***STREPTOMYCES* GENE EXPRESSION TOOL DEVELOPMENT AND BEYOND**

# DEVELOPMENT OF A NOVEL LUCIFERASE REPORTER TOOL FOR HIGH THROUGHPUT GENE EXPRESSION ANALYSIS IN *STREPTOMYCES*

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# ABSTRACT

Streptomycetes biology and genetics encompasses a variety of interesting features including multicellular growth, rich secondary metabolite production, and extensive environmental sensory and response systems. The characteristically large genomes of streptomycetes makes studying the diverse external stimuli intricate and internal regulation of these gene systems a challenge. Currently, there does not exist an efficient, cost-effective method of high throughput gene expression analysis in streptomycetes. Luciferase reporters have been used successfully in *Streptomyces coelicolor* to measure select promoter activity, however, they have demonstrated limited success in other strains and are not favourable to gene expression studies on a larger scale. Here, I present pLHR, a novel luciferase-based reporter tool designed specifically for high throughput gene expression studies in streptomycetes as well as the preliminary results which support the further development of this tool for gene expression profiling in *S. coelicolor*. Once developed, pLHR may be used to generate libraries of *Streptomyces* reporter stains to measure promoter activity repeatedly under variable conditions for the duration of the organism's complex lifecycle.

# ACKNOWLEDGEMENTS

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# ABBREVIATIONS

*egfp*: Enhanced Green Florescent Protein

GUS: β-Glucuronidase

MCS: Multiple Cloning Site

SCO: *Streptomyces coelicolor*

PCR: Polymerase Chain Reaction

*aac(3)IV*: apramycin resistance cassette

DMSO: Dimethyl Sulphoxide

BLAST: Basic Local Alignment Search Tool

CPS: Counts per Second

TFR: TetR-Family Regulators

# DECLARATION OF ACADEMIC ACHIEVEMENT

The totality of the work presented in this thesis was completed by me unless otherwise cited.

# 1.0 INTRODUCTION

## 1.1 *Streptomyces* Biology and Genetics

In the bacterial domain, streptomycetes possess many distinctive characteristics of interest to researchers. In addition to being a major producer of pharmaceutically significant molecules, they undergo a complex filamentous, spore-forming life cycle, and are ubiquitous to terrestrial environments which require them to respond to a variety of environmental and biological cues (Hopwood, 2006). During the *Streptomyces* life cycle, intricate regulation is used to manage secondary metabolite production and developmental cycling in a highly competitive, nutrient poor environment (Bentley *et al.*, 2002; Vetsigian *et al.*, 2011). With the increasing number of *Streptomyces* genomes being sequenced, the genetic resources available to investigate on a larger scale how streptomycetes utilize these treasure troves of secondary metabolites in relation to development and interactions with their diverse environments are becoming more available.

The first *Streptomyces* genome was published in 2002 for the model organism *Streptomyces coelicolor A3(2)* (Bentley *et al.*, 2002). The chromosome was found to be 8.7 Mb, the largest bacterial genome at the time, providing a new platform for more advanced genetic analysis of the genus and other actinomycetes. A large, linear chromosome with high GC content helps to characterize the Gram-positive streptomycetes (Hopwood, 2006; Bentley *et al.*, 2002). The genome comprises two "arms" flanking a "core" of essential genes (Hopwood, 2006). The genes located near the ends of the chromosome can undergo large deletions in

the lab (Hopwood, 2006). It is proposed that the many auxiliary genes and biosynthetic pathways located in the more genetically variable chromosome arms allow these organisms to cope with changing environments and to promote competitive advantages against the many other organisms that make up diverse soil microbiomes (Hopwood, 2006; Bentley *et al*., 2002; Vetsigian *et al*., 2011).

*1.1.1 Lifecycle*

The complex multicellular lifecycle presents an interesting gene regulation element to *Streptomyces* development. The organism must tightly regulate sporulation gene expression based on nutrient availability and other environmental conditions to ensure optimal development through the multiple stages of its lifecycle. Premature or latent passage to the subsequent stage may seriously reduce colony survivorship (Kieser *et al*., 2000; McCormick and Flardh, 2012). The *Streptomyces* lifecycle starts when the spore is in a nutrient rich environment favorable to colony growth and spore germination is triggered (Figure 1). Upon spore germination cell wall growth at the tip of a germ tube occurs rapidly relative to DNA replication leading to germ tube elongation. The resulting multichromosomal filamentous tissue branches laterally forming a network of substrate mycelium into the growth medium. Septation of the substrate mycelium into large segments occurs away from the rapid growth activity at the hyphal tip. Upon a change in the growth conditions, such as localized nutrient resource depletion, the colony projects aerial mycelium out of the growth medium. This

tissue differentiation step from substrate mycelium to aerial mycelium is the stage

in the *Streptomyces* lifecycle when many secondary metabolites are produced

(Kieser *et al.*, 2000). Aerial mycelium then undergo cell division, segmenting the

multichromosomal filamentous tissue into uninucleoid exospore compartments.

These compartments mature into desiccation resistant spores ready for dispersal.
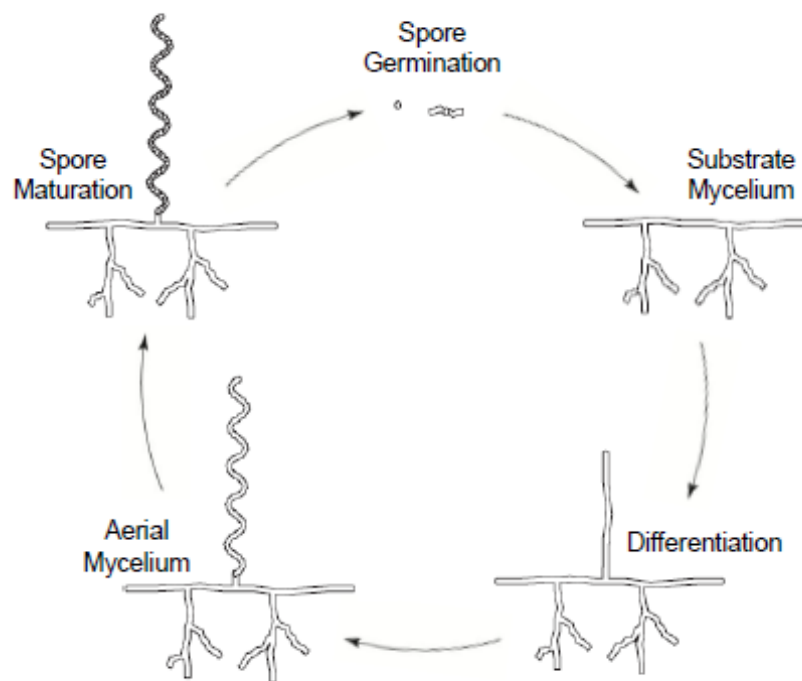


**Figure 1. *Streptomyces* lifecycle.** The *Streptomyces* lifecycle starts with spore germination in favourable growth conditions. Substrate mycelium grows into the growth medium before the colony undergoes tissue differentiation, grows out of the medium and forms aerial mycelium. Individual compartments form from the aerial mycelium and eventually mature into spores. (Image adapted from Dr. Leslie Cuthbertson)

*1.1.2 Secondary Metabolism*

Streptomycetes are well known for their ability to produce an impressive array of diverse secondary metabolites many of which are used clinically as antibiotics, antifungals, antiparasitics, anticancer and immunosuppressive agents (Nett *et al.*, 2009). Though these secondary metabolites are discovered and used as medically relevant natural products, it is not clear what biological function these non-essential molecules serve for the bacteria themselves. As genomic databases grow, the opportunities to mine streptomycetes and other actinomycetes genomes (Nett, *et al.* 2009; Bentley, *et al.* 2002) and mobile elements (Kinashi *et al.*, 2011) increases.

In attempts to identify novel antibiotics and other natural products, significant attention has been placed on better understanding the regulatory networks of antibiotic biosynthetic clusters (Lui *et al.*, 2013; Martin and Liras, 2012; van Wezel *et al.*, 2011). This may allow for the identification of novel antibiotics and to shed light on their biological function by establishing under what growth conditions these secondary metabolites are expressed (Martin and Liras, 2012; Nieselt *et al.*, 2010).

*1.1.3 Environmental Stimuli and Response*

Streptomycetes are soil-dwelling bacteria and are ubiquitous in nature (Kieser *et al.*, 2000). In addition to being found in terrestrial and aquatic environments as free-living organisms, a number of *Streptomyces* species have

been isolated from symbiotic interactions with plants, animals, and fungi (Seipke *et al.*, 2011). As a genus, streptomycetes are able to survive in a wide variety of conditions and interact with other organisms, but they also exist in highly diverse and competitive soil ecosystems resulting in complex interaction networks among streptomycetes and other soil-dwelling organisms (Vetsigian *et al.*, 2011). These interactions may be mutually beneficial or in direct competition for limited resources. It is believed that the many secondary metabolites produced by streptomycetes are produced to respond to low nutrient conditions (Martin and Liras, 2012), symbiotic interactions (Seipke *et al.*, 2011), colony growth (McCormick and Flardh, 2011), and other environmental triggers (Lui *et al.*, 2013).

All organisms sense and respond to their environment. There are two major categories of regulatory systems which allow bacteria to identify changes in their environment and initiate an appropriate response, one-component systems and two-component systems. One-component regulatory systems comprise a transcriptional regulator which contains both a ligand binding domain and a DNA binding domain (Ulrich *et al.*, 2005). Upon binding its ligand the regulator will either bind to the promoter region of its target gene(s) thus blocking transcription of that gene, or be released from the promoter region and allow transcription of its target gene. A typical representative of one-component systems is TetR and its target gene *tetA*, which codes for an efflux pump (Hillen and Berens, 1994; Orth *et al.*, 2000; Ramos *et al.*, 2005). Together these genes confer resistance to the antibiotic tetracycline. When tetracycline is not present in the cytosol, TetR binds to the

promoter region of *tetA* repressing transcription of the gene. However, in the presence of tetracycline, TetR binds to the antibiotic, undergoes a conformation change releasing TetR from the promoter region of *tetA*, and *tetA* is transcribed. The expression of TetA confers resistance to tetracycline by pumping the antibiotic out of the cell.

Two-component regulatory systems, in contrast, involve two separate proteins, a sensor kinase and a response regulator (Jung *et al*., 2012; Hutchings *et al*, 2004; Wuichet *et al*., 2010). Sensor kinase is a membrane-bound protein which has a sensor ligand binding domain and an ATPase with a conserved histidine motif. Response regulators have a conserved aspartate box and a DNA binding domain.  Signaling is initiated when the sensor domain either binds to a ligand or another stimulus such as changes in temperature and light (Hutchings *et al*., 2004) causes the conserved histidine box to be autophosphorylated. A phosphoryl group is then transferred from the histidine box in the sensor kinase to the aspartate box in the response regulator. The phosphorylated response regulator undergoes a conformational change allowing it to bind to the promoter region of its target gene(s). This is the structure of a prototypical two-component system, however, unmatched sensor kinases, orphaned response regulators, accessory proteins, scaffold proteins, and phosophorelays which support and diversify the signaling achieved by these systems may also play a role (Jung *et al*., 2012). An example of a standard two-component system is VanRS, which regulates resistance genes to the antibiotic vancomycin (Hutchings *et al*., 2004;

Pootoolal *et al.*, 2002). VanS senses vancomycin and phosphorylates VanR which binds to the promoter of the vancomycin resistance gene cluster (*vanRS*, *vanJ*, *vanK*, and *vanHAX*) upregulating its transcription (Pootoolal *et al.*, 2002). Expression of the vancomycin resistance gene cluster confers resistance to the antibiotic by reprogramming the structure of the cell wall such that vancomycin can no longer interfere with cell wall assembly.

Both one-component and two-component systems contribute to a bacterium's ability to sense changes in their environments. The size of a genome is a good indicator of the regulatory tools available to the bacteria as the number of one-component and two-component systems in a chromosome is proportional to the genome size (Ulrich *et al.*, 2005). Therefore, the many one-component and two-component systems present in the large chromosomes of streptomycetes help to facilitate the regulation of a complex developmental lifecycle, diverse metabolism, and fluctuating environmental conditions and microbial interactions. In order to study these transcriptional regulatory systems in a high throughput manner, cost effective and efficient gene expression tools are required to better understand expressional profiles of transcriptional regulators and their target genes.

## 1.2 Reporter Genes in *Streptomyces*

The highly dynamic nature of streptomycetes biology requires intricate gene expression regulation of developmental, secondary metabolite, and environmental

response genes. A number of reporter genes have been used in streptomycetes as attempts to develop tools for detecting promoter activity for genes of interest. Such gene expression profiles are particularly beneficial when studying streptomycetes as promoter activity can be read over the complete lifecycle of the organism and under varied conditions rather than at a single moment during the lifecycle. Here, the advantages and disadvantages of each reporter system will be discussed briefly.

*1.2.1 Luciferase Bioluminescence*

Bacterial bioluminescence genes have been used as a transcriptional reporter extensively in molecular biology (Meighen, 2003). Light is produced when the luciferase enzyme (*luxA* and *luxB*) catalyzes an oxidation reaction with the long-chain fatty aldehyde substrate (*luxC*, *luxD*, and *luxE*) and host flavin mononucleotide (FMNH$_2$) (Meighen, 2003).

Bioluminescence expression assays have been used in *Streptomyces* in two ways (Rodriguez-Garcia *et al.*, 2005; Craney *et al.*, 2007). Either only the luciferase genes (*luxAB*) were expressed and the n-decanal substrate was added to the culture, or the full operon (*luxCDABE*) was expressed in the host. Rodriguez-Garci *et al.* 2005 used a codon optimized version of *luxAB* as a reporter system for measuring an artificially regulated transcriptional promoter. In this system, light was produced when the substrate n-decanal was added to the culture to test for luciferase activity. The more recent *lux* reporter in *Streptomyces* was developed by

Craney *et al.* 2007 and uses the complete *luxCDABE* operon to express both the luciferase and tetradecanal substrate in the host. The proposed advantages of this system include avoiding adding n-decanal substrate separately to the culture for each light emission reading. This is done for two reasons, the permeability of the different *Streptomyces* may be varied among the different cell types over the organism's lifecycle, and the additional step of adding luciferase substrate is less advantageous for high throughput time trials. Both *lux* reporter systems utilize genes which are codon optimized for expression in *Streptomyces* and other high-GC organisms.

### 1.2.2 Fluorescent Proteins

A number of fluorophores have been developed for improved protein localization imaging in cell biology (Shaner, *et al.*, 2004). Two fluorescent proteins, *egfp* and *mCherry*, have been used successfully in *Streptomyces* to create green and red autofluorescent tags (Willemse and van Wezel, 2009; Willemse *et al.*, 2012; Flardh, 2010; Sun *et al.*, 1999). However, there are some drawbacks, to using these proteins in streptomycetes, primarily high autofluorescence and photobleaching when exposed to UV light which can make imaging proteins expressed at low levels difficult (Willemse and van Wezel, 2009). To overcome the challenge of autofluorescence in *Streptomyces* a derivative of *S. coelicolor* M145 with reduced autofluorescence levels was identified allowing for more accurate fluorescent microscopy in *Streptomyces* (Willemse and van Wezel, 2009). This

improvement however is limited to studies in *S. coelicolor* M145 and no other streptomycetes. Though fluorescent proteins are invaluable markers for protein expression, trafficking and localization in *Streptomyces*, other reporters may serve as better transcriptional reporters for gene expression studies (Myronvskyi *et al.*, 2011).

### *1.2.3 β-Glucuronidase*

Most recently a reporter using β-glucuronidase (GUS) activity was presented as a sensitive and versatile gene expression tool in actinomycetes (Myronvskyi *et al.*, 2011). The β-glucuronidase enzyme hydrolyzes several β-glucuronides and is encoded by *gusA* from *Escherichia coli*. As with *luxCDABE*, the *gusA* gene was codon optimized for expression in *S. coelicolor*. Myronvskyi and authors (2011) report many advantages of this system, including the stability and specificity of GUS activity, no host cofactors are required for GUS activity, GUS is not native to most actinomycetes, and may be used as an N-terminal or C-terminal translational fusions (Myronvskyi *et al.*, 2011). Additionally, depending on the substrate used, different assays for GUS activity – spectrophotometry, fluorimetry, chemiluminescence, and histochemical, may be used (Myronvskyi *et al.*, 2011). This feature allows for increased versatility of the reporter when using different organisms and assaying a variety of genes. An additional facility of GUS for *Streptomyces* genetics is as a screening tool for double cross over reactions using colorimetric screening.

Similar to the *luxAB* reporter, however, this system also requires that an enzyme substrate is added to the culture for readings, cell wall permeability to the substrate may change during the lifecycle of the organism, and it is less efficient for the purposes of high throughput time course assays. This reporter system shows promise, but additional evidence may be required to demonstrate how versatile it is as a high throughput tool for *Streptomyces* gene expression studies.

*1.2.4 Others Reporters*

A number of promoter-less reporter genes have been explored as candidates for gene expression tools in streptomycetes, however, to date only luciferase and *egfp* have been widely used in *Streptomyces* studies (Cuthbertson *et al.*, 2013; Xu *et al.*, 2012; Ahn *et al.*, 2012; Willemse *et al.*, 2012; Santos-Beneit *et al.*, 2011; McKenzie and Nodwell, 2009; Lamp *et al.*, 2013; Heichlinger *et al.*, 2011). Previous to these two reporters several transcriptional other reporters were developed for use in *Streptomyces* including *xylE*, encoding catechol 2,3-dioxygenase (Ingram *et al.*, 1989), *melC*, encoding a tryosinase (Paget *et al.*, 1994), and *amy*, encoding amylase (Flores *et al.*, 2003), but have not predominated as efficient reporter tools for future studies.

**1.3 Research Goals**

It is the intent of this report to 1. present a novel gene expression tool for use in streptomycetes using the *luxCDABE* reporter, 2. the progress to date on

testing its efficacy and accuracy as a transcriptional reporter, and 3. discuss proposed future directions for continued work on the topic. Ultimately, the tool presented may be used for high throughput directed analysis of gene expression patterns in *S. coelicolor* and other streptomycetes under various conditions for the extent of the entire lifecycle. This approach may provide insight into the biological function of secondary metabolites, the genes that regulate their production and the interplay of these molecules involved in cell development, differentiation, interactions with other organisms, and in response to environmental changes.

## 2.0 BACKGROUND

### 2.1 Present Tool: pLHR

The current *lux* reporter, pFlux, is used to measure promoter activity in *Streptomyces coelicolor* (Craney *et al.,* 2007) (Figure 2). With this tool, the promoter of interest is inserted into the Multiple Cloning Site upstream of the *luxCDABE* operon, creating a promoter-*lux* fusion. When the promoter is active, the *luxCDABE* operon will be expressed and light production can be quantified. The promoter of interest is first introduced into the MCS of pFlux in *E. coli*, at which point it is introduced to *E. coli* ET12567 [pUZ8002], before being introduced in *S. coelicolor* via conjugation (Flett *et al.*, 1997). This *lux* reporter employs a φBTI integrase to integrate the reporter into the *Streptomyces* chromosome at the bacterial attachment site (attB) (Gregory *et al.*, 2003).
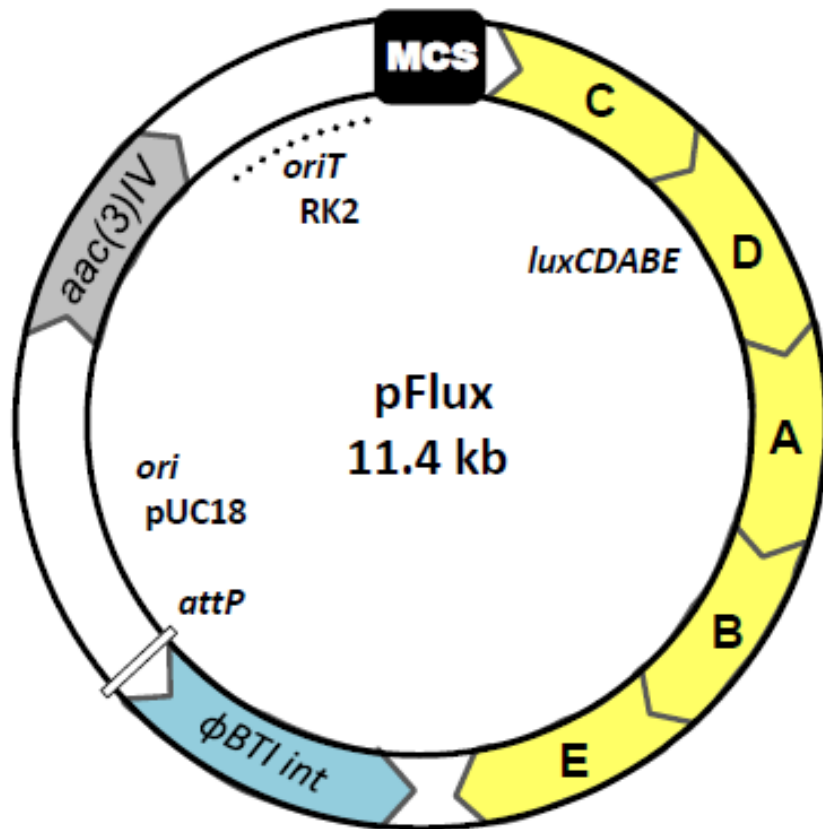
MCS:<u>GATATC</u>/<u>GGATCC</u>TCTAGAATGCAT<u>GGTACC</u>AGATCTGACTGAGTGAACCAA**AGGAGG**C
GGACAT**ATG**ACGAAG

**Figure 2. pFlux, an integrative *lux* reporter that uses ϕBT1 integrase to integrate into the bacterial attachment site (attB) in the chromosome.** *ϕBT1 int* , phaged integrase; *attP*, phage attachment site; *ori*, origin of replication for *E. coli* from pUC18; *aac(3)IV*, apramycin resistance cassette that is selectable for in both *E. coli* and *Streptomyces*; *oriT*, origin of transfer from RK2 for conjugation into *Streptomyces*. The MCS contains three unique restriction enzyme sites (*Eco*RV, *Bam*HI, and *Kpn*I) underlined. The MCS is followed by a ribosome binding site depicted in bold. Figure modified from Craney *et al.*, 2007.

This system has been very useful in a number of separate studies (Craney

*et al.*, 2007; Cuthbertson *et al.*, 2013; Xu *et al.*, 2012; Ahn *et al.*, 2012), however,

some limitations have been identified. First, this tool has had limited success when

used in *S. venezuelae*. Both *S. coelicolor* and *S. venezuelae* are used regularly as model systems and it would be beneficial to have a gene expression tool that is more versatile in terms of the organisms with which it is compatible.  Second, the attB site that the vector integrates into the chromosome interrupts a gene of unknown function, *SCO4848* (Gregory *et al.*, 2003). Though this gene disruption does not appear to affect the cell negatively, it is not known what affects this gene disruption has on cell growth and development. Third, the ϕBTI integrase may integrate into pseudo attachment sites in the bacterial chromosome which may skew data if more than one promoter-*lux* copy is present, or an unknown gene is disrupted. Finally, the vector is 11.4 kb in size, making it challenging to transform and conjugate, thus a smaller vector would be beneficial. These considerations may be taken into account when designing a new gene expression tool for use in *Streptomyces*.

## 2.2 Proposed Tool: pLHR

The new gene expression tool, pLHR, is also designed to measure promoter activity and gene expression using a *luxCDABE* operon readout, however, instead of using a phage integrase to integrate the vector into the bacterial chromosome it will rely on host systems for homologous recombination (Figure 3). pLHR was constructed by designing and ordering a MCS compatible with *Streptomyces* plasmid pOJ260 and inserting the MCS into the vector. The *luxCDABE* operon was then inserted into a designated area of the downstream end of the MCS. In order

to quantify gene expression using pLHR, a homologous region of your gene of interest is cloned into the MCS, the vector is then introduced into *E. coli* ET12567 [pUZ8002] before transforming *Streptomyces* with the vector via conjugation (Flett *et al.*, 1997). Single recombinants are selected for using antibiotic resistance markers.
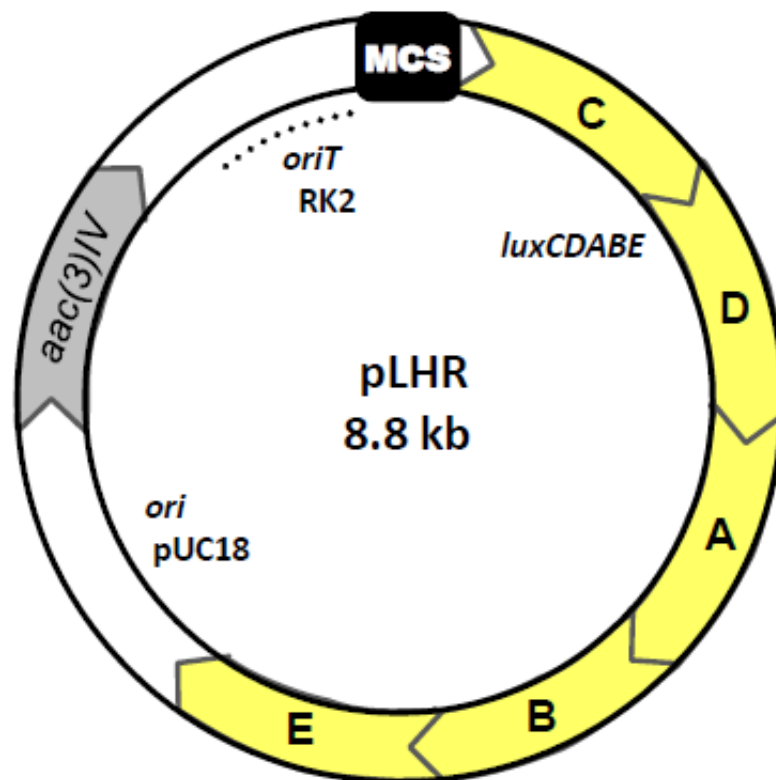


MCS:TTAATTAA<u>CCTGCAGG</u>GGCTAGC<u>GAATTC</u>TTCGAA<u>CCTAGG</u>GATATC<u>AACGTT</u>CTTAAG<u>CA</u>
<u>ATTG</u>GGGATCC<u>TCTAGA</u> ATGCATGGTACC AGATC**TGACTGAGTGA**CCAA**AGGAGG**CGGACAT
**ATG**ACGAAGAAGA

**Figure 3. pLHR, an integrative *lux* reporter that uses homologous recombination to integrate into the bacterial chromosome.** *ori*, origin of replication for *E. coli* from pUC18; *aac(3)IV*, apramycin resistance cassette which is selectable for in both *E. coli* and *Streptomyces*; *oriT*, origin of transfer from RK2 for conjugation into *Streptomyces*. The MCS contains 12 unique restriction enzyme sites (*Pac*I, *Sbf*I, *Nhe*I, *Eco*RI, *Bst*BI, *Avr*II, *Eco*RV, *Acl*I, *Afl*II, *Mfe*I, *Bam*HI, and *Nde*I) are designated by alternating underlines. The MCS is followed in-frame stop codons and a ribosome binding site show in bold.

There are several potential advantages to using homologous recombination to create a *lux* fusion for your gene of interest. Homologous recombination allows the *luxCDABE* operon to be placed directly downstream of any promoter or gene of interest. It is also possible to selectively knockout only part of the downstream region of the gene or create a gene-*lux* fusion operon, which preserves the native system by placing the *lux* reporter directly downstream of your gene of interest. Therefore, this tool may be multifaceted in how it is used to measure gene expression in relation to the promoter-gene system being investigated. pLHR is also 8.8 kb which is approximately three kilobases smaller than pFlux and easier to manipulate and work with.

Something to consider while testing this tool is whether having the plasmid backbone integrated into the chromosome will alter regulation of surrounding genes. This should not be an issue in many cases; however, it will likely need to be considered on a case-by-case basis when choosing a homologous region and where the *luxCDABE* operon and vector backbone will be placed following homologous recombination.

The most pressing question becomes how big does the homologous region need to be for efficient and accurate homologous recombination into a *Streptomyces* chromosome? To address this, an inducible one-component system conferring antibiotic resistance will be used to test this new gene expression tool first in *S. coelicolor.*

## 2.3 Test System: The Kijanimicin Story

Kijanimicin is an antibiotic produced by the soil bacterium *Actinomadura kijaniata* (Waitz *et al.*, 1981; Zhang *et al.*, 2007). A mechanism for kijanimicin resistance, comprising two genes, *kijR* and *kijX*, was identified and tested in *S. coelicolor* (Cuthbertson *et al.*, 2013). Together these genes function as a one-component system for the regulation and activation of kijanimicin resistance in the presence of the antibiotic. The transcriptional regulation of *kijX* by KijR is not unlike the transcriptional regulation of *tetA* by TetR that confers resistance to tetracycline (Cuthbertson *et al.*, 2013). In this system, KijR acts as a transcriptional repressor of *kijX*, and when bound to kijanimicin, KijR undergoes a conformation change releasing it from the promoter of *kijX*. The expression of *kijX* produces an enzyme that degrades kijanimicin by deglycosylation. In the absence of kijanimicin, KijR remains bound to the promoter of *kijX*, and no KijX is produced.

It has been shown experimentally by Cuthbertson *et al.* 2013 that the expression of *kijX* is induced by kijanimicin in *S. coelicolor*. Therefore, this *kijX* inducible promoter system in *S. coelicolor* is a good system to test the efficacy of the tool in the model organism. To do this, homologous regions of decreasing lengths of 1.0 kb, 0.8 kb, 0.6 kb, 0.4 kb, and 0.2 kb will be used (Figure 4). These *kijX* homologous regions will be PCR amplified and cloned into pLHR. Rather than disrupting the *kijX* gene, the homologous regions are selected to homologous recombination at the downstream end of the gene to produce a *kijX-lux* fusion reporter (Figure 5).
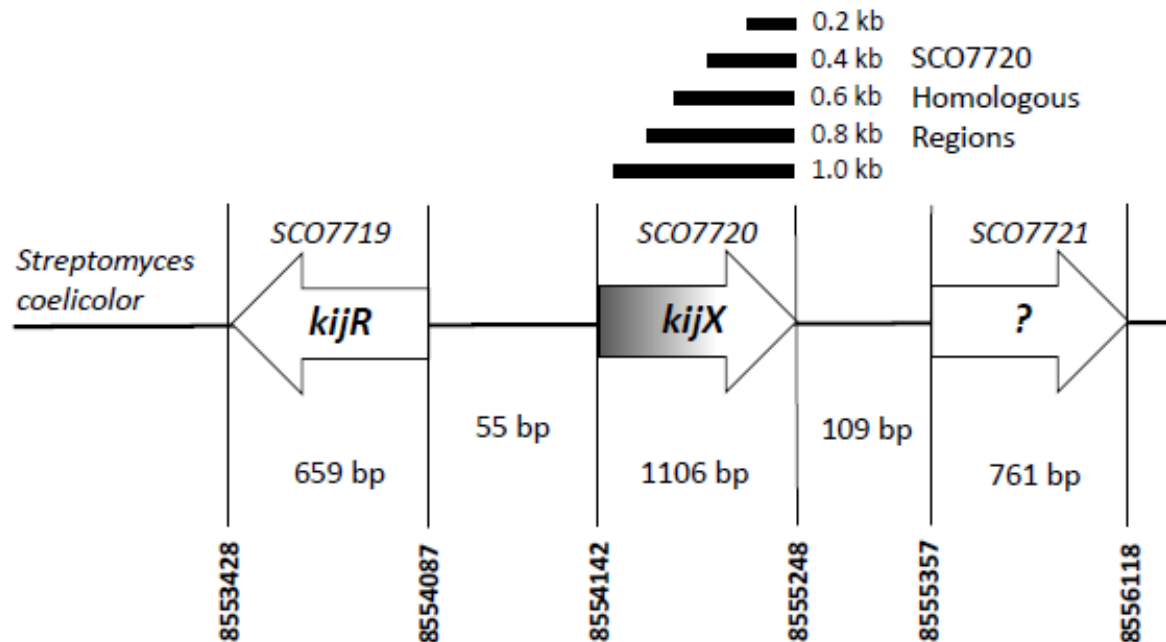
**Figure 4. Schematic diagram of the *kijX* homologous regions used to create *kijX-lux* fusion reports.** The five *kijX* homologous regions ranging from 0.2 kb to 1.0 kb in size are PCR amplified from *S. coelicolor* M145 genomic DNA. Homologous recombination at these locations in the *kijX* sequence will produce a *kijX-lux* fusion as the product.
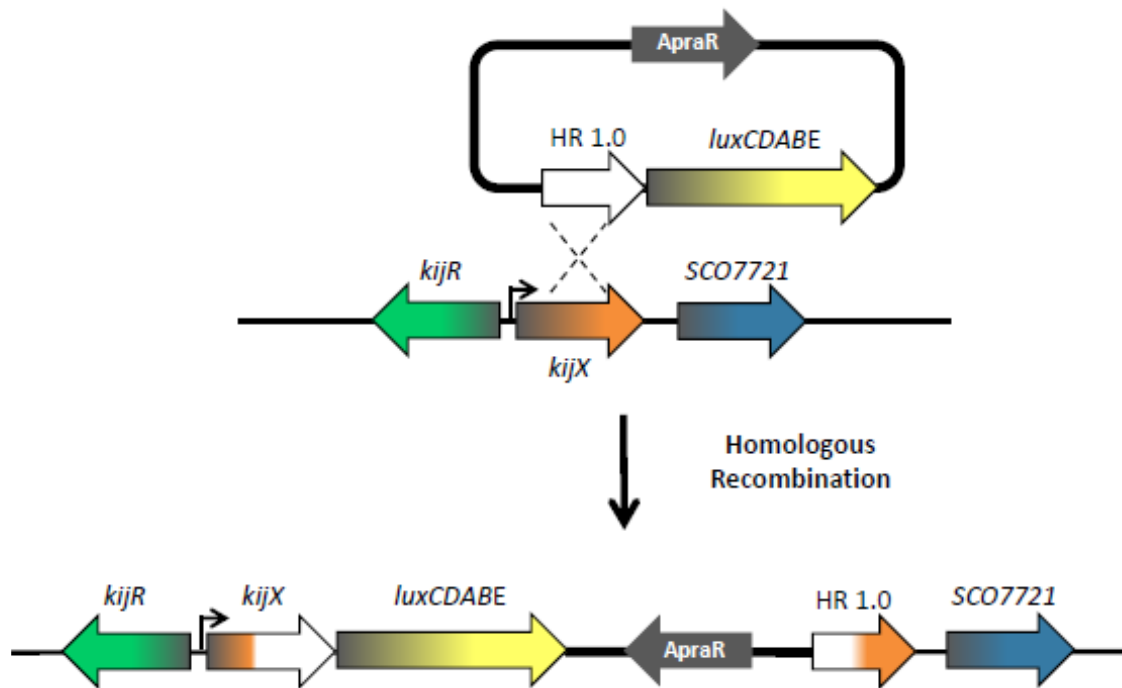
**Figure 5. Schematic representation of integrated pLHR::HR reporter into the *Streptomyces* chromosome.** *Streptomyces* exconjugants containing pLHR::HR will undergo homologous recombination at the downstream end of *kijX* as indicated by the dotted lines. The resulting product is a *kijX-lux* fusion integrated into bacterial chromosome. The *kijX* promoter is indicated by the arrow. In this case, the *kijR, kijX* system is not disrupted and because the homologous region (HR 1.0) does not contain a promoter, a truncated copy of *kijX* will not be transcribed.

Each of the five pLHR::HR *lux* reporter candidates will be evaluated on two characteristics, the number of exconjugants and *kijX-lux* induction by kijanimicin. The number of exconjugants will indicate how efficient the vector recombines into the bacterial chromosome. Exconjugants will be selected for using the apramycin resistance gene in the pLHR backbone. Induction of the *kijX-lux* fusion with kijanimicin will indicate that the *luxCDABE* operon is regulated by the *kijX* promoter and whether the vector has recombined elsewhere in the chromosome. If necessary, additional homologous region lengths may be tested to determine more specifically the minimal homologous region needed to optimize.

## 3.0 Materials & Methods

### 3.1 Culturing Conditions of Bacterial Strains

Strains and plasmids used in this study are listed in Table 1. *E. coli* stain XL1 Blue was used as a cloning vector in this study. All plasmids introduced into *S. coelicolor* via conjugation were first transformed into *E. coli* strain ET12567 [pUZ8002] ensuring appropriate methylation of the DNA (Flett *et al*., 1997). All *E. coli* strains were grown with agitation at 37°C in Luria-Bertani (LB) broth with antibiotic to maintain positive selection of pFlux, pLHR and all pLHR-derived plasmids. *S. coelicolor* M145 strain was cultured on solid R2YE or Mannitol Soya Flour (MS) media at 30°C. *S. coelicolor* M145 strain was also grown in R2YE and Yeast Extract-Malt Extract (YEME) broth at 30°C with agitation. Antibiotic concentrations were used in the following final concentrations where appropriate: 100 μg/mL ampicillin, 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, 50 μg/mL tetracycline, 50 μg/mL apramycin and 25 μg/mL nalidixic acid.

Plasmids used in this work were propagated in *E. coli* XL1 Blue and were obtained from the preparation of an overnight culture using a miniprep or midiprep kit (Qiagen).

**Table 1. Bacterial strains and plasmids used in this work**

| Strain or plasmid | Relevant genotype or characteristics | Reference |
|---|---|---|
| *Strains* | | |
| *S. coelicolor* M145 | Prototrophic, SCP1-. SCP2- | Kieser *et al.*, 2001 |
| *E. coli* XL1 Blue | *recA1 endA1 gyrA96 hsdR17 supE44 relA1 lac{F' proAB lacIQZ_M15Tn10 (Tetr)}* | Stratagene |
| *E. coli* ET12567 [pUZ8002] | *dam13::Tn9 dcm-6 hsdM hsdR recF143 zjj-201::*Tn*10 galK2 galT22 ara14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtII glnV44*, containing the nontransmissible *oriT* mobilizing plasmid pUZ8002 | Flett *et al.*, 1997 |
| *Plasmids* | | |
| λ DNA | *Ac*I, *Avr*II, *Mfe*I restriction enzyme sites | NEB |
| pSWEET | *Pac*I restriction enzyme site | Bhavsar *et al.*, 2001 |
| pFlux | *luxCDABE* operon, *aac(3)IV, ori* pUC18, *oriT* RK2, φBTI integrase | Craney *et al.*, 2007 |
| pOJ260 | Cloning vector – *aac(3)IV, ori* pUC18, *oriT* RK2, *lacZα* | Bierman *et al.*, 1992 |
| pOJ260::MCS | MCS inserted | This work |
| pLHR | pOJ260::MCS with *luxCDABE* inserted | This work |
| pLHR::0.2HR | SCO7720 0.2 kb homologous region inserted | This work |
| pLHR::0.8HR | SCO7720 0.8 kb homologous region inserted | This work |
| pLHR::1.0HR | SCO7720 1.0 kb homologous region inserted | This work |

Note: pLHR is the completed tool comprised of pOJ260::MCS::*luxCDABE*
Abbr: NEB; New England Biolabs Ltd.

## 3.2 *In silico* Digests and Restriction Enzyme Digests

Sequences for pOJ260 and *luxCDABE* were analysed for common zero cutting restriction enzyme sites using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) to identify restriction enzyme candidates for the MCS. It is important that restriction enzyme sites used in the MCS produce single digest products and do not digest the plasmid backbone. Both pOJ260 and *luxCDABE* were digested with candidate restriction enzymes using a positive control template when the enzyme did not cut either sequence and an alternative template was used to ensure restriction enzyme activity. All FastDigest enzymes

were ordered from Thermo Fisher Scientific Inc., however all other restrictions enzymes were ordered from New England Biolabs Ltd. All restriction enzyme digests were conducted following the manufacturer's instructions unless otherwise noted. DNA was visualized using a 1% agarose gel electrophoresis.

### 3.3 Building pLHR

Construction of the pLHR tool required two steps: inserting an *in situ* designed MCS into the cloning vector pOJ260 and inserting the *lux* operon downstream of the MCS. The MCS was generated by annealing two complimentary *de novo* sequences, MCS_1 and MCS_2. The following 1 mL annealing solution: 0.1 M NaCl, 0.1 M Tris-HCl, pH 8, 1 µM EDTA, 5 nM MCS_1 and 5 nM MCS_2 was heated to 95°C for 15 min and allowed to cool in the heating block to room temperature. The resulting double stranded product was designed to have a 5' *Bgl*II sticky end and a 3' *Mfe*I sticky end. pOJ260 vector was prepared by digestion with *Bgl*II and *Eco*RI following the manufacturer's instructions. The double digested vector was gel purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc.) to remove any undigested vector. Digested pOJ260 and annealed MCS were ligated using a standard ligation protocol with T4 DNA ligase (New England Biolabs Ltd.), before being transformed into $CaCl_2$ chemical competent *E. coli* XL1 Blue by heat shock transformation. Each ligation reaction was added to a thawed 200 µL aliquot of competent cells and incubated on ice for 30 min, heated to 42°C for 45 s, returned to ice for 2 min before 900 µL of LB media

was added. Cells were incubated at 37°C for 1 h and plated on LB agar plates supplemented with apramycin and incubated at 37°C. Undigested pOJ260 and sterile water were used as positive and negative transformation controls respectively. Potential pOJ260::MCS clones were verified by restriction digest and sequencing.

All DNA sequencing analysis was performed by MOBIX Lab (Hamilton, Canada). Query sequences were compared to a construct template sequence using Align Sequence Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The reverse complement sequence for reverse sequences were generated using an online reverse complement application (http://www.bioinformatics.org/SM S/r ev_comp.html).

Once pOJ260::MCS was confirmed the *luxCDABE* operon was inserted into the designated downstream region of the MCS to complete the pLHR. pOJ260::MCS was first digested with *Not*I overnight, the linearized vector was gel purified before being digested with Fast Digest *Bam*HI to ensure efficient double digestion of the vector. pFlux was digested with *Bam*HI, *Not*I, and *Bst*XI to generate the double digested *luxCDABE* operon. The products of this digestion were the *luxCDABE* operon (5.5 kb) and the pFlux backbone digested by *Bst*XI to produce two fragments of 3.2 kb and 2.5 kb. Digestion with *Bst*XI allows for gel purification of the double digested *luxCDABE* operon. Linearized pOJ260::MCS and *luxCDABE* were ligated as described and transformed into *E. coli* XL1 Blue via

heat shock transformation. Successful pOJ260::MCS::*luxCDABE* constructs were

identified by luminescence detection, restriction digestion, and sequencing.

**Table 2. Oligonucleotides used in this work**

| Oligonucleotides | Oligonucleotide Sequence 5'→3' |
|---|---|
| *MCS* | |
|    MCS_1 | GATCT<u>TTAATTAA</u>CCTGCAGG<u>GCTAGC</u>GAATTC<u>TTCGAA</u>CC<u>TAGG</u><u>GATATC</u>AACGTT<u>CTTAAG</u>CAATTG<u>GGATCC</u>**ACGATA**G<u>CGGCCGC</u>C |
|    MCS_2 | AATTG<u>GCGGCCGC</u>**TATCGT**<u>GGATCC</u>CAATTG<u>CTTAAG</u>AACGTT<u>GATATC</u>CCTAGG<u>TTCGAA</u>GAATTC<u>GCTAGC</u>CCTGCAGG<u>T</u><u>TAATTAA</u>A |
| | |
| *Sequencing* | |
|    pOJseqF | CGTATGTTGTGTGGAATTGTG |
|    pOJseqR | TTCGCGGAGCTGGTGAAGTA |
|    *luxCDABE*_DW_F | TTCTTTGAGGACGAGATGCAG |
|    *luxCDABE*_UP_R | TAGCCCATGTACTTCTTCAGG |
|    pOJseqF_P1 | GAAAGAACATGTGAGCAAAAG |
|    pOJseqF_P2 | CTAGAAGAACAGTATTTGGTA |
|    pOJseqF_P3 | GATGGGCCACTTGGACTGATC |
|    pOJseqF_P4 | GAAGGAAGGTCCAGTCGGTC |
|    pOJseqF_P5 | ATAGCGATTTTTTCGGTATAT |
| | |
| *SCO7720 Homologous Region Amplification* | |
|    SCO7720_HR_P1.1 | GTATAGC<u>GGATCC</u>TTTCGGTCACCGCCTGTAC |
|    SCO7720_HR_P2.1 | GTATAGC<u>TTAATTAA</u>ACAGCGCGTTCGTGTTCCT |
|    SCO7720_HR_P3.1 | GTATAGC<u>TTAATTAA</u>GAAGCCGCTCGCCGTCAC |
|    SCO7720_HR_P4.1 | GTATAGC<u>TTAATTAA</u>AGCTCGGGTCGCGCATCAA |
|    SCO7720_HR_P5.1 | GTATAGC<u>TTAATTAA</u>ACCCCGAGCCGATCCTCAC |
|    SCO7720_HR_P6.1 | GTATAGC<u>TTAATTAA</u>ATTCGTCGTGCACGGCCAG |

Note: Restriction enzyme cleavages sites where applicable are underlined within the primer sequence and alternating underlines in the MCS sequences. Sequences in bold were designed for the insertion of luxCDABE after the MCS was inserted into pOJ260. All oligonucleotides were ordered from Sigma-Aldrich.

## 3.4 Generating SCO7720 Homologous Region (pLHR::HR) Reporters

Homologous regions of SCO7720 were PCR amplified from a *S. coelicolor*

genomic DNA preparation using the appropriate primers for homologous regions

of differing lengths (0.2 kb, 0.4 kb, 0.6 kb, 0.8 kb, and 1.0 kb). Genomic DNA was prepared from *S. coelicolor* using the DNeasy Blood & Tissue Kit (Qiagen). FastDigest BamHI and FastDigest PacI were used to prepare pLHR and complimentary restriction sites were designed in the primers used to amplify the SCO7720 homologous regions. PCR amplification was performed using Vent polymerase (New England Biolabs Ltd.) and buffer. Various DMSO concentrations were tested for each PCR to optimize amplicon yield obtained from a high GC template. All PCR reactions were conducted using the following conditions: denaturation at 95°C for 2 min, 3 cycles of denaturation for 30 s, annealing at *x*°C for 30 s, and extension at 78°C for 90 s, where each 3 cycles stage is completed for a reducing temperature gradient (where x is 62°C, 60°C, then 58°C) before 18 cycles of denaturation for 30 s, annealing at 56°C for 30 s, and extension at 78°C, followed by a final extension for 10 min at 78°C. The gradual reduction of annealing temperatures allows for increased PCR amplicon yield obtained. PCR amplicons were PCR purified using a PCR purification kit (Qiagen) to remove primers, the DNA was digested with FastDigest *Bam*HI and FastDigest *Pac*I, and PCR purified again to remove the restriction enzymes. Ligation of the double digested PCR product and pLHR vector and transformation of the DNA into *E. coli* XL1 Blue were conducted as stated previously. Successful pLHR::HR constructs were verified by restriction digest and sequencing.

## 3.5 Conjugal Transfer of SCO7720 Homologous Region Constructs into *S. coelicolor*

To transfer the pLHR::HR plasmids into *S. coelicolor* by conjugation, the vectors were first transformed into $CaCl_2$ competent *E. coli* ET12567 [pUZ8002] using the same heat shock protocol. Transformed *E. coli* ET12567 [pUZ8002] were plated on LB agar supplemented with chloramphenicol, kanamycin, and apramycin. A single *E. coli* ET12567 [pUZ8002] colony was used to inoculate 10 mL LB containing chloramphenicol, kanamycin, and apramycin then grown overnight. The overnight culture was used to inoculate 10 mL LB plus antibiotics using a dilution factor of 1:100. The culture was grown until an $OD_{600}$ of 0.4 is achieved. Due to the slower doubling time of the *E. coli* ET12567 [pUZ8002] cells were left overnight to obtain cell cultures with sufficient optical density. Cells were then washed twice with 10 mL LB to remove antibiotics and resuspended in the approximately 500 µL residual liquid. *S. coelicolor* spores were prepared by suspending 40 µL of frozen spore stock (approx. $10^{12}$ spores/µL) in 500 µL 2 x YT media during the *E. coli* wash steps. The *E. coli* suspension was added to the spore stock producing 1 mL mix from which a $10^{-1}$ to $10^{-4}$ dilution series of 100 µL in 900 µL sterile water was prepared. Each dilution and the original mix was plated on MS agar and incubated at 30°C for 36 hrs. Plates were then overlayed with 1 mL water containing 0.5 mg nalidixic acid and 1.25 mg apramycin and returned to incubate at 30°C. Six exconjugant colonies were streaked for individual colonies on R2YE plates containing nalidixic acid and apramycin. Successful exconjugants constructs were identified by luminescence detection.

### 3.6 Kijanimicin Induced *luxCDABE* Expression Assay

An 8 hour culture of *S. coelicolor* containing integrated pLHR::1.0HR was cultured at 30°C with shaking in 2xYT media supplemented with apramycin. A white 96-well plate was prepared by adding 99 µL of *S. coelicolor* culture and 1 µL kijanimicin to each well with a small glass bead (3 mm) for aeration. Kijanimicin was suspended in DMSO at varying working concentrations (1 mM, 100 µM, 10 µM, 1 µM and 100 nM). DMSO was used as a negative control. The plate was incubated at 30°C with shaking and luminescence was measured every 30 mins using a SynergyHybrid plate reader H1 (BioTek Instruments, Inc.).

### 3.7 Media Recipes

### Mannitol Soya Flour Media (MS)

| | |
|---|---|
| Agar | 20 g |
| Mannitol Soya Flour | 20 g |
| Tap Water | up to 1000 mL |

Mannitol Soya Flour was dissolved in water with heat and the medium was autoclaved. After remelting the medium 5 mL $MgCl_2$ was added for a final concentration of 10 mM $MgCl_2$.

### R2 Medium

| | |
|---|---|
| Sucrose | 103 g |
| $K_2SO_4$ | 0.25 g |
| $MgCl_2 \cdot 6H_2O$ | 10.12 g |
| Glucose | 10 g |
| Difco Casamino acids | 0.1 g |
| Distilled Water | 800 mL |

Agar was added and the medium was autoclaved. The medium was melted the following as listed was added 400 mL of R2 Medium.

| | |
|---|---|
| 0.5% $KH_2PO_4$ | 5 mL |
| 3.68% $CaCL_2 \cdot H_2O$ | 40 mL |
| 20% L-proline | 7.5 mL |
| 5.73% TES Buffer (pH 7.2) | 50 mL |
| Trace Element Solution | 1 mL |
| 1M NaOH (IN) | 2.5 mL |

Trace Element Solution ($L^{-1}$)

| | |
|---|---|
| $ZnCl_2$ | 40 mg |
| $FeCl_3 \cdot 6H_2O$ | 200 mg |
| $CuCl_2 \cdot 2H_2O$ | 10 mg |
| $MnCl_2 \cdot 4H_2O$ | 10 mg |
| $Na_2B_4O_7 \cdot 10H_2O$ | 10 mg |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 10 mg |

Trace Element Solution was filter sterilized.


**R2YE Medium**

R2YE Medium was prepared in the same way as R2 Medium, however, 5 ml of Difco Yeast Extract (10%) was added to each 100 ml flask at time of use.


**2 x YT Medium**

| | |
|---|---|
| Difco Bacto Tryptone | 16 g |
| Difco Bacto Yeast Extract | 10 g |
| NaCl | 5 g |
| $H_2O$ | up to 1000 mL |


**Yeast Extract-Malt Extract (YEME) Medium**

| | |
|---|---|
| Difco Yeast Extract | 3 g |
| Difco Bacto-peptone | 5 g |
| Oxoid Malt Extract | 3 g |
| Glucose | 10 g |
| Sucrose | 340 g |
| Distilled Water | up to 1000 mL |

After autoclaving the following were added:
- $MgCl_2 \cdot 6H_2O$ (5mM final)
- Glycine (0.5% final)

## 4.0 RESULTS

### 4.1 Designing the MCS for pLHR

*In silico* analysis of both pOJ260 and *luxCDABE* sequences generated a list of 28 zero cutting restriction sites as possible candidate sites for a MCS in pLHR. These sites were analyzed for reaction condition compatibility such as temperature and activity in specific buffers, as well as common usage, cost, blunt cutting, AT-rich restriction sites, and sequence length. Based on these criteria, a list of 11 restriction sites were identified (Table 3). Additional restriction enzymes were used to identify digestion patterns for pOJ260 and pFlux, and used to clone the MCS and *luxCDABE* into pLHR as reported.

**Table 3. Restriction enzymes used to design the MCS for pLHR**

| Restriction Enzyme | pOJ260 Cuts | pFlux Cuts | Unit Assay Substrate | Comments |
|---|---|---|---|---|
| *Acl*I | 0 | 0 | λ DNA | Candidate restriction enzyme |
| *Afl*II | 0 | 0 | φX174 RF I DNA[a] | Candidate restriction enzyme |
| *Avr*II | 0 | 0 | λ DNA | Candidate restriction enzyme |
| *Bam*HI[b] | 1 | 1 | | Candidate restriction enzyme |
| *Bgl*II | 1 | 8 | | Used to insert MCS |
| *Bst*BI[d] | 0 | 2 | | Candidate restriction enzyme |
| *Dde*I | 3 | 29 | | Diagnostic digest to identify pOJ260 |
| *Eco*RI[cd] | 1 | 2 | | Candidate restriction enzyme |
| *Eco*RV[bd] | 1 | 1 | | Candidate restriction enzyme |
| *Mfe*I | 0 | 0 | λ DNA | Candidate restriction enzyme |
| *Nde*I | 0 | 1 | | Exists upstream of *luxCDABE* |
| *Nhe*I[d] | 0 | 2 | | Candidate restriction enzyme |
| *Not*I | 1 | 1 | | Used to insert *luxCDABE* |
| *Pac*I | 0 | 0 | pSWEET | Candidate restriction enzyme |
| *Sbf*I[d] | 0 | 2 | | Candidate restriction enzyme |
| *Xba*I | 1 | 4 | | Diagnostic digest to identify pFlux |

[a] Template φX174 RF I DNA was not available during these digests. *Afl*II was not analyzed by digestion.

[b] *Bam*HI and *Eco*RV sites were digested out of pOJ260 when the vector was double digested with *Bgl*II and *Not*I.

[c] *Eco*RI site in the pOJ260 backbone was destroyed upon ligation with *Mfe*I.

[d] *Bst*BI, *Eco*RI, *Eco*RV, *Nhe*I, and *Sbf*I are all present in the backbone of pFlux, but do not exist within the *luxCDABE* sequence and are therefore still candidates for the MCS.

To ensure that the sequences used for the *in silico* analysis of restriction enzymes were accurate, both pOJ260 and pFlux were digested with each enzyme to establish the predicted digestion patterns were achieved (Table 3). *Acl*I and *Avr*II did not digest either pOJ260 or pFlux but both enzymes digested λ DNA (Figure 6A). *Mfe*I also did not digest either pOJ260 or pFlux but cleaved λ DNA (Figure

6B). These results indicated that *Acl*I, *Avr*II, and *Mfe*I were all good candidates for the MCS. pOJ260 digested with *Bam*HI produced a single band around 3.5 kb (Figure 6B). This result confirmed the *in silico* digest prediction, however the *Bam*HI restriction site was removed from pOJ260 when the vector was digested with *Bgl*II and *Eco*RI and therefore *Bam*HI is a good candidate for the MCS. pFlux was not digested with *Bam*HI here as the single restriction site in pFlux was repeated confirmed when *Bam*HI was used to cleave *luxCDABE* out of pFlux. *Bst*BI did not cleave pOJ260 and produced two bands (10.8 kb and 0.55 kb) when used to digest pFlux (Figure 6B). These results confirmed the *in silico* digest with *Bst*BI. The two *Bst*BI restriction sites are located in the pFlux backbone and not in *luxCDABE* therefore *Bst*BI is a good candidate for the MCS.

Pacl did not digest either pOJ260 or pFlux but linearized pSWEET (Figure 6C). This confirmed *Pac*I as a good candidate for the MCS. *Eco*RI digests of both pOJ260 and pFlux were not complete. This may be due to duration of the digestion reaction or the buffer choice. Additional *Eco*RI digests were used to address this issue. *Eco*RV linearized both plasmids (Figure 6D), however both restriction sites were removed when *luxCDABE* was cloned into the backbone of pOJ260, and therefore *Eco*RV was a good candidate for the MCS. Both *Nde*I and *Nhe*I produced partially linearized pOJ260. This did not correspond with the *in silico* analysis that identified no restriction sites in pOJ260. It is possible that a restriction site exists in pOJ260 that is not present in the pOJ260 sequence or star activity occurred. This may not be an issue since the location of the potential sites is unknown and the

sites may be been lost when the MCS was added to pOJ260. Though *Nde*I and *Nhe*I were still considered candidates for the MCS, they were not the first choice restriction enzymes for cloning. *Sbf*I did not digest pOJ260 and digested pFlux producing two bands around 10 kb and 0.8 kb (Figure 6E). There appeared to be some undigested pOJ260 from the adjacent lane. The longer reaction time for *Eco*RI (Figure 6E) still produced incomplete digestion of both pOJ260 and pFlux. However, the FastDigest *Eco*RI linearized pOJ260 and digested pFlux producing two bands at 6.9 kb and 4.5 kb as predicted, therefore *Eco*RI was a good candidate for the MCS.

Once the list of 11 restriction sites was established, care was taken in distancing common enzymes in the sequence to ensure restriction enzymes would not be competing for the same area when digesting, and double digestion reactions would be more efficient (See Figure 3 for pLHR MCS sequence).
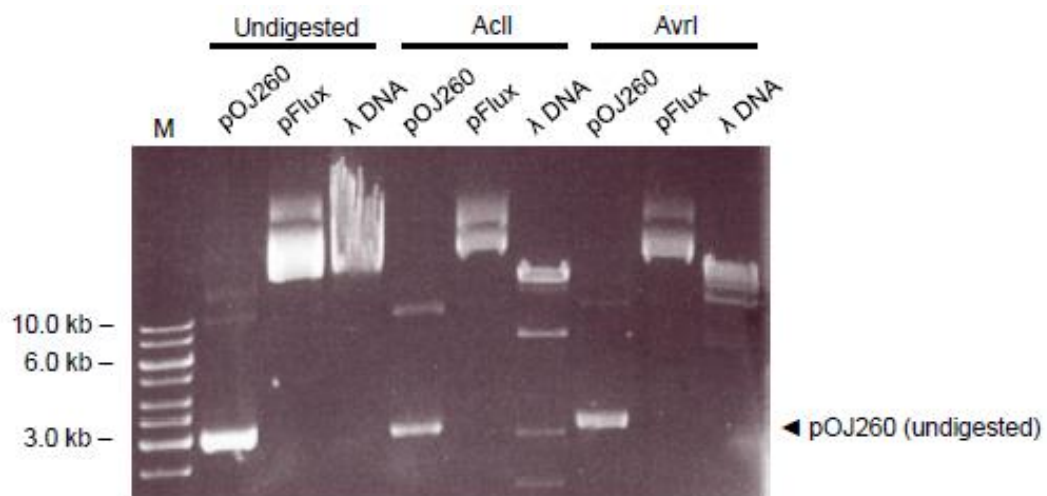


**Figure 6A. Restriction digests of pOJ260, pFlux and λ DNA with *Acl*I and *Avr*I.**
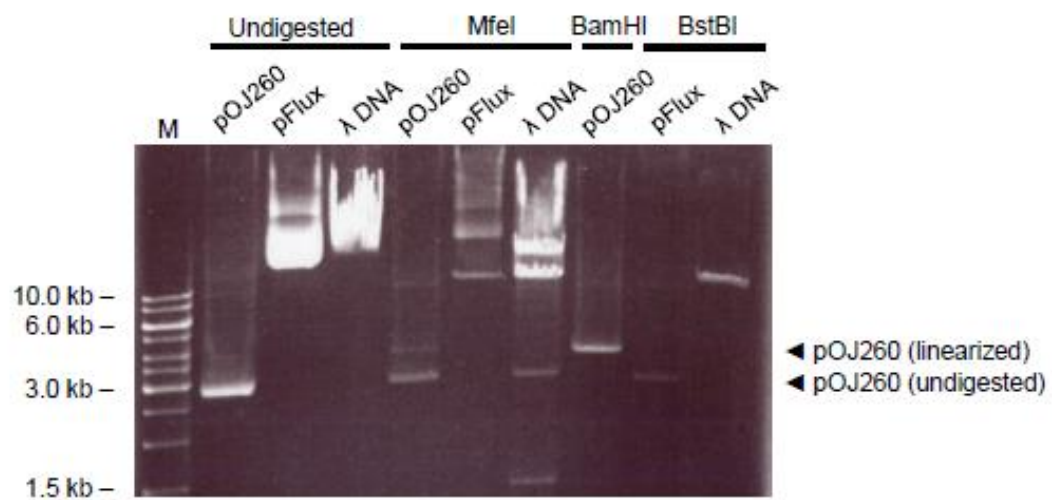
**Figure 6B. Restriction digests of pOJ260, pFlux and λ DNA with *Mfe*I, pOJ260 digested with *Bam*HI, and pFlux and λ DNA digested with *Eco*RV.**
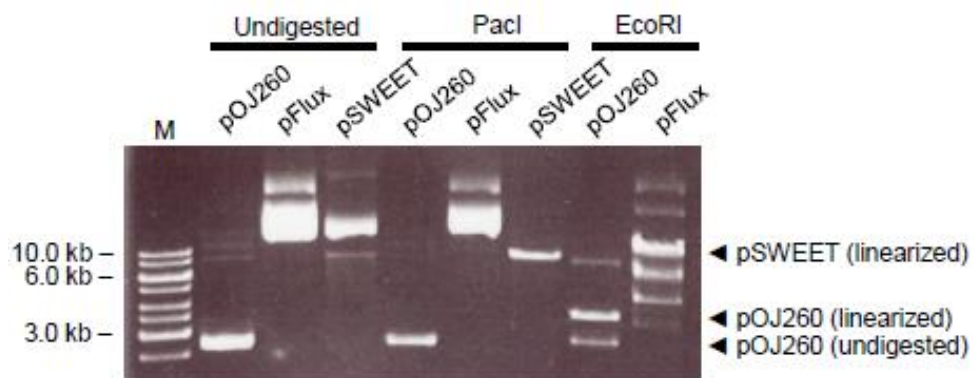


**Figure 6C. Restriction digests of pOJ260, pF*lux* and pSWEET with *Pac*I, pOJ260 and pFlux digested with *Eco*RI.**
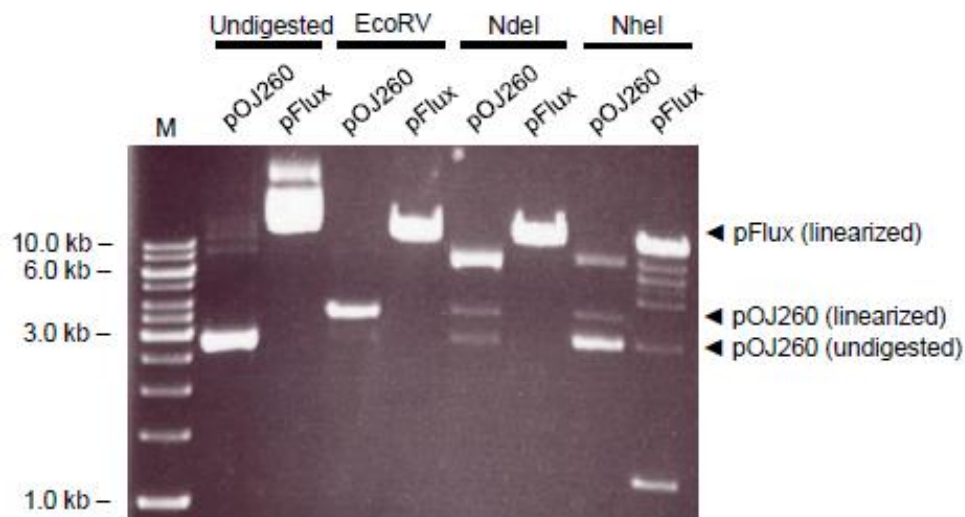
**Figure 6D. Restriction digests of pOJ260 and pFlux with *Eco*RV, *Nde*I and *Nhe*I.**
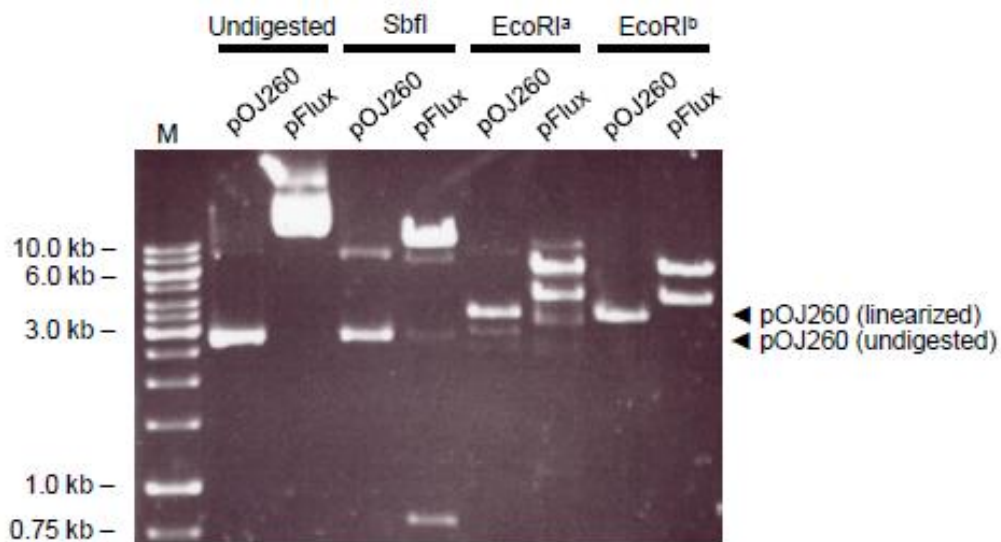


**Figure 6E. Restriction digests of pOJ260 and pFlux with *Sbf*I and *Eco*RI.** [a] *Eco*RI was digested longer than the previous *Eco*RI reaction (overnight instead of 6 hours). [b] *Eco*RI FD was the FastDigest restriction enzyme from Thermo Fisher Scientific.

## 4.2 The New Tool: pLHR

The new gene expression tool, pLHR, was constructed by designing and ordering a MCS compatible with *Streptomyces* plasmid pOJ260 and inserting the MCS into the vector. The *luxCDABE* operon was then inserted into a designated area of the downstream end of the MCS. The resulting vector was 8.8 kb and featured an apramycin cassette, pUC18 origin of replication, RK2 origin of transfer for conjugal transfer from *E.coli* ET12567 to *Streptomyces* species. The *lacZα* gene was lost from pOJ260 when the MCS was cloned into the vector. This was done in an effort to make the tool as small as possible, considering the size of the *luxCDABE* operon (5.5 kb), and easy to work with.

This tool uses homologous recombination to integrate the vector into the bacterial chromosome creating a site specific *lux*-fusion reporter strain. The homologous region of your gene of interest would be cloned into the MCS of pLHR and the vector then introduced into *E.coli* ET12567 [pUZ8002] before transforming *Streptomyces* with the vector via conjugation. Single recombinants would then be selected for using antibiotic resistance markers and luminescence output.

## 4.3 Constructed Kijanimicin *lux*-Fusion Reporters in *E. coli*

To address the question of how big the homologous region needs to be determined for efficient and accurate recombination into *S. coelicolor* chromosome. Five plasmids must be constructed with various homologous regions of *kijX* cloned into pLHR. These plasmids would then be introduced into *S.*

*coelicolor* via conjugation and the number of successful exconjugants would be analyzed. To date, three of these reporters have been built in *E. coli*: pLHR::0.2HR, pLHR::0.8HR, and pLHR::1.0HR (Table 4).

**Table 4. Status of *kijX-lux* reporter collection in *E. coli***

| *kijX-lux* Reporter | Achieved to date |
|---|:---:|
| pLHR::0.2HR | ✓ |
| pLHR::0.4HR | ✗ |
| pLHR::0.6HR | ✗ |
| pLHR::0.8HR | ✓ |
| pLHR::1.0HR | ✓ |

## 4.4 Conjugal Transfer of pLHR::HR Reporters into *S. coelicolor*

Using the conjugation protocol presented in this report, successful exconjugants were obtained for the pLHR::1.0HR reporter. Exconjugants were not obtained for pLHR::0.8HR and pLHR::0.2HR using an alternative conjugation protocol with a shorter incubation time before antibiotic selection. This might have been due to the homologous regions not being sufficiently large enough for homologous recombination, but if the optimized conjugation protocol is used, exconjugants may be achieved using the pLHR::HR reporters to test how large the homologous region needs to be for efficient and accurate homologous recombination.

**4.5 Kijanimicin Dependent Induction of *kijX-lux* Reporter Assay**

Three exconjugants with the highest levels of basal luminescence readings, designated 4.1, 4.2 and 4.3, were chosen for an initial test to establish whether the kijanimicin inducible *kijX-lux* reporter was functional. One of the three exconjugants showed basal luminescence levels compared to the DMSO negative control (Figure 7A), however, the other two exconjugants demonstrated increased luminescence levels with 1 µM kijanimicin (Figure 7B) and 10 µM kijanimicin (Figure 7C). These preliminary results suggested the *kijX-lux* fusion was functioning as a gene expression reporter for *kijX* for two of the three exconjugants. For the exconjugant that did not produce increased luminescence levels it was possible that the *luxCDABE* operon was lost from the vector and the vector backbone remained in the chromosome, or the vector integrated into another site in the chromosome and therefore the *luxCDABE* operon was not under the control of the *kijX* promoter and was not inducible with kijanimicin. This could be avoided by doing a genome preparation of the selected exconjugants following screening for basal luminescence levels and PCR amplifying regions of integration.

Both exconjugants 4.2 and 4.3 produced increased luminescence readings when induced with kijanimicin. Though the concentration of kijanimicin was different between the two it is likely due to differing culture concentrations. Further analyses of this protocol and the characteristics of *luxCDABE* expressions using pLHR are needed to confirm these preliminary results.
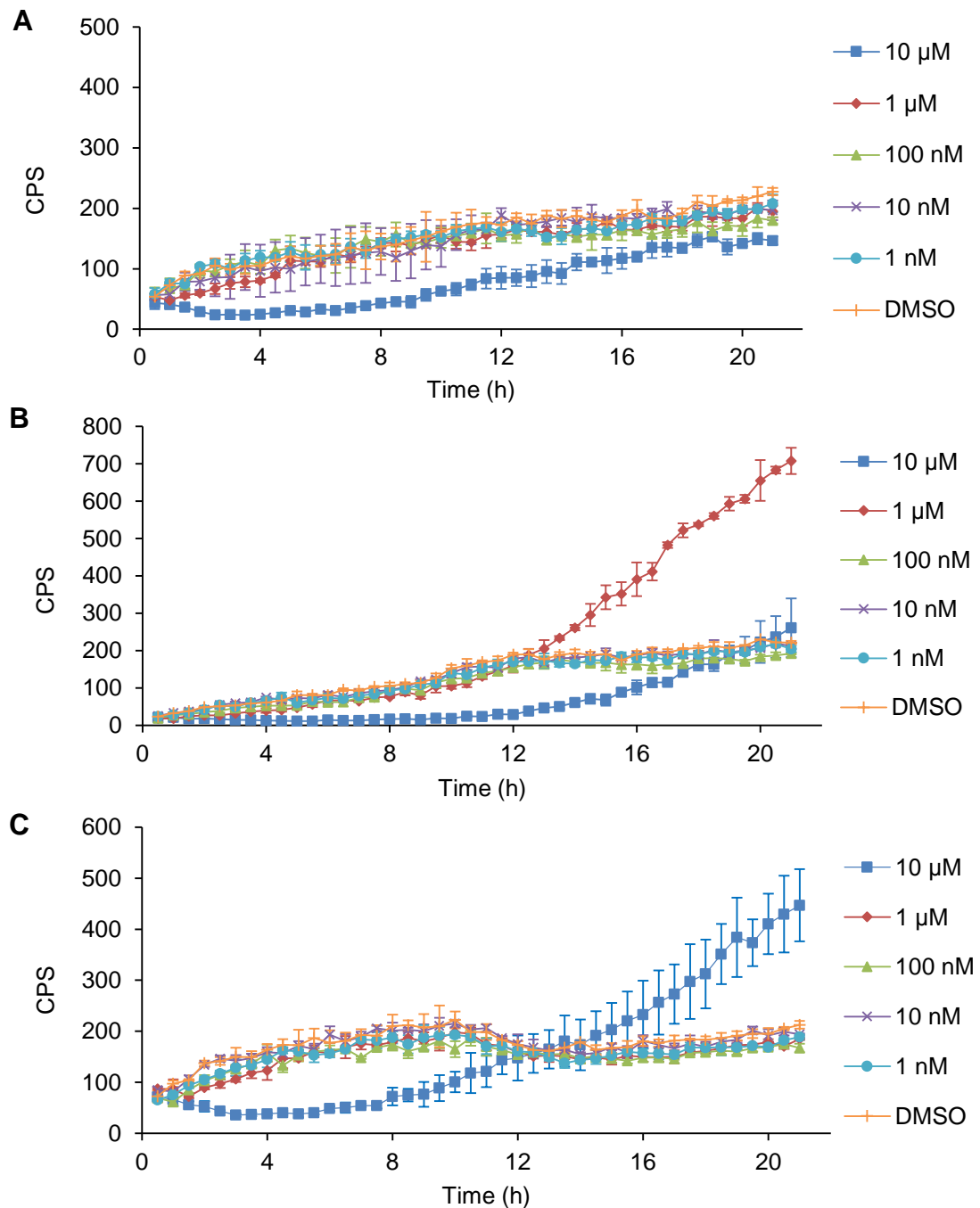
**Figure 7. Kijanimicin induction of *luxCDABE* in three *S. coelicolor* pLHR::1.0HR exconjugants, 4.1, 4.2 and 4.3.** A. No luminescence was measured from exconjugant 4.1 when induced with kijanimicin. B. Luminescence was observed when exconjugant 4.2 was incubated with 1 μM kijanimicin. C. Luminescence was observed when exconjugant 4.3 was incubated with 10 μM kijanimicin. Error bars represent the standard deviation of biological triplicates with the exception of exconjugant 4.2 incubated with 10 μM kijanimicin for which the data reported is of biological duplicates due to extraneous results.

# 5.0 DISCUSSION

Gene expression analyses in *Streptomyces* is currently limited to costly and labour intensive methods which are not favourable for high throughput analyses. A number of transcriptional reporter genes have been explored for their use in *Streptomyces*, and though significant advances have been made to improve these gene expression reporters, an efficient reporter system for high throughput gene expression profiling in *Streptomyces* is still needed. Here, I present a novel integrative luciferase reporter vector, pLHR, which may be used to develop a collection of transcriptional reporter stains. Such a strain collection would be useful for conducting repeated gene expression studies over the entire lifecycle of the organism under varying growth conditions or against a molecule library.

## 5.1 Continued Development of pLHR

The work to date on pLHR has presented a promising foundation for the development of the tool as a high throughput gene expression reporter in *Streptomyces.* Successful integration of pLHR was achieved using a 1.0 kb homologous region using the optimized conjugation protocol. Previous conjugation attempts with pLHR::0.8HR and pLHR::0.2HR were not successful. This may be due to insufficient homologous region length required for integration, or the optimized conjugation protocol may be required to produce more accurate results. Completing the *kijX-lux* reporter collection (pLHR::0.4HR and pLHR::0.6HR) is necessary to establish what length of homologous regions is optimal for efficient

and accurate homologous recombination using the optimized conjugation protocol. This information is important for streamlining the conjugation and integration process when considering high throughput studies.

*Streptomyces coelicolor* with pLHR::1.0HR exconjugants obtained were initially screened for basal luminescence levels and three were tested for luminescence induction with kijanimicin. This assay provides good preliminary evidence that pLHR integrated downstream of *kijX* and luminescence readings may be used to measure *kijX* expression, but this assay needs to repeated to support these results.

In summary, there are four outstanding questions which need to be addressed to ensure pLHR functions as intended:

1. What is the optimal size of the homologous region to ensure efficient and accurate homologous recombination of pLHR into the bacterial chromosome?

2. Does the conjugation protocol presented here produce reliable and consistent results that are required for high throughput methods?

3. How sensitive are pLHR::HR reporters for measuring promoter activity?

4. Will this protocol prove successful in other streptomycetes, namely *S. venezuelae*?

With additional work to confirm the preliminary results here, pLHR may be used on a larger scale to tag multiple genes in *S. coelicolor* and eventually *S. venezuelae* for extensive gene expression profiling studies.

## 5.2 Potential Applications for pLHR

There are an estimated 7846 genes in *S. coelicolor* alone (http://strepdb.streptomyces.org.uk/) and it therefore becomes important to identify which gene groups to develop expression profiles for. Depending on the particular research interests of the lab, strain collections of related genes can be developed to study gene expression profiles of related genes. Here, three potential gene groups are presented to use pLHR to address questions related to ligands of one-component systems, secondary metabolite production, and environmental triggers of two-component systems.

*5.2.1* Streptomyces *Conserved TFRs*

TetR-family regulators (TFRs) are abundant in *Streptomyces* genomes (Cuthbertson *et al.*, 2013). A comparative analysis by Dr. Leslie Cuthbertson (personal communication) of all available *Streptomyces* species genomes has revealed ten TFRs that are conserved among all sequenced *Streptomyces*. An additional two TFRs were identified in all species, but not all *Streptomyces* strains. This project would involve creating TFR-*lux* fusion reporters for each of these highly conserved TFRs and using a natural products library to identify ligands that bind to these regulators. The TFR-*lux* fusion reporters may also be used to elucidate when during the developmental lifecycle of *Streptomyces* these genes are being expressed. Due to the highly conserved nature of these transcriptional regulators among sequenced *Streptomyces*, it would be interesting to determine if

these genes are essential to the cell and if they provide any insight into the evolution of streptomycetes.

### 5.2.2 Secondary Metabolite Biosynthetic Clusters

There have been 30 secondary metabolite gene clusters identified in *S. coelicolor* (Nett *et al.*, 2009). These secondary metabolites have been of much interest for their potential medical or technological applications, however, our understanding of their native functions on bacterial physiology is poorly characterized. This project would be designed to create a *lux* reporter for each gene cluster to quantify expression of these biosynthetic pathways. Screening such a collection of gene cluster-*lux* fusion reporters against a natural products library and evaluating culture growth under varying conditions such as nutrient starvation or enrichment may provide clues as to what stimulates secondary metabolite gene expression. Learning what ligands or other stimuli induce expression of these secondary metabolites and at what stage of the *Streptomyces* lifecycle the secondary metabolites are produced may suggest a more specific biological purpose for each chemical.

Ultimately, the power in a survey of these molecules is increased with the number of organisms used for comparison. *S. avermitilis* is reported to have 37 secondary metabolite gene clusters, and *S. griseus* contains 36 gene clusters (Nett *et al.*, 2009). By creating *lux* reporters for the secondary metabolite gene clusters of multiple *Streptomyces* species you can begin to correlate secondary metabolite

responses to the same stimuli and look for trends in cellular functionality of each of these secondary metabolites.

### 5.2.3 Response Regulators of Two-Component Systems

A search of the *S. coelicolor* genome identified 67 sensor kinase- response regulator pairs that make up two-component systems (Hutchings *et al.,* 2004). There are two major families of response regulators in *S. coelicolor*, NarL and OmpR.  There are 41 paired NarL response regulators and 21 paired OmpR response regulators. Included in the OmpR family of response regulators are AfsQ1 (AfsQ1Q2), VanR (VanRS), and PhoR (PhoRP). Both AfsQ1Q2 and PhoRP have been shown to regulate either directly or indirectly secondary metabolite expression (Nett *et al.*, 2009), and VanRS regulates antibiotic resistance to vancomycin (Hutchings *et al.*, 2004; Pootoolal *et al.*, 2002). PhoRP is triggered by limited phosphate availability and VanRS responds to the presence of VanRS; however, the signal for many sensor kinases is unknown. The pLHR *lux* reporter system may be used to elucidate under what conditions these two-component systems are expressed.

The OmpR family of response regulators may be a good place to start because it contains some characterized two-component systems including VanRS which is inducible with vancomycin. This assay would be done by integrating the *luxCDABE* operon downstream of the sensor kinase and response regulator genes for the OmpR family response regulators. A brief survey of the gene orientation and

clustering of these two-component systems suggests that they are autoregulated. Therefore in the presence of an activating signal, the response regulator will target multiple gene clusters including the gene cluster which contains the sensor kinase and response regulator genes upregulating the expression of the response regulator.

Finding a signal which activates these two-component systems may provide a glimpse into the biological relevance of the genes which are activated directly or indirectly by the response regulators of two-component systems.


## 5.3 Conclusion

Streptomycetes appear to have the genetic tools available to adapt and respond to many different environmental conditions and interactions. These regulatory systems are used to tightly regulate diverse metabolic reactions and a complex developmental lifecycle in highly variable, competitive microbiomes. Using gene expression tools, such as pFlux and pLHR, we may learn more about when genes are active for gene groups or signaling pathways throughout the *Streptomyces* lifecycle. With an understanding of how the bacteria use regulatory systems to manage secondary metabolism and cell development in ever changing natural environments we may gain some insight into the biology and genetics of *Streptomyces* and the metabolites they produce.

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