773 containing the apramycin disruption cassette from an overnight culture of DH5α cells was completed using a miniprep kit (QIAGEN) as per the manufacturer's instructions. Confirmation of expected plasmid size was completed through 1% agarose gel electrophoresis. The desired disruption cassette was then excised using *Eco*RI and *Hind*III in a single restriction enzyme digest. This reaction was incubated in a 37°C water bath for one hour; the composition of this reaction is shown in Table IX. Subsequent precipitation allowed this entire reaction to be run in one lane on an agarose gel for scalpel excision and gel purification of the desired band.

4.2.2 PCR amplification of disruption cassette

Generation of the apramycin disruption cassette for a specific cluster knockout was achieved using long PCR primers designed for in-frame gene deletion once the disruption cassette was introduced into *S. coelicolor,* with 5′ ends complimentary to 39 nucleotides flanking the gene(s) to be deleted, and 3′ ends complimentary to the cassette (see Table XIII). The purified disruption cassette was used as the template in the PCR reaction to amplify the disruption cassette. The iProof PCR system from Bio-Rad was used according to instructions, and the specialized PCR program used is shown in Table XV (see Table VII for the contents of a typical PCR reaction). Amplification was verified by gel electrophoresis, before precipitation and purification of the cassette was carried out as described above.

4.2.3 PCR targeted knockout of targeted genes on a *S. coelicolor* cosmid

E. coli BW25113/pIJ790, carrying a *S. coelicolor* genomic cosmid (Table XIV) with the desired gene(s) to be inactivated, was electro-transformed with the

purified disruption cassette to permit gene disruption through homologous recombination.

From an overnight culture, 400 µL of *E. coli* was grown in 40 mL of SOB lacking $MgSO_4$ (Hanahan, 1983) with the appropriate antibiotics and 10 mM of L-arabinose for 3-4 hours at 30°C until an OD_{600} between 0.4-0.6 was reached. Cells were recovered with three rounds of washing with 10% glycerol, as follows: spin at 4000 rpm for 5 minutes in a table-top centrifuge, followed by repetitive steps of glycerol resuspension and centrifugation with successive volumes of 10 mL, 10 mL, and 5 mL of 10% glycerol. The final pellet was resuspended in approximately 100 µL of the supernatant. In an ice-cold 1.5 mL Eppendorf tube, 50 µL of the cell suspension was gently mixed with 2 µL of the purified cassette, before the DNA-cell suspension was transferred to an ice-cold electroporation cuvette. Electroporation was performed using a BioRad MicroPulser™ (expected time constant of 4.5-5.2 ms), with 1 mL of ice cold LB added immediately afterwards. The electroporated cells were grown by shaking for one hour at 37°C, before being spun down and spread on LB plates containing selective antibiotics. Successful introduction of the disruption cassette into the desired cosmid was verified through cosmid isolation and subsequent restriction analysis.

4.2.4 Introduction of the mutant cosmid into *S. coelicolor* strain M145

As *S. coelicolor* possesses a strict restriction system that destroys methylated (deemed foreign) DNA, the mutant cosmid containing the desired knockout gene needed to first be introduced through a non-methylating *E. coli* host (such as the ET12567/pUZ8002 strain; MacNeil *et al.,* 1992; Kieser *et al.,* 2000). An electro-transformation was used to introduce the mutant cosmid into the ET12567/pUZ8002 strain. The desired transformants were selected for with the appropriate antibiotics on agar plates, and used as the inoculum for conjugation (as described in chapter 1) into *S. coelicolor.*

Replica plating to screen for colonies of the expected antibiotic genotype was carried out on Difco nutrient agar (DNA) plates containing naladixic acid and apramycin, compared to growth on naladixic acid and kanamycin. This identified colonies with the desired apramycin^{R}kanamycin^S resistance phenotype. PCR analysis (60°C, 90 seconds) using different combinations of primers were used to confirm the loss of the targeted gene(s). Once candidate colonies were verified through antibiotic sensitivity, and PCR, spore stocks were created for long-term storage and use.

4.2.5 In-frame removal of the disruption cassette

In order to utilize the same antibiotic disruption cassette for multiple knockout targets, the disruption cassette was removed in such a way as to leave only a small, in-frame "scar" sequence behind in the genome. This was mediated

through homologous recombination between the FRT (FLP recognition target) sites flanking the apramycin cassette in the presence of the FLP recombinase enzyme. Two similar methods were used in this regard, one which facilitated the FLP procedure in *E. coli,* and the other in a *Streptomyces* host. The creation of a "scar" plasmid in *E. coli* before being introduced into *S. coelicolor* via protoplast transformation, is described first in the following section.

A 10 mL LB overnight culture of *E.coli* DH5α cells containing the temperature sensitive (30°C) BT340 plasmid (Datsenko and Wanner, 2000) with appropriate antibiotic selection was used to start a 10 mL LB subculture at 30°C. Electroporation with the mutant cosmid lacking the knockout target was carried as described above. Transformants were incubated at 30°C for approximately 48 hours, before a single colony was streaked on a non-selective LB plate for single colonies. Overnight growth at 42°C resulted in expression of FLP recombinase, as well as subsequent loss of the temperature-sensitive BT340 plasmid.

Colonies sensitive to apramycin (due to loss of the disruption cassette) were then identified by streaking single colonies on a LB agar plate containing apramycin, followed by a plate containing kanamycin. Overnight growth at 37°C provided candidate colonies whose cosmids could subsequently be verified through PCR and restriction analysis.

Similarly to section 4.2.4, introduction of a cosmid into *S. coelicolor* and subsequent homologous recombination was the method used to replace the chromosomal cassette with the vector-bound "scar" sequence. In this case, protoplast transformation was required to introduce the "scar" cosmid as it no longer contained the cassette-bound *oriT* sequence. Passage of the cosmid through *E. coli* ET12567/pUZ8002 (MacNeil *et al.,* 1992; Kieser *et al.,* 2000) yielded unmethylated DNA ready to be transformed, in a procedure adapted from that described by Gust and colleagues (2002). Wildtype spores were grown at 30°C in a 10 mL solution of equal parts YEME and TSB (Table V) for one day, before being subcultured in a baffled flask to a final volume of 40 mL for the second day. This was incubated for approximately 24-30 hours. The culture was then centrifuged at 3000 rpm for 5 minutes, before three successive washes with 10.3% sucrose.

The resultant pellet was resuspended in 4 mL of filter sterilized lysozyme solution (3 mg/mL in P (protoplast) buffer; (Kieser *et al.,* 2000) and incubated in a 30°C water bath for 45 minutes. A 5 mL serological pipette was used to thrice quickly draw up and expel the solution, before continued incubation for another 15 minutes. The same mixing and incubation was repeated after an additional 5 mL of P buffer was added to the reaction. The protoplasts were then filtered through a 10 mL syringe plugged with cotton into a 15 mL falcon tube. Centrifugation at 3000 rpm for 7 minutes and subsequent resuspension of the protoplasts in approximately 500 μL of supernatant yielded cells receptive to transformation.

After 5 µL of the cosmid to be transformed was added to a 500 µL volume of protoplasts, it was immediately mixed by tapping the tube. Two hundred microlitres of fresh 25% polyethylene glycol (PEG) in P buffer was added next, and this was mixed by drawing the solution in and out of the pipette four times. The mixture was immediately divided and spread on two R2YE plates before incubation for 14-20 hours at 30°C. Subsequent flooding with 1.25 mg of kanamycin (selective for colonies containing the cosmid) and growth for 3-4 days provided selectable colonies that were streaked for single colonies on non-selective MS media and grown for 3-4 days at 30°C. Replica plating was carried out to score for colonies with the desired antibiotic phenotype.

The second method of generating an unmarked mutation involved expressing a synthetic (high GC content) FLP recombinase in *Streptomyces,* thus preventing the need for generating a scar cosmid in an *E. coli* host. Conjugation introduced pUWLFLP (Fedoryshyn *et al.,* 2008) into *Streptomyces.* Under the constitutive *ermE** promoter, FLP recombinase was immediately functional, and isolates with the correct antibiotic phenotype were screened as described above. Typically, several rounds of growth on selective hygromycin were necessary before correct exconjugants that were apramycin sensitive (signifying the loss of the antibiotic cassette from the chromosome) were found.

4.2.6 Genomic DNA preparation for PCR checks of the knockout strains (modified from Pospiech and Neumann, 1995)

S. coelicolor was cultured for 24-28 hours as described above, in 10 mL of YEME/TSB (1:1) broth, before being harvested in a centrifuge at 3000 rpm for 5 minutes. The cell pellet was resuspended in 500 µL of GET solution (1% glucose, 20 mM pH8 EDTA, 50 mM ph 8 Tris-HCl). To lyse cells, 10 µL of lysozyme (30 mg/mL) and 10 µL of RNaseA (5 mg/mL) were added to the culture before incubation at 37°C for 30 minutes. Approximately 10 µL of 10% SDS were mixed into the solution, before the addition of one volume of phenol/chloroform/IAA in an eppendorf tube. After gentle mixing, the mixture was spun at 13 000 rpm for 5 minutes to separate into two phases. The aqueous phase was transferred to a new tube, and this extraction step repeated until a clear interface was achieved. At this point, genomic DNA was precipitated out by the addition of 1 mL of 95% ethanol to the final aqueous phase. This tube was set at room temperature for approximately 5 minutes, before the DNA was recovered by centrifugation and resuspension of the resultant pellet in 50 µL of sterile water. Genomic DNA was subsequently used to perform PCR checks of the knockout strains.

Table XIII: List of oligonucleotides sequences used in this chapter. The cassette-specific primer sequence is underlined in each oligonucleotide used to create a knockout cassette.

Description of PCR oligonucleotide	**Oligonucleotide sequence (5'-3')**
Amplification of apramycin knockout cassette (actinorhodin cluster)	Upstream: GCCCCCAGGAGACGGAGAATCTCGACGGGGGCGC AGATGATTCCGGGGATCCGTCGACC Downstream: TCAGGGTGTGGACCACCCGGTGTTCTCCCGGCCGG CTCATGTAGGCTGGAGCTGCTTC
Verification of loss of targeted actinorhodin genes	Upstream: TCGGAAGCCTCGACCACTGC Downstream: TCCTGGCGGAACGTCACCC Internal to gene cluster: ATGCGAAATTACCAGGGACCG
Amplification of apramycin knockout cassette (undecylprodigiosin cluster)	Upstream: GTCCTGTGTTGAGGCCCGAATCCGATCGTTCGGTG GATGATTCCGGGGATCCGTCGACC Downstream: GTACGGTCCCCATCCTTCCTGGAC GAAAGTCAACGTATGTGTAGGCTGGAGCTG CTTC
Verification of loss of targeted undecylprodigiosin genes	Upstream: CGTTTGCGTCGTTCAGTTC Downstream: TAAGTTTGGCCGCATGAGG Internal to gene cluster: CGCCGCGCAGGAACATCG
Amplification of apramycin knockout cassette (calcium-dependent antibiotic cluster)	Upstream: AACTGAGCGCGGAGTCGAGCCCGGAGGGCGTCAG GGATGATTCCGGGGATCCGTCGACC Downstream: GAGTCTCCAGCCCGACGCTCCCGGAAGGAATGCG ACGTGTGTAGGCTGGAGCTGCTTC
Verification of loss of targeted CDA genes	Upstream: GGACGCGGGCAAGACGATGCC Downstream: GGTGGACCGGCCGTTCCTGC Internal to gene cluster: TCACCGGGTCGCCGGGCTGC

Amplification of apramycin knockout cassette (*scbA* gene)	Upstream: AACAGCTCGGCATCACATGATTCCTGGGGGGGAC CCATGATTCCGGGGATCCGTCGACC Downstream: CGCGAACCGGCGATGTTCGCGCCCCGGGGCCGGT GCTCATGTAGGCTGGAGCTGCTTC
Verification of loss of targeted *scbA* gene	Upstream: TTGGTAGCCCTGCTTCTCG Downstream: GCGCAGACCTCGACGTTCG Internal to gene cluster: ACTTGAGGACTGGTGAAGC

Table XIV: *Streptomyces* cosmids used in this chapter as templates to amplify knockout antibiotic selection cassettes (Redenbach *et al.*, 1996).

Targeted knockout region	**Cosmid**
Actinorhodin (Act): *SCO5085-5089* (~4000 bp)	StBAC28G1
Calcium-dependent antibiotic (CDA): *SCO3216-3218* (~5000 bp)	StE8
Undecylprodigiosin (Red): *SCO5877-5881* (~6000 bp)	St2E9
scbA: SCO6266 (~1000 bp)	StAH10

Table XV: PCR Program for amplification of extended disruption cassette. Steps 2-4 and 5-7 are repeated for a total of 15 cycles each.

Step	**Temperature (°C)**	**Duration**
1) Denaturation	94	2 minutes
2) Denaturation	94	45 seconds
3) Anneal	55	45 seconds
4) Extension	72	1.5 minutes
5) Denature	94	45 seconds
6) Anneal	60	45 seconds
7) Extension	72	1.5 minutes
8) Final extension	72	5 minutes

4.3 Results

4.3.1 Single and double knockout mutants were created to generate a host S. coelicolor strain for improved metabolite titres

The pigmented antibiotic clusters of *S. coelicolor* were selected to be the first two targeted knockouts. They were created simultaneously using the PCR-targeted technology described above (Gust et al, 2002). Targeting of the blue-pigmented actinorhodin cluster resulted in the deletion of five genes (*SCO5085-5089*), including its pathway specific regulator, *actII-ORF4* (*SCO5085*); similarly, regulators *redD* and *redZ* of the red-pigmented undecylprodigiosin cluster were among the genes deleted from the Red cluster (*SCO5877-5881*). As seen in Figure 12A, along with digest and PCR checks during construction, the generation of these mutants was easily detected by visual screening.

Once the above mutants were created, work immediately focused on excising the selective antibiotic cassette from both strains, using the *Streptomyces* in vivo methodology (Fedoryshyn *et al.,* 2008). The lost of apramycin resistance in a mutant then allowed for a cassette-containing mutant cosmid of the other antibiotic to be introduced and selected for. It was in this way that both antibiotic knockouts were combined into a common strain lacking two of its secondary metabolic clusters. An apramycin-resistant undecylprodigiosin knockout was introduced into the apramycin-sensitive actinorhodin knockout, which produced a colourless mutant strain (see Figure 12A). To check that the ensuring chromosomal area between both gene clusters was still intact, PCR amplification of an intervening gene, *absB* (*SCO5572*) was conducted, and found to be present.

As a third knockout target, the single gene knockout of *scbA* (*SCO6266*), a protein involved in the activation of a cryptic biosynthetic cluster, was introduced into the double antibiotic knockout stain (Takano *et al.,* 2005). The knockout host strain was modified to be apramycin-sensitive, thus this antibiotic was able to be used as the selective marker for the introduction of the mutant *scbA* cosmid. At current, this knockout has not been successfully incorporated into the double knockout yet.

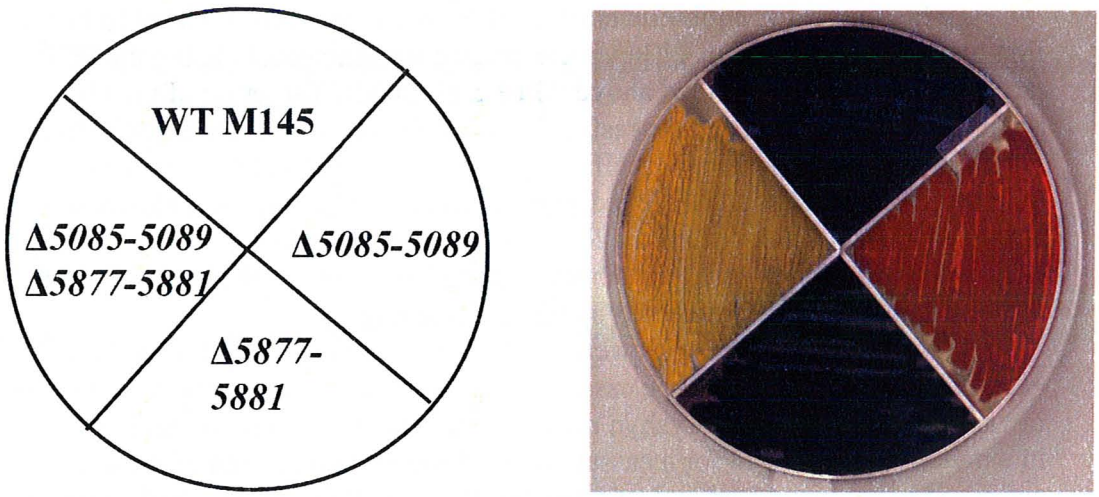


Figure 12: Phenotypic analysis of the various *S. coelicolor* knockout strains created in this work, as seen from the bottom of the plate. A) Phenotypic analysis of the single and double antibiotic knockout strains on R2YE agar after 5 days incubation. Knockout regions were: Δ*5085-5089* (Δactinorhodin), and Δ*5877-5881* (Δundecylprodigiosin).

4.4 Discussion

There are many advantages to the heterologous production of bacterial metabolites, both from genetic and industrial perspectives. Some producer organisms are not amenable to the large scale-up necessary for industrial fermentation, may not have very robust growth, and/or may be difficult to work with for other reasons. When manipulation of the producer is not possible or effective, it becomes desirable to use a well-defined heterologous host to express products of interest (Zhou *et al.*, 2004).

The creation of a universal host for antibiotic production came about as a natural complement to the antibiotic work initiated in chapter 3. By generating a host that could dedicate its cellular resources to producing a specific compound of interest, this would facilitate the purification and characterization of any molecules of interest.

A strain of *S. coelicolor*, with mutations that abolished the production of two key metabolites was created; the knockout targets were actinorhodin and undecylprodigiosin. These knockout targets were chosen due to their close ties with antibiotic production, and also for the available knowledge concerning characterized regulatory genes that helped direct the most advantageous knockout regions. Loss of both was expected to generate a surplus of precursors that could be diverted towards other processes.

Recently, Komatsu and colleagues (2010) successfully created a strain of the industrially significant *S. avermitilis* which lacked the chromosomal area housing the biosynthetic genes for its major secondary metabolites. By losing the full compliment of its usual metabolites, this strain displayed higher efficiency in the heterologous production of molecules from introduced biosynthetic gene clusters. In addition to providing good support for the validity of our work, this work was significant for a number of other reasons. Their work supported findings from other groups in underlining considerations for conducting heterologous expression experiments. This includes compatibility with host codon usage, and the presence of specifically elevated levels of regulators for a given metabolite cluster. Addressing the latter point, Komatsu *et al* (2010) realized that just cloning a biosynthetic cluster of pladienolide (from *S. platensis*) was not sufficient for pladienolide production – it also required a higher expression level of a particular regulator, *pldR*. The need to clone additional regulatory elements was also found when the fredericamycin cluster from *S. griseus* could only be expressed in *S. lividans* with the constitutive production of activator FdmR1 (which was not transcribed from the cluster in *S. lividans*) (Chen *et al.*, 2008). Furthermore, fredericamycin production was upregulated even more with the overexpression of another FDM regulator in *trans*, once again underlying the complex regulatory ties in place within a biosynthetic cluster.

Although S. *avermitilis* is also a well-studied actinomycete due to its industrial significance, *S. coelicolor* at present remains the model organism for

this genus. Creating an expression host of the latter would provide the opportunity to work with the organism with the most widely available and accessible information. In the future, the rational engineering of this universal host strain can continue with of the removal of additional metabolites; two reasonable candidates would be that of the calcium-dependent antibiotic (CDA) cluster and the coelibactin cluster. A CDA knockout has already been initiated, while the coelibactin cluster is interesting, as its deregulation negatively affects both morphological and chemical differentiation (Hesketh *et al.,* 2009; Kallifidas *et al.,* 2010).

The "genome-minimized" *S. avermitilis* was able to heterologously produce levels of streptomycin above that of wildtype S. *avermitilis* and natural producer *S. griseus*. This supports the notion that cellular resources were freed from the demands of native *S. avermitilis* secondary metabolites, allowing for incorporation into heterologous products (Komatsu *et al.,* 2010). This phenomenon has been observed previously for a variety of *Streptomyces* (Butler *et al.,* 2003; Ou *et al,* 2009). As touched upon above, purification (along with overproduction) of metabolites is an added benefit of genome-minimized strains – Penn and colleagues (2006) noted that metabolic products of the *S. lividans* native host complicated downstream production and analysis of molecules of interest using high-performance liquid chromatography. Interestingly, they found that deletion of actinorhodin genes allowed for the use of a medium advantageous to their molecule of interest, while also suppressing other host molecules. This represents further support that growth medium (and added factors) is yet another important consideration in the optimization of antibiotic production (Kim *et al.*, 2001; Penn *et al.*, 2006; Tanaka *et al.,* 2010).

As seen above, there are many avenues to pursue that may compliment deletions in this engineered *S. coelicolor* strain to produce copious amounts of a desired compound. As a research tool, the creation of this strain may prove valuable for the efficient production of molecules and metabolites of interest in a model organism. With the lost of its two pigmented antibiotics, it becomes particularly helpful in monitoring the production of pigmented products.

CHAPTER 5: OVERALL CONCLUSIONS AND SIGNIFICANCE

A greater understanding of the potential of ectopic expression of regulatory genes for antibiotics would greatly benefit not only the field of metabolite optimization, but also provide valuable information about the regulatory networks in place in *Streptomyces*. This work revealed the regulatory considerations necessary in increasing heterologous gene expression at both the transcriptional and translational levels – the synergistic use of both an efficient promoter and ribosome-binding site – providing a basis for future construction of overexpression vectors. In the process of screening for novel antimicrobial activity upon the introduction of heterologous regulatory genes in the Cu#39 strain, we found large wide-spread activity against two pathogenic bacteria of great clinical importance. While this did not definitively support our working hypothesis, it did provide insight into possible considerations of the potential of this strategy on a whole. Finally, the engineering of a *S. coelicolor* strain as a host for the production of heterologous compounds was initiated as a tool to aid in the production of increased yields of bioactive molecules for downstream characterization. Overall, this thesis contributed to the field of antibiotic regulation via various approaches, and the insight gained will aid in directing future investigations.

The timeliness of this research is due to the pharmaceutical and biotechnological industries driven by the need for efficiency, and the increasing societal concern of bacterial illnesses. Expansion of this research holds the possibility of inexpensively producing higher yields of known and previously uncharacterized antibiotics, as well as the mass production of other bacterial metabolites.

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APPENDICES

APPENDIX A: Quantification of the intensity of RT-PCR bands for *actII-orf4* (Figure 8) in the *atrA* overexpressing strains and its empty vector control. The average intensity (using ImageJ software) was calculated based on two independent RT-PCR reactions, and have been normalized to the corresponding 16S rRNA sample to control for RNA levels. None of the differences seen below were statistically significant; however, given the large standard deviation values, use of additional samples would provide a clearer picture of this phenomenon.

Strain	Average Intensity	Standard Deviation
Control (18.5h)	0.561217	0.124776
Control (24h)	1.109227	0.621051
Control (42.5h)	0.630168	0.126576

Strain	Average Intensity	Standard Deviation
atrA overexpression (18.5h)	0.722967	0.254389
atrA overexpression (24h)	0.972944	0.000347
atrA overexpression (42.5h)	1.156439	0.42012