HTP ENGINEERING AND *LUX*-BASED SCREENING OF *STREPTOMYCES*

THE DEVELOPMENT OF PROTOCOLS TO ENGINEER AND SCREEN *STREPTOMYCES* IN HIGH THROUGHPUT TO TEST FOR THE ACTIVATION OF CRYPTIC CLUSTERS BY "HETEROLOGOUS EXPRESSION OF PLEIOTROPIC REGULATORS"

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TITLE: The Development of Protocols to Engineer and Screen *Streptomyces* in High Throughput to Test for the Activation of Cryptic Clusters by the Heterologous Expression of Pleiotropic Regulators

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ABSTRACT

The Gram-positive, soil dwelling bacteria of the genus *Streptomyces* produce greater than 50% of the clinically relevant antibiotics in use today. Thanks to the falling price of DNA sequencing, *Streptomyces* genomes are revealing that they encode more secondary metabolites (potential antibiotics) than they produce under standard laboratory conditions. By heterologously overexpressing the known pleiotropic regulators of antibiotic expression from *Streptomyces coelicolor* in several other *Streptomyces* species it has been shown that the secondary metabolite profile of these species can be influenced. While present-day methods of introducing genes (conjugation) and screening for antibiotics work well on a small scale, the low throughput nature of these protocols stand as a barrier to testing this hypothesis on a larger scale. The focus of the research presented here was to develop high throughput (HTP) methods of engineering and screening *Streptomyces*. With these two technologies in place, an attempt was to made to introduce three plasmids (pSET152-*ermE**p-null, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$) into 120 wild-isolate *Streptomyces* species from the Wright Actinomycete Collection. Exconjugants were successfully obtained for all three plasmids in 48 species of *Streptomyces* and were screened for increased antimicrobial activity using a HTP, *lux*-based bioassay. Numerous strains showed increased antimicrobial activity but WAC00206, WAC00230 and WAC00263 with pSET152-*ermE**p-$lsr2_{NTD}$ showed the most promising improvement in antimicrobial activity. These hits have been designated as high priority for future investigation. These results suggest that HTP

conjugation and the *lux*-based bioassay are powerful methods for introducing plasmids into and screening engineered streptomycetes.

DEDICATION

To my Family, Friends, and Brothers in Battle.
Without you, I am nothing.

ACKNOLEDGEMENTS

First and foremost, I would like to thank Dr. Justin Nodwell. He has been an eternal fountain of knowledge and is always willing to listen to an outlandish idea. If there is one thing that Justin has taught me, it is that every experiment, no matter how mundane, needs to have controls! Thank you for everything, Justin.

I would like to thank Dr. Yingfu Li and Dr. Gerry Wright for serving on my committee. Your experiences, comments and thoughts have been pivotal in how I understand my work. Thank you.

To the Wright Lab, thank you for the use of your strain collection. I cannot even begin to imagine the countless hours it took to collect these. Thank you! Thank you! Thank you!

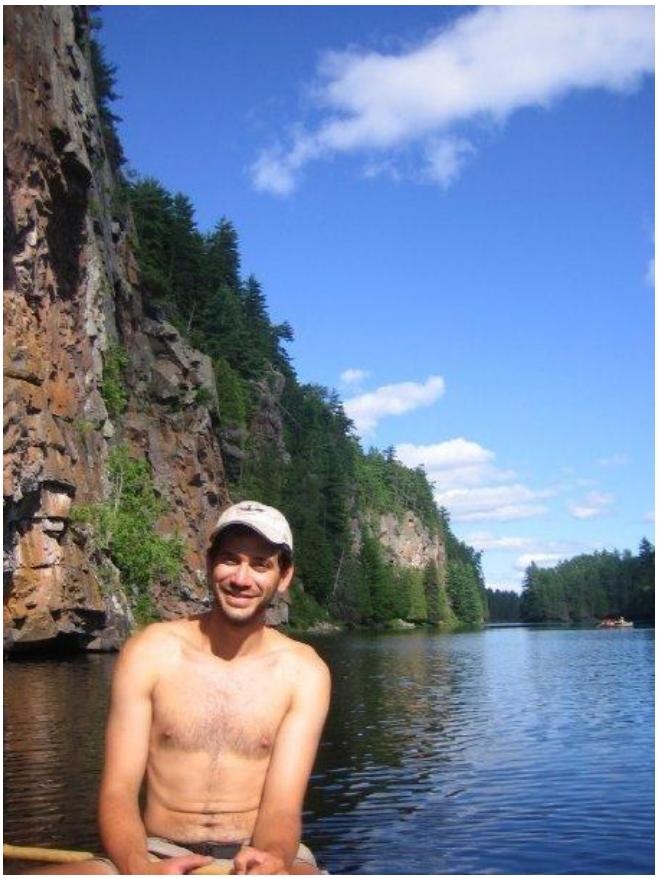
I would like to thank my lab mates. You have always been there for a good laugh and to help me puzzle through a problem. To Patricia Pak, thank you for your constant willingness to discuss this project and the humble smile you always greeted me with. To Kyle Salci, Daniel Socko, Mike Hewak and Yang Lei, it has been a pleasure knowing and working with all of you. To Leslie Cuthberton, thank you for being the epitome of the perfect scientist and having the highest scientific standards. I aspire to be more like you. Thank you to Arryn Craney, a veritable text book on all things *Streptomyces*. Thank you to Steven Ahn, your willingness to help and teach others (especially in regards to cloning) is invaluable. Thank you to Charul Jani, your warm smile, keen mind and sharp retorts make you a constant pleasure to be around. Thank you to Sheila Pimentel-Elardo, your willingness to teach me about the arcane science of analytical chemistry is invaluable.

Thank you to Salman Ahmed, your lighthearted laugh and willingness to chat make the lab an indefinitely better place to work. Thank you to Stef Mak, your dry humour keeps me on my toes and always smiling. Thank you to Ye Xu, you are more talkative than you let on and I look forward to each conversation.

I would like to thank Leesa, you are my transition state analogue. You have always been there to support me and are always willing to tell me the hard truth when I need to hear it. Thank you for everything.

Finally, I would like to thank my family. Mama, you are always up for a good conversation and always willing to offer me advice and a hug. Tete, you always want me to be better than I am. Thank you for pushing me and always supporting me. Micheal, you are the most intelligent person I have ever met. I look forward to each conversation we have regardless of the topic. Ariana, you are a pleasure to spend time with and your no BS attitude always keeps my ego from running away with itself. Marcus, you always have a goofy smile on your face. You make coming home for the weekend a treat.

IN MEMORY OF MICHAEL HART (1986 – 2011)



To the memory of a friend and a scientist who helped establish the foundation of this project.

I'm a great believer in luck and I find the harder I work, the more I have of it.

Thomas Jefferson

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ABBREVIATIONS

BLAST: basic local alignment search tool

BSL: biosafety level

HTP: high throughput

LB: Luria Bertani broth

LC-MC: liquid chromatography-mass spectrometry

m/z: mass to charge ratio

MIC: minimum inhibitory concentration

PCR: polymerase chain reaction

RLU: Relative Luminescence Units

DECLARATION OF ACADEMIC ACHIEVEMENT

I have personally completed all work in this thesis with the exception of the following: Patricia Pak and Michael Hewak of the Elliot Lab created the three plasmids pSET152-*ermE**p-null, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$; the Wright Lab collected the WAC strains.

CHAPTER 1: INTRODUCTION

1.1 *The Life Cycle of* Streptomyces

Streptomyces are found ubiquitously throughout soil and marine sediments (Kieser *et al.*, 2000; Pathom-Aree *et al*., 2006). The life cycle of these filamentous, Gram-positive bacteria is unlike that of any other (Figure 1). The life cycle starts when a spore settles in a nutrient rich area. This stimulates the spore to exit its dormant state and undergo germination giving rise to either one or two germ tubes. After the germ tubes emerge from the spore, they grow by tip elongation. Despite genomic replication as the tubes grow, the cell does not undergo binary fission. Instead, infrequent cross-sectional walls are synthesized resulting in long, multi-genomic compartments. Through extension and branching, the germ tubes give rise to a dense network of filaments which grow into and across the surface of an agar plate. This network is called the substrate mycelium. As the colony continues to grow, the mycelium in the centre of the colony begins to differentiate. Differentiation results in the formation of a new cell type, the aerial hyphae. When skyward growth of the spiralling, multi-genomic aerial hyphae stops, the aerial hyphae undergo synchronous cell division giving rise to monoploid compartments each of which will develop into a desiccation-resistant spore (Kieser *et al.*, 2000).

Differentiation also marks the onset of secondary metabolism (Kieser *et al.*, 2000). Secondary metabolites are molecules that provide a non-critical advantage to the producing organism (Madigan and Martinko, 2006). Secondary metabolites differ from primary metabolites (*e.g.* nucleotides and amino acids) in four ways: 1) they are not

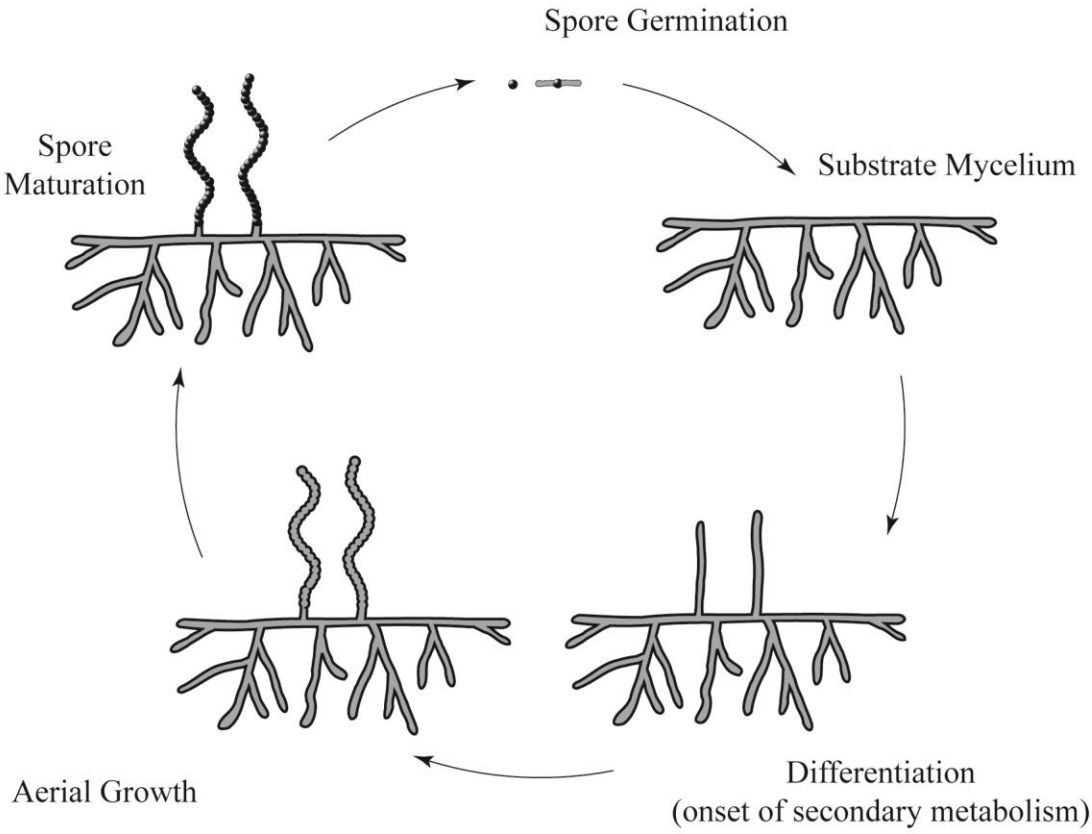


Figure 1. The life cycle of *Streptomyces coelicolor*. The life cycle of *Streptomyces coelicolor* begins when a spore finds itself in a nutrient rich area. This results in germination and the development of a dense network of filaments known as the substrate mycelium. As the substrate mycelium ages, it undergoes differentiation and gives rise to aerial hyphae which develop into spores. Differentiation is also important as it makes the onset of secondary metabolism.

essential for growth, 2) their formation is dependent on growth conditions (*i.e.* growth medium), 3) they are often produced as groups of closely related molecules, and 4) it is often possible to overproduce these molecules (Madigan and Martinko, 2006). Secondary metabolites of *Streptomyces* are of particular interest for their medicinal properties. While the most familiar secondary metabolites produced by *Streptomyces* are antibiotics, they are also capable of producing numerous other medically-relevant compounds (Keiser *et al*., 2000). Some include anti-fungal agents (*e.g.* nystatin) (Hazen and Brown, 1950), anti-

cancer agents (*e.g.* doxorubicin) (Arcamone *et al*., 1969) and immunomodulatory agents (*e.g.* rapamycin) (Martel *et al*., 1977). In addition to the secondary metabolites that are used as drugs, *Streptomyces* also produce other molecules (*e.g.* siderophores), many that have unknown function (*e.g.* geosmin, the molecule which gives soil its 'earthy' smell) and could serve as future drugs (Gerber and Lechevalier, 1965; Challis and Ravel, 2000).

1.2 *The Need for New Antibiotics*

Since the early 1940s, more than 10^7 microbes have been screened for antimicrobial activities, a massive effort that has yielded more than 2000 antibiotics (Baltz, 2006). Of the clinically useful antibiotics, more than 50% are derived from *Streptomyces* (Keiser *et al*., 2000). The most productive years of antibiotic discovery were between 1945 and 1960 (Watve *et al.*, 2001). Since then, the rate of discovery has rapidly declined, suggesting that those early fruitful years constituted a "golden era" for antibiotic discovery (Watve *et al.*, 2001; Wright, 2007). Despite continued efforts to find new antibiotics, numerous problems plague the process. Antibiotics like streptothricin, actinomycin, streptomycin and tetracycline are frequently re-discovered from unrelated species of *Streptomyces*, an issue referred to as the "dereplication problem" (Baltz, 2006). Due to their common occurrence, these antibiotics also act to obscure the activities of less common, undiscovered antibiotics (Baltz, 2006; Madigan and Martinko, 2006). During the 1970s, due to diminishing discoveries and a paradigm shift toward medicinal chemistry, screening for natural products was largely abandoned and drug companies turned toward chemically modifying existing antibiotics to combat resistance (Baltz,

2006). Medicinal chemistry is capable of extending the lifespan of an antibiotic by making modifications which can overcome resistance mechanisms used by bacteria to the previous version. This method is effective but is starting to be stretched thin. Even with the use of synthetic drugs, resistance mechanisms and genes which can be modified to form resistance mechanisms (the aggregate of which is called the "resistome") are already present in the environment (D'Costa *et al*., 2006). This suggests that resistance to a drug is inevitable and that it is merely a matter of time before resistance is acquired by pathogens (D'Costa *et al.,* 2006). If we are to avoid returning to a pre-antibiotic era, novel antibiotics must be discovered. Despite the stagnation in *Streptomyces*-based antibiotic discovery, this genus may still be a rich source of new antibiotics (Baltz, 2006).

1.3 Streptomyces *are a Potential Source of Novel Antibiotics*

In 2001, it was estimated that about 97% of antibiotics from *Streptomyces* remained to be found (Watve *et al*., 2001). The release of the genome sequence of the model streptomycete, *Streptomyces coelicolor,* a year later gave data to support this (Bentley *et al.,* 2002). By looking for the tell-tale sequences of secondary metabolite biosynthetic machinery in the genome, it appeared that *S. coelicolor* was capable of producing twice the number of secondary metabolites it was known to (Bentley *et al*., 2002; Nett *et al.,* 2009). Similar analyses of the genomes of *Streptomyces avermitilis* and *Streptomyces griseus* showed that 60% and 75%, respectively, of the biosynthetic clusters in these organisms coded for unobserved secondary metabolites (Ikeda *et al.,* 2003; Ohnishi *et al*, 2008; Nett *et al*., 2009). It is unknown why so many molecules go

unexpressed under laboratory culture conditions or if these molecules are even capable of being expressed. Since the products of these unexpressed clusters are unknown, they are often referred to as "cryptic clusters" (van Wezel *et al*., 2009). Given that these three well-studied species possess numerous cryptic clusters, it seems likely that poorly-studied and wild-isolate strains of *Streptomyces* will also possess them. As a result of the large numbers of unexpressed molecules and the habit of *Streptomyces* to produce antimicrobials, it is likely that some of these clusters code for novel antibiotics. By understanding the regulation of antibiotic expression in *S. coelicolor* and other lab strains of *Streptomyces*, it may possible to activate the cryptic clusters of studied and unstudied *Streptomyces* alike through genetic manipulation (McKenzie *et al*., 2010). Such a method may be an effective way to discover new antibiotics.

1.4 *Regulation of Antibiotic Production in* Streptomyces

The regulation of antibiotic production in *Streptomyces* occurs in a top down manner (van Wezel *et al.,* 2009). "Pleiotropic regulators" hold the top position in this hierarchy by regulating, either directly or indirectly, the expression of numerous "pathway-specific regulators of antibiotic production" (van Wezel *et al*., 2009). The pathway-specific regulators directly control expression of an antibiotic's biosynthetic machinery and thus the antibiotic (Narva and Feitelson, 1990; Fernandez-Moreno *et al*., 1991). Numerous pleiotropic regulators have been identified in *S. coelicolor*. A list of some include the two-component systems AbsA1/2 and AfsQ1/2 (Ishizuka *et al.*, 1992; Anderson *et al.*, 2001; Sheeler *et al*., 2005; McKenzie and Nodwell, 2007; McKenzie and

Nodwell, 2009; Shu *et al*., 2009), the γ-butyrolactone synthase ScbA (Takano *et al.*, 2001; Butler *et al*., 2003; D'Alia *et al*, 2011), the TetR-family regulator AtrA (Uguru *et al.*, 2005; Hong *et al.*, 2007; Hirano *et al*., 2008), the AfsK-AfsR-AfsS signalling pathway (Stein and Cohen, 1989; Horinouchi and Beppu, 1992; Vogtli *et al*., 1994; Horinouchi, 2003; Lee *et al*., 2007; Lian *et al*., 2008; Santos-Beneit *et al*., 2009; Santos-Beneit *et al*., 2011) and the S-adenosylmethionine synthetase MetK (Kim *et al*., 2003; Okamoto *et al*., 2003; Zhao *et al*., 2006; Zhao *et al*., 2010). Each system has different levels of influence and polarity (activator/repressor) in determining the expression of an overlapping set of secondary metabolites. The promoter region of the pathway-specific regulators appears to serve as a convergence point for many pleiotropic regulators (Uguru *et* al., 2005; McKenzie and Nodwell, 2007; Rigali *et al*., 2008). Here, the ultimate decision to express the pathway-specific regulator, and thus the antibiotic, is made. Despite efforts to understand these signalling pathways, the external and developmental signals which control antibiotic expression and the degree of interconnectedness of the pathways is still poorly understood.

1.5 *Screening for Antimicrobial Activity in* Streptomyces

There are a number of ways to screen for antibiotics in *Streptomyces*. One common way of screening is a method referred to as a colony diffusion assay (Figure 2). In this method of screening, a fixed number of spores are spotted onto an agar plate. As the *Streptomyces* grow, they secrete antibiotics into the growth medium. After a predefined amount of time (usually ranging from a single day to more than a week), the

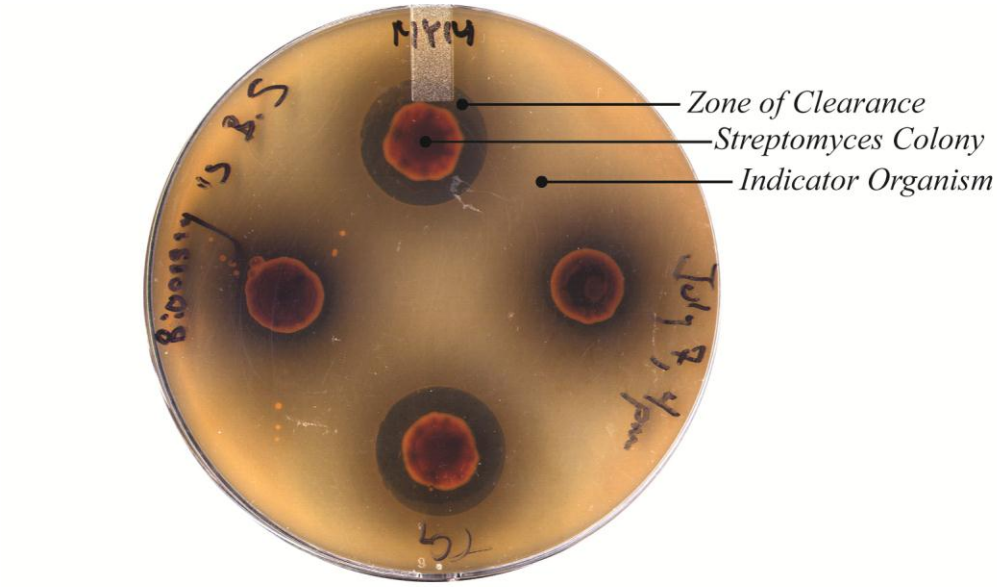


Figure 2. An example of a colony diffusion assay. When performing a colony diffusion assay, a fixed number of *Streptomyces* spores are spotted onto a plate. The plate is then left to grow, allowing antibiotics produced by the *Streptomyces* to be secreted into the media. The plate is overlaid with an indicator organism (in this example, *Bacillus subtilis*) suspended in soft agar. The plate is then left overnight to allow the indicator organism to grow. Antibiotics produced by the *Streptomyces* diffuse up into the overlay media and generate a zone of clearance. Larger zones of clearance indicate increased antibiotic production or a more potent antibiotic.

plate is overlaid with an indicator organism suspended in molten agar. The agar is allowed to solidify and then placed in an incubator overnight. Antibiotics produced by the *Streptomyces* prior to overlay with the indicator organism diffuse from the initial growth medium up into the LB overlay. The antibiotics are then able to act against the indicator organism. Antibiotic activity is observed as a zone of clearance surrounding the *Streptomyces* colony (Figure 2). As a general rule, the larger the circle, the more — or increased potency of the — antibiotic produced. *Streptomyces* are subject to large variations in secondary metabolite profile based on the growth medium (Madigan and

Martinko, 2006). A very classical and still prominent way to screen for antibiotics is to test a *Streptomyces* strain on numerous media types.

1.6 *The Heterologous Expression of Pleiotropic Regulators*

Presently, our lab is studying several approaches to activate cryptic secondary metabolites. Given the large numbers of *Streptomyces* that have already been screened and the problem of dereplication, it seems unlikely that many new antibiotics are going to be discovered with classical methods (Watve *et al*, 2001; Baltz, 2006). Attempting to mine cryptic clusters for new molecules may be a more effective way of discovering novel antibiotics (Nett *et* al., 2009; McKenzie *et al*., 2010). An emerging technique for accessing cryptic clusters is through the heterologous expression of pleiotropic regulators (McKenzie *et al*, 2010). This technique takes advantage of known pleiotropic regulators by heterologously overexpressing them in other *Streptomyces* species (e.g. the *S. coelicolor*-derived pleiotropic regulator *absA1* could be introduced into and overexpressed in *Streptomyces lividans*). The presence of orthologues of *S. coelicolor* pleiotropic regulators in other *Streptomyces* species suggests that there are commonalities between the pathways used to regulate antibiotic expression (Stein and Cohen, 1989; Ishizuka *et al*., 1992; Vogtli *et al*., 1994; Uguru *et al*, 2005; Zhao *et al*., 2011). Based on this assumption, a foreign pleiotropic regulator has the potential to influence the regulation of antibiotics within the host strain (Maharjan, *et* al., 2008; Makitrynskyy *et al*, 2010; McKenzie *et al*., 2010). In this manner, the regulator could induce the otherwise inappropriate expression of cryptic clusters. The use of this technique to activate cryptic

clusters is still quite novel, but there have been several reports of using pleiotropic regulators to boost the expression of known secondary metabolites (Maharjan *et* al., 2008; Makitrynskyy *et al*., 2010).

Boosting the production of an antibiotic is an important process which allows for increased production of a known molecule often for commercial purposes. Common ways to boost antibiotic production are by meticulous optimization of growth media and mutagenizing of the producer strain (Karalovcava *et al*, 1984; Wu *et al*., 2010). Thanks to increasing knowledge about pleiotropic regulators, a number of examples of using heterologously-expressed regulators to boost antibiotic production exist (Maharjan *et* al., 2008; Makitrynskyy *et al*., 2010). A first example comes from a group interested in boosting the production of moenomycin in *Streptomyces ghanaensis* (Makitrynskyy *et al*., 2010). By expressing the *S. coelicolor* pleiotropic regulator AfsS in *S. ghanaensis* they were able to boost production of moenomycin by 1.25-fold (Makitrynskyy *et al*., 2010). While the increase is not extraordinary, it does show that pleiotropic regulators are capable of influencing antibiotic regulation outside of their native host. Another example showed more sizable gains (Maharjan *et* al., 2008). This group used the pleiotropic regulators MetK and AfsR from *Streptomyces peucetius* (orthologues to those of *S. coelicolor*) and used them to boost the expression of pikromycin in *Streptomyces venezuelae* (Maharjan *et* al., 2008). This group was capable of increasing pikromycin by 1.6-fold and 2.6-fold with MetK and AfsR, respectively (Maharjan *et* al., 2008). Again, this example highlights the ability of heterologously expressed pleiotropic regulators to influence antibiotic expression.

The only example of a cryptic cluster being activated with this technique comes from our lab (McKenzie *et al*., 2010). McKenzie *et al.* (2010) introduced an allele of the *S. coelicolor* pleiotropic regulator *absA1* (*absA1*$_{H202A}$) (McKenzie and Nodwell, 2007) into nine characterized and six environmental isolates of *Streptomyces* (McKenzie *et al*., 2010). In an attempt to overcome the dereplication problem (*i.e.* re-discovery a known molecules), screening for antimicrobial activity was carried out against antibiotic resistant bacteria (McKenzie *et al*., 2010). In one particular example, the regulator enabled *Streptomyces flavopersicus* to generate a zone of clearance against the pathogen *Burkholderia cenocepacia*, an activity that was not present in the control strain (Figure 3A) (McKenzie *et al*., 2010). The activity was found to be due the previously known antibiotic pulvomycin (Figure 3B) (McKenzie *et al*., 2010). Although this molecule was not novel, what is exciting is that *S. flavopersicus* had never been shown to produce pulvomycin — the *absA1* allele was activating the expression of a cryptic cluster (McKenzie *et al*., 2010). This example is important for two reasons. First, it shows that the activation of cryptic clusters by way of heterologous expression of pleiotropic regulators is possible. Second, it shows it is possible for a cryptic cluster to code for an antibiotic.

This initial work done by McKenzie *et al.* has laid the foundation for a considerable amount of follow-up work which is being done by our lab in collaboration with the laboratory of Dr. M. Elliot (Dept. of Biology, McMaster University). The goal of our collaboration is to test the hypothesis that the heterologous expression of pleiotropic regulators is an effective way to modify secondary metabolism and ultimately discover

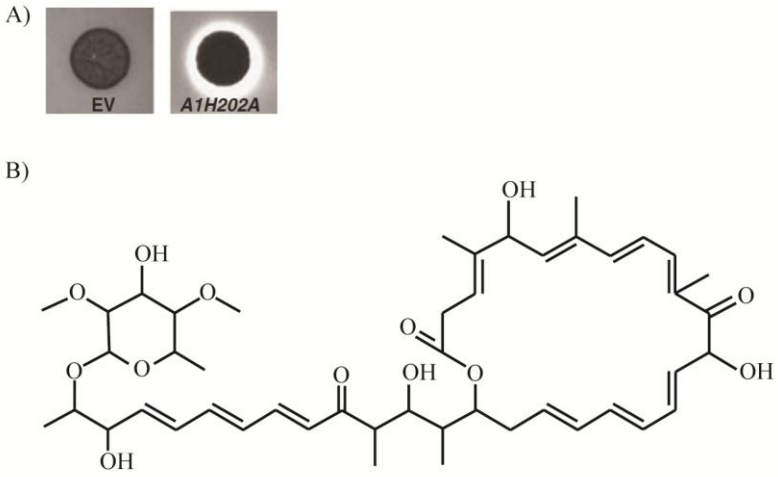


Figure 3. The activation of a the cryptic cluster in *S. flavopersicus.* (A) A colony diffusion assay showing activation of a novel activity produced by *S. flavopersicus* expressing the H202A allele of the pleiotropic regulator *absA1*. The indicator organism used was *B. cenocepacia.* Extraction and elucidation of the activity reveaded it to be pulvomycin (B). This image was reprinted by permission from Macmillan Publishers Ltd: Journal of Antibiotics (McKenzie *et al*., 2010).

new antibiotics. Early efforts have been aimed at testing the ability of numerous pleiotropic regulators to influence antibiotic expression in a limited number of strains. The efforts of four colleagues were to introduce the regulators $absA1_{H202A}$ (McKenzie *et al*., 2010), *abaB* (Scheu *et al*., 1997), *absB* (Price *et al*., 1999), *afsQ1* (Ishizuka *et al*., 1992), *afsR* (Stein and Cohen, 1989), *afsS* (Vogtli *et al*., 1994), *eshA* (Kawamoto *et al*., 2001), *scbR* (Takano *et al*., 2001), *metK* (Okamoto *et al*., 2003), *ppk* (Chouayekh and Virolle, 2002), *scbA* (D'Alia *et al*., 2011), *atrA* (Uguru *et al*., 2005) and $lsr2_{NTD}$ (Chen *et al.*, 2008) into the sequenced strain *S. venezuelae* and the uncharacterized strains *Streptomyces* sp. Ja#2b, *Streptomyces* sp. PV20b, *Streptomyces* sp. Co#7 and *Streptomyces* sp. Cu#39. Various antibiotic activities were observed for each strain against *Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Bacillus subtilis*

and *Burkholderia cepacia* . The results of their experiments again confirmed that the pleiotropic regulators are capable of influencing antibiotic production (M. Hart and J.R. Nodwell, personal communication; Pak, 2010; K. Salci and J.R. Nodwell, personal communication; M. Hewak and M. Elliot, personal communication). This can be seen exceptionally well in Figure 4A where the strain expressing the regulator AfsQ1 is producing a larger zone of clearance than the wild type (M. Hart and J.R. Nodwell, personal communication). Additionally, the results also suggest that despite these regulators being characterized as inducers of antibiotic expression, they may not always have this effect when introduced into other strains (M. Hart and J.R. Nodwell, personal communication; Pak, 2010; K. Salci and J.R. Nodwell, personal communication; M. Hewak and M. Elliot, personal communication). This was also apparent in the work of McKenzie *et al.* (2010) who saw that, despite having characterized the allele $absA1_{H202A}$ as a potent activator of actinorhodin (McKenzie and Nodwell, 2007), the regulator generated a species-specific response in each of the 15 strains tested. Some species showed increased antimicrobial activity, others showed decreased activity and some experienced no change at all (McKenzie *et al*, 2010). Of all the results from the Nodwell/Elliot collaboration, the one that was most interesting was the ability of the strain *S.* sp. Ja#2b to generate considerable antibiotic activity against vancomycin-resistant *Enterococcus* and a highly resistant clinical isolate of *Acinetobacter baumanii* (M. Hart and J.R. Nodwell, personal communication). LC-MS has shown that the wild-type strain also produces this molecule (personal unpublished results), but overexpression of the regulators $absA1_{H202A}$ and *afsQ1* resulted in increases to the amount of antibiotic

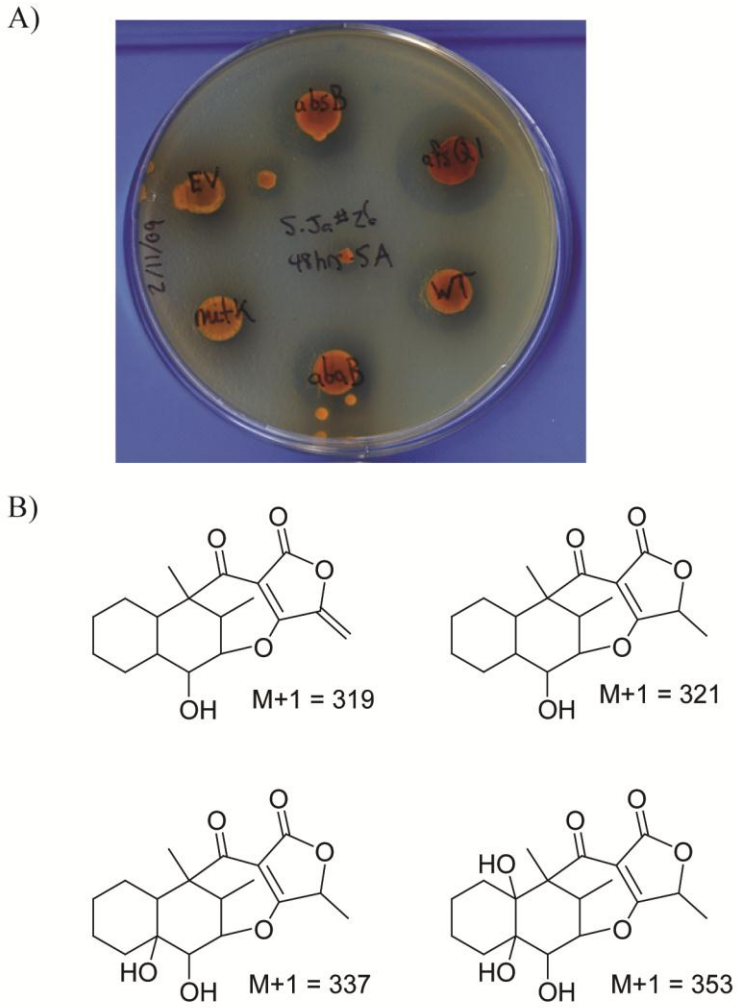


Figure 4. The effect of pleiotropic regulators on antibiotic expression in *S.* sp. Ja#2b and the discovery of hartinomycin. (A) A colony diffusion assay which shows the effects of heterologously overexpressing numerous pleiotropic regulators. A considerable increase in antibiotic activity caused AfsQ1 can be seen. *Staphylococcus aureus* was used as the indicator organism (B) *S.* sp. Ja#2b showed considerable activity against a highly antibiotic resistant *Acinetobacter baumanii.* Isolation of that activity lead to the discovery of a new antibiotic, hartinomycin. The primary species in hartinomycin has a m/z of 319.

produced. The antibiotic, tentatively named hartinomycin, has a m/z of 319 (Figure 4B). Three additional, less abundant variations of this molecule were also identified in extracts (M. Hart and J.R. Nodwell, personal communication).

1.7 *Conjugation of DNA from* E. coli *to* Streptomyces

One challenge to further studying the heterologous expression of pleiotropic regulators on a large scale is the means by which DNA is introduced into *Streptomyces*. The most common method of introducing DNA into *Streptomyces* for genetic manipulation and molecular biology is by conjugation (Mazodier *et al*., 1989). Conjugation is the process in which DNA is passed from one bacterium to another through a pilus or bridge formed between the two cells (Madigan and Martinko, 2006). Although conjugation is often perceived as taking place between cells of the same genus or species, plasmids can also be passed between Gram-negative and Gram-positive organisms, a fact which was not known until 1987 (Trieu-Cuot *et al*., 1987). The protocol for conjugation between *E. coli* and *Streptomyces* was published in 1989 and has adopted only one major change since then (Figure 5) (Mazodier *et al*., 1989; Flett *et al*., 1997). The conjugation process utilizes the donor *E. coli* ET12567 [pUZ8002] (NacNeil, *et al*., 1992; Flett *et al*., 1997). ET12567 is a methylation deficient strain of *E. coli* (NacNeil, *et al*., 1992). Many *Streptomyces* possess potent restriction systems which prevent them from taking up foreign DNA (MacNeil, 1988). *S. coelicolor*, among other species, will restrict incoming methylated DNA (MacNeil, 1988). Using a donor *E. coli* that does not methylate its DNA increases the number of *Streptomyces* species which can accept DNA

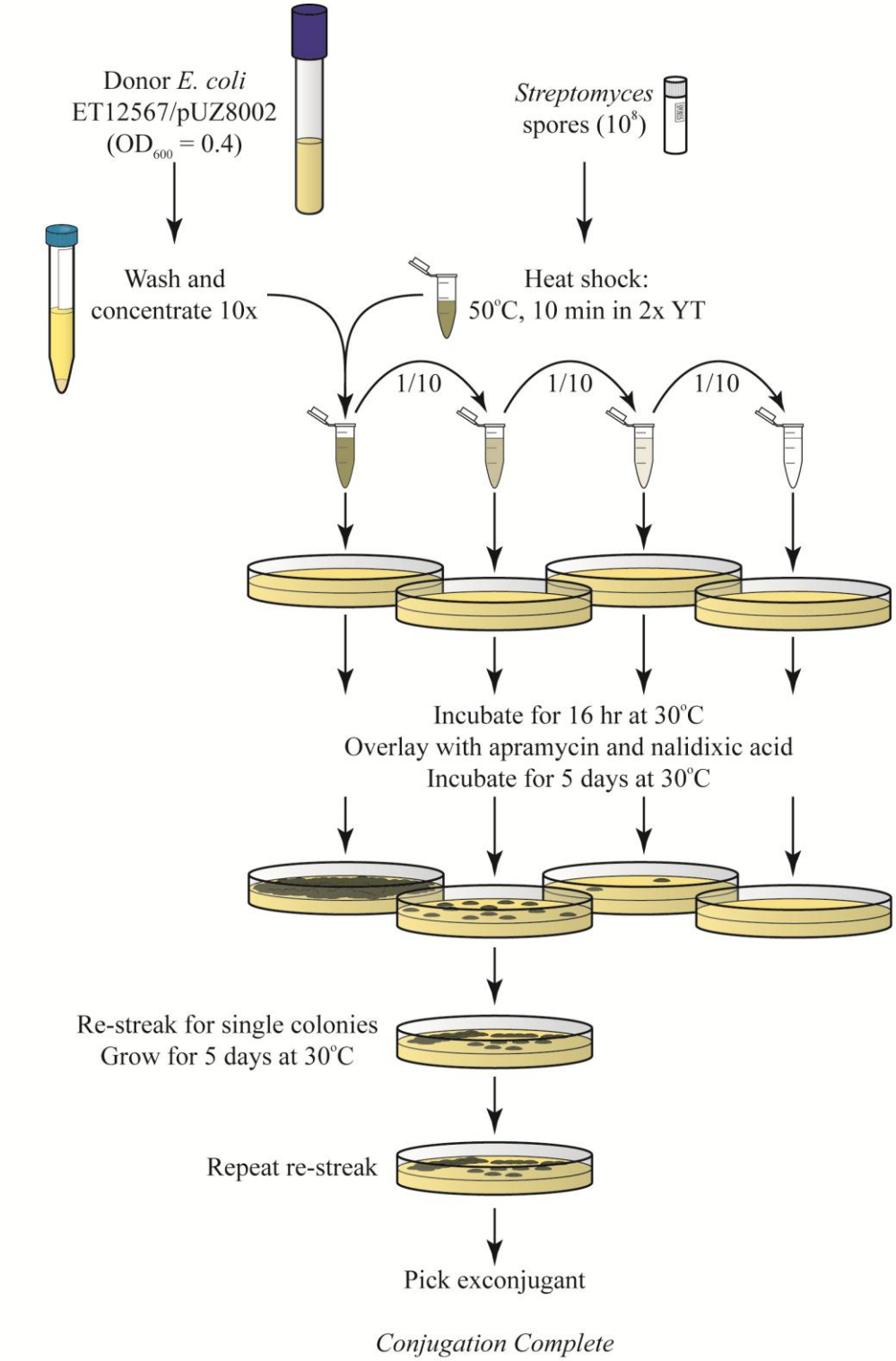


Figure 5. Diagrammatic representation of the low throughput conjugation protocol. The protocol is described in the text of Chapter 1.

by conjugation (MacNeil, 1988; MacNeil *et al*., 1992; Flett *et al*., 1997). The plasmid pUZ8002 is a non-transferable, mobilisation plasmid (Paget *et al*., 1999). It encodes the cellular machinery that is required for the donor to make the pilus and transfer a plasmid containing an RK2 *oriT* (origin of transfer) (Paget *et al*., 1999). pUZ8002 is a derivative of the RK2 plasmid, a member of the incompatibility group P plasmids (Paget *et al*., 1999; Thomas and Smith, 1987).

The entire conjugation protocol takes almost two weeks to complete (Figure 5). On the first day, a concentrated culture of donor *E. coli* and heat-shocked *Streptomyces* spores are mixed and a dilution series plated on the media MS. Since the frequency of successful conjugation differs with different species of *Streptomyces* the dilution series ensures the generation of single colonies of exconjugants (Lezhetskyy *et al*., 2006). Around 12 to 16 hr after the dilution series has been plated, the plate is overlaid with nalidixic acid and apramycin. Nalidixic acid is used to select against the *E. coli* as *Streptomyces* possess natural resistance to this broad-spectrum DNA gyrase inhibitor (Sugino *et al*, 1977; Keiser *et al*., 2000). The apramycin selects against any *Streptomyces* which did not receive the pSET152 plasmid. Plates are left to grow for a number of days until colonies appear. Since nalidixic acid is bacteriostatic, a major challenge associated with conjugation is getting rid of any contaminating *E. coli* (Deitz *et al*., 1966). This is accomplished by several rounds of clonal selection on fresh media. Conjugation with the integrating pSET152 plasmid has been shown to be an effective way to introduce DNA into many lab strains and several wild-isolates of *Streptomyces* (Bierman *et al*., 1992; Lezhetzkyy *et al*., 2006).

1.8 *Future Work to Test the Heterologous Expression of Pleiotropic Regulators*

The results of our work studying the heterologous expression of pleiotropic regulators to enhance antibiotic production have shown considerable promise. To date, the collaboration has shown that pleiotropic regulators introduced into other species of *Streptomyces* are capable of influencing antibiotic expression. Since the effect of each regulator varies between species, large samples of data need to be generated to identify which regulators are most effective at boosting antibiotic expression (M. Hart and J.R. Nodwell, personal communication; Pak, 2010; K. Salci and J.R. Nodwell, personal communication; M. Hewak and M. Elliot, personal communication). While all of this work can be done with the presently established low throughput methods, the labour required to attempt dozens or even hundreds of conjugations followed by screening with colony diffusion assays is not practical in an academic lab. Instead, high throughput (HTP), miniaturized methods for both conjugation and screening are needed. Once such protocols are in place, it will be possible to generate large libraries of *Streptomyces* expressing various pleiotropic regulators. Screening these libraries will allow us to answer two central questions regarding the pleiotropic regulator hypothesis: 1) is this an effective way to access cryptic clusters, and 2) if so, which pleiotropic regulators would be most effective at doing so. This thesis more thoroughly explores the abilities of two pleiotropic regulators: *atrA* and $lsr2_{NTD}$.

1.9 *AtrA*

AtrA was identified in *S. coelicolor* as a positive regulator of the antibiotic actinorhodin (Uguru *et al*., 2005). It has been shown to bind to two sites near the promoter of *actII-ORF4* (the pathway-specific regulator for actinorhodin) (Uguru *et al*., 2005). There are 25 AtrA responsive elements on the *S. coelicolor* genome (Nothaft *et al*, 2010). Three of these sites positively control expression of the *N*-acetylglucosamine (GlcNAc) uptake system (Figure 6) (Nothaft *et al*., 2010). As GlcNAc is taken up into the cell it is phosphorylated and converted to glucosamine-6-phosphate (GlcN-6-P), an allosteric effector of DasR that abolishes DasR's DNA binding ability (Rigali *et al*., 2006; Nothalt *et al*., 2010). DasR, which negatively regulates actinorhodin and the GlcNAc transporter, has been shown to have 250 binding sites in the *S. coelicolor* genome (Rigali *et al*., 2004; Rigali *et al.*, 2008; Nothaft *et al*., 2010). The indirect ability of AtrA to regulate DasR suggests that AtrA might be an especially advantageous pleiotropic regulator for heterologous expression. *S. griseus* has an orthologue of AtrA which has been shown to act as a positive regulator for streptomycin biosynthesis (Hirano *et al*, 2008). Interestingly, when *S. coelicolor*'s AtrA is heterologously expressed in *S. griseus* it acts to repress streptomycin biosynthesis (Hong *et al*., 2007). Expression of AtrA in *S. lividans* stimulates production of actinorhodin while expression in *Streptomyces rimosus* has no effect on the production of oxytetracycline or rimocidin (Hong *et al*., 2007). Work by Pak and Elliot (2010) has shown that AtrA is able to boost antibiotic activity of *S.* sp. Cu#39 against *A. baumanii* by 36% (as compared to wild type) but completely repressed activity of *S.* sp. Cu#39 against *B. cenocepacia*. Orthologues of *atrA* exist in all

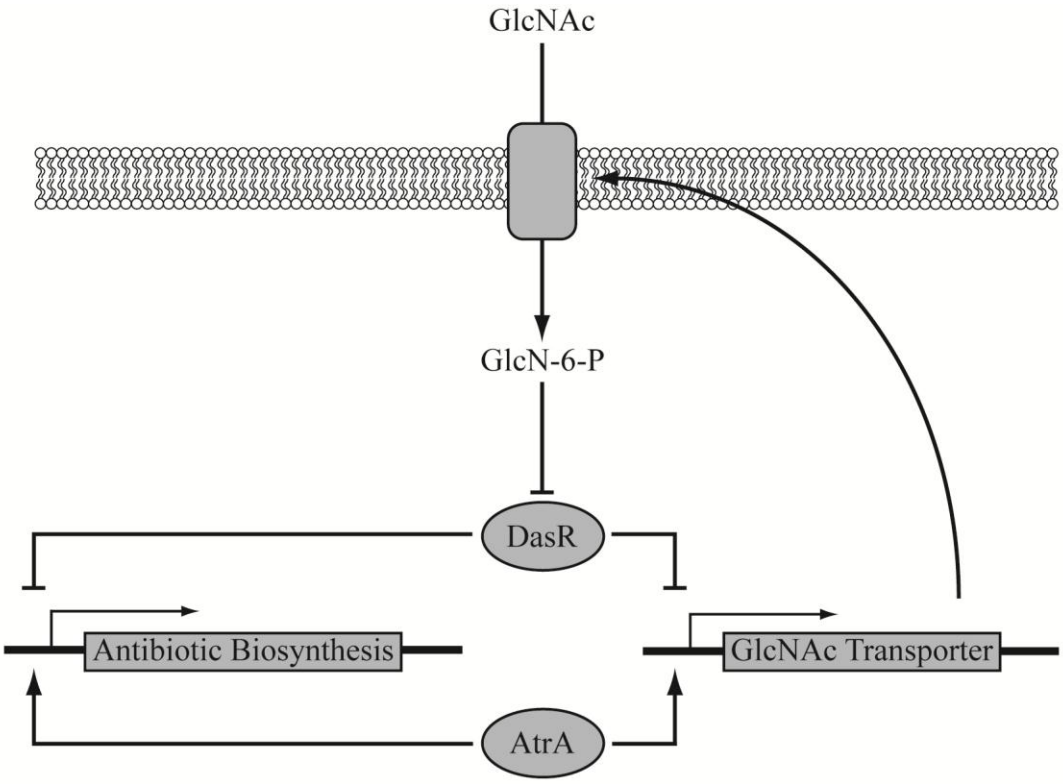


Figure 6. AtrA is able to influence the expression of DasR through the GlcNAc transporter. AtrA has been shown to be a positive regulator of antibiotic expression, the *N*-acetylglycosamine (GlcNAc) transporter and has been shown to bind to a total of 25 sites on the *S. coelicolor* genome. Activation of the GlcNAc transporter allows uptake of GlcNAc which is converted into glucosamine-6-phosphate (GlcN-6-P), and allosteric effector of DasR which abolishes its DNA binding ability. DasR has been shown to have 250 binding sites in the *S. coelicolor* genome.

Streptomyces sequenced to date. The ability of *S. coelicolor*'s AtrA to cause a phenotype in *S. griseus*, *S. lividans* and *S.* sp. Cu#39, the presence of orthologues in other *Streptomyces* species, and the indirect ability to regulate DasR suggests that heterologous expression of *atrA* in other *Streptomyces* may activate cryptic clusters.

1.10 *Lrs2*

The role of Lsr2 (SCO3375) in *Streptomyces* has not been examined but data regarding its putative function can be deduced from studies of the Lsr2 orthologues in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Lsr2 is classified as an H-NS-like protein (Gordon *et al*., 2008). H-NS functions in *E. coli* and *Salmonella enterica* by bridging adjacent loops of DNA (Dame *et al*., 2000). Looping results in condensation of the DNA which occludes RNA polymerase thus negatively regulating the expression of more than 400 genes in *E. coli* (Dame *et al*., 2000; Navarre *et al*., 2006). Lsr2, like H-NS, acts to regulate gene expression by binding non-specifically and looping AT-rich sections of DNA (Figure 7) (Chen *et al*., 2008; Gordon *et al*., 2010; Gordon *et al*., 2011). Lsr2 is composed of an N-terminal dimerization domain and a C-terminal DNA binding domain (Gordon *et al*., 2010; Gordon *et al*., 2011). Over expression of the N-terminal dimerization domain of Lsr2 ($Lsr2_{NTD}$) is suggested to generate a dominant negative phenotype thus giving RNA polymerase access to normally repressed genes (Chen *et al*., 2008; Hewak and Elliot, personally communication). What is most fascinating is that preliminary analysis of the *S. coelicolor* genome has revealed that many secondary metabolite pathway specific regulators are relatively AT-rich compared to the rest of the genome (C. Yin and M. Elliot, personal communication). This suggests that Lsr2 might play an important role in antibiotic expression. Work by Hewak and Elliot (personal communication) in *S. griseus* has shown that $Lsr2_{NTD}$ was able to induce novel activity against methicillin-resistant *S. aureus* and caused a two-fold increase in activity against *K. pneumoniae*. Introduction into *S.* sp. Ja#2b also resulted in a two-fold increase in

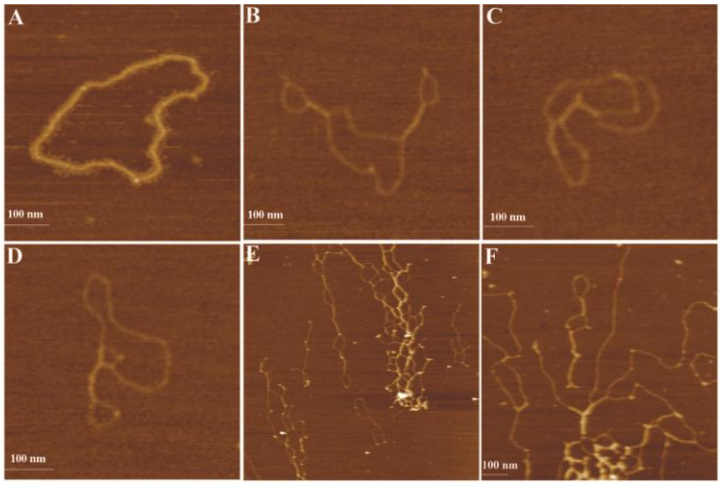


Figure 7. Atomic force microscopy images of protein-DNA complexes formed by Lsr2 and relaxed circular DNA. Lsr2 acts by bridging adjacent loops of AT-rich DNA. In this way, Lsr2 is able to condense DNA and exclude RNA polymerase. (A) Relaxed circular plasmid without Lsr2. (B-D) Lsr2–plasmid complexes at low protein/DNA ratios (1 dimer per 65 bp). The dimesnion for (E) is 2 μm × 2 μm. The same colour scale ranging from 0.0 to 5.0 nm (from dark to bright) was used for all images. Figure reproduced from Chen *et al.* by permission of Oxford University Press.

activity against *B. subtilis* and *B. cenocepacia* and a novel activity for *S. coelicolor* against *S. aureus*. This pleiotropic regulator is atypical as a regulator for heterologous expression in that it is exceptionally ill studied in *Streptomyces* and does not belong to a signalling pathway. Its unique mechanism of action may be able to activate biosynthetic machinery not accessible to other pleiotropic regulators.

1.11 *Research Objectives*

In order to assess the potential usefulness of heterologous expression, the hypothesis that pleiotropic regulators are able to effectively activate cryptic clusters must be tested in a larger number of *Streptomyces* strains. There is a distinct need for HTP methods to engineer *Streptomyces* and screen the resulting exconjugants for antimicrobial

activity. This thesis reports on the design of two HTP protocols: HTP conjugation and a HTP, *lux*-based bioassay for screening exconjugants. Using these protocols, the regulators *atrA* and *lsr2*$_{NTD}$ were successfully introduced into 48 wild-isolates of *Streptomyces* and the changes in antibiotic expression were examined. Although the data provided insufficient evidence to suggest that the regulators successfully activated cryptic clusters, the regulators did show considerable influence over antibiotic expression. This thesis assesses the efficacy of the heterologous expression of pleiotropic regulators to access cryptic clusters and examines the usefulness of AtrA and Lsr2$_{NTD}$ as regulators for this technique.

CHAPTER 2: MATERIALS AND METHODS

2.1 *Bacterial Strains and Growth Conditions*

All the strains of *E. coli* and plasmids used in this study are listed in Table 1. All strains of Actinomycetales used to characterize HTP conjugation (Chapter 3) are listed in Table 2. The 120 strains from the Wright Actinomycete Collection into which HTP conjugation was attempted (Chapter 5) are listed in Table 3. *E. coli* XL-1 Blue strains and *E. coli* ET12567 [pUZ8002] were cultivated in liquid Luria-Bertani (LB) media or on solid LB at 37°C. Liquid cultures were shaken at 200 rpm. *Streptomyces* were cultivated as described in Keiser *et al*., 2000. All actinomycetes and WAC strains were grown on MYM agar (recipe reported in Appendix 1) unless otherwise stated. Conjugations utilized MS media (Kieser *et al.*, 2000). HTP conjugations used MS and SGED (developed for this thesis; reported in Appendix 1). *lux*-based bioassays used MYM, MS, R2YE (Keiser *et al*., 2000), BEN (also Bennett's agar, Keiser *et al*., 2000) and SAM (Wright Lab, Personal Communication; recipe reported in Appendix 1). All streptomycetes were cultured at 30°C. The exception to this is during the *lux*-based bioassay, where the *Streptomyces* and the *E. coli* XL-1 Blue + pCM-Str indicator organism are grown together at 37°C. Where appropriate, the following antibiotics were used at these final concentrations: apramycin (50 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL) and nalidixic acid (25 μg/mL).

Table 1. Plasmids and *E. coli* strains used in this thesis.

Strain or plasmid	Genotype or description	Reference
Plasmids		
pCS26-Pac	Promoterless *luxCDABE*, Kan^r	Bjarnason *et al*., 2003
pCM-Str	*lux*-based bioassay, Kan^r	This work
pSET152-*ermE**p-null	Empty vector, Apr^r	Pak and Elliot, 2010
pSET152-*ermE**p-*atrA*	*atrA* plasmid, Apr^r	Pak and Elliot, 2010
pSET152-*ermE**p-$lsr2_{NTD}$	$lsr2_{NTD}$ plasmid, Apr^r	Hewak and Elliot, 2011
E. coli		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 glnV44 hsdR17 supE44 relA1 lac [F' proAB lac1^{q}ZΔM15* Tn*10* (Tet^r)]	Statagene
ET12567 [pUZ8002]	*dam::*Tn*9 dcm6 hsdM hsdR recF143 galK2 galT22 zjj201::*Tn*10 ara14 lacY1 syl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44 pUZ8002, chl^r, kan^r*	Flett *et al*., 1997

2.2 *Low Throughput Conjugation of* Streptomyces

Low throughput conjugation (as described by Keiser *et al*., 2000) was used in an attempt to introduce the plasmid pSET152-*ermE**p-null into the strains listed in Table 2. Spores stocks of all the strains were generated by streaking a single colony for a lawn on MYM, allowing it to grow for 7 days at 30°C and then gently dislodging the spores from the substrate mycelium with the use of a 10 mL volume of 0.85% saline and gentle scraping with a wooden stick. The spores recovered from the plate were vortexed for 30 seconds. The spore suspension was filtered through a cotton filled syringe into a sterile 15 mL conical tube. The spores were collected by centrifugation for 10 minutes at 1560 × g in a table top centrifuge. The supernatant was decanted and the pellet suspended in 1 mL of 20% glycerol and 0.85% saline. Spore stocks were kept at -80°C. Where performing a

Table 2. Actinomycetales strains used to characterize high throughput conjugation.

Bacterial Strain	Genotype or description	Reference
Broad Strains		
Kutznernia sp. 744	Kutznerides producer	Broad Institute
Micromonospora africana ATCC 39149	Chloramphenicol producer	Broad Institute
Streptomyces pristinaespiralis	Pristinamycin producer	Broad Institute
Streptomyces albus J1074	Molecular biology strain	Broad Institute
Streptomyces clavuligerus ATCC 27064	Clavulanate producer	Broad Institute
Streptomyces ghanaensis ATCC 14672	Moenomycin producer	Broad Institute
Streptomyces griseoflavis Tü4000	"Tailoring" enzymes	Broad Institute
Streptomyces hygroscopicus ATCC 53653	"Tailoring" enzymes	Broad Institute
Streptomyces lividans TK24	Molecular biology strain	Broad Institute
Streptomyces roseosporus 11379	Daptomycin	Broad Institute
Streptomyces roseosporus 15998	Daptomycin	Broad Institute
Streptomyces sp. SPB74	Beetle symbiont	Broad Institute
Streptomyces sp. SPB78	Beetle symbiont	Broad Institute
Streptomyces sviceus ATCC 29083	"tailoring" enzymes	Broad Institute
Streptomyces viridochromogenes DSM40736	Phosphinothricin producer	Broad Institute
Streptomyces sp. MG1	Alaska	Broad Institute

Unknown Strains		
Streptomyces sp. Co#7	Wild isolate	Wright Actinomycete Collection
Streptomyces sp. CP#23	Wild isolate	Wright Actinomycete Collection
Streptomyces sp. Ja#2b	Wild isolate	Wright Actinomycete Collection
Streptomyces sp. Kar#7	Wild isolate	Wright Actinomycete Collection
Streptomyces sp. PV20b	Wild isolate	Wright Actinomycete Collection
Streptomyces sp. WASP	Wild isolate	Wright Actinomycete Collection
Lab Strains		
Saccharopolyspora erythraea	Erythromycin producer	Levaditi *et al.*, 1953
Streptomyces coelicolor M145	Wild type prototroph; SCP1$^-$, SCP2$^-$	Keiser *et al.*, 2000
Streptomyces flavopersicus	NRRL 2820; spectinomycin producer	Stumpp *et al.*, 2005
Streptomyces venezuelae	Chloramphenicol, jadomycin producer	Nodwell Lab
Streptomyces lividans 1326	Wild Type	Nodwell Lab

Table 3. Wild isolate strains used in this research. All strains are Canadian wild isolates that were taken from the Wright Actinomycete Collection.

00161	00187	00208	00230	00254	00275
00164	00188	00209	00231	00255	00276
00165	00189	00210	00232	00256	00277
00166	00190	00211	00235	00257	00278
00167	00191	00212	00236	00258	00280
00170	00192	00213	00237	00259	00282
00171	00193	00215	00238	00260	00283
00172	00194	00217	00239	00261	00284
00173	00195	00218	00240	00262	00285
00174	00196	00219	00241	00263	00286
00175	00197	00220	00242	00265	00287
00177	00198	00221	00243	00266	00288
00178	00199	00222	00245	00267	00289
00179	00201	00223	00246	00268	00290
00180	00202	00224	00247	00269	00291
00182	00203	00225	00248	00270	00292
00183	00204	00226	00249	00271	00293
00184	00205	00227	00250	00272	00294
00185	00206	00228	00251	00273	00295
00186	00207	00229	00253	00274	00296

low throughput conjugation, an overnight culture of *E. coli* ET12567 [pUZ8002] + pSET152-*ermE**p-null was grown in LB supplemented with chloramphenicol, kanamcyin and apramycin. The culture was diluted 1 in 100 in the morning and allowed to grow until an OD_{600} of 0.4. At this time, the donor *E. coli* culture was washed three times in LB containing no antibiotics (cells were collected at 1560 × g in a table top centrifuge). The donor *E. coli* were suspended in a tenth the original volume after the final wash. *Streptomyces* spore stocks were thawed on ice and 10 µL of spores (approximately 10^8 spores) were taken from the spore stocks and mixed with 500 µL of 2×YT broth. Spores

were heat shocked at 50°C for 10 minutes and left at 24ºC for 10 minutes. 500 µL of the concentrated donor *E. coli* were mixed with the spore suspension. The mixture was collected by centrifugation at 21 130 × g for 30 seconds in a microcentrifuge and 950 µL of the supernatant was removed. A dilution series of the cells was made ranging from 10^{-1} to 10^{-5} and 100 µL of each dilution was spread on MS agar with glass beads. The plates were incubated for 16 hr at 30°C. The MS agar/conjugation plates were overlaid with 0.5 mg of nalidixic acid and 1.25 mg of apramycin suspended in 1 mL of water. The antibiotic overlay was dried in a laminar air flow hood. The plates were incubated for an additional 5 days at 30°C. The presence of colonies on the plates indicated a successful conjugation. Conjugations which were to be made into spore stocks were twice streaked on MYM agar plates supplemented with apramycin and nalidixic acid for single colonies before streaking for a lawn. Positive and negative controls were performed with the conjugations. Positive controls were conjugations in which the antibiotic overlay did not contain apramycin. Negative controls were conjugations in which the donor *E. coli* was excluded from the conjugation.

2.3 *High Throughput Conjugation of* Streptomyces

The step-by-step protocol for HTP conjugation is reported in Appendix 2. All strains of *Streptomyces* used with HTP conjugation were arrayed into the inner 60 wells of 96-well MYM agar plates. The 96-well agar plates possessed 200 µL of MYM per well. The plates were left at 30°C for 7 to 10 days to allow all the strains to sporulate. If required, 96-well pin tools were used to duplicate the master plate onto other 96-well

MYM agar plates which were incubated at 30ºC for 7 to 10 days until sporulated. The day before the HTP conjugation, an overnight culture of *E. coli* ET12567 [pUZ8002] + pSET152-*ermE**p-null or pSET152-*ermE**p-*atrA* or pSET152-*ermE**p-$lsr2_{NTD}$ (Figure 8) was grown in LB supplemented with chloramphenicol, kanamycin and apramycin. The day of the conjugation, the culture was diluted 1 in 100 in LB supplemented with the same antibiotics and grown to an OD_{600} of 0.4. The culture was washed three times in LB containing no antibiotics in a table top centrifuge by collecting the cells at 1560 × g for 10 minutes. The donor *E. coli* was suspended in a tenth of the original volume of the LB. 10 µL of the *E. coli* solution was overlaid into each well of a 96-well MS agar plate (containing 200 µL of MS agar per well). The plates were allowed to dry under laminar air flow until no more fluid was present on surfaces of the plate. Sterile 96-well pin tools were used to pin from the master plate to the conjugation plate containing the donor *E. coli*. A single master plate was used to distribute spores to more than one conjugation plate. The plates conjugation plates were placed at 30°C overnight. After 12 to 14 hr, each well of the plate was overlaid with 30 µL of antibiotic solution (0.125 mg/mL nalidixic acid and 0.313 mg/mL apramycin). The plates were left under laminar air flow until the liquid on the surface of the plate had dried (usually 2 to 3 hr). The plates were incubated for 7 to 10 days at 30ºC to allow the *Streptomyces* to sporulate. Sterile pin tools were used to transfer spores from each conjugation plate to a 96-well SGED agar plate supplemented with apramycin and nalidixic acid. The SGED plates were left at 30ºC for an additional 7 to 10 days to allow them to sporulate. Exconjugants which were reproducible in two of three replicates selected by hand and arrayed into a new master 96-

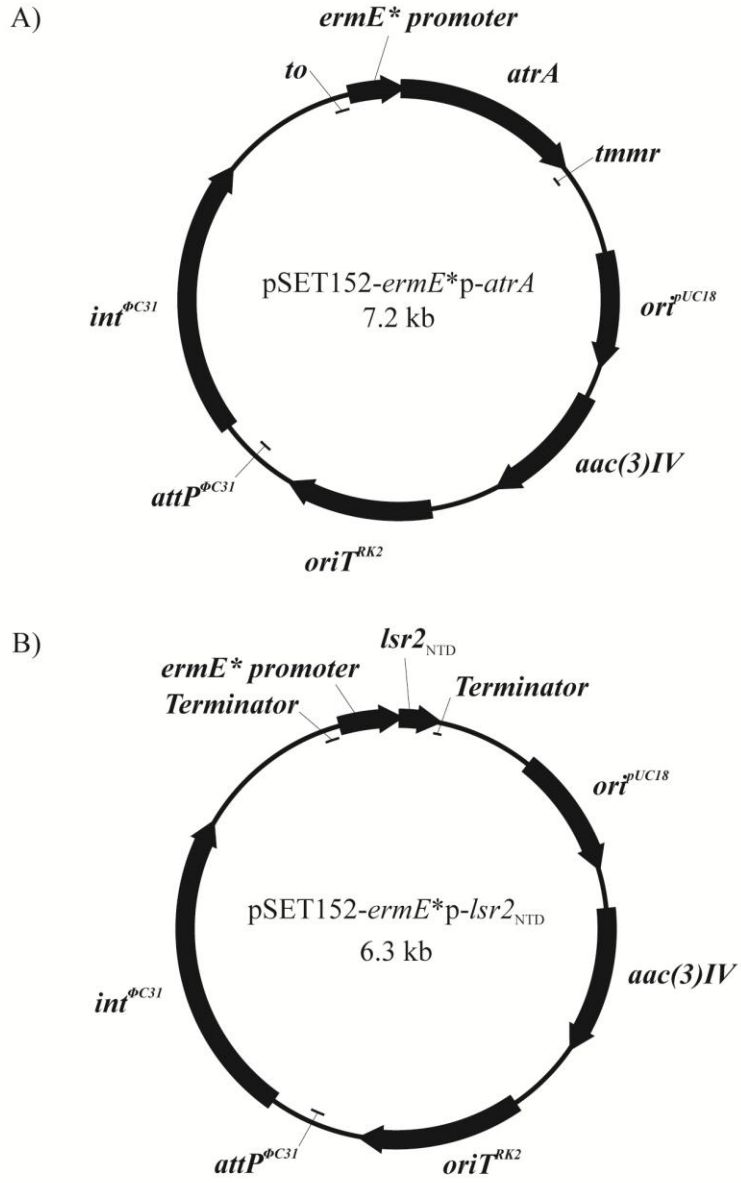


Figure 8. The *atrA* and $lsr2_{NTD}$ containing plasmids which were used in this thesis. The plasmids posssess the *oriT* from RK2, the *ori* from pUC18, the integrase gene and *attP* site from ΦC31, apramycin resistance and a strong promoter driving expression of the pleiotropic regulators. The plasmids were graciously donated for use in this thesis by Patricia Pak and Michael Hewak.

well plate. HTP conjugations were performed with a positive and negative control. The positive control did not apramycin in the antibiotic overlay. The negative control is a HTP conjugation which excludes the donor *E. coli*. The negative control identifies any strains which possess natural apramycin resistance. Any strains which grew on the negative control were not arrayed onto the new master 96-well plate.

2.4 *PCR Confirmation of High Throughput Exconjugants*

Chromosomal DNA from an exconjugant was prepared by inoculating spores directly from a colony into 2 mL of MYM broth in a test tube. A single glass bead was placed into each culture tube to improve aeration. The cultures were incubated at 30°C with shaking at 200 rpm for 2 days. 1 mL of culture was taken from each tube and the chromosomal DNA was extracted with the Qiagen DNeasy kit. PCR was performed on the chromosomal DNA to confirm the presence of the plasmid in the chromosome. The oligonucleotides HTPc fwd and HTPc rev3 (Table 4) were designed to amplify a 565 bp fragment if the pSET152-*ermE**p-null plasmid was present in the chromosome. The oligonulceotides were ordered from Mobix Labs at McMaster University. PCR was performed on approximately 50 ng of chromosomal DNA with Vent polymerase (New England Biolabs) without supplementation with dimethylsulfoxide. Other reagents were set up according to the manufactures instructions. PCR was performed as follows: 2 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 52°C, 40 seconds at 72°C followed by a final extension period of 2 minutes at 72°C.

Table 4. Oligonucleotides used in this thesis. All oligonucleotides were ordered from Mobix Labs at McMaster University.

Name	Sequence (5' → 3')
HTPc fwd	ACG ACG GCC AGT GCC AAG CT
HTPc rev3	CGG CTC GTA TGT TGT GTG GAA TTG T
J23119P top	TCG AGT TGA CAG CTA GCT CAG TCC TAG GTA TAA TGC TAG C
J23119P bot	GAT CGC TAG CAT TAT ACC TAG GAC TGA GCT AGC TGT CAA

2.5 *Construction of the pCM-Str Plasmid*

The pCM-Str plasmid (Figure 9B) is a derivative of the plasmid pCS26-Pac (Figure 9A) (Bjarnason *et al*., 2003). A promoter was inserted into the *Xho*I and *Bam*HI sites upstream of the promoterless *lux*CDABE cassette. The promoter was synthesized *de novo* by annealing the oligonucleotides J23119P top and J23119P bot (Table 4). Oligonucleotides were ordered from Mobix Labs at McMaster University. 1 mL of primer annealing solution (0.1 M NaCl, 0.1 M Tris·Cl pH 8.0, 1 μM EDTA, 5 nM J23119P top, 5 nM J23119p bot) was created and placed in a heat block at 95°C for two minutes. The heat block was then turned off and the reaction allowed to cool to room temperature. The primers were designed to possess a 5′ *Xho*I site and a 3′ *Bam*HI compatible site once annealed. The pCS26-Pac plasmid was isolated from *E. coli* XL-1 Blue by using a Qiagen Miniprep Kit. 500 ng of the plasmid was digested with *Xho*I and *Bam*HI (NEBuffer 3) in a 20 μL total volume. Digestion was performed for 1 hr at 37°C. The digestion was purified using a Qiagen PCR purification kit to remove the small drop out fragment from between the *Xho*I and *Bam*HI restriction sites. The annealed promoter was then ligated into the *Xho*I and *Bam*HI sites of pCS26-Pac overnight at room temperature using T4 DNA ligase (New England Biolabs) according to manufactures instructions. Ligation of the promoter into the plasmid was attempted at four different dilutions: 10^{0}, 10^{-1}, 10^{-2} and

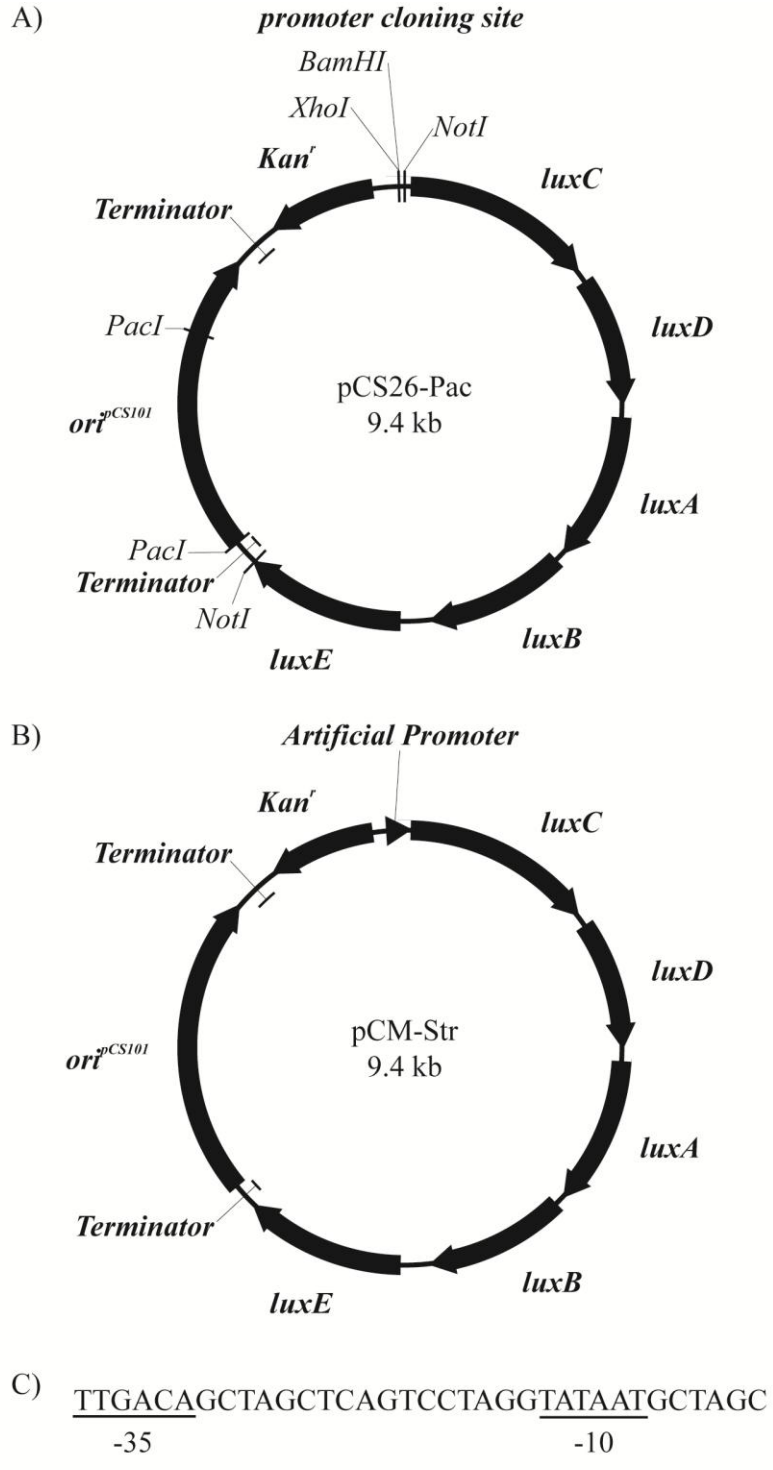


Figure 9. The plasmid pCM-Str (B) is a derivative of the plasmid pCS26-Pac (A). pCM-Str was made by inserting a strong, artificial, constitutive σ^{70} promoter (C) into the XhoI and BamHI sites of pCS26-Pac. The promoter possesses the consensus -35 and -10 sequences of σ^{70} promoters

10^{-3}. Each of the four ligations were transformed into chemically competent *E. coli* XL-1 Blue by mixing 5 μL of the ligation with 100 μL of chemically competent *E. coli* XL-1 Blue. The transformation was allowed to sit on ice for 30 minutes, heat shocked at 42°C for 45 seconds and placed on ice for an additional 2 minutes. 250 μL of LB broth was then added to the transformation and the transformation was placed at 37°C with shaking at 200 rpm for 1 hr. The transformation was then plated on LB supplemented with kanamycin and incubated at 37°C for 16 hr. Colonies which formed on the plate were cultured in 5 mL of LB broth supplemented with kanamycin overnight at 37°C with shaking at 200 rpm. Each culture was screened for luminescence. The candidate with the highest luminescence was selected and the plasmid it possessed was designated pCM-Str. This strain of *E. coli* XL-1 Blue + pCM-Str was also used in the *lux*-based bioassay.

2.6 *Minimum Inhibitory Concentration of* E. coli *XL-1 Blue + pCM-Str*

A 10 mL culture of *E. coli* XL-1 Blue + pCM-Str was grown in LB supplemented with kanamycin. A 1 in 100 dilution was made and the culture grown to an OD_{600} of 0.4. The culture was diluted 1 in 100 and then 1 in 1000 (total dilution of 1 in 10^5). 198 μL of this culture was added to each well (except those wells in column 12) of a 96-well plate. Column 12 contained 198 μL of LB broth and served as a sterility control. An antibiotic master plate was made by putting each of the antibiotics to be tested in rows A through H. Column 1 and 12 of each row contained only the solvent used to dissolve the antibiotic. Columns 2 to 11 had two-fold dilutions of the antibiotics starting at 6.4 mg/mL and ending at 12.5 μg/mL. 2 μL from each well of the antibiotic master plate were transferred

to the corresponding well of the 96-well plate containing the dilute *E. coli* XL-1 Blue + pCM-Str. The plate was incubated at 37°C for 20 hr in a stationary incubator. OD_{600} and luminescence were measured from each well and the MIC of each antibiotic was determined. The antibiotics tested were ampicillin, kanamycin, trimethoprim, apramycin, nalidixic acid, rifampicin and tetracycline.

2.7 *Lux-based Bioassay*

Streptomyces to be tested were arrayed into the inner 60 wells of a 96-well MYM agar plate supplemented apramycin and were incubated at 30°C for 7 to 10 days until sporulated. Sterile pin tools were used to create replica plates as needed. Sterile pin tools were used to transfer spores from a sporulated master plate to white, 96-well agar plates. The white 96-well agar plates contained 100 µL of MYM, MS, R2YE, SAM or BEN. The inoculated plates were incubated for 48 hr at 30°C. The plates were then overlaid with 100 µL of 55°C, extra soft LB agar (5 g/L of agar) containing *E. coli* XL-1 Blue + pCM-Str at an OD_{600} of 0.01. Once the overlay was distributed, the plate was incubated for 24 hr at 37°C. The plate was then removed from the incubator and read without the lid by a Victor X Light Luminescence Plate Reader (Perkin Elmer). Each well of the plate was read for 1 second and relative luminescence units (RLUs) were collected. All data points were expressed relative to the positive control included on each plate. Positive controls were which possessed no *Streptomyces*. Negative controls were completed on separate plates by excluding the *E. coli* XL-1 Blue + pCM-Str from the overlay.

CHAPTER 3: DEVELOPMENT OF A PROTOCOL FOR THE HIGH THROUGHPUT CONJUGATION OF *STREPTOMYCES*

3.1 *Rationale*

My hypothesis states that the heterologous overexpression of *S. coelicolor*-derived pleiotropic regulators in other species of *Streptomyces* is an effective way to activate expression of cryptic clusters. The discovery by McKenzie *et al*. (2010) that *absA1*$_{H202A}$ was able to activate expression of pulvomycin in *S. flavopersicus* showed that pleiotropic regulators can be used to activate cryptic clusters. Additionally, it also showed that cryptic clusters can encode antibiotics. I am presently investigating two primary questions to test my hypothesis: 1) is this an effective way to activate cryptic clusters, and 2) if so, which regulators will best function to this end. The use of large data sets is the best way to address both of these questions as it will give a clearer picture of the situation than that provided by only a few strains. The demand for large sets of data requires HTP methods for generating and screening engineered strains of *Streptomyces*. Routinely, inter-species conjugation is used to introduce cloned genes into single strains of *Streptomyces*. This method is an effective and easy way to introduce plasmids in a low throughput manner. There has been no report of a protocol that enables the introduction of plasmids into *Streptomyces* in a HTP manner. It was a primary objective of my research to establish a HTP conjugation protocol compatible with a diverse array of *Streptomyces* species. The HTP conjugation protocol reported here (Figure 10) was based on the low throughput conjugation protocol reported by Flett *et al*. (1997) (Figure 5). The HTP conjugation

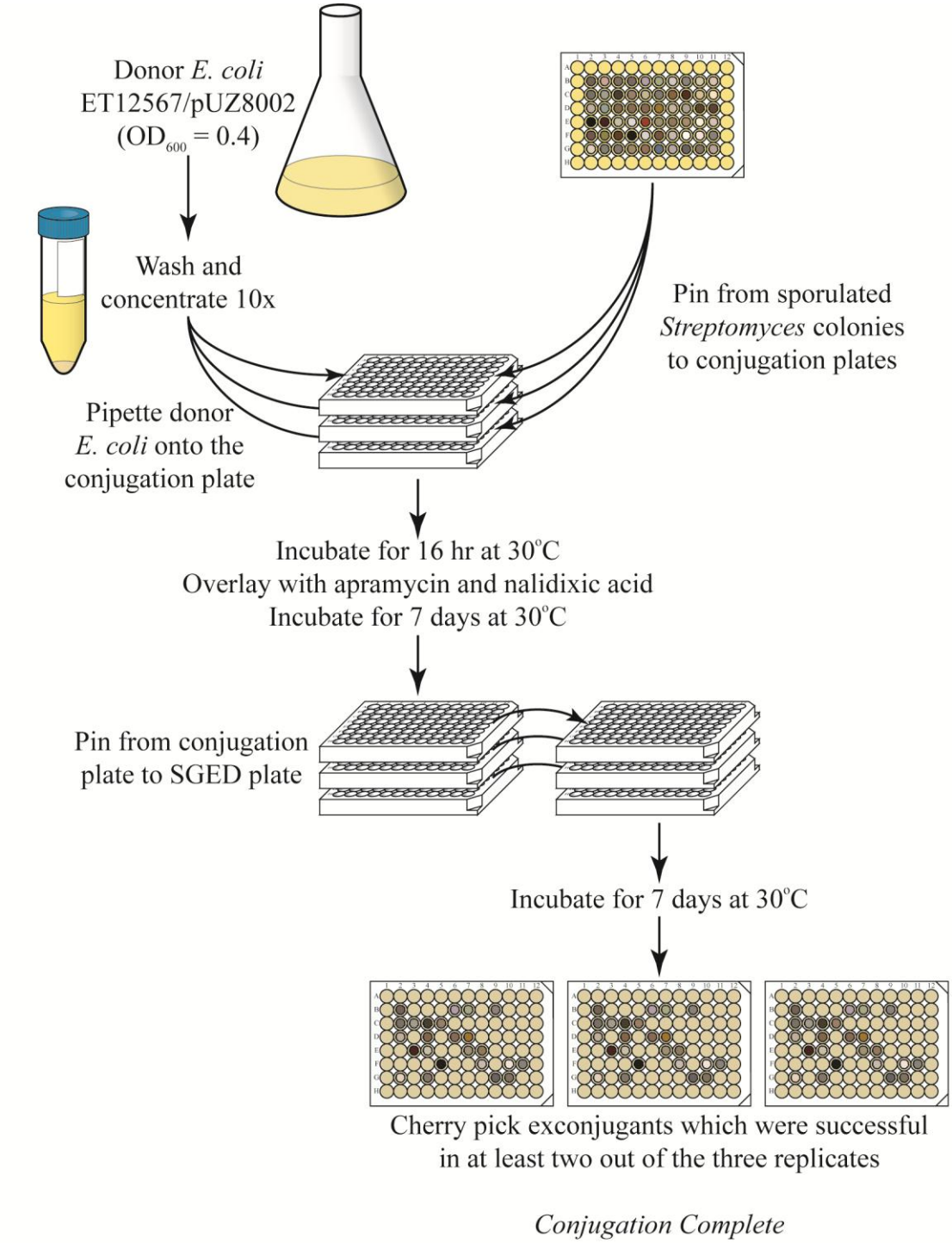


Figure 10. Diagrammatic representation of the high throughput conjugation protocol. The protocol is described in the text of Chapter 3.

protocol allowed introduction of DNA into large numbers of *Streptomyces* without the gross amounts of work, time and incubator space that would be needed if an equivalent number of conjugations were done using existing low throughput methods.

3.2 *Description of High Throughput Conjugation of* Streptomyces

On the first day of the HTP conjugation protocol (Figure 10), *Streptomyces* spores and the donor *E. coli* containing the pSET152-based plasmid to be transferred were combined upon a 96-well conjugation (MS media) plate. The *E. coli* culture was prepared by washing it in fresh LB to remove antibiotics present in the growth medium. The culture was then concentrated and pipetted onto the conjugation plate. Typically, the donor *E. coli* was placed on the plate first as this reduced the number of pipette tips needed to distribute the *E. coli*. The plates were then dried under laminar air flow. It was important to ensure that the plates did not have any excess fluid left on their surface as conjugation does not efficiently take place in liquid (Mazodier *et al*., 1989). This is due to the susceptibility of the IncP-type conjugative pilus to shearing forces (Bradley, 1980). Over drying of the plate also needed to be avoided as *Streptomyces* are unable to develop at high osmotic potentials (Holt, 1989). The spores for conjugation came from a master plate prepared in advance. The master plate was created by arraying the *Streptomyces* species to be conjugated in a 96-well agar plate and leaving them to sporulate. Pin tools were then used to transfer spores from the master plate to the *E. coli*-covered conjugation plates. A single master plate was used as the source of spores for numerous conjugation plates. Once both the donor *E. coli* and the *Streptomyces* spores had been transferred to

the conjugation plate, the plate was left overnight at 30ºC so that conjugation could take place. On the second day, an antibiotic overlay was used to select against the donor *E. coli* and any unconjugated *Streptomyces*. As with the low throughput protocol, nalidixic acid was used to select against the *E. coli* and apramycin against bacteria without the pSET152-based plasmid. The plates were then left for seven to ten days at 30ºC to sporulate. When performing the conjugation, it was important to include both positive and negative controls. In the positive control, only the donor *E. coli* was selected against during the antibiotic overlay (*i.e.* no apramycin). This ensured that the *Streptomyces* spores were viable and able to grow on the conjugation plate. The negative control (no donor *E. coli*) tested for naturally apramycin resistant species of *Streptomyces*. Such *Streptomyces* were discarded when collecting the final exconjugants.

One of the major challenges associated with the low throughput conjugation protocol is the isolation of the *Streptomyces* exconjugants from the donor *E. coli*. This challenge is due to the fact nalidixic acid is bacteriostatic (Deitz *et al*., 1966). The low throughput protocol accomplishes this separation through two rounds of clonal selection. In this way, the exconjugants are physically separated from any contaminating *E. coli*. Due to the use of pin tools and 96-well plates, the HTP protocol could not advantage of clonal selection. Instead, once the conjugation plates had sporulated the exconjugants were pinned to SGED media plates. SGED media was created for use with the HTP conjugation protocol and was designed to eliminate the donor *E. coli* ET12567 by taking advantage of the fact that they are auxotropic for histidine and leucine. The media—inspired by yeast drop-out media—was designed to lack these amino acids. Aside from

the use of a defined nitrogen source, the rest of the media was designed to mimic MYM (the primary *Streptomyces* growth media used in this thesis). Experiments have shown that *E. coli* plated on SGED did not form visible colonies. Furthermore, *E. coli* plated on SGED and left for 7 days at 30ºC was unable to be recovered. This contrasted with LB which required a minimum of 16 μg/mL of nalidixic acid to attain comparable results. Actinomycetes grown on SGED showed similar, albeit slightly slower, growth compared to MYM (Figure 11). A few species from the test set showed a marked difference in growth on SGED. For example, *Micromonospora africana* and *Streptomyces clavuligerus* are two strains that were unable to grow on SGED.

After incubating the SGED plates for seven to ten days at 30ºC, successful exconjugants were selected and re-arrayed into new master plates. When using 96-well plates, well-to-well contamination was a concern. Due to the use of laminar air flow hoods and the tiny nature of spores, it was conceivable that spores could be blown 'downwind' into other wells. For this reason, HTP conjugation was done in triplicate and once the *Streptomyces* had sporulated on the SGED plate, a survey of the plates was made and each conjugation was compared across the triplicates. Only if two of the three conjugations were successful did that specific exconjugant get selected and arrayed into the new master plate. By ensuring that at least two of the three conjugations were successful I was more confident that the exconjugant isolated was the species that it was supposed to be. Using a test set of 27 species of actinomycetes (Table 2), HTP conjugation successfully introduced the plasmid pSET152-*ermE**p-null into 16 of the 27 species (Table 5). This suggested the HTP conjugation protocol should be able to

Figure 11. Comparison of the growth of 27 actinomycetes on MYM and SGED. MYM is a generic medium for growing *Streptomyces* which supports vigorous growth and sporulation. Growth on SGED is generally less vigorous. It can be observed that neither *S. clavuligerus* nor *S. africana* appeared to grow on SGED. SGED was developed for use with the HTP conjugation protocol. It is designed to select against *E. coli* ET12567 by taking advantage of the fact that they are auxotrophic for both histidine and leucine. This image depicts 7 days of growth at 30°C.

Table 5. Comparison of the ability of low throughput and HTP conjugation to introduce the plasmid pSET152-*ermEp-null into 27 actinomycetes.** Check marks (✓) denote exconjugants which were able to successfully be conjugated.

Bacterial Strain	Traditional Conjugation	High Throughput Conjugation
Kutznernia sp. 744	✓	
Micromonospora africana ATCC 39149		
Saccharopolyspora erythraea		
Streptomyces coelicolor M145	✓	✓
Streptomyces pristinaespiralis		✓
Streptomyces albus J1074		✓
Streptomyces clavuligerus ATCC 27064		
Streptomyces flavopersicus		
Streptomyces ghanaensis ATCC 14672	✓	✓
Streptomyces griseoflavis	✓	
Streptomyces hygroscopicus ATCC 53653		
Streptomyces lividans 1326	✓	✓
Streptomyces lividans TK24		✓
Streptomyces roseosporus 11379		✓
Streptomyces roseosporus 15998		
Streptomyces sp. Co#7	✓	✓
Streptomyces sp. CP#23	✓	✓
Streptomyces sp. Ja#2b	✓	✓
Streptomyces sp. Kar#7		✓
Streptomyces sp. MG1	✓	✓
Streptomyces sp. PV20b	✓	✓
Streptomyces sp. SPB74		
Streptomyces sp. SPB78	✓	✓
Streptomyces sp. WASP		
Streptomyces sviceus ATCC 29083	✓	✓
Streptomyces venezuelae	✓	✓
Streptomyces viridochromogenes DSM40736		

introduce plasmids into 59% of all species it is attempted with (*i.e.* it is compatible with 59% of species). The test set of strains was comprised of 5 common lab strains, 5 wild isolate strains and 17 Broad strains. Of these, three were not from the genus *Streptomyces*. Slight decreases in the compatibility of two related plasmids, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-lsr2$_{NTD}$, introduced into the test set of strains was noted though the differences were small. Exconjugants of *S. lividans* 1326, *S.* sp. Ja#2b,

S. coelicolor M145 and *S. venezuelae* were confirmed by PCR to ensure that exconjugants generated by the HTP conjugation protocol were not spontaneous apramycin-resistance mutants (Figure 12).

3.3 *Contrast of the Low Throughput and HTP Conjugation Protocols*

There exist a number of differences between the low throughput and the HTP conjugation protocol that are worth noting. There seemed to be a slight difference in the compatibility of each species for each protocol (Table 5). The compatibility of the low throughput protocol with the tested strains was 48%. This contrasted to the 59% reported for the HTP conjugation protocol. Five species favoured the HTP conjugation protocol: *Streptomyces pristinaespiralis, Streptomyces albus, S. lividans* TK24, *Streptomyces roseosporus 11379* and *Streptomyces* sp. Kar#7. There are a few possible reasons why these strains favoured the HTP protocol. HTP conjugation used spores that had never been frozen (they were taken directly from a colony) and did not heat shock the spores before use. It is possible that the repeated freeze-thaw treatment of the spore stocks used for the low throughput conjugations reduced their viability. Alternatively, the spores of some species may stay in a state of reduced activity for a period of time after being thawed thus preventing them from being conjugated. *K.* sp. 744 and *Streptomyces griseoflavus* were unique in that they were conjugable by the low throughput but not by the HTP protocol. Observation of *K.* sp. 744 on MS (the nutrient agar used for conjugations) and MYM agar showed that it did not sporulate well on either media type (Figure 13). This may have resulted in the pin tools failing to efficiently transfer the

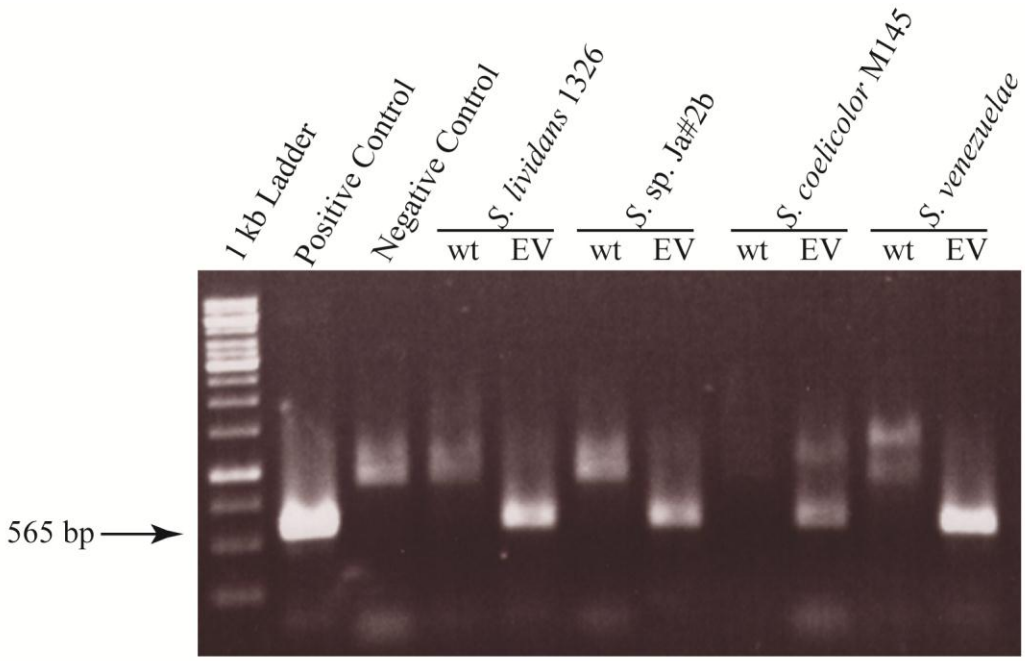


Figure 12. PCR confirmation of the presence of the pSET152-*ermEp-null plasmid in the chromosome of four different *Streptomyces* exconjugants generated by HTP conjugation.** A band at 565 bp denotes the presence of the plasmid in the chromosome. The positive control used purified pSET152-*ermE**-null plasmid as the template. The negative control contains no template DNA. The PCR product at 1 kb is likely a contaminant. "wt" denotes the wild type strain and "EV" denotes the pSET152-*ermE**-null containing strain. It is expected that all empty vector strains should generate a 565 bp band.

spores between plates during the HTP conjugation. It also highlights the role that media plays in compatibility. HTP conjugation used three different media types. If a strain of *Streptomyces* did not sporulate on any one of these media types, it was likely to be lost (*i.e.* incompatible as opposed to unconjugatable). The reason for *S. griseoflavus* incompatibility is unknown.

One of the largest differences between the HTP and the low throughput protocols was the lack of selection for individual colonies in the HTP protocol. Traditional microbiological methods suggest only clones of an isolate should be used. The pin tools,

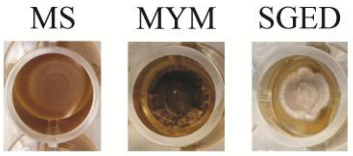


Figure 13. Comparison of the ability of *Kutznernia* sp. 744 to grow on MS, MYM and SGED. The poor sporulation of *K.* sp. 744 on MS is the most likely reason for its failure to conjugation with the HTP conjugation. The lack of spores suggests that the pin tools are unable to transfer the strain from the MS plate to the SGED plate. Its ability to sporulate on MYM is also poor though it sporulates with vigor on SGED.

though, pick up any spores they contact thus transferring more than one exconjugant from the well of a conjugation plate. This lack of streaking for individual colonies presents an interesting point for discussion. A proximal disadvantage of not streaking for single colonies was the difficulty in separating the donor *E. coli* from the exconjugants, a problem remedied through the use of SGED (discussed previously in Chapter 3.2). The point of intrigue though is the consequence of a HTP exconjugant representing a population as compared to the clone generated by the low throughput protocol. Since all the species of *Streptomyces* used in HTP conjugation originally came from clones there should have been no initial genetic diversity. The pSET152 plasmid has been shown to integrate into multiple sites of the *S. coelicolor* genome (Combes *et al*., 2002). It is thus reasonable to suggest that that there exists numerous integration sites in the genomes of most the species used. As an extension of this idea, any one HTP exconjugant is thus composed of a finite number of "sub-strains" each of which may be phenotypically distinct from the other. When screening a population, the result would represent the average phenotype of all the sub-strains. This can be advantageous if integration into a particular site results in an extreme phenotype (e.g. the complete inhibition of antibiotic

expression). Thus, the use of a population will average the positional effects of the plasmid's integration. This can be contrasted against single clones which may exhibit extreme, integration-site-related phenotypes that can be confused with the effect of an introduced gene. While use of a population is advantageous in that respect, populations can cause problems with reproducibility. A pin tool may not always pick up or distribute an equal number of spores from each sub-strain. As a result, a founder effect can take place resulting in a skewed number of a particular sub-strain being placed in a well. If these sub-strains exhibit integration-site-related phenotypes, this could cause differences between replicates.

There were several more differences between the low throughput and HTP conjugation protocols. These relatively superficial final differences included the use of 96-well plates, using colonies as the source of spores and the concentration of the antibiotic overlay used. A 96-well plate supported up to 60 conjugations per plate (outer wells were excluded due to edge effect, note Figure 10). Use of 96-well plates simplified organisation of large numbers of strains and reduced space requirements in an incubator. The HTP conjugation protocol also chose not to use spore stocks as a source of *Streptomyces* spores. Instead, spores were taken directly from a colony. This mechanism is advantageous as it reduced dependence on spore stocks. Instead, a single master plate could be replicated numerous times to give a second generation of master plates each which could be used for multiple conjugations. By taking spores directly from a colony though, it was not possible to accurately estimate the number of spores being pinned to a plate. The use of numerous species, especially unstudied ones, compounded this problem

as they each produced spores with varying vigour. Additionally, since one master plate was used to distribute spores to more than one conjugation plate, the number of spores printed to a 96-well plate likely decreased with each successive plate. In regards to the antibiotic overlay, the concentration of the antibiotics in the overlay solution was three times more dilute in the HTP conjugation protocol. This concentration allowed for the use of a 30 µL volume of overlay solution per well. Smaller volumes of overlay solution did not always cover the full surface of the agar, potentially allowing the growth of *Streptomyces* that did not possess apramycin resistance. The final concentration of antibiotic in each well ended up being equal to the concentration of antibiotic used in the low throughput protocol.

3.4 *Possible Reasons for Incompatibility of HTP Conjugation with Certain* Streptomyces *species*

There are several potential reasons that HTP conjugation did not work with a particular strain of *Streptomyces*. pSET152 is an integrating plasmid that inserts into the *attB* site (as well as other pseudosites) utilized by the *Streptomyces* phage ΦC31 (Combes *et al*., 2002). All species that were compatible with the HTP conjugation protocol must possess an *attB* site or a pseudosite. Integration of the pSET152 plasmid is necessary as it does not have a *Streptomyces* origin of replication. It is conceivable that some of the species were compatible with all aspects of the HTP conjugation protocol except the ability to integrate the plasmid into their genome. The use of other plasmids (non-integrative or that integrate into different sites) may reveal that the HTP conjugation

protocol is compatible with a larger percentage of species than reported here. Interestingly, a basic local alignment search tool (BLAST) search of one of the species that was unable to be conjugated, *M. africana,* showed that it does not possesses an *attB* site. The lack of *attB* site could be the reason for its inability to be conjugated. *S. erythraea,* on the other hand, was also unable to be conjugated yet it possesses an *attB* site.

There exist a number of other potential reasons why conjugation, let alone HTP conjugation, did not work in some species. Given that very little is known about the actually process of conjugation from *E. coli* to *Streptomyces*, there may be problems with the receptor used by the donor pilus. Additionally, restriction systems may be preventing the uptake of the plasmid. This was the case with conjugation to *S. coelicolor* until the use of *E. coli* ET12567, a strain of *E. coli* that does not methylate its DNA (MacNeil *et al*., 1992; Flett *et al*., 1997). *S. coelicolor* has a strong restriction system against methylated DNA which largely prevented introduction of DNA into *S. coelicolor* by conjugation before the use of *E. coli* ET12567 (MacNeil, 1988; Flett *et al*., 1997). Strong restriction systems may exist in some of the *Streptomyces* species that are unable to be conjugated. A final potential reason for incompatibility considers the effect of the gene expressed from the plasmid as the problem. Since I used the empty vector for these experiments this is not directly applicable, but it is possible that the expressed regulator is interfering with essential processes in the *Streptomyces* and making the exconjugants unviable (e.g. aberrant intracellular signalling, inhibition of sporulation, etc.). Additionally, regulators may induce antibiotics but not the cognate resistance resulting in suicidal exconjugants.

3.5 *Conclusion*

With this HTP conjugation protocol in place, it is now possible to introduce DNA into large numbers of *Streptomyces* with relative ease, something that was not attainable before this point. The HTP conjugation protocol has been shown to be compatible with approximately 59% of the actinomycetes tested. The HTP conjugation protocol will now allow us to introduce multiple pleiotropic regulators into libraries of *Streptomyces*.

CHAPTER 4: METHOD OF *LUX*-BASED SCREENING WITH ENGINEERED STREPTOMYCETES

4.1 *Rationale*

Thanks to the development of HTP conjugation I am now able to introduce plasmids into large numbers of *Streptomyces.* This capability was used to introduce the plasmids pSET152-*ermE**p-null, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$ into a small library of *Streptomyces* (reported in Chapter 5). These efforts hope to start answering two questions related to our hypothesis: 1) is heterologous introduction of pleiotropic regulators an effective way to access cryptic clusters, and 2) if so, which pleiotropic regulators would be most effective in this regard. To start answering these questions, the exconjugants generated by HTP conjugation need to be screened for antibiotic activity. One of the presently accepted ways to screen *Streptomyces* species for antibiotic activity is by colony diffusion assays (Figure 2). Due to the need to visually inspect and measure each zone of clearance this method of screening is not efficient for large numbers of strains. To test large numbers of exconjugants for antibiotic activity a HTP, *lux*-based bioassay was developed. The bioassay took advantage of 96-well plates and luminescence to enable screening of hundreds of exconjugants with a fraction of the effort and incubator space required to screen an equal number of exconjugants by colony diffusion assays.

4.2 *Description of the HTP,* lux*-Based Bioassay*

The *lux*-based bioassay (Figure 14) was designed to follow the same principles as the colony diffusion assay—growth of *Streptomyces* on agar and then overlaying with an indicator organism—but has been adapted to 96-well plates and HTP quantification. After HTP conjugation, the exconjugants were arrayed into a 96-well master plate. The colonies were allowed to grow until the point where they had all sporulated, usually about 7 to 10 days. The *Streptomyces* spores were then pinned from the master plate to a white, 96-well plate containing 100 µL of one of five different growth mediums: MYM, MS, R2YE, SAM and BEN. Multiple growth media were tested since *Streptomyces* are known to have altered secondary metabolite profiles on different media (Ruiz *et al*., 2010). The plates were then left at 30ºC for 48 hr to allow the *Streptomyces* to grow and secrete antibiotics into the growth medium. Independent observations by M. Hart (personal communication), Pak (2010) and me have shown that 48 hr of growth gives the most consistent results and the largest differences in antibiotic production between regulator containing and empty vector strains. 100 µL of extra-soft, molten LB (LB agar that uses a quarter of the agar typically called for) mixed with a constitutively luminescent indicator organism was overlaid into each well. The use of extra-soft LB increased the ease of pipetting the molten media. The overlay was allowed to solidify and are placed at 37ºC for 24 hr. Antibiotics present in the growth media diffused into the LB overlay and interacted with the indicator organism. Any antibiotics that were capable of killing or inhibiting the growth of the organism were perceived as a decrease in luminescence as compared to the positive control (Figure 15). All luminescence values generated with the

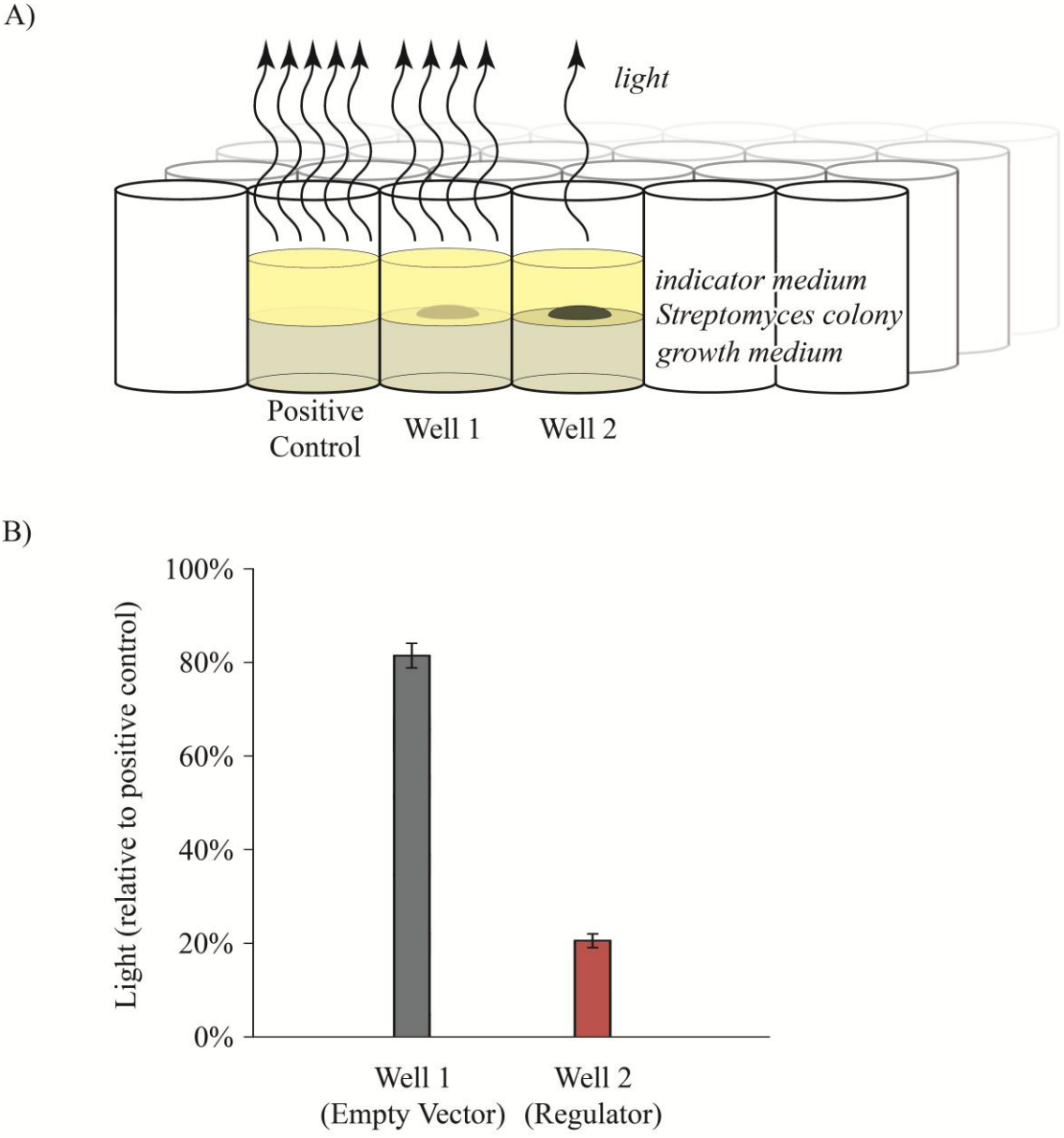


Figure 14. The *lux*-based bioassay allows HTP screening of *Streptomyces* for antibiotic activity. (A) A diagram of how the *lux*-based bioassay functions. A colony of *Streptomyces* is grown on a plug of agar in a 96-well plate. After two days, the plate is overlaid with the luminescent *E. coli* XL1 Blue + pCM-Str plasmid suspended in LB (the "indicator medium"). Antibiotic activity of the *Streptomyces* is inferred based on the amount of luminescence from each well. Less luminescence implies increased antibiotic activity. (B) An ideal hit as generated by the *lux*-based bioassay is one in which the empty vector strain has very little antibiotic activity (lots of luminescence) and the regulator containing strain has high antibiotic activity (very little luminescence).

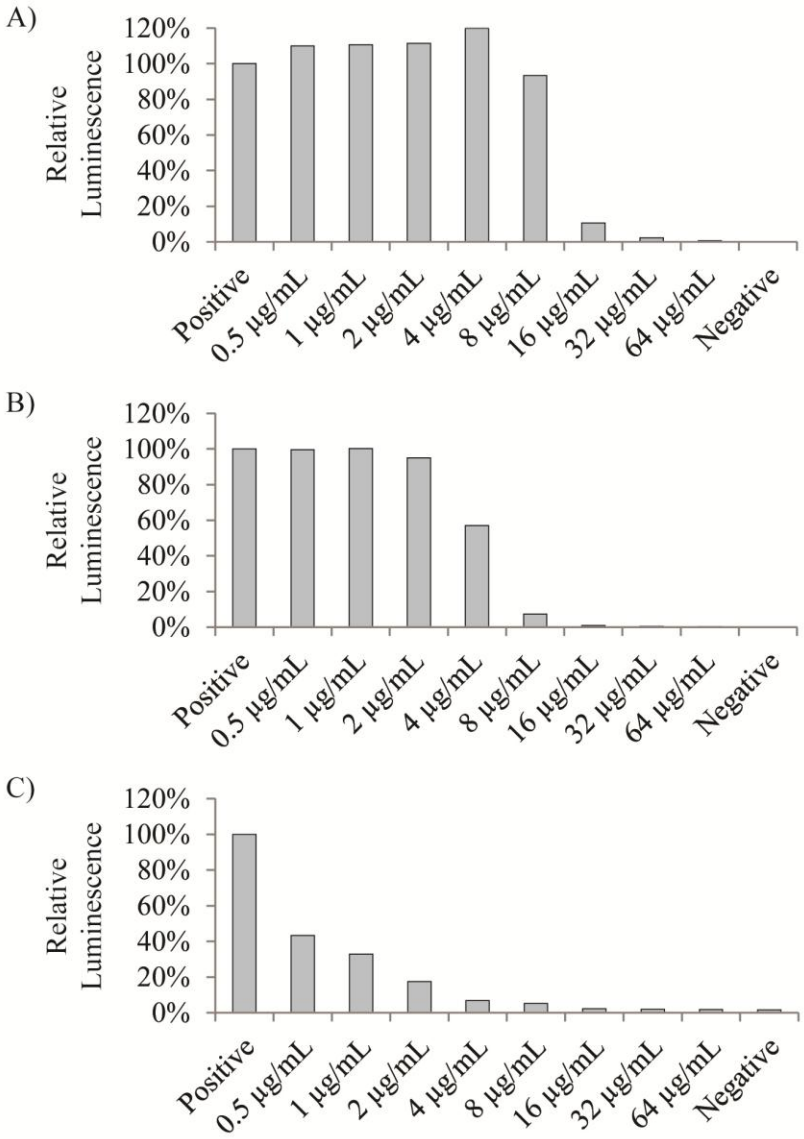


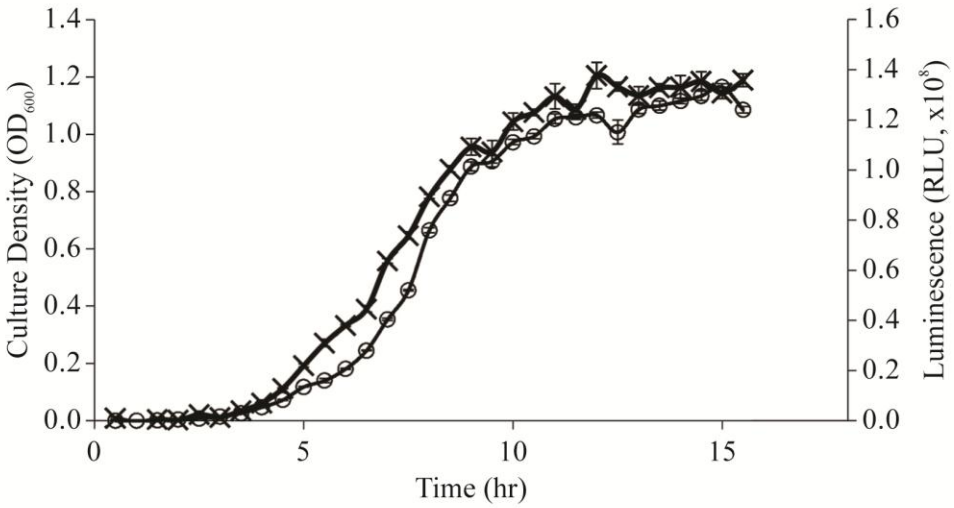
Figure 15. An increased concentration of antibiotics in the *lux*-based bioassay results in decreased luminescence. Antibiotics ampicillin (A), apramycin (B) or rifampicin (C) were added to wells of a 96-well plate in place of *Streptomyces*. Concentrations ranged from 0.5 ug/mL to 64 ug/mL. The indicator strain used was *E. coli* XL1 Blue + pCM-Str and the growth media type used was MYM. Positive controls did not have antibiotic added to them. Negative controls had no antibiotic or indicator bacteria added to them. All luminesence is represented as a percentrage of the positive control.

lux-based bioassay were represented as a percentage of the positive control. Setting all values relative to the positive control allowed for comparison between different growth media and for compensation of plate-to-pate variations (e.g. slightly different concentrations of indicator organisms in the overlay, variations in luminescence caused by plate temperature, *etc.*). Since *Streptomyces* experience variability in antibiotic expression between replicates the *lux*-based bioassay was completed with multiple replicates (Siebenberg *et al*., 2010).

4.3 *Luminescent Indicator Strain used in the* lux*-Based Bioassay*

The organism used for the *lux*-based bioassay was *E. coli* XL-1 Blue + pCM-Str. The plasmid pCM-Str (Figure 9B) was created for use with the *lux*-based bioassay. It is a derivative of pCS26-Pac (Figure 9A), a low copy-number *E. coli* plasmid containing a promoterless *luxCDABE* cassette, a kanamycin resistance cassette and the origin of replication from pSC101 (the first cloning vector) (Cohen *et al*., 1973; Bjarnason *et al*., 2003). pCM-Str contains an artificial *E. coli* σ^{70} consensus promoter which drives expression of the *luxCDABE* cassette (Figure 9C). This promoter (properly named BBa_J23119) was created by Dr. J. Christopher Anderson of Berkeley University (Boston, MA) from a combinatorial library and possesses the -35 and -10 consensus sequences of the *E. coli* housekeeping sigma factor, σ^{70} (Figure 9C). The expression of the *luxCDABE* cassette by this promoter allows growth of the *E. coli* XL-1 Blue to be closely tracked by luminescence (Figure 16A). An interesting observation is that luminescence generated by a culture of the strain is bright enough to be seen by the naked

A)



B)

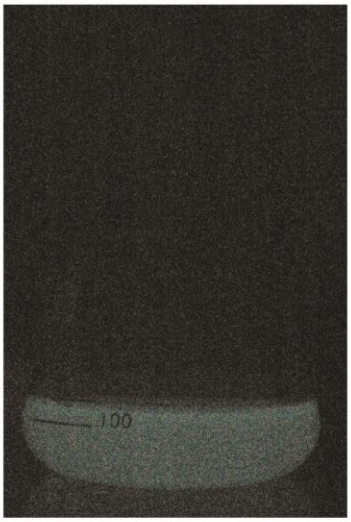


Figure 16. Luminescence produced by *E. coli* XL1 Blue containing pCM-Str is able to track culture growth. (A) The luminescence (circles) generated by the culture is able to closely track optical density (×). (B) A culture of this strain is capable of luminescing brightly enough to been seen with the naked eye in a dark room. This image was taken when the culture was in stationary phase. The photograph has been enhanced for purposes of printing. The culture was grown in LB broth at 37°C with shaking.

eye (Figure 16B). *E. coli* XL-1 Blue + pCM-Str was characterized to have the following MICs: 2 µg/mL ampicillin, >64 µg/mL kanamycin (recall, pCM-Str has kan^r), 0.5 µg/mL trimethoprim, 8 µg/mL apramycin, 32 µg/mL nalidixic acid, 4 µg/mL rifampicin and >64 µg/mL tetracycline. When calculating MICs based on luminescence instead of OD_{600}, only trimethoprim varied with an MIC of 1 µg/mL.

The use of this strain of *E. coli* as an indicator strain had a number of consequences that are pertinent to how the results of a *lux*-based bioassay are perceived. The outer membrane of Gram-negative bacteria gives them natural resistance to a number of antibiotics that Gram-positive organisms are susceptible to (MacAlister *et al*., 1972). Since Gram-negatives are innately more difficult to kill, I was able to filter out many of the hits that would be registered against a Gram-positive indicator. This reduces the number of hits but also means that Gram-positive-specific antibiotics cannot be observed (e.g. daptomycin) (Eliopoulos *et al*., 1986). Despite *E. coli*'s inherent resistance to Gram-positive-specific antibiotics, *E. coli* XL-1 Blue is used for molecular biology and thus sensitive to many antibiotics. This allows for the detection of a large number of Gram-negative-active molecules. Since the ultimate goal is to test for the activation of cryptic clusters by the pleiotropic regulators, the use of a highly antibiotic resistant organism may reduce the frequency of observing a hit to the point where it is not possible to tell if the regulator is actually having an effect (the strain is not sensitive enough). The innate resistance of *E. coli* XL-1 Blue yet its susceptibility to numerous antibiotics allows it to tread a fine line between being resistant enough to observe meaningful changes yet susceptible enough to be sensitive to those changes. Additionally, given its status as a

biosafety level (BSL) 1 organism, *E. coli* XL-1 Blue could be used on the bench top. Any hits from the *lux*-based bioassay can be followed up with colony diffusion assays against highly resistant organisms at a later time.

4.4 *The Ideal Hit: Defining What Constitutes an Interesting Hit*

The ultimately purpose of developing HTP conjugation and the *lux*-based bioassay was to test the hypothesis that heterologous overexpression of pleiotropic regulators is an effective way to access cryptic clusters. McKenzie *et al*. (2010) introduced *absA1* alleles into numerous *Streptomyces* and explored regulator-stimulated antimicrobial activities. With *S. flavopersicus* they were able to activate a completely novel activity against *B. cenocepacia*, thus showing the activation of a cryptic cluster. By screening for this kind of pattern (low antimicrobial activity from empty vector-containing strains, high antimicrobial activity from regulator-containing strains), it is possible to screen for activation of antibiotic cryptic clusters. Minimal antimicrobial activity from the empty vector strain is important because it increases the likelihood that the observed activity is generated from a cryptic cluster (as opposed to up regulation of something already being expressed). An example of an ideal hit is provided in Figure 14B. Assuming that "Well 1" of Figure 14B represents an empty vector-containing strain of *Streptomyces* and "Well 2" represents the regulator-containing strain of *Streptomyces*, we observe a considerable decrease in luminescence as caused by the presence of the regulator's introduction. This decrease may signify the activation of a cryptic cluster that encodes an antibiotic. Alternatively, the regulator may be grossly up regulating an already expressed antibiotic.

Even though this graph represents the best possible circumstance (a 60% difference in relative luminescence would be considered ideal), any species in which the regulator-containing strain causes less luminescence than the empty vector strain is of interest. As a side note, all data in this thesis was analysed manually but a proposed equation for automating hit identification has been provided in Appendix 3.

4.5 *Conclusions*

The development of the *lux*-based bioassay allows the screening of hundreds of *Streptomyces* for antimicrobial activity. The indicator organism used in this bioassay was *E. coli* XL blue + pCM-Str. The use of a non-multi-resistant Gram-negative strain reduces the effects of the dereplication problem but still allows for the sensitivity needed to observe changes in antibiotic production. When screening, the ideal hit is one in which the empty vector has close to 100% relative luminescence (little to no antimicrobial activity) and the regulator-containing strain generates about 60% less relative luminescence.

CHAPTER 5: GENERATION OF A LIBRARY OF ENGINEERED STREPTOMYCETES

5.1 *Rationale*

The purpose of developing both HTP conjugation and the *lux*-based bioassay was to allow us to start testing the hypothesis that heterologous expression of pleiotropic regulators is an effective way to access cryptic clusters. With both of these protocols in place, I am now able to generate and screen libraries of *Streptomyces* possessing multiple plasmids. This enables us to begin exploring two important questions regarding our hypothesis: 1) is this an effective way to access cryptic clusters, and 2) if so, which regulators will best function to activate these cryptic clusters. Reported in this chapter is the generation of a library of 48 wild-isolate *Streptomyces* each containing the plasmids pSET152-*ermE**p-null, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$ and the subsequent screening of that library with the *lux*-based bioassay. This led to the identification of three promising candidates for follow-up.

5.2 *Generation of a Library of Engineered* Streptomyces *and a Primary Screen*

One-hundred and sixty strains of uncharacterized, wild isolate actinomycetes (most of which are suggested to be *Streptomyces*) were acquired from the Wright Actinomycete Collection (WAC) at McMaster University. The 160 strains were streaked on MYM. The first 120 strains that were capable of growing on the MYM were arrayed into the inner 60 wells of two 96-well plates (Table 3). Using these master plates as a

source of spores, HTP conjugation was used to introduce the three plasmids pSET152-*ermE**p-null (also referred to as empty vector), pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$ into these isolates. Of the 120 isolates, 48 (40%) were successfully conjugated with all three plasmids (Table 6). Observing each plasmid separately, 59 species (49%) were successfully conjugated with pSET152-ermE*p-null, 72 (60%) with pSET152-*ermE**p-*atrA* and 71 (59%) with pSET152-*ermE**p-$lsr2_{NTD}$. This data stands apart from the 59% reported in Chapter 3 as this used a considerably larger data set and did not use any lab strains of *Streptomyces*. It suggests that a wild isolate *Streptomyces* taken at random has a 40% chance of generating exconjugants of all three plasmids with the HTP conjugation protocol. Of the strains attempted, only five (4%) showed apramycin resistance. Each of the three plasmid-containing strains from the 48 species were arrayed into 96-well agar plates. Additionally, the wild type strains of these 48 species were also arrayed into a 96-well plate.

Using the wild-type, empty vector, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$-containing strains (a total of 48×4=192 different strains), a primary screen was completed in triplicate using the *lux*-based bioassay. The strains were grown for 48 hr at 30°C on five different growth media (MYM, MS, R2YE, SAM and BEN) before overlaying with the *E. coli* XL-1 Blue + pCM-Str indicator medium. Luminescence was read 24 hr later. Positive controls (no *Streptomyces* colony) were assigned to the 12 unfilled wells left in each plate. All luminescence readings were expressed relative to positive control. Averages and standard deviations were graphed and differences between strains of the same species were recorded. Differences were

Table 6. The 48 Wright Actinomycete Collection (WAC) strains from which exconjugants containing the plasmids pSET152-ermE*p-null, pSET152-ermE*p-*atrA* and pSET152-ermE*p-*lsr2*$_{NTD}$ were created. These strains were used for the *lux*-based bioassays completed in Chapter 5.

00161	00198	00239	00272
00165	00201	00242	00274
00166	00204	00246	00278
00170	00205	00247	00282
00171	00206	00254	00283
00175	00210	00257	00284
00177	00212	00258	00286
00179	00213	00263	00289
00180	00229	00266	00290
00182	00230	00269	00291
00190	00231	00270	00292
00196	00238	00271	00293

determined based on whether or not the empty vector or regulator-containing strains' error bars overlapped (statistical analysis was not completed due to the small sample number). There are a number of initial observations to be made from this primary screen. The first observation is that the gross majority of the wild type *Streptomyces* possessed antibiotic activity on all the media types (Table 7). Since most of the wild type strains showed antibiotic activity, it can be assumed from the start that there is only a very small chance that an 'ideal hit,' as described in Chapter 4.4, will be found. A point of interest is that all of the species of *Streptomyces* tested were observed to produce antibiotics on MYM and MS. This makes these media interesting as they may have a particular feature which induces antibiotic activity. The second observation is that the empty vector has a tremendous effect on the antibiotic activity of the *Streptomyces* species (Figure 17). Furthermore, this effect seems to be skewed toward reducing antibiotic activity. It is

Table 7. Number of wild type strains which showed no antibiotic activity. Wild type strains which showed no antibiotic activity were defined as those which had luminescence values similar to the positive control. This data was collected from the primary screen.

Media	Number of Species
MYM	0
MS	0
R2YE	7
SAM	1
BEN	5

possible that the use of the pSET152 plasmid may be hindering my efforts to activate cryptic clusters. The *S. coelicolor* gene that pSET152 integrates into is SCO3798 (Combes, P. *et al*., 2002). This protein is a putative chromosome condensation protein that is orthologous to the mammalian protein Pirin (Bentley *et al*., 2002) and the *E. coli* protein YhhW (Adams and Jia, 2005).

From this screen, there are 21 strains in which the regulator boosts antibiotic activity beyond both the wild type and the empty vector (Figure 18). A more detailed look at these reveals a number of hits that may be worth following up on. Of the AtrA hits (Figure 18A), WAC00182 on R2YE, WAC00201 on MS, WAC00283 on R2YE and WAC00286 on SAM appear promising due to the relatively large differences between wild type/empty vector and the *atrA*-containing strain. Hits potentially worth following up from $Lsr2_{NTD}$-contain species are WAC00201 on MS, WAC00205 on R2YE, WAC00230 on SAM and WAC00284 on MS (Figure 18B).

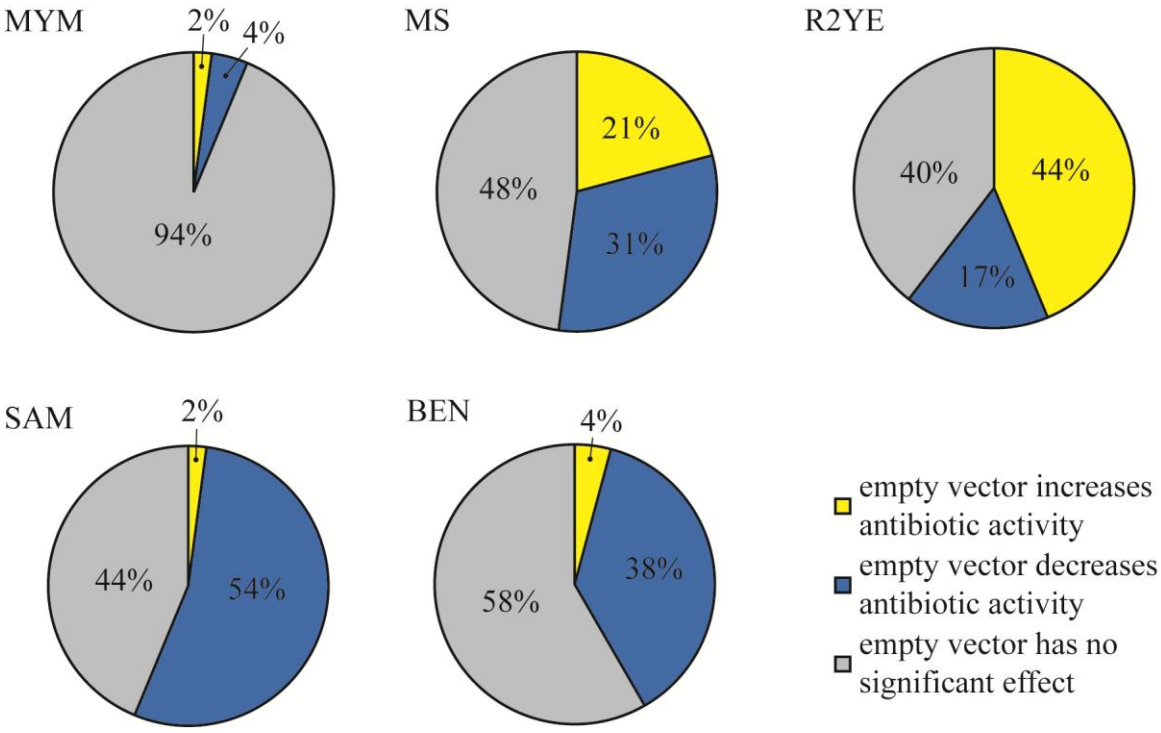


Figure 17. The empty vector plasmid has a considerable effect upon antibiotic expression. The data here shows a summary of the effect that the empty vector had, as compared to the wild type strain, on antibiotic production. As a general rule, the empty vector decreased antibiotic activity. This doesn't hold true with either R2YE or MYM. On R2YE, the empty vector generally increases antibiotic activitiy. On MYM, there are very few differences between the empty vector and wild type. This data was taken from the primary screen.

5.3 *Analysis of the Primary Screen*

The purpose of generating and screening this library of streptomycetes was to begin testing on a large scale the hypothesis that heterologous expression of pleiotropic regulators is an effective way to access cryptic clusters. I have started to answer two questions about my hypothesis: 1) is this an effective way to access cryptic clusters, and 2) if so, which regulators will best function to this end. Since only a very small subset of secondary metabolites can be observed with the *lux*-based bioassay (namely, Gram-

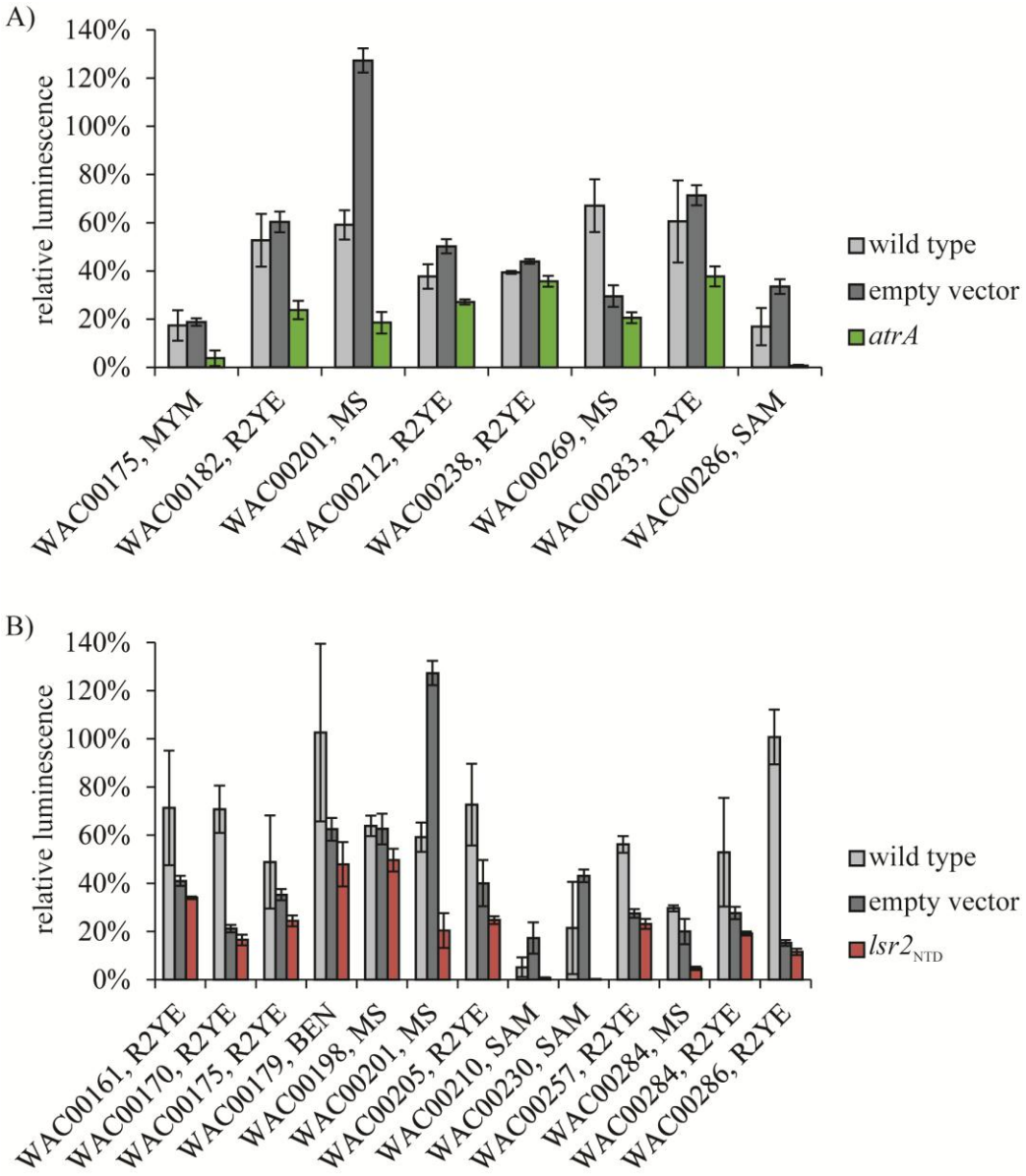


Figure 18. Summary of the hits identified from the primary screen. (A) The 8 hits identified that were generated by pSET152-*ermE**p-*atrA* plasmid. The hits WAC00182 on R2YE, WAC00201 on MS, WAC00283 on R2YE and WAC00286 on SAM were identified as interesting hits for potential follow-up. (B) The 13 hits identified that were generated by the by pSET152-*ermE**p-$lsr2_{NTD}$ plasmid. The hits WAC00201 on MS, WAC00205 on R2YE, WAC00230 on SAM and WAC00284 on MS were identified as interesting hits for potential follow-up.

negative-active antimicrobials) it is difficult to fully assess the capabilities of this technique or either regulator, but I can make conclusions regarding their ability to influence Gram-negative-active antibiotics. By looking at the ability of the regulators to either increase or decrease antibiotic expression compared to 1) both the wild type and empty vector, 2) the empty vector alone, and 3) the wild type alone I can start to understand the capabilities of these regulators.

When asking whether this was effective at activating cryptic clusters, I consider the effect of the regulator-containing plasmids (pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$) as compared to both the wild type and the empty vector (Figure 18). By looking at the ability of regulator-containing plasmids to cause changes compared to both the empty vector and the wild type, it is clear that neither plasmid possessed the overwhelming ability to stimulate antimicrobial activities on all media (Table 8). Viewing this same data from a different angle, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-$lsr2_{NTD}$ were able to influence—either by increasing or decreasing—antibiotic activity in these species 12% of the time regardless of media type (Table 8). Considering these statistics more closely, we can observe that the regulator-containing plasmids were capable of increasing antibiotic expression in 18 of the 48 (38%) species tested (Figure 18). Decreased antibiotic activity is observed in 20 of the 48 (42%) species tested. When accounting for species that experienced both increased and decreased antibiotic expression, I am left with 30 of 48 species (63%) in which the regulator-containing plasmids successfully changed antibiotic activity compared to both the wild type and the empty vector. These are impressive statistics that speak to the considerable effect that

Table 8. Summary of the abilities of AtrA and $Lsr2_{NTD}$ to either increase or decrease antibiotic production compared to both the empty vector and wild type strains. This data has been taken from the primary screen. Each number represents the number of *Streptomyces* strains which showed a significant increase or decrease in antibiotic production compared to both the wild type strain and the empty vector strain. The percentage of the sum is taken by dividing the sum by 240 (48 species × 5 medias).

Media	AtrA		$Lsr2_{NTD}$	
	Increase	Decrease	Increase	Decrease
MYM	1	4	0	2
MS	2	3	3	2
R2YE	4	0	7	3
SAM	1	9	2	2
BEN	0	5	1	7
Sum:	**8 (3%)**	**21 (9%)**	**13 (5%)**	**16 (7%)**

heterologous expression of pleiotropic regulators can have on antibiotic expression. Unfortunately, no 'ideal hits' were identified making it difficult to infer whether any of these hits were the result of the activation of a cryptic cluster. Future bioassays and liquid chromatography-mass spectrometry (LC-MS) data with some of the hits will more quantitatively show whether this technique is successfully activating cryptic clusters. Until then it will suffice to say that heterologous expression is a very potent way to influence antibiotic activity.

When assessing the usefulness of each regulator to increase antibiotic expression, it appears that pSET152-*ermE**p-$lsr2_{NTD}$ was more likely to increase antibiotic expression (relative to wild type and empty vector) as compared to pSET152-*ermE**p-*atrA* (Table 8). By looking at regulator-induced increases in antibiotic activity compared to the empty vector, we are able to assess the capabilities of the regulator alone (as opposed to the regulator *and* the plasmid) (Table 9). $Lsr2_{NTD}$, in this regard also, appeared to be slightly

Table 9. The number of strains which, when expressing either AtrA or $Lsr2_{NTD}$, experienced either an increase or decrease in antibiotic activity as compared to the empty vector strain. These results are taken from the primary screen. The percentage of the sum is taken by dividing the sum by 240 (48 species x 5 medias).

Media	AtrA		$Lsr2_{NTD}$	
	Increase	Decrease	Increase	Decrease
MYM	2	8	0	5
MS	6	11	11	16
R2YE	22	7	23	7
SAM	1	29	3	23
BEN	2	23	3	27
Sum:	**33 (14%)**	**78 (33%)**	**40 (17%)**	**78 (33%)**

more effective at boosting antibiotic expression than AtrA (Table 9). Since it was shown that the pSET152-*ermE**p-null plasmid had considerable effects upon antibiotic expression (Figure 17) the ability of the regulator and plasmid as a whole must also be assessed. This was done by comparing each plasmid's ability to induce antibiotic expression as compared to the wild type (Table 10). It is important to note the subtle difference between comparing to *wild type alone* (Table 10) and *wild type and empty vector* (Table 8). By comparing to wild type alone, the effect of the plasmid and the regulator together can be observed. By comparing to wild type *and* empty vector, an additional screening requirement is added that no longer isolates the effects of the plasmid-regulator combination. Looking at Table 10, it can be seen that the pSET152-*ermE**p-$lsr2_{NTD}$ appeared to have the upper hand in increasing antibiotic activity compared to wild type. In conclusion, it appeared that between AtrA and $Lsr2_{NTD}$, $Lsr2_{NTD}$ was slightly more capable of positively influencing antibiotic expression. Since, no ideal hits were identified with this bioassay, so it is difficult to infer the true capacity

Table 10. The number of strains which, when expressing either AtrA or $Lsr2_{NTD}$, experienced either an increase or decrease in antibiotic activity as compared to the wild type strain. These results are taken from the primary screen. The average of the sum is taken by dividing the sum by 240 (48 species x 5 medias). AtrA represents strains in which

Media	AtrA		$Lsr2_{NTD}$	
	Increase	Decrease	Increase	Decrease
MYM	8	8	3	8
MS	7	7	14	7
R2YE	7	2	14	4
SAM	4	11	8	3
BEN	6	9	3	11
Sum:	**32 (13%)**	**37 (15%)**	**42 (18%)**	**33 (14%)**

of either regulator to activate cryptic clusters. This will need to be further investigated in the future.

5.4 *Follow-up screen of pSET152-ermE*p-null and pSET152-ermE*p-lsr2$_{NTD}$*

Since the pleiotropic regulator *lsr2*$_{NTD}$ appeared to be more successful than *atrA* at inducing antibiotic activity, a 10 replicate *lux*-based bioassay was completed on all empty vector and pSET152-*ermE**p-*lsr2$_{NTD}$*-containing strains. The strains were pinned onto the same five media types (MYM, MS, R2YE, SAM and BEN) and were overlaid with the *E. coli* XL-1 Blue indicator medium after 48 hr growth at 30ºC. Luminescence was read 24 hr later. To highlight the HTP capabilities of the *lux*-based bioassay, it should be noted that this experiment constituted 6000 data points (of which 4800 were *Streptomyces* and the remaining 1200 were positive controls). As with the primary screen, the averages and standard deviations were graphed and compared. If the standard deviations of the empty vector and pSET152-*ermE**p-*lsr2$_{NTD}$*-containing strains did not overlap they were

Table 11. Summary of the abilities of $Lsr2_{NTD}$ to either increase or decrease antibiotic production relative to the empty vector. Results were taken from the follow up screen. Each number represents the number of *Streptomyces* strains which showed a significant increase or decrease in antibiotic production compared to both the empty vector strain. The percentage of the sum is taken by dividing the sum by 240 (48 species x 5 medias).

Media	Increase	Decrease
MYM	2	12
MS	1	2
R2YE	14	5
SAM	2	10
BEN	1	1
Sum:	**20 (8%)**	**30 (13%)**

considered different. In total, *lsr2*$_{NTD}$ increased antibiotic expression in 20 species and decreased antibiotic production in 30 species as compared to the empty vector (Table 11). This is half the number of hits as compared to the primary screen (Table 9). Of the 20 hits in which antibiotic production was increased (Figure 19), four were reproducible between the primary bioassay and this bioassay: WAC00161 on R2YE, WAC00170 on R2YE, WAC00205 on R2YE and WAC00230 on SAM. Other interesting hits are those generated by WAC00206, WAC00230 and WAC00263 (Figure 20). These strains generated hits on more than one media type. This suggests that the regulator may be playing more than a media-related role in the increased antibiotic activity. A non-pooled t-test (95% confidence) showed that of the seven hits generated by these three species all except WAC00206 on R2YE are statistically significant. Regardless, WAC00206, WAC00230 and WAC00263 are high priority for follow-up. This is especially true of WAC00230, which was reproducible between the primary and the secondary screen. The strains WAC00161, WAC00170 and WAC00205 may also be worth following up due to

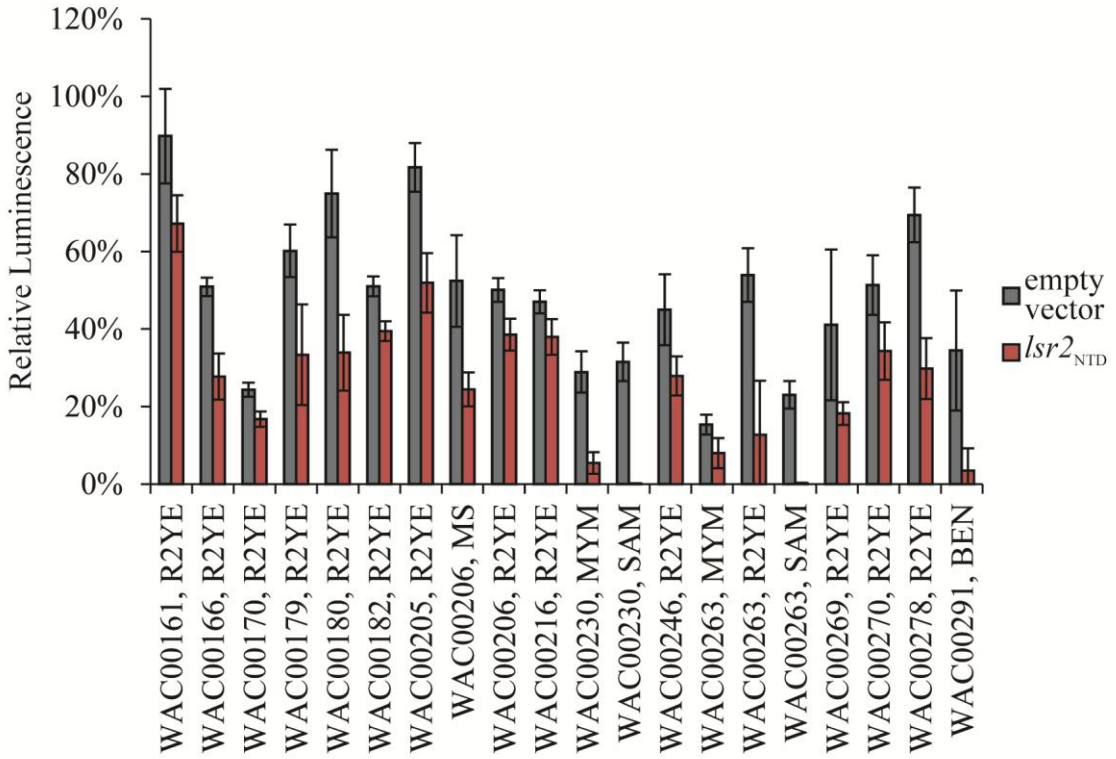


Figure 19. Hits identified from the follow up screen using the *lux*-based bioassay. Of these hits, WAC00161, WAC00170, WAC00205 and WAC00230 were reproducible between the primary screen and this screen. WAC00206, WAC00230 and WAC00263 generated hits on multiple media types thus were considered the most promising hits generated from this screen.

their reproducibility between the primary and the follow-up screens but the differences between the empty vector and the *lsr2*$_{NTD}$-containing strain are small making them less interesting (Figure 19).

The hits WAC00230 on SAM and WAC00263 on SAM are unique in that they are the only strains in this assay that reduced luminescence to approximately 0%. SAM contains NaCl, $CaCO_3$ and glycerol, all components that are unique to this media type. It is possible that the combination of Lsr2$_{NTD}$ and one or more of these media components is activating antibiotic activity. Comparison of SAM to MYM and R2YE (the other media

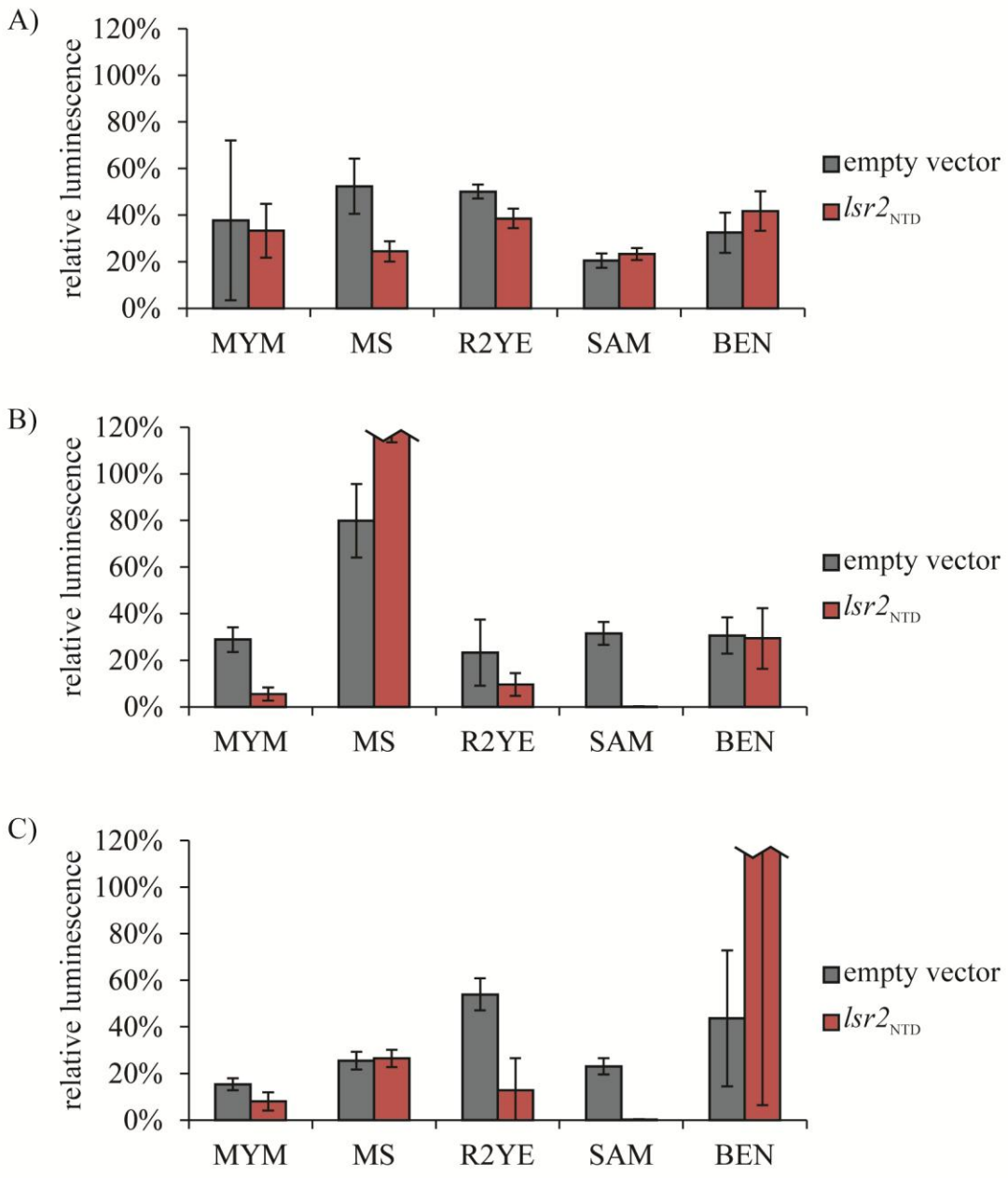


Figure 20. Summary of the antibiotic activities of the three hits generated from the follow up *lux*-based bioassay across all the media types tested. (A) WAC00206 showed hits on MS and R2YE of which only MS was statistically significant. (B) WAC00230 showed satistically significant hits on MYM and SAM. The hit on SAM was reproducible from the primary screen. (C) WAC00263 showed statistically significant hits on MYM, R2YE, and SAM.

types that WAC00230 and WACO00263 registered hits on) shows few commonalities, again suggesting the regulator is playing a major role in the induced activity. In regards to WAC00230, the only common element between MYM and SAM is yeast extract. Extrapolation of the media components might allow the suggestion that these media also share glucose as a carbon source (MYM uses maltose, a disaccharide of glucose) and, as much more liberal stretch, both contain plant seed products (malt extract by MYM and soy peptone by SAM). It is worth noting that WAC00230 (both empty vector and pSET152-*ermE**p-$lsr2_{NTD}$) generated high antibiotic activity on all media types except MS (Figure 20B). MS is the only media that does not contain yeast extract. Additionally, MS is the only media that possesses both mannitol and soy flour (though, similar to the afore mentioned liberties, soy flour may be considered similar to soy peptone). The MS-associated decrease in antibiotic activity does not hold true when looking at the activity of WAC00263 on all the media types (Figure 20C). Instead, BEN showed more luminescence over the other media types. Despite this BEN-associated luminescence, the considerable standard deviation of the data suggests that results for WAC00263 on BEN should not be trusted. Overall, no firm conclusions can be developed regarding media type and antibiotic production. Given that consideration has been given to how the media type affected the *Streptomyces*, a note should also be made regarding how the growth medium affected the indicator *E. coli*. Despite the fact that the *E. coli* are suspended in LB, it is reasonable to assume that nutrients in the growth medium will, much like the antibiotics are expected to, diffuse into the indicator medium. How the growth medium affects the luminescence of the indicator can be observed by graphing the positive

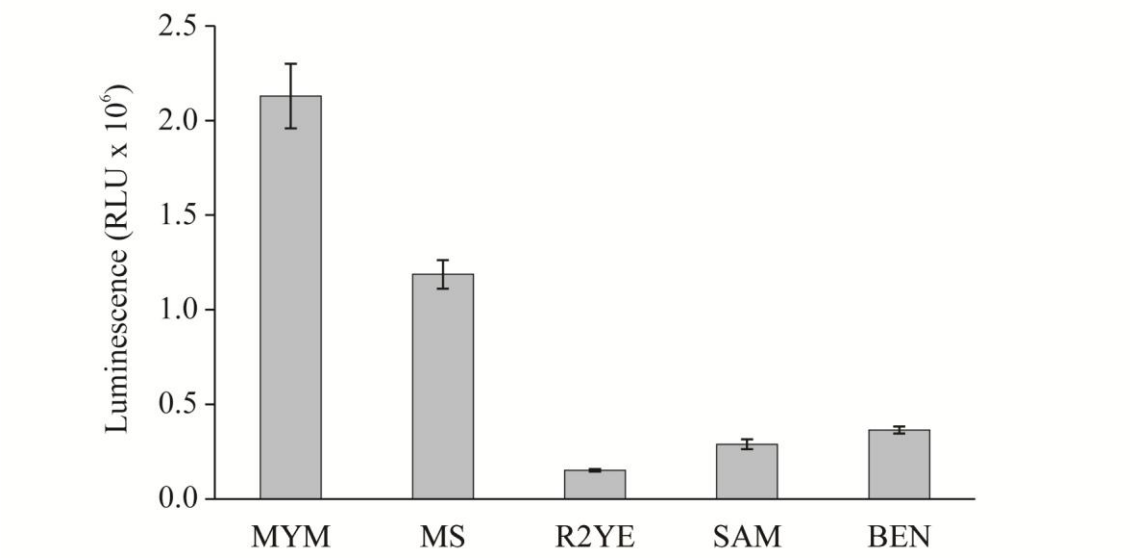


Figure 21. The growth media used for the *Streptomyces* has a considerable effect upon luminescence of the *E. coli* XL1 blue + pCM-Str. Full 96-well plates of each media used in the *lux*-based bioassay were tested for their ability to affect the luminescence of the *E. coli* XL1 Blue indicator organism in the absence of *Streptomyces*. Only the inner 60 wells were measured.

controls (Figure 21). These gross differences in luminescence either represent differences in the ability of the indicator *E. coli* to grow or alterations in the *E. coli*'s ability to luminesce. Both suggest that the properties of the *E. coli* may be different with each growth medium used. Given that media type is known to affect the secondary metabolite profile in *Streptomyces* (Ruiz *et al*., 2010), there is no reason to assume that the susceptibility of the *E. coli* to certain antibiotics is constant on each medium. This suggests that media-based differences between regulators may be just as much a result of altering the secondary metabolite profile of *Streptomyces* as it is the altered susceptibility of the *E. coli* to those molecules.

The most important hit from this screen that should be followed up on is WAC00230. It shows considerable antibiotic activity on two media types and was

reproducible between the primary and the follow-up bioassays. Other hits of high interest are WAC00263 and WAC00206. Additional hits that may merit follow-up are WAC00161, WAC00170 and WAC00205.

5.5 *Conclusions*

Due to the considerable ability of the heterologous expression of pleiotropic regulators to modify antibiotic expression, this hypothesis warrants further resources and efforts. Between AtrA and $Lsr2_{NTD}$, the data suggests that $Lsr2_{NTD}$ is presently the more effective regulator at activating antibiotic activity. Despite this, neither regulator seems as if it will serve as a 'silver bullet' for activating antimicrobial cryptic clusters (though this may be harsh judgement for the first two regulators tested in such a large number of strains). To quantitatively assess the ability of the regulators to activate cryptic clusters, a more analytical method (*e.g.* LC-MS) needs to be used to analyse the exconjugants. Such efforts will begin with the pSET152-*ermE**p-$lsr2_{NTD}$-containing strains of WAC00230, WAC 00206 and WAC00263. This data will start to truly reveal whether heterologous expression of pleiotropic regulators is an effective way to access cryptic clusters.

CHAPTER 6: CONCLUSIONS

6.1 *Conclusions*

The hypothesis that heterologous expression of pleiotropic regulators is an effective way to access cryptic clusters is still in the process of being tested. The desire to test the hypothesis in large numbers of species generated a need for HTP protocols to engineer *Streptomyces* and screen the resulting exconjugants. To meet this need, HTP conjugation and the *lux*-based bioassay were developed. The HTP conjugation protocol and the *lux*-based bioassay are extremely powerful techniques which allow the engineering and screening of *Streptomyces* on a scale not previously possible. HTP conjugation allows the introduction of multiple plasmids into hundreds of species of *Streptomyces* with considerably less effort than required by present low throughput techniques. Moreover, HTP conjugation is compatible with 40% of *Streptomyces* species. Donor *E. coli* is eliminated with the use of SGED media and PCR has been used to confirm exconjugants generated with this protocol. Exconjugants from HTP conjugation can be efficiently screened with the *lux*-based bioassay. The plasmid pCM-Str was created to track growth of the indicator organism *E. coli* XL-1 Blue allowing luminescence to be used to indirectly measure antibiotic production by *Streptomyces*. The *lux*-based bioassay is an especially potent way to screen *Streptomyces* in large numbers. In one bioassay reported here, 6000 data points were collected in a single screen. With both of these HTP technologies in place, a library of 48 *Streptomyces* containing the plasmids pSET152-ermE*p-null, pSET152-*ermE**p-*atrA* and pSET152-*ermE**p-*lsr2*$_{NTD}$

was created. These three strains and the wild type were screened for antibiotic activity. The results show that the heterologous expression of pleiotropic regulators is an extremely potent way to influence secondary metabolism. Of the two regulators, $Lsr2_{NTD}$ was found to be the more dominant one. From this bioassay and a follow-up bioassay, three enticing hits were identified: WAC00230, WAC00206 and WAC00263. Follow-up work on these three hits may yield a new Gram-negative antibiotic.

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APPENDICES

Appendix 1: Media Recipes

MYM

Maltose	4 g
Yeast Extract	4 g
Malt Extract	10 g
Bacto Agar	20 g

- Make in 1 L of 50% tap water, 50% reverse osmosis water
- After autoclaving, add 2 mL of R2 trace elements (Keiser *et al*., 2000) per 1 L of media

SAM

Glucose	15 g
Soy Peptone	15 g
NaCl	5 g
Yeast Extract	1 g
Glycerol	2.5 mL
Agar	20 g

- Make in 1 L of reverse osmosis water

SGED

D-(+)-Maltose Monohydrate, *Sigma-Aldrich*	4 g
Yeast Synthetic Drop-out Media Supplement (w/o his, leu, trp, ura), *Sigma*	1.4 g
Yeast Nitrogen Base, Without: Amino acids, With Ammonium Sulfate, *BioShop*	6.74 g
Uracil, *Sigma*	20 mg
L-Tryptophan, *BioShop*	20 mg
Bacto Agar, *BD*	20 g

- Make in 1 L of reverse osmosis water
- After autoclaving, add 2 mL of R2 trace elements (Keiser *et al*., 2000) per 1 L of media

Appendix 2: Step-by-step Protocol for High Throughput Conjugation

1. Have prepared: a) 96-well MS plates (200 µL of MS Agar + 10mM $MgCl_2$/well, 1 mL 1M $MgCl_2$/100ml MS agar), and b) sporulated colonies of to-be-conjugated *Streptomyces* arrayed in 96-well format (usually on 96-well MYM plates).
    - If mold is likely to be a problem, 50 µg/ml of cyclohexamide may be added to the media

2. Grow up an overnight culture of *E. coli* ET12567/pUZ8002 + pSET152 in LB + 50 ug/mL apramycin, 25 ug/mL chloramphenicol, and 50 ug/ml kanamycin. Grow to an OD_{600} of ~0.4.

3. Sterilize a cryoreplicator by leaving it on an extremely hot "hot plate" for 10 minutes (this can be done in a non-sterile manner). Transfer the cryoreplicators to a sterile hood and allow to cool for 30 minutes. *Proceed to next step*.

4. *While the cryoreplicators are sterilizing and cooling.* Wash *E. coli* culture 3 times in an equal volume of LB (no antibiotics) to remove antibiotics which could inhibit growth of *Streptomyces*. Wash at 3000 RPM for 10 minutes in a table top centrifuge. Resuspend in $1/10^{th}$ original volume of LB. Alternatively, 10 mL of culture can be spun down and resuspended in 1 mL of LB and transfer to a microfuge tube. The *E. coli* culture can then be washed in the microfuge tube. Due to the reduced volume of LB for washing (less capability to dilute antibiotics clinging to cells), increase the number of washes to 5 or 6. Wash at 15000 RPM for 1 minute in a microcentrifuge.

5. Using a multi-channel pipette, overlay 10 µl of the 10x donor *E. coli* culture onto the MS plates. Allow the plate to dry. This usually takes between 30 minutes to an hr depending on the prior condition of the conjugation plates (how old they are, how long they were dried after initially pouring, etc.).

6. Using a cryoreplictor, pin from *Streptomyces* colonies to the 96-well MS conjugation plates.

7. Grow for 12-14 hr (overnight) at 30°C, no shaking, face up.

8. Using a multi-channel pipette (an automated fluid distributer, e.g. µFill, can also be

used), overlay with 30 µL of solution containing 0.125 mg/ml naladixic acid and 0.313 mg/ml apramycin (master mix: 250 µl of 25 mg/ml naladixic acid and 313 µl of apra into 50 mL of sterile H_2O). Overlay the positive control with naladixic acid only (master mix: 25 µl of nalidixic acid in 5 mL of sterile H_2O). Allow to dry under laminar air flow (usually 2 to 3 hr).

8. Leave plates at 30°C until colonies sporulate. Judge this based on the positive control, as it is expected that not all the conjugations will be successful. This process usually takes a week.

9. Sterilize the cryoreplicators as before. Pin from the conjugation plates onto 96-well SGED-AN plates (SGED + 50 µg/ml apramycin, 25 µg/ml nalidixic acid).

10. Leave plates at 30°C until colonies to sporulate.

11. Cherry pick successful exconjugants from the SGED-ANC plate and array them onto a 96-well MYM + apramycin plate (50 µg/ml apramycin). Note: When doing conjugations, it is wise to do the conjugations in triplicate. As a general rule, I compare the conjugation plates to each other and will only cherry pick exconjugants which appear in 2 of the 3 conjugations plates.

Appendix 3: Using ψ to help identify interesting hits

$$\psi = \frac{E - R}{E} - |1 - E|$$

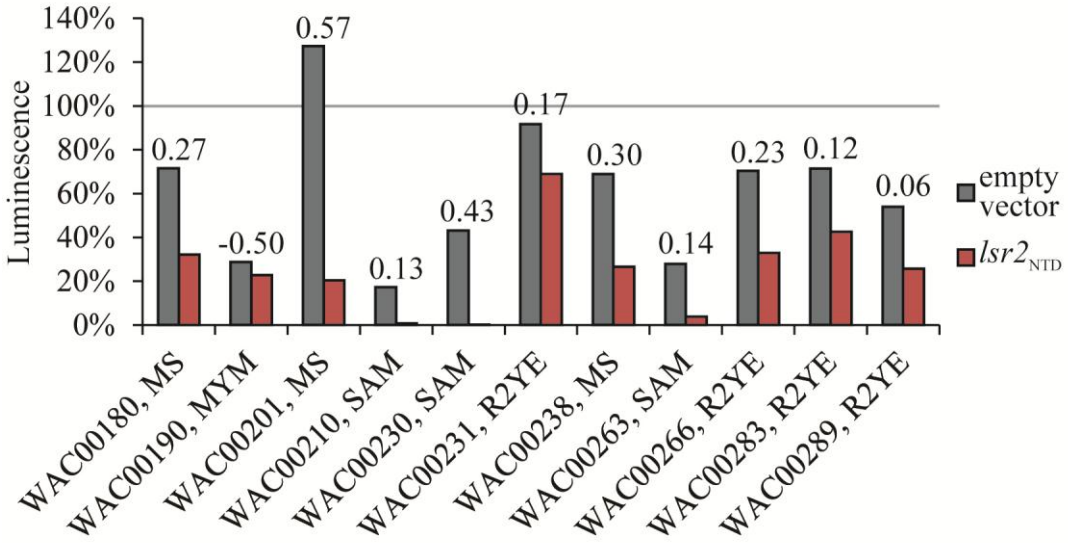
Where,

ψ = the "indicator" value for a particular regulator and its cognate empty vector
E = average luminescence allowed by a species' empty vector strain expressed relative to the plate's positive control
R = average luminescence allowed by a species' regulator-containing strain expressed relative to the plate's positive control

While looking at graphs can serve as an appropriate way to analyse data in a low throughput manner, large quantities of data need to be pre-proccessed by a computer. As a result of the need to automate the analysis of data generated by the *lux*-based bioassay, an equation was developed which calculates an "indicator" value (ψ) (see equation above) for each strain. ψ is a real number that ranges from $-\infty$ to 1. Based on ψ, potential hits can be highlighted in a large set of data. The equation functions by calculating a value for each species based on how well the empty vector and regulator-containing strains conform to the "ideal hit" (lots of luminescence from the empty vector strain, very little luminescence from regulator-containing strain). The more closely a potential hit resembles the ideal hit, the closer the ψ value is to 1. The ideal hit (100% luminescence from the empty vector, 0% luminescence from the regulator-containing strain) has a value of exactly 1. Once potential hits have been identified by their ψ value, they are validated manually (i.e. by looking at the graph).

The ψ equation can be divided into two components. The left hand component of the equation, (E-R)/E, takes the difference in luminescence between the empty vector and the regulator-containing strain and represents it as a fraction of the empty vector strain. In other words, it describes the fold change between the empty vector and the regulator-containing strain. This component of the equation is incapable of independently identifying interesting hits though. In a situation in which, for example, the empty vector strain allows 1% luminescence and the regulator-containing strain allows 0% luminescence, the equation would produce the value 1, the highest possible value. This suggests that this is an extremely strong hit. In fact, this hypothetical species, although potentially interesting, does not follow the requirements of an interesting hit as described above. The fact that the empty vector strain is already producing a strong antibiotic makes the visualization of the activation of a cryptic cluster impossible. Thus the amount of luminescence allowed by the empty vector strains must also be accounted for. This is where the second component of the equation, |1-E|, plays an important role. Initially, the second component of the equation was an iteration of the first component except that the positive control was treated as the empty vector (E) and the empty vector was treated as the regulator (R). Akin to the first component, the second component of the equation simply represents the fold change between the positive control and the empty vector. The

simplification of this component comes from the fact that since all luminescence readings are relative to the positive control, the value of the positive control must always be 1. The absolute value of the second component ensures that the number generated cannot be a negative number (this occurs when the empty vector increases light over that of the positive control, i.e. E > 1). Since the second component is subtracted from the first, a negative number generated by the second component (without taking the absolute value) gives rise to a positive number. This increases the ψ of species whose empty vector strains manage to increase luminescence. The absolute value of this component acts to reduce the ψ of these strains in the same way it does to a strain whose empty vector strain allows very little luminescence.



This graph gives an example of the potential hits that ψ would generate if used with the primary screen. The data for AtrA has not been included here. An arbitrary cut off of 0 was decided as the limit for identifying hits. No hits were identified on MYM with a cut off of 0, so the cut off was relaxed to −0.5 for MYM only. This method identified only 3 hits in common with the method presented in Chapter 5: WAC00201 on MS, WAC00210 on SAM and WAC00230 on SAM. This method obviously suffers by not accounting for distribution of data points.